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REVISITING THE TAXONOMY OF *TARENNA FLAVA* ALSTON (RUBIACEAE: IXOROIDEAE)

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Abstract

The present paper discusses the identity and distribution of *Tarenna flava* Alston (Rubiaceae: Ixoroideae) in India. Detailed description with colour photographs are provided along with morphological comparison of the allied species *Tarenna asiatica* (L.) Kuntze ex K. Schum. Here we also designated the lectotypes for the names *Tarenna flava* and *Stylocoryna webera* A. Rich var. *montana* Thwaites.

Keywords: Lectotypification, south India, Sri Lanka, *Tarenna asiatica*, *T. flava*

Introduction

The genus *Tarenna* was first described by J. Gaertner (1788: 139) on the basis of *T. zeylanica* Gaertner (1788: 139), which was known to the natives of Ceylon (= Sri Lanka) in vernacular name as ‘Tarennae’ (Merrill 1920). *Tarenna* is placed under the tribe Pavetteae, subfamily Ixoroideae, and in the section *Euwebera* on the basis of the number of ovules per locule (Hooker 1880). The genus consists of 192–203 species (POWO 2022, Davis *et al.* 2009) and is distributed widely in tropical Asia and Africa (De Block & Robbrecht 1998) and commonly occur from low to high elevation (Kesonbua & Chantaranonthai 2008). In India, 15 taxa are recorded so far (Hooker 1880, Gamble & Fischer 1923, <https://efloraindia.bsi.gov.in>), and of which, 8 are present in southern Western Ghats with 6 endemics (Sasidharan & Sivarajan 1990, Sasidharan 2013). Compared to many other Rubiaceae genera, the genus *Tarenna* is very poorly known in India. In many of the herbaria, the specimens were misidentified with other genera viz. *Ixora* Linnaeus (1753: 110) and *Pavetta* Linnaeus (1753: 110). *Tarenna* has long been treated in a broad sense by various authors and separated the infrageneric elements of the genus mainly based on placentation and seed type (Bridson 1979, De Block *et al.* 2001).

As a part of the ongoing taxonomic revision on the genus *Tarenna* in the Western Ghats, the authors encountered an interesting specimen from the margins of shola and evergreen forests in the states of Kerala and Tamil Nadu. Critical studies perusal of the available literature (Thwaites 1859, Alston 1931, Matthew 1996, 1999, Ridsdale 1998, Murugan 2020) have revealed the identity of the collected specimen as *Tarenna flava* Alston (1931: 150), a little known species recorded from Sri Lanka and south India. Its identity has been confirmed after the comparison with its type specimens available at Kew (K) and Meise Botanic Garden Herbaria (BR). Detailed descriptions with colour photographs are provided along with morphological comparison of the allied species *T. asiatica* (Linnaeus) Kuntze ex K. Schumann (1902:332) to facilitate its easy identification.

It is also pertinent to mention that, the name *Tarenna flava* and its heterotypic synonym *Stylocoryna webera* A. Rich (1834:248) var. *montana* Thwaites (1859:158) were without precise original material, which have been lectotypified here following Art. 9.3 of the ICN (Turland *et al.* 2018).

Taxonomy

Tarenna flava Alston (1931: 150). (Figures 1 & 2)

Type (lectotype, designated here):—Ceylon (=Sri Lanka). Nuvara Eliya, 6000 ft, s.d., *Gardner* 337 K000031409 [digital image!]; isoelectotype, BR532715 [digital image!] **Figure 3.**

Heterotypic synonym : *Stylocoryna webera* A. Rich (1834: 248) var. *montana* Thwaites (1859: 158).

Type (lectotype, designated here):—Ceylon (=Sri Lanka). s.d., *Thwaites* 158 K000031410 [digital image!]; isoelectotypes, K000031411 [digital image!], BM014118049 [digital image!], P02273269 [digital image!], P03804015 [digital image!], P03804014 [digital image!], BR000000532748 [digital image!], BR000000552374 [digital image!]. **Figure 4.**

Description: Erect shrub, 3–12 m tall. Stem branched, woody, angular, green, glabrous; internodes 1.2–3 cm long. Stipules interpetiolar, linear, 6.9–7 × 5.9–6 mm, base broad, margin entire, acute at apex, green, glabrous. Leaves porrect, petiolate; petioles 1–1.1 cm long, thick, green, glabrous; lamina leathery, elliptic, 7–11 × 3.5–6 cm, base attenuate, margins entire, apex acuminate, dark green, turns dark brown on drying, glabrous on both sides; midrib canalculated on upper surface, secondary veins 8–11 pairs, glabrous. Inflorescence terminal, corymbose cymes; 24–27 flowers, peduncle glabrous, 1.8–2 cm long, thick; bracts and bracteoles distinct; bracts lanceolate, 6–6.5 mm long, glabrous; bracteoles short, ca. 1 mm long, scale like, glabrous. Pedicel terete, 1.8–2.1 mm long, shiny, glabrous. Flower hermaphrodite, pentamerous, homostylous, 14–17 mm long, white to yellowish-white, glabrous; flower bud green at apex, remains white and covered with waxy exudation. Calyx cup-shaped; lobes 5, lanceolate, 4–5 mm long, apex acute, margin entire, green, glabrous. Corolla infundibuliform, 10–12 mm long, white to yellowish-white, glabrous outside; corolla tube short, completely concealed in the calyx cup, throat and basal part of lobes are pubescent; lobes ovate-lanceolate, 5–6 × 2.5–3 mm, prominently veined. Stamens 5, eipetalous, exerted; filaments inconspicuous, ca. 0.15 mm long, arising from the corolla tube hair ring, glabrous; anthers oblong, ca. 5 mm long, connective mucron curved outwardly with extended protrusion. Style short, stout, 3–4 mm long, glabrous; stigma club shaped with longitudinal grooves, 2.9–3.2 mm long, glabrous. Disc cylindrical, 0.2–0.3 mm long, glabrous; ovary 2-celled with many ovules in each cell, glabrous. Fruit berry, bilocular, globular, 0.6 × 0.6 cm, calyx persistent, dark green, glabrous always. Seeds 6–8, angular, black at maturity.

Phenology: Flowering October–April, Fruiting December–June.

Distribution and Ecology:—Restricted to Sri Lanka and south India (so far collected from Kerala and Tamil Nadu). In the protologue, Alston (1931) mentioned the distribution area as south India without any precise locality. Since then, no representative collections were made by any subsequent researchers from south India until the collection by Matthew from Palni hills of Tamil Nadu (1999). Recently, Murugan (2020) discussed the distribution of *Tarenna flava* in Kerala based upon the voucher specimens deposited in TBGT herbarium from Agasthyamala hills of Thiruvananthapuram district collected by Mohanan in 1988. But an elaborated taxonomic description along with colour photographs still lacking for this taxon yet, to delineate the species from its close resemblance *Tarenna asiatica*. During the present study, we succeeded to collect and record many more good populations in high altitude forests ranges of Elival hills of Muthikulam, Palakkad district of Kerala; Pettimudi, Idukki district of Kerala and different forest regions of Nilgiris in Tamil Nadu. During the field investigation it is also observed that, in Elival hills, the plants are grown in the patches of shola grass lands and grows upto a height of 3 m. But in Nilgiris, they are grown into a tree like habit in the evergreen forest's patches and shola upto a height of 12 m. In Pettimudi they are growing as tall shrubs upto a height of 3m in the patches of evergreen forests.

Taxonomic Affinity:—*Tarenna flava* differs from *T. asiatica* by its porrect leaves (vs. non porrect), glabrous petiole (vs. puberulous), 24–27 flowers per inflorescence (vs. 50–65), glabrous pedicel and calyx (vs. puberulous), presence of waxy exudation on the young flower bud (vs. without waxy exudation), completely concealed corolla tube in the calyx cup (vs. partially protruded out), anther connective extended and protruding out (vs. very short protrusion), fruit with persistent calyx (vs. calyx deciduous at maturity), 6–8 seeds per fruit (vs. 4–5). More delineation attributes are provided in Table 1.

Specimens examined:—INDIA. **Kerala:** Palakkad district, Elival hills, Muthikulam, 1845 m, 10°56'12" N, 76°38'26" E, 22 November 2013, *K. M. Prabhukumar* 7667 (CMPR); 11 November 2016, *K. M. Prabhukumar* & *P. M. Binu Prakash* 9898 (CMPR). Idukki district, Thekkadi, 1650 m, 25 February 2021, *Maya S. Nair* 5031 (SD College Herbarium); Munnar, Pettimudi outpost, 1852 m, 27 February 2022, *Maya S. Nair* 11143 (KUBH); Thekkadi to Devikulam, 1500 m, 16 June 1976, *A. J. G. H. Kostermans* 26129 (L). Thiruvananthapuram district,

way to Agastyarkudam, 900 m, 05 March 1980, *M. Mohanan* 66036 (MH); Pongalapara, 1500 m, 07 February 1988, *N. Mohanan* 9510 (CALI & TBGT). **Tamil Nadu:** Near Border semi evergreen forest, close to 43/5 mile marker, 19 October 1974, *D. D. Tirvengadam* & *A. J. G. H. Kostermans*, 718/118 (L); Palni Hills, 1700–2100 m, s.d., *K. M. Matthew* 52511 (RHT).



FIGURE 1. *Tarennia flava*. A. Habit (Tree form); B. Habit (Shrubby); C. Single flower (Inset); D. Inflorescence; E. Infructescence.

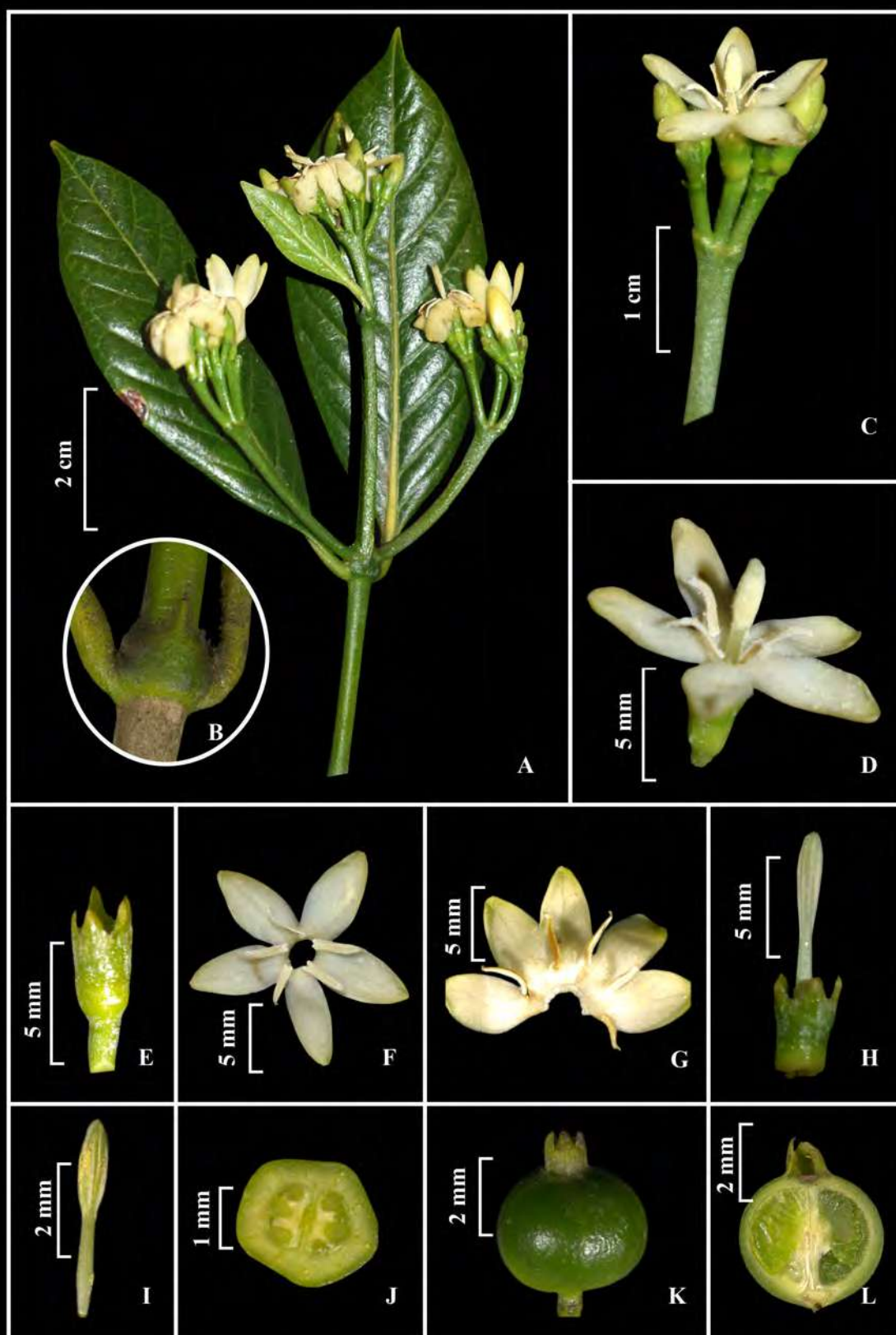


FIGURE 2. *Tarennia flava*. A. Flowering twig; B. Stipule (inset); C. Inflorescence; D. Flower; E. Calyx; F & G. Corolla with stamens; H. Gynoecium with calyx; I. Gynoecium; J. C.S. of Ovary; K. Fruit; L. L.S. of Fruit.

TABLE 1. Morphological comparison of *Tarenna flava* with its allied species *T. asiatica*.

Character	<i>T.asiatica</i>	<i>T.flava</i>
Leaves	non porrect, lamina elliptic-oblong	porrect, lamina elliptic
Leaf petiole	puberulous	glabrous
Number of flowers per inflorescence	50–65	24–27
Pedicle	puberulous	glabrous
Bracteole	brownish	green
Flower bud	white, waxy exudation absent	green at apex and remains white, waxy exudation present
Calyx	puberulous	glabrous
Corolla tube	partially protruded out of calyx cup	completely concealed in the calyx cup
Anther	connective incurved with short protrusion	connective curved outwardly with long protrusion
Fruit	pubescent at young stage, glabrous at maturity, whitish-green, calyx deciduous at maturity	glabrous always, dark green, calyx persistent
Number of seeds per fruit	4–5	6–8



FIGURE 3. Digital image of the lectotype designated for the name *Tarenna flava*. ©copyright of the Board of Trustees of the Royal Botanic Gardens, Kew.



FIGURE 4. Digital image of the lectotype designated for the name *Stylocoryna webera* var. *montana*. © copyright of the Board of Trustees of the Royal Botanic Gardens, Kew.

SRI LANKA. Nuwara Eliya, 1850 m, 17 June 1969, C. F. Beusekom & R. J Beusekom 1398 (US); Pidurutalagala, Nuwara Eliya District, Central Province, 1990 m, 22 September 1974, D. D. Tirvengadam & Shelton Waas 546 (K).

Lectotypification of the names *Tarenna flava* and *Stylocoryna webera* var. *montana*

Alston (1931) proposed the name *Tarenna flava* based on the Gardner's collection from Nuvara Eliya, Sri Lanka (Ceylon), and he also mentioned "Also in S. India". It appears that, Alston may also be seen the specimens from south India while describing the novelty. But our efforts to trace out any specimen from south India before 1931 in any of the repositories either in India or abroad are in vain. During the study, we have traced out the multiple sheets of Gardner's homogenous collection from K (K000031409) and BR (BR532715) herbaria. Of which, the specimen kept in K, with barcode K000031409 is well preserved and perfectly fit with the protologue (having fruits with persistent calyx), which is designated here as lectotype.

During the study, it is also noticed that, the name *Stylocoryna webera* A. Rich var. *montana* Thwaites is presently considered as a synonym of *T. asiatica* (POWO 2022). But the persistent calyx on fruits clearly distinct the former from the latter. Hence the former should be considered as a synonym under the name *T. flava*. Thwaites proposed the

var. *montana* in 1859 based on collection (collection number 158, Ceylon Plants Number 1649) from Sri Lanka. In the protologue, Thwaites mentioned that, the proposed variety is very abundantly distributed in the Islands and other more elevated localities than var. *webera*. During the present study, we have traced out the multiple sheets of this homogenous collection from Kew (K000031410, K000031411), The Natural History Museum (BM014118049), Meise Botanic Garden Herbaria (BR000000532748, BR000000552374) and Paris (P02273269, P03804015, P03804014) herbaria respectively. Of which K000031410 is perfectly matching with the protologue with well preserved reproductive parts (flower buds and fruits) is considered to be the best choice and designated here as the lectotype.

Acknowledgments

MSN and SKT express sincere thanks to the Principal and PG & Research Department of Botany, Sanatana Dharma College, Alappuzha, Kerala, India and Kerala University, Kerala, India for the support. KMP express his gratitude to the Director, CSIR-National Botanical Research Institute, Lucknow, India and Director, Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal for the support. MSN also thankful to Dr. Thomas V. P., Department of Botany, Catholicate College, Pathanamthitta, Kerala for the help rendered during the research. Authors express sincere gratitude to the Curators and staff of K, BR, BM, E, P, CAL, MH, CALI, TBGT and CMPR herbaria for their support during herbarium reference. The authors further thank the authorities of K, BM and BR for providing the images of the specimens and Kerala Forest Department for permitting access to the forest.

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- <https://doi.org/10.12705/Code.2018>



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To,

Maya. S. Nair
Department of Botany
N.S.S College
Cherthala.

Sub:- Permission to do Psychopharmacological Evaluation of Biophytum
Sensitivum in mice.

Sir,

Ms. Ayishu Mansoor 4th Semester M. Pharm Pharmacology
student of St. Joseph's College of Pharmacy, Cherthala would like to do
Psychopharmacological Evaluation of Biophytum Sensitivum in mice
as a part of M. Pharm thesis work. Kindly grant permission to do the works.

For T. S. Nair

Principal

Dr. Sr. Daisy. P. A
Principal

St. Joseph's College of Pharmacy
Dharmagiri College Campus
Cherthala - 688 524

For guide
[Signature]



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Department of Pharmacology

10.10.2022

To

The Head of the department,

Department of botany,

NSS College,

Cherthala.

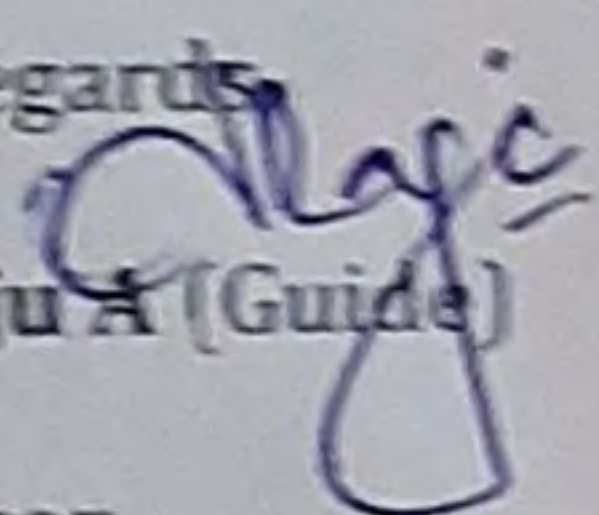
Dear sir/madam,

Greeting from St. Joseph's College of Pharmacy, Cherthala.

We are sending our student Ms. **Aryananda S**, III semester M. Pharm (Pharmacology) to get plant identification and authentication certificate from the department of botany, NSS College, Cherthala. She is doing research (PG) on a natural plant-based study for the need for identification and authentication certificate to ensure the plant and also a part of her plan of research work. Kindly help the same.

Thanking You.

With regards,

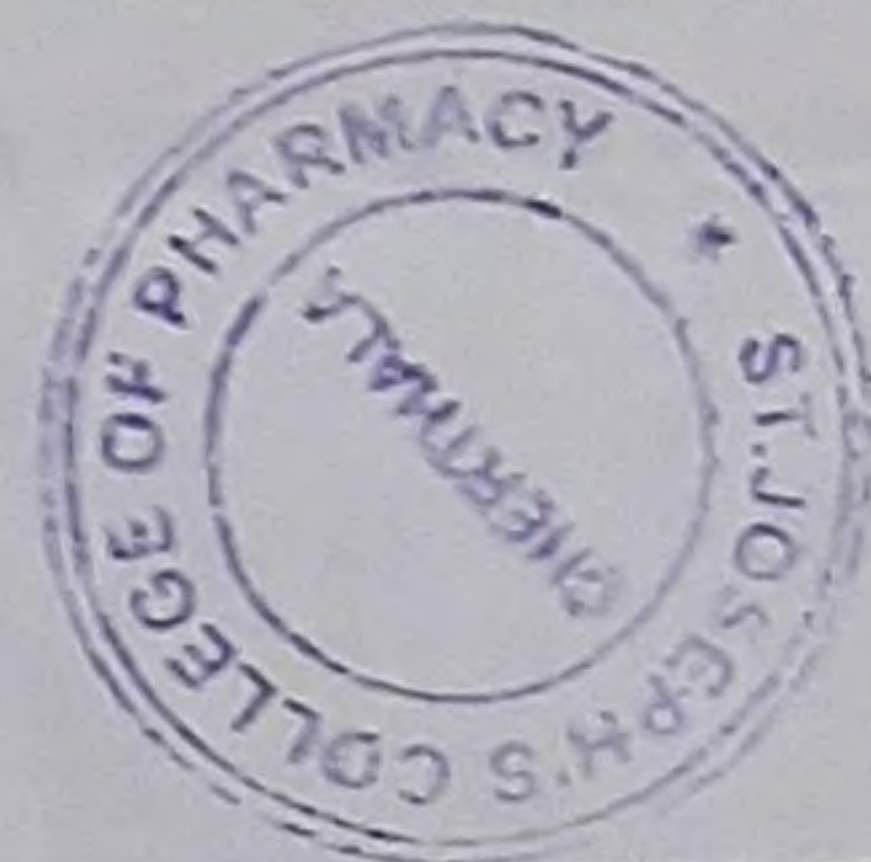

Dr. Raju A (Guide)

Professor,

Department of Pharmacology,

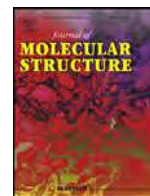
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Crystal structure, DFT studies, Hirshfeld surface and energy framework analysis of 4-(5-nitro-thiophen-2-yl)-pyrrolo [1, 2-a] quinoxaline: A potential SARS-CoV-2 main protease inhibitor

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ARTICLE INFO

Article history:

Received 3 August 2021

Revised 7 October 2021

Accepted 12 November 2021

Available online 15 November 2021

Keywords:

Pyrrolo[1,2-a] quinoxaline

Crystal structure

Hirshfeld surface

DFT

ABSTRACT

The title compound 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) was obtained by a straightforward catalyst-free reaction of 5-nitro-2-thiophene carboxaldehyde and 1-(2-aminophenyl) pyrrole in methanol and was structurally characterized by FT IR, UV-Vis, NMR spectroscopic techniques and elemental analysis. The structure of the compound has been confirmed by the single-crystal X-ray diffraction technique. The compound crystallizes in a monoclinic crystal system with space group P2₁/c. Unit cell dimensions: $a = 12.2009(17)$ Å, $b = 8.3544(9)$ Å, $c = 13.9179(17)$ Å and $\beta = 104.980(5)^\circ$. Hirshfeld surface analysis was carried out to understand the different intermolecular interactions. The two-dimensional fingerprint plot revealed the most prominent interactions in the compound. Theoretical calculations were executed using Density functional theory (DFT) by Gaussian09 software to develop optimized geometry and frontier molecular orbital analysis. Molecular docking studies revealed that the title compound is a potent inhibitor of Main protease 3CL^{pro} with PDB ID: 6LU7, the viral protease which is responsible for the new Corona Virus Disease (COVID-19).

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1. Introduction

SARS-CoV-2 outbreak poses a serious threat to humanity all over the world today. It was declared as a pandemic by World Health Organization on 11 March 2020 [1]. Currently, there are no effective drugs developed for the treatment of this disease. Researches are going on for developing vaccines, druggable molecules, monoclonal antibodies, and cell-based therapies [2]. The chymotrypsin-like cysteine protease also known as Main protease 3CL^{pro} plays an important role in viral replication of SARS-CoV-2 [3]. 3CL^{pro} is the key target in anti-COVID-19 drug design due to its non-similarity with human proteins [3,4].

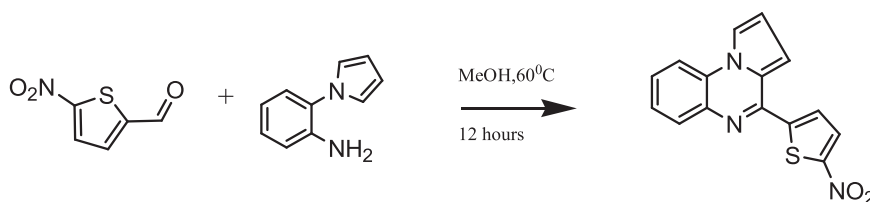
Compounds with quinoxaline ring systems are extensively studied for the last two decades due to their wide range of biological properties. Several quinoxaline derivatives have been reported to possess remarkable pharmacological properties which fight against several diseases with few side effects. They have been described as anti-inflammatory [5], antimalarial [6], antidepressants [7], antiviral [8], antimicrobial [9,10] as antifungal and antibacterial agents.

Pyrrolo [1, 2-a] quinoxalines, an important heterocyclic compound bearing quinoxaline moiety is characterized by a broad range of biological properties [11]. Derivatives of these tricyclic systems have received a great deal of attention for their miscellaneous use in the medicinal field as antileukemic [12], anti-tuberculosis [13], antimalarial [14], anti-Leishmania [15] antidiabetic [16,17] and antibacterial [18] agents. Also, well-established evidence showed that several pyrrolo [1,2-a] quinoxaline-based compounds act as inhibitors against human protein kinase CK2, AKT kinase [19]. Furthermore, suitably functionalized quinoxaline derivatives can act as promising antiviral drugs [8]. At present, research on the synthesis, characterization, and development of new candidates which can effectively bind the Covid 19 Main protease is flying up [4]. In this context, we have been enthused to screen, *in silico*, the interaction between the main protease (6LU7) active site with a pyrrolo [1, 2-a] quinoxaline-based compound.

Methods to synthesize pyrrolo [1,2-a] quinoxalines often involve the use of functionalized aniline precursors [20], transition metal catalysts [21–23], and harsh reaction conditions. We aimed to develop a simple method that avoids the use of expensive transition metal catalysts and dangerous oxidizers. In this paper, we have described the synthesis of a new pyrrolo [1, 2-a] quinoxaline compound, 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) in good yield through the simple coupling of

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Scheme 1. Synthesis of 5NO₂TAAPP

1-(2-aminophenyl) pyrrole and 5-nitro-2-thiophene carboxaldehyde. We also report the single-crystal X-ray structure, spectral characterization, Hirshfeld Surface analysis, and from the docking studies, predicted the binding affinity of this compound to the active site of 6LU7.

2. Experimental

2.1. General characterization techniques

1-(2-aminophenyl) pyrrole and 5-nitro-2-thiophene carboxaldehyde were purchased from Sigma Aldrich. All the chemicals and reagents were of analytical grade and used without further purification. Electronic spectra of the compound were recorded in DMF on a Thermo electron Nicolet evolution 300 UV-Vis spectrophotometer. FT-IR spectra of the compound were recorded as KBr pellets with a JASCO-8000 FT-IR spectrophotometer in the 400–4000 cm⁻¹ range. Elemental analyzes of the compound were done using an Elementar Vario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India. ¹H and ¹³C NMR spectra were recorded in CDCl₃ on a Burkert Advance DRX 300 FT-NMR spectrometer with TMS as the internal standard.

2.2. Synthesis of 5NO₂TAAPP

A methanolic solution (10 mL) of 5-nitro-2-thiophene carboxaldehyde (2 mmol) and 1-(2-amino phenyl) pyrrole (2 mmol) was refluxed in an oil bath at 60 °C for 12 h. On completion of the reaction as observed using TLC (20:80; Ethyl acetate: Hexane), the solvent was evaporated. The crystalline precipitate formed was collected through filtration using a vacuum pump, washed with cold methanol, dried, and recrystallized from methanol. The light-yellow single crystals were collected. The yield and melting point of the product were determined. Yield 80%; m. p 125–127 °C; IR (KBr, cm⁻¹): 3070(CH), 1607(C=N), 1495(NO₂); UV Vis (λ, nm): 228, 315; ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 7.9(1H, J = 4.4 Hz, d), 7.4(1H, J = 12 Hz, d), 7.1(1H, J = 4 Hz, d), 6.7–6.9(4H, m), 6.2(1H, J = 3.2 Hz, t), 6.01(1H, J = 2.8 Hz, d); ¹³C NMR (DMSO-d₆, δ ppm): 157.9, 149.9, 145.1, 134.8, 130.4, 126.8, 125.5, 125.2, 124.6, 119.4, 116.2, 115.8, 115.2, 110.7, 106.1; Anal. Calcd. For: C₁₅H₉N₃O₂S (295.04) C, 61.01; H, 3.07; N, 14.23; S, 10.86. Found: C, 60.80; H, 3.12; N, 14.16; S, 10.52.

2.3. Crystal structure determination and refinement

Single crystal of the compound 5NO₂TAAPP, suitable for X-ray structure analysis was obtained by slow evaporation at room temperature from its methanolic solution over 24 h. The single-crystal X-ray diffraction studies were carried out using a Bruker AXS Kappa Apex 2 CCD diffractometer, with graphite monochromator Mo Kα radiation (*k* = 0.71073 Å). The unit cell dimensions and intensity data were recorded at 296 K. The structure was solved with the direct method using SIR 92 [24] and refinement was carried out by full-matrix least-squares on F² using SHELXL-97

[25]. The program SAINT/XPREF was used for data reduction and APEX2/ SAINT for cell refinement [26]. Software used for computing molecular graphics ORTEP 3 [27] and Mercury [28]. Software used to prepare material for publication SHELXL-97.

2.4. DFT and Hirshfeld surface analysis

The density functional theory calculations were performed with Gaussian 09 package [29] with the B3LYP exchange-correlation functional and the 6–31 G (d, p) basis set. For the 5NO₂TAAPP optimized structure, Frontier molecular orbitals (HOMO, LUMO) and Mulliken atomic charges were generated using GaussView05 [30]. Hirshfeld surfaces were created using the Crystal Explorer 17 program [31]. The energy framework and interaction energies of the 5NO₂TAAPP molecule were calculated using the TONTO program [32], which is inherent in Crystal Explorer 17 software. The initial geometries were taken from the X-ray data CIF file of 5NO₂TAAPP and used as input files for DFT and Hirshfeld surface analysis.

2.5. Molecular docking

The structure of 3CL^{pro} protein having PDB ID: 6LU7 was retrieved from RCSB Protein Data Bank [33]. An online server, ExPASy protparam which provides all information regarding the protein was used for its characterization. Molecular docking was performed using Autodock 4.2.6 [34]. Both protein and ligand were prepared in pdbqt format. Polar hydrogens and Gasteiger charges were added to the receptor. Grid centered at −19.173, 20.969, 68.039 Å⁰ along x, y, z axes was prepared with 60 X 60 X 60 Å^{0 3} with spacing 0.375 Å⁰. The genetic algorithm was employed as a search parameter with 50 runs, 300 population size and 27,000 number of generations, respectively.

3. Results and discussion

3.1. Characterization

The title compound 4-(5-nitro-thiophen-2-yl)-pyrrolo [1, 2-a] quinoxaline (5NO₂TAAPP) was synthesized (Scheme 1) and characterized. The synthesized compound was crystalline, non-hygroscopic, insoluble in water but soluble in methanol, DMF and DMSO. The compound was characterized by elemental analysis, IR, UV-Visible spectra and NMR analysis. UV-Visible, IR and NMR (¹H and ¹³C) spectra of the title compound are shown in Figs. S1, S2, S3, S4, respectively in the supporting information file.

3.2. Crystal structure of 5NO₂TAAPP

The compound could be crystallized from the slow evaporation of its methanolic solution at room temperature. ORTEP diagram of the compound 5NO₂TAAPP with atom numbering scheme is given in Fig. 1(a). Crystal data and refinement parameters of the compound are given in Table 1. X-ray crystallographic analysis revealed that the compound crystallizes in a monoclinic crystal system with space group P2₁/c. Unit cell dimensions of the crystal are *a* = 12.2009(17) Å, *b* = 8.3544(9) Å, *c* = 13.9179(17) Å

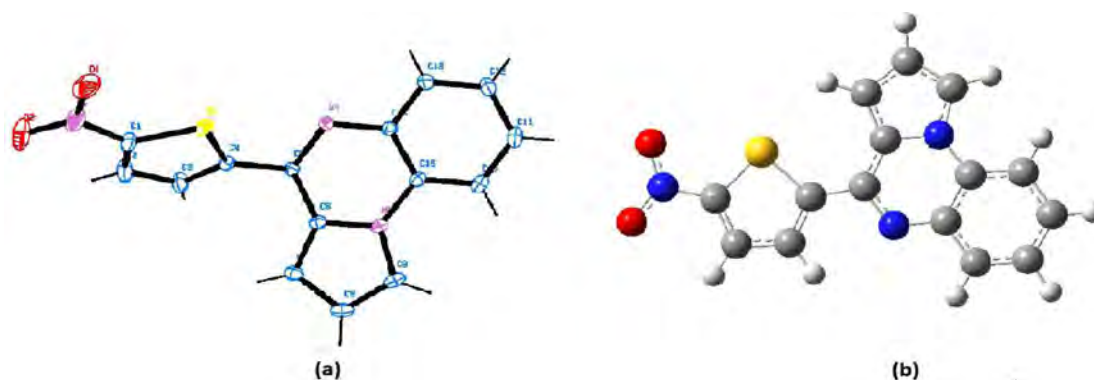


Fig. 1. (a) ORTEP diagram of the compound with thermal ellipsoids drawn at 50% probability level (b) The optimized structure of the compound

Table 1
Crystal data and details of the structure refinement for the title compound.

Parameters	Compound
CCDC number	2,093,318
Empirical formula	C ₁₅ H ₉ N ₃ O ₂ S
Formula weight	295.32
Temperature (K)	296(2)
Crystal system	Monoclinic
Space group	P 2 ₁ /c
a (Å)	12.2009(17)
b (Å)	8.3544(9)
c (Å)	13.9179(17)
Volume (Å ³)	1370.5(3)
Z	4
Calculated density (mg/cm ³)	1.431
μ (mm ⁻¹)	0.244
F(000)	608
Crystal size (mm ³)	0.300 × 0.200 × 0.200
Θ range for data collection (°)	1.728 to 28.420 deg
Index ranges	−16 ≤ h ≤ 11, −10 ≤ k ≤ 10, 18 ≤ l ≤ 18
T _{min} , T _{max}	0.931, 0.953
Reflections collected/unique	11,100 / 3405 [R (int) = 0.0335]
Completeness to theta	25.242, 99.9%
Data/restraints/parameters	3405 / 0 / 190
Goodness-of-fit on F ²	1.130
Final R indexes [I ≥ 2σ (I)]	R1 = 0.0576, wR2 = 0.1683
Final R indexes [all data]	R1 = 0.0855, wR2 = 0.1924
Largest diff. peak/hole (e Å ⁻³)	0.631 and −0.360

Table 2
Selected bond lengths (Å) and angles (°) of the crystal structure of the compound.

Bond	Length (Å)	Bond	Angle (°)
C(1)–S(1)	1.701(3)	N(3)–C(5)–C(4)	108.88(17)
C(4)–C(5)	1.502(3)	N(3)–C(5)–C(6)	108.40(17)
C(4)–S(1)	1.711(2)	C(6)–C(5)–C(4)	111.32(18)
C(5)–N(3)	1.474(3)	C(5)–C(4)–S(1)	120.24(16)
C(6)–N(2)	1.369(3)	C(3)–C(4)–S(1)	112.24(19)
C(9)–N(2)	1.375(3)	C(14)–N(3)–C(5)	116.96(17)
C(14)–N(3)	1.398(3)	C(6)–N(2)–C(15)	122.75(19)
C(15)–N(2)	1.406(3)	C(1)–S(1)–C(4)	89.55(13)
N(1)–O(1)	1.221(4)	O(1)–N(1)–C(1)	117.2(3)
N(1)–O(2)	1.217(4)	O(2)–N(1)–C(1)	118.5(4)

and $\beta = 104.980(5)^\circ$. Bond lengths and bond angles obtained from crystal data are summarized in Table 2.

In the crystal structure of the compound, all C–C and C–H bond lengths in the benzene ring are in the normal range and bond angles are approximately 120° . The bond lengths and bond angles in the pyrrole and thiophene ring are comparable to other previously reported crystal structures [35]. The angle between the benzene ring and quinoxaline ring C(13)–C(14)–N(3) and C(10)–C(15)–N(2)

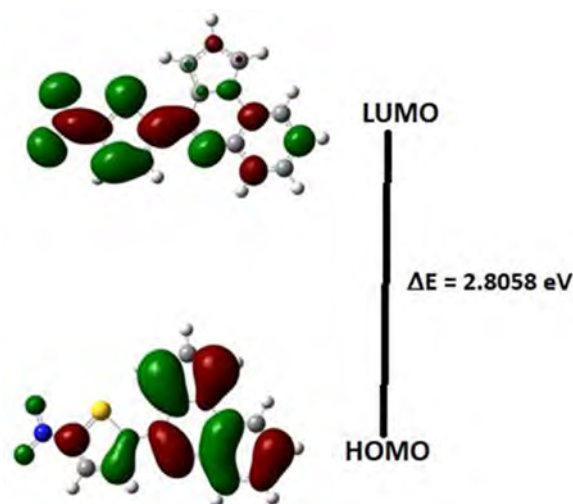


Fig. 2. HOMO-LUMO and energy gap of the title compound

are 120.6° and 122.7° , respectively. The thiophene ring joined to the quinoxaline ring through the bonds N(3)–C(5)–C(4), C(6)–C(5)–C(4) at an angle of 108.88° , 111.32° , respectively. The torsion angle between the quinoxaline ring and the thiophene ring is -25.3° and -85.1° for the atoms S(1)–C(4)–C(5)–N(3) and C(3)–C(4)–C(5)–C(6), respectively. CCDC deposition number 2,093,318 contains supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

3.3. Geometry optimization, frontier molecular orbitals and mulliken charges distribution

The optimized geometry of the compound is given in Fig. 1(b). Frontier molecular orbital analysis was carried out which provide an insight into its biological potential and chemical reactivity [36,37]. The highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energies were calculated to be $E_{\text{HOMO}} = -6.0504$ eV and $E_{\text{LUMO}} = -3.2446$ eV. The HOMO – LUMO energy gap is 2.8058 eV. By using frontier molecular orbital energy values, the global reactivity descriptors such as hardness (η), chemical potential (μ), softness (S), electronegativity (χ) and electrophilicity index (ω) have been defined [38, 39]. The frontier molecular orbital and their energy gap for the compound is shown in Fig. 2. Table 3 lists the energy gap and the other global reactivity descriptors for the title compound.

Mulliken charge distribution within the molecule is very significant because it affects the overall stability, reactivity, electronic

Table 3
HOMO –LUMO and global reactivity descriptors of the title compound.

Parameter	Value
E_{HOMO}	−6.0504 (eV)
E_{LUMO}	−3.2446 (eV)
Energy gap (ΔE)	2.8058 (eV)
Ionization potential (I)	6.0504 (eV)
Electron Affinity (A)	3.2446 (eV)
Chemical Hardness (η)	1.4029 (eV)
Global softness (σ)	0.3564 (eV ^{−1})
Electronegativity (X)	4.6475 (eV)
Chemical potential (μ)	−4.6475 (eV)
Electrophilicity (ω)	7.698 (eV)

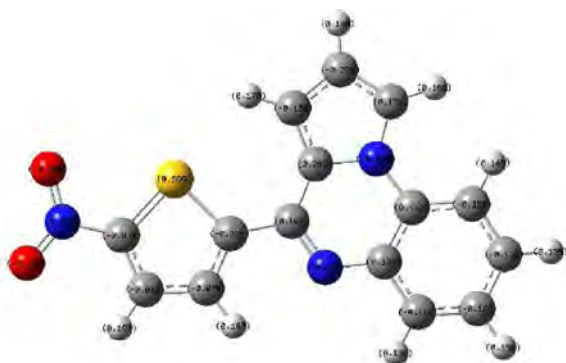


Fig. 3. Mulliken charge distribution for the optimized compound

structure and more properties of the molecular system [40]. The net atomic charges 5NO₂TAAPP molecule obtained by using Mulliken population analysis, are plotted in Fig. 3. The result indicates that the S atom has the highest positive charge, with the corresponding value of charge +0.599. The two nitrogen atoms in the quinoxaline ring have the highest electronegativity and the lowest net atomic charge values (−0.848 e and −0.477 e). Therefore, it is anticipated that the quinoxaline ring as a whole or the individual nitrogen atoms within the ring can interact through hydrogen bonds or other intermolecular forces with the suitably positioned amino acid residue in the target protein. In addition, the carbon atoms in the thiophene ring (−0.321, −0.039, −0.074, −0.079 e) and oxygen atoms of the nitro group (−0.302, −0.295 e) also have relatively low atomic charges, may also be involved in prolific binding interactions with the enzymes.

3.4. Hirshfeld surface analysis

Hirshfeld surface analysis enables us to understand the packing modes, the surface of the molecular system and intermolecular interactions in crystals. Hirshfeld surface volume and surface area of the compound are 335.91 Å³ and 312.47 Å², respectively. The globularity and the asphericity for the shape are 0.748 and 0.213, respectively. In the Hirshfeld surface mapped over normalized contact distance d_{norm} (Fig. 4), the white surface area indicates the contact with distances equal to the sum of van der Waals radii, and the red and blue colours indicate the distances shorter (in close contact) or longer (distinct contact) than the van der Waals radii, respectively [41].

The 2D fingerprint plot permit us to calculate the percentage contribution of each type of contacts for the total Hirshfeld surface area. The H . . . H contacts are responsible for the largest contribution (30.6%) to the Hirshfeld surface. Beside these contacts, H . . . O/O . . . H (24.8%), H . . . C/C . . . H (14.4%), H . . . S/S . . . H (7.3%), H . . . N/N . . . H (5.6%), C . . . C (5.5%), N . . . C/C . . . N (4.6%) and S . . . C/C . . . S (3.1%) interactions contribute considerably to the total Hirshfeld

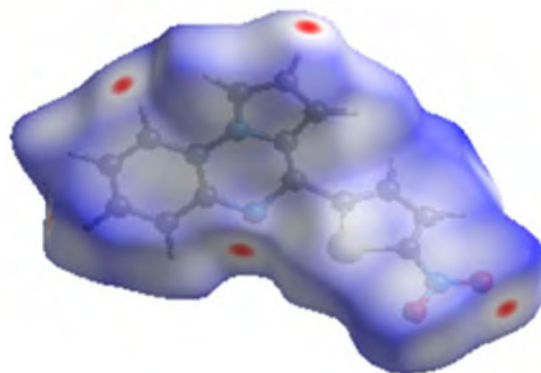


Fig. 4. Three-dimensional Hirshfeld surface of the title compound plotted over d_{norm} in the range −0.1039 to 1.4949 a. u.

surface area. The 2D fingerprint plot for all major contacts are depicted in Fig. 5. The contributions of other contacts are only minor and add to N . . . N (0.1%), S . . . O/O . . . S (0.1%) and S . . . N/N . . . S (0.1%).

Hirshfeld surface plotted over shape index, curvedness and electrostatic potential map (Fig. 6.) give more details about the shape and molecular packing in crystals. The shape index map (Fig. 6a.) of the title compound was generated in the range of −1 to 1 Å². The surface around the acceptor atoms are indicated by the blue colour regions and the surface around the donor atoms are indicated by red colour regions in the shape index map. The curvedness map (Fig. 6b.) of the title compound was generated in the range −4 to 4 Å². The large green regions indicate a planar surface area, while the blue regions reveal the areas of curvature. The presence of π – π stacking interactions is also evident as the flat regions around the thiophene and benzene ring on the Hirshfeld surface plotted over curvedness. In the electrostatic potential map (Fig. 6c.) over the Hirshfeld surface, the electropositive regions (around hydrogen bond donor) are indicated by blue colour; whereas red colour regions are electronegative regions (around hydrogen bond acceptor) [42].

3.5. Energy framework analysis

The intermolecular interaction energies for the title compound was calculated using CE-B3LYP/6–31 G(d, p) energy model available in crystal explorer with scale factors $k_{\text{ele}} = 1.057$, $k_{\text{pol}} = 0.740$, $k_{\text{disp}} = 0.871$, $k_{\text{rep}} = 0.618$, respectively [31]. The different interaction energies, coulombic interaction energy (red), dispersion energy (green), total interaction energy (blue) of the compound are depicted in Fig. 7. The magnitude of the interaction energy is proportional to the radii of the corresponding cylinder. Table 4 list the result of different interaction energies of the title compound, rotational symmetry operations to the reference molecule (Symop), the centroid to centroid distance between the reference molecule and the interacting molecule (R) and the number of pairs of the interacting molecule to the reference molecule (N).

The calculated electrostatic, polarization, dispersion, repulsion energies are −45.0 KJ/mol, −22.3 KJ/mol, −179.1 KJ/mol and 82.7 KJ/mol, respectively. The calculated total energy of the molecule is −154.7 KJ/mol. The result revealed that dispersion energy is predominant over the other interaction energies and have a key role in the total forces in the crystal packing.

3.6. Docking analysis

The purpose of this docking analysis was to assess the binding affinity of the amino acids within the 3CLP^{pro} active site to the target ligand 5NO₂TAAPP. The Auto Dock tool was used to perform the

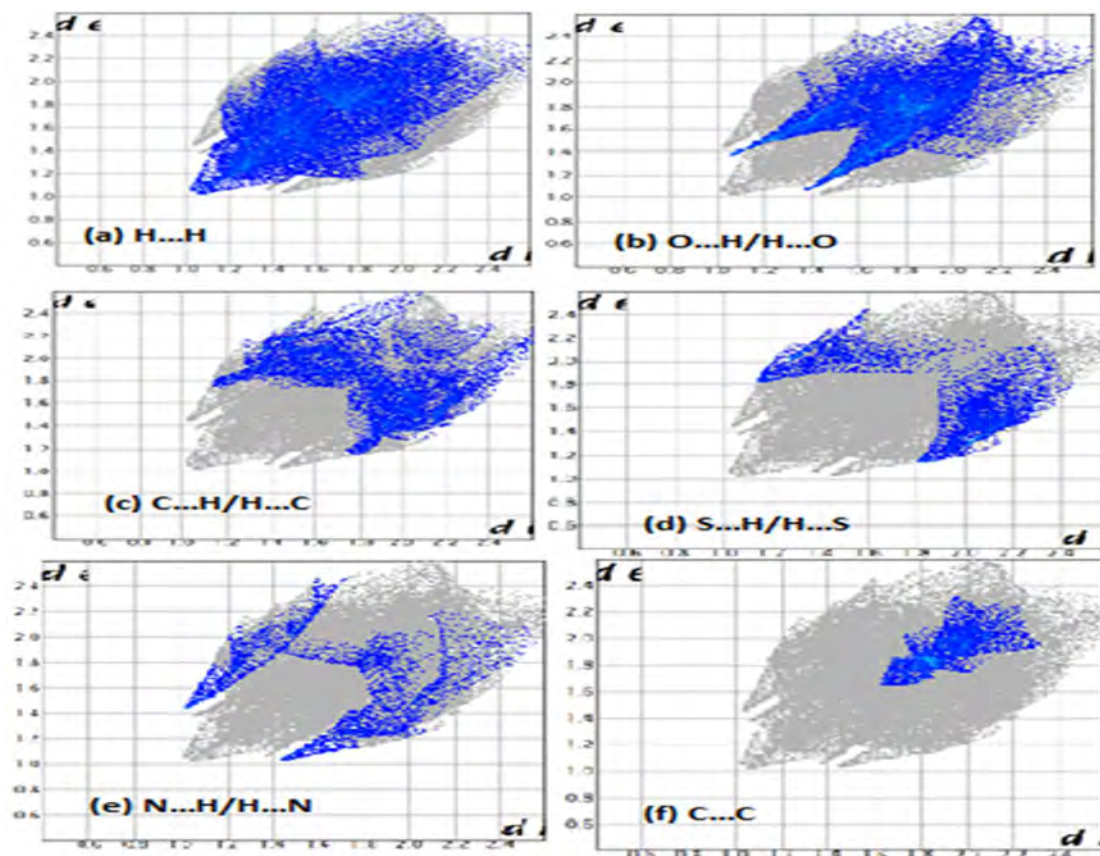


Fig. 5. 2D fingerprint plots for all major contacts on Hirshfeld surface. d_i and d_e denote the closest internal and external distances in Å from a point on the surface.

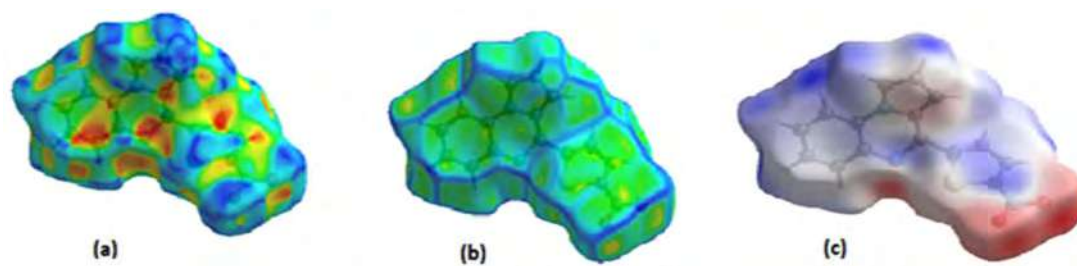


Fig. 6. Hirshfeld surface mapped over (a) shape index (b) curvedness (c) electrostatic potential

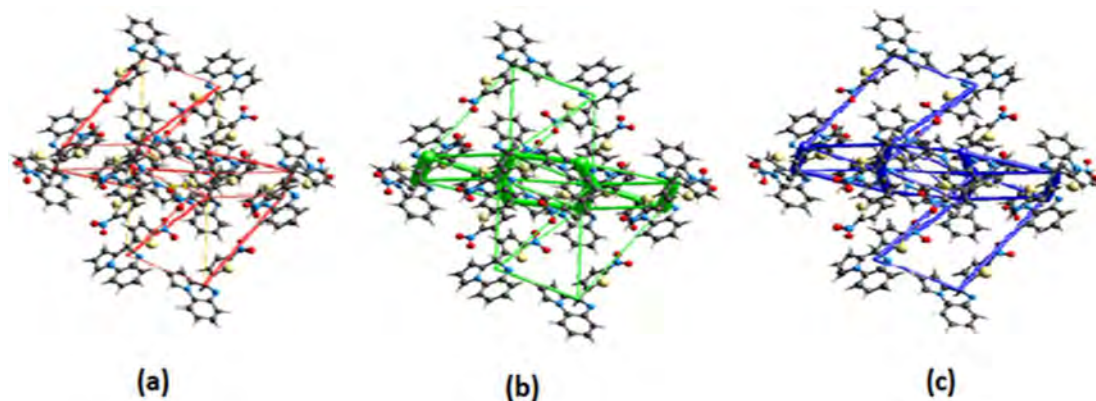


Fig. 7. Graphical representation of electrostatic interactions (a) coulomb interaction energy (b) dispersion energy (c) total energy of the title compound

Table 4
Different interaction energies of compound in KJ/mol.

N	Symop	R (Å ⁰)	Eele	Epole	Edis	E rep	Etot
1	-x, -y, -z	13.42	2.1	-0.5	-3.1	0.0	-0.9
2	x,-y + 1/2, z + 1/2	12.65	-15.6	-3.7	-8.3	5.3	-21.4
2	x, y, z	12.20	5.4	-2.4	-10.1	5.7	-0.6
2	-x,y + 1/2,-z + 1/2	13.80	0.5	-0.2	-2.0	0.0	-1.4
1	-x, -y, -z	7.21	-12.4	-2.9	-15.8	5.3	-24.5
2	-x,y + 1/2,-z + 1/2	4.29	-8.7	-4.0	-71.2	34.5	-47.6
1	-x, -y, -z	7.20	1.0	-4.0	-36.7	20.5	-18.0
2	x,-y + 1/2, z + 1/2	7.43	-6.1	-2.1	-18.8	9.8	-16.7
1	-x, -y, -z	11.56	-7.9	-2.2	-9.6	1.5	-16.9
1	-x, -y, -z	9.76	-3.3	-0.3	-3.5	0.1	-6.7

**Fig. 8.** The pictorial representation of the compound 5NO₂TAAPP bound inside the pocket of SARS-CoV-2 main protease 3CL^{pro}.**Table 5**
Characterization of protein.

Properties	Values
molecular weight	333,797.64 KDa
energy	-16,473.465 kJ mol ⁻¹
Number of amino acids	306
Theoretical pI	5.95
Instability index	27.65
Aliphatic index	82.12
GRAVY	-0.019
Resolution	2.16 Å

docking of the ligand into the catalytic binding site of SARS-CoV-2 3CL^{pro} with PDB ID: 6LU7 (Fig. 8). The selected protein was characterized by primary and secondary structure analysis as shown in Table 5.

The isoelectric point (pI value) 5.95 indicates that the protein is slightly acidic. The relative volume of a protein occupied by its aliphatic side chains is termed as the aliphatic index (AI). The aliphatic index plays role in protein thermal stability. A high

**Fig. 9.** Interaction of 5NO₂TAAPP with SARS-CoV-2 main protease.

aliphatic index specifies that the protein is thermally stable over a wide temperature range. Aliphatic index in the range of 66.5 to 84.33 indicate high thermal stability and hydrophobicity of protein, which help them for biological membrane perturbation [43]. The Grand average of hydropathy (GRAVY) value is a measure of the hydrophilic nature of protein [44]. The value obtained for GRAVY is -0.019, which is close to zero, indicate the hydrophobic nature of the protein. The instability index is an assessment of the stability of a protein experimentally. A protein whose instability index is smaller than 40 is predicted as stable [45]. These results indicated that 6LU7 is a stable, hydrophobic protein.

The binding affinity of the inhibitor (ligand or drug) is an important parameter, which determines the strength of its binding interaction with the target protein or the biomolecule. These interactions can be of many kinds such as hydrogen bonding, electrostatic interactions, hydrophobic and van der Waals forces. The 2D interaction diagram of the compound with the target 6LU7 is shown in Fig. 9. Compound forms two hydrogen bonds with GLY 143 and CYS 145 through an oxygen atom of the nitro group; van der Waals interactions with GLU 166, HIS 164, GLN 189, ASP 187, ARG 188, PRO 52, TYR 54, HIS 41, SER 144, ASN 142; π -sulphur interaction by Sulphur atom of CYS 44 with the π electron cloud of benzene ring; and a π -donor hydrogen bond interaction with the -SH group of CYS 145. MET 165 and MET 49 form π -alkyl bonds with the compound.

The binding affinity of a ligand with a protein is measured in terms of binding energy. The negative value of binding energy indicates a release of energy while forming a protein–ligand docked complex, which imparts stability. The more negative the binding energy, the higher will be the stability and binding affinity [46]. The binding energy obtained for the compound 5NO₂TAAPP

with 6LU7 is $-7.95 \text{ Kcal mol}^{-1}$. Hydroxychloroquine and remdesivir, which were approved drugs as inhibitors to SARS-CoV-2 having binding affinity values -6.06 and $-4.96 \text{ Kcal mol}^{-1}$, respectively [47]. In general, molecular docking results reveals that there should be effective hydrogen bonding and other hydrophobic interactions between the compound and the target protein. These interactions together with the predicted binding affinity, indicating that the compound 5NO₂TAAPP should show significant SARS-Cov-2 inhibitory activity.

4. Conclusion

The new biologically active compound, 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) was synthesized and structurally characterized by FT IR, UV-Vis, NMR and elemental analysis techniques. Single crystal X-ray diffraction analysis was used to confirm the three-dimensional conformation of the compound. Hirshfeld surface analysis revealed the important intermolecular interactions and contacts within the crystal structure. Using DFT calculations, its molecular structure was optimized, frontier molecular orbitals were deduced. Molecular docking experiments disclosed that the compound formed important binding interactions with the amino acid residues within the active site of 3CL^{pro}, the main protease of SARS-CoV-2. Hydrogen bonding and many other hydrophobic interactions are responsible for the binding affinity of the compound with the target protein. The result revealed that the studied compound has a comparable binding affinity for 3CL^{pro} to that of approved drugs for COVID-19 such as remdesivir and favipiravir. This suggests that the compound can be chosen for further studies as a potential therapeutic candidate for COVID-19.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

CRediT authorship contribution statement

K.M. Divya: Writing – original draft, Formal analysis, Visualization. **D.P. Savitha:** Formal analysis, Writing – review & editing. **G. Anjali Krishna:** Formal analysis, Visualization. **T.M. Dhanya:** Formal analysis. **P.V. Mohanan:** Project administration, Writing – review & editing.

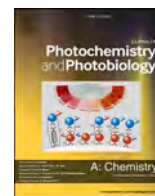
Acknowledgments

The author wishes to thank SAIF-STIC, CUSAT, Kerala for their help in single-crystal XRD analysis.

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A thiophene based pyrrolo [1, 2-a] quinoxaline fluorescent probe as a “turn-off” sensor for the selective nanomolar detection of sodium ion

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ARTICLE INFO

Keywords:

Thiophene based fluorescent sensor
Fluorescence turn-off
Nanomolar
Sodium ions

ABSTRACT

A novel thiophene based pyrrolo [1, 2-a] quinoxaline (TAAPP) fluorescent sensor, suitable for the nanomolar detection of sodium ions was successfully developed and characterized by various spectroscopic techniques. The fluorescent sensor showed excellent selectivity for sodium ions over other biologically and environmentally important metal ions/biomolecules by a remarkable quenching of fluorescence. Stern-Volmer studies reveal that static quenching predominates over dynamic quenching. The detection limit was found to be 2.219 nM within the linear range of 3 to 30 nM. DFT studies were performed to validate the results. The applicability of the probe can be extended to quantify sodium ions in real samples.

1. Introduction

Sodium is the primary cation in the extracellular fluids of animals and humans, which play a substantial role in many physiological processes acting to control blood volume, blood pressure, osmotic equilibrium and pH [1]. The concentration of sodium in the blood is around 130–150 mM [2], while that in intracellular fluid is only 5–30 mM [3]. Any irregularities in its concentration may enhance the risk of strokes, heart failure, diabetes, hyponatremia and kidney problems [4]. The salinity of water and soil is mainly due to the excessive concentration of sodium salts [5]. A high concentration of sodium in plant cells can disturb the water structure, inhibit enzymes and create nutritional imbalance [6]. So the development of a selective chemosensor for the quantification of sodium plays a vital role in biological and environmental monitoring.

Recognition of sodium ions can be achieved by using various techniques such as colourimetry, electrochemical methods, flame photometry, and inductively coupled plasma-atomic emission spectrometry [7–13]. In contrast to these techniques, fluorescence spectroscopy received greater attention in this field due to its simplicity of operation, high sensitivity, high selectivity and rapid response [14]. Various types of fluorescent sensors for sodium-based on small molecule chelators [15–17], nanoparticles [18,19], DNazymes [20] and DNA aptamer [21] have been developed.

Fluorescent thiophene based materials have been explored as sensing

materials for various metal ions [22–24], anions [25,26] and small molecules [27–29]. The ease of synthesis of pyrrolo[1,2-a] quinoxalines from thiophene 2- carboxaldehyde by a one-pot bicomponent cyclo condensation reaction and the fluorescent nature of the obtained product inspired us to explore this scaffold as a fluorescent probe. The present report describes an efficient and highly sensitive thiophene based probe, which offers an excellent fluorescent turn-off sensing scheme for the selective monitoring of sodium ions. The practical utility of the probe can be expanded to the estimation of sodium ions in biological and environmental samples.

2. Experimental details

2.1. Materials and methods

1-(2-amino phenyl) pyrrole and thiophene-2-carboxaldehyde were purchased from Sigma-Aldrich. Methanol, acetonitrile and DMF were purchased from Spectrochem and used without further purification.

Fluorescence and lifetime measurements were acquired on Horiba Fluorolog 3 (FL-1057). UV-visible spectra of the compounds were recorded in DMF on a Thermo Scientific™ Evolution 201 UV-Vis spectrophotometer. FT-IR spectra of the compounds were recorded as KBr pellets with a JASCO-8000 FT-IR spectrophotometer in the 400–4000 cm⁻¹ range. ¹H and ¹³C NMR spectra were measured on a Burkert Advance DRX 300 FT-NMR spectrometer with chemical shifts reported

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<https://doi.org/10.1016/j.jphotochem.2022.114046>

Received 26 February 2022; Received in revised form 17 May 2022; Accepted 25 May 2022

Available online 28 May 2022

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in ppm (in DMSO- d_6 ; TMS as the internal standard). Elemental analysis of the synthesized compound was done using an Elementar Vario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India. LC-MS spectrum was recorded on Waters 3100 Mass Detector utilizing ESI-MS procedure at Cochin University of Science and Technology, Kochi, India. Cyclic voltammetry studies were carried out using a CH analyser.

2.2. Synthesis of thiophene based pyrrolo [1, 2-a] quinoxaline (TAAPP)

Compound TAAPP was synthesized according to our previously reported synthetic procedure with slight modification [30]. Detailed synthetic procedure and scheme is outlined in [supporting information](#).

3. Results and discussion

3.1. Characterization and optical properties of TAAPP

The ligand was characterized by ^1H and ^{13}C NMR, LC-MS and IR spectra (see the ESI, [Fig. S1-S4](#)). The optical and photophysical properties of the ligand were also explored. The UV-Visible spectrum of TAAPP (1 mM) in DMF shows an absorption peak at 312 nm, which may be due to intramolecular charge transfer (ICT). The ligand showed a distinct fluorescence emission band at 470 nm upon excitation at 340 nm in DMF. The absorption and emission spectra of TAAPP in DMF are shown in [Fig. S5](#). Quantum yield (QY) is an important parameter which describes how proficiently a fluorophore transforms the excitation light into fluorescence. The quantum yield of the compound was determined to be 75% (see [Fig. S6](#)).

3.2. Fluorescent detection for Na^+

Using UV-visible and fluorescence spectroscopy, we studied the interaction of various metal chlorides (Na^+ , Li^+ , K^+ , Fe^{2+} , Fe^{3+} , Mn^{2+} , Co^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} and Al^{3+} , 60 nM) with the ligand to assess the effective interaction between the ligand and the metal ions. The study shows that the ligand selectively identifies Na^+ ions. Upon the addition of Na^+ ions, the UV-Visible spectrum of TAAPP changes considerably and absorption maxima shifted to 360 nm ([Fig. 1](#)). The shift in absorption band may be due to the formation of a ground-state complex by charge transfer from the ligand to the analyte.

The emission intensity of the ligand is not affected by the addition of various metal ions except Na^+ ions. The fluorescence emission intensity of TAAPP underwent significant quenching in presence of Na^+ ions ([Fig. 2a](#)). It may be due to the high binding affinity of the nitrogen atom (present in the quinoxaline ring) to Na^+ ions than the other metal ions

through a ground state donor- acceptor charge transfer process.

Fluorescence titrations were carried out at room temperature to understand the coordination behaviour of TAAPP. The progressive addition of Na^+ (0–60 nM, DMF) to the probe TAAPP (0.6 nM, DMF) caused a significant decrease in emission intensity at 470 nm, indicating the “turn-off” behaviour of the sensor ([Fig. 2b](#)). A significant linear relationship is attained over a range of 3–30 nM and the detection limit is found to be 2.219 nM ($R^2 = 0.9909$) from the detection limit calculation ([Fig. 2c](#)).

3.3. Selectivity and stability studies

The selectivity and sensitivity of the probe towards Na^+ ions in the presence of other metal ions (Li^+ , K^+ , Fe^{2+} , Fe^{3+} , Mn^{2+} , Co^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} and Al^{3+}) was confirmed through the competition experiment. Accordingly, solutions of various metal ions (60 nM, DMF) were added to TAAPP solution (0.6 nM, DMF) in the presence of 60 nM Na^+ ion solution under the same experimental conditions, and their fluorescence intensity was recorded at 470 nm. Fluorescent quenching is obtained for Na^+ without significant interference from the other metal ions ([Fig. 3](#)).

To extend the analytical application of the probe in the biological environment, the influence of the various co-existing biomolecule on the emission of the probe-analyte mixture has been investigated. The biomolecules such as aspartic acid, ascorbic acid, urea, glutamic acid, glucose, creatinine and ammonia (60 nM, DMF/ H_2O ; 9:1 v/v) did not interfere in the analysis. Thus, TAAPP act as an excellent fluorescent detection probe which shows distinct selectivity toward Na^+ ion in the competitive environment ([Fig. S7](#)).

After selectivity studies of the probe, the effect of counter ions was studied by using different salts of sodium such as sodium chloride, sodium fluoride, sodium acetate, sodium nitrite, sodium sulphite, sodium carbonate, and sodium hypochlorite. The emission intensity of the probe was quenched effectively with all these salts of sodium indicating that the counter ions did not affect the selectivity of the probe ([Fig. S8](#)).

The emission profile of the probe was recorded in the presence and absence of the analyte for 8 h to explore its stability. No substantial change was observed in the emission profile of the probe which proved stability ([Fig. S9](#)). The sensing performance of the probe to Na^+ under different pH was studied by varying the pH values from 2 to 10. Emission intensity is maximum at a pH of 7.0 and then decreases gradually in alkaline pH ([Fig. S10](#)). The result suggests that the probe could be effectively used in physiological pH for biological applications.

3.4. Nature of binding and stoichiometry

The binding stoichiometry of the probe with Na^+ was examined by Job's continuous variation method [31]. The binding isotherm ([Fig. 4](#)) indicates a maximum value of 0.5 which correspond to a 1:1 stoichiometry between TAAPP and Na^+ . The association constant of TAAPP with Na^+ was determined as $1.51 \times 10^3 \text{ M}^{-1}$ using the Benesi-Hildebrand equation [32] ([Fig. 5](#)).

3.5. Mechanism of fluorescent quenching

Fluorescent quenching can occur mainly either by static or dynamic processes. Dynamic quenching is caused by the diffusive encounters between the fluorophore and the quencher during the lifetime of the excited state [33]. Since the dynamic quenching affects only the excited state, the absorption spectrum of the fluorophore is not expected to change [33,34]. Static quenching takes place through the formation of the nonfluorescent ground-state complex between the fluorophore and the quencher and hence absorption spectrum of the fluorophore is expected to change in the presence of the quencher [33].

To understand the fluorescence quenching mechanism, emission quenching results were quantitatively subjected to the Stern-Volmer

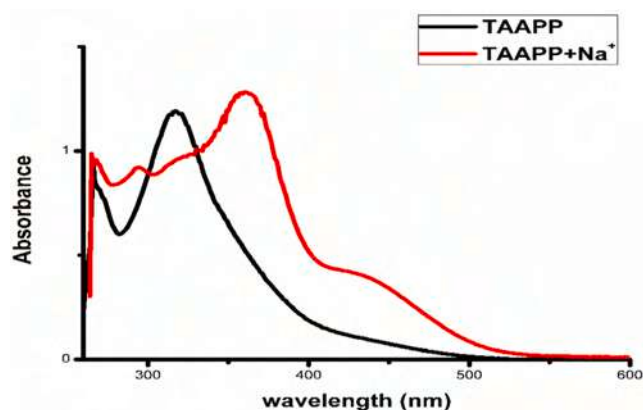


Fig. 1. UV-visible spectra of TAAPP (black line) and TAAPP + Na^+ (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

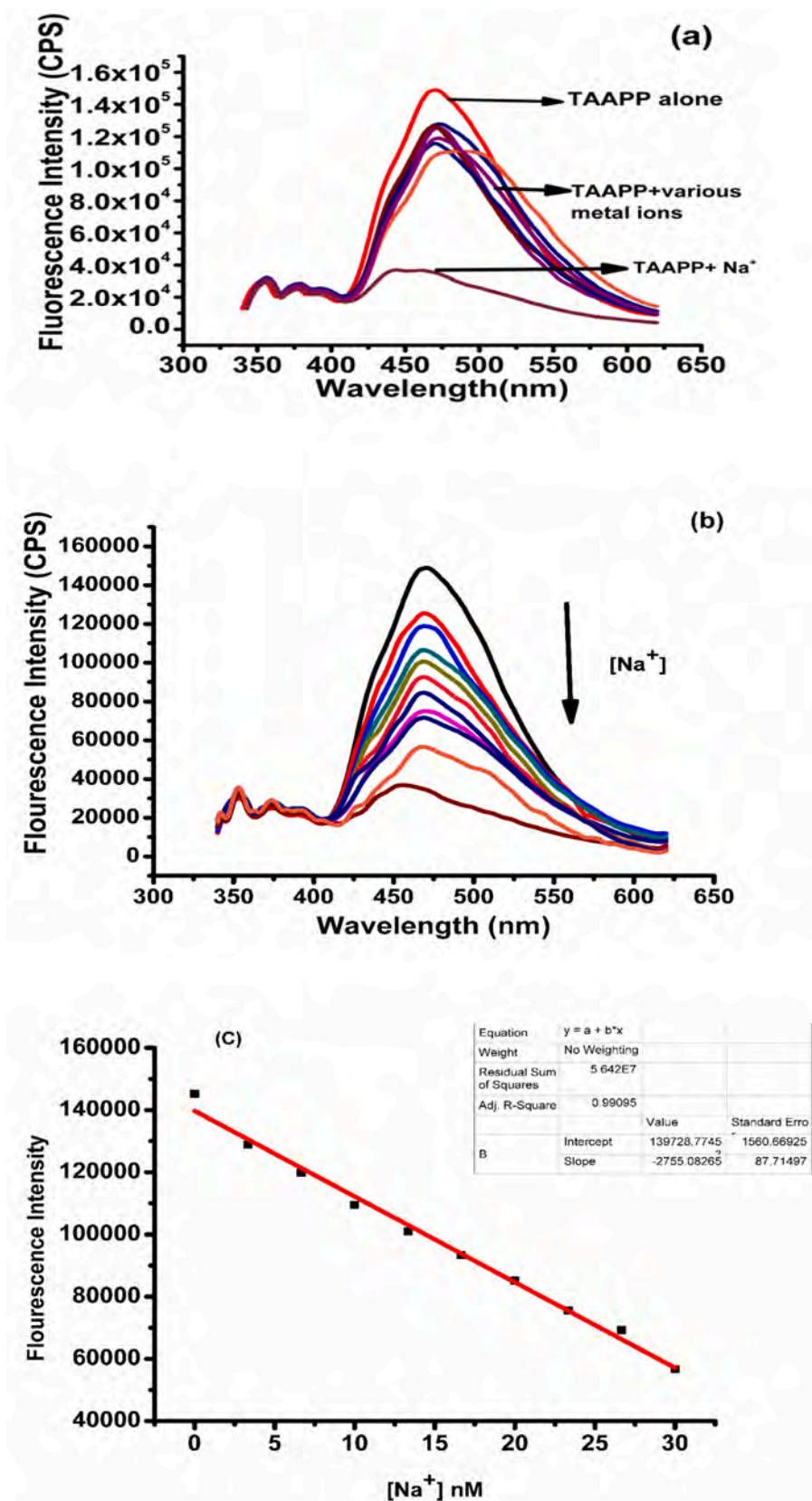


Fig. 2. (a) Fluorescence spectral change of TAAPP (0.6 nM) in the presence of various metal ions (Li⁺, K⁺, Fe²⁺, Fe³⁺, Mn²⁺, Co²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Al³⁺, 60 nM) in DMF (b) Fluorescence spectral change of TAAPP (0.6 nM) upon the addition of various concentrations of Na⁺ ion (0–30 nM) in DMF (c) The Linear relationship of fluorescence intensity at 470 nm and Na⁺ concentration (TAAPP: 0.6 nM; λ_{ex} : 340 nm).

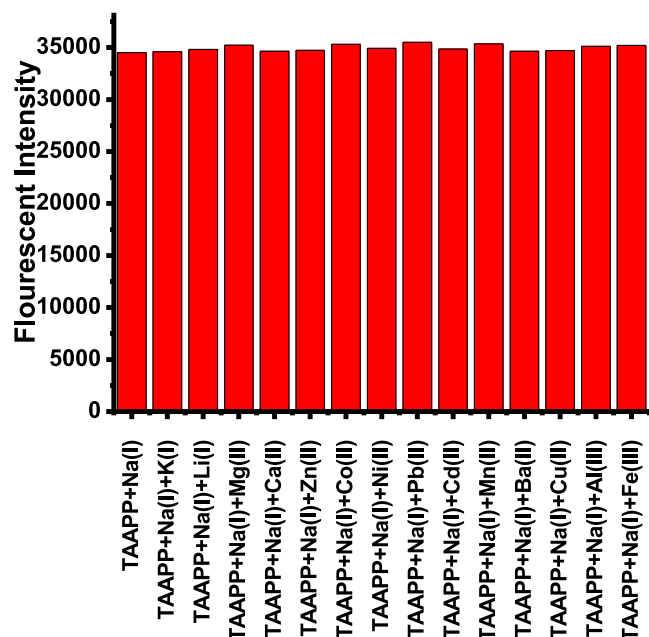


Fig. 3. Fluorescence response of TAAPP in the presence of Na^+ with various metal ions in DMF (TAAPP: 0.6 nM; λ_{ex} : 340 nm).

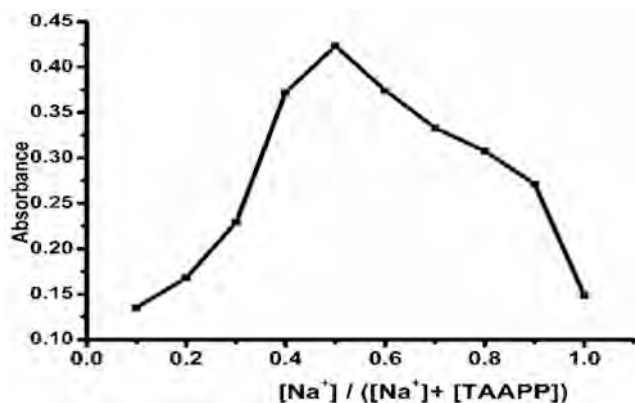


Fig. 4. Job's plot for the determination of binding stoichiometry of TAAPP with Na^+ obtained from absorbance data.

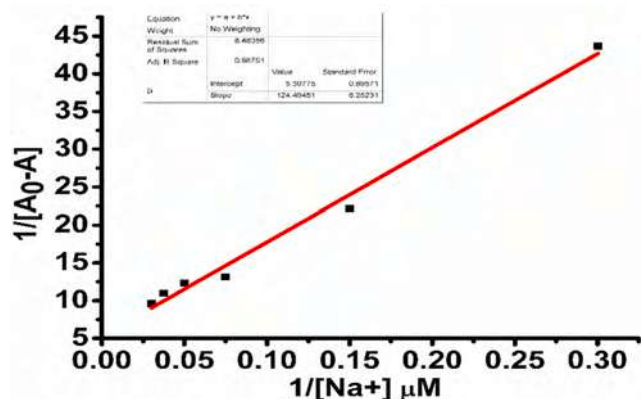


Fig. 5. B-H plot of TAAPP assuming 1:1 stoichiometry for the association between TAAPP and Na^+ .

equation [33].

$\frac{F_0}{F} = 1 + K_{\text{sv}}[Q]$, Where F and F_0 indicate the emission intensities with and without the addition of Na^+ ions, $[Q]$ is the concentration of Na^+ ions and K_{sv} is the Stern-Volmer quenching constant. The Stern-Volmer plot of emission quenching of TAAPP versus concentration of Na^+ ions is given in Fig. 6. The linear SV plot indicates a static quenching mechanism in which emission quenching of TAAPP is directly related to the quantity of Na^+ ions. From the slope of the SV plot, the Stern-Volmer quenching constant is found to be $3.407 \times 10^7 \text{ mol}^{-1} \text{ dm}^3$, which discloses a strong emission quenching of TAAPP with Na^+ ions.

Fluorescent lifetime analysis is the most conclusive method to understand the mechanism of quenching. For static quenching $\tau_0/\tau = 1$ and dynamic quenching $F_0/F = \tau_0/\tau$ [33]. To get a clear understanding of the fluorescent quenching mechanism, the fluorescent lifetime decay analysis of TAAPP before and after the addition of Na^+ ion was examined. The time-resolved fluorescence spectra of TAAPP in the presence of Na^+ (3–30 nM) solution (Fig. 7a) and Stern-Volmer plot of TAAPP in terms of lifetime measurements (Fig. 7b) are shown below.

The fluorescence lifetime of TAAPP alone is 1.52 ns. Soon after the addition of Na^+ ions (30 nM) to TAAPP, the average fluorescence lifetime of TAAPP with Na^+ ion is slightly reduced from 1.52 ns to 1.36 ns. The small change in the average lifetime value in the presence of Na^+ ions suggests a static quenching process rather than a collisional quenching process [35]. The quenching constant k_q is calculated to be $2.505 \times 10^{16} \text{ M}^{-1} \text{ s}^{-1}$ by substituting the value of τ in the equation, $k_q = \frac{K_{\text{sv}}}{\tau}$, where K_{sv} is the Stern-Volmer quenching constant [36]. The static process behind the quenching is confirmed, since the obtained quenching constant value is higher than the maximum scattering collision quenching constant ($2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [36,37].

3.6. Theoretical studies

To get an idea about the proposed binding mode and the optical response of the probe TAAPP upon the addition of sodium ion, DFT studies were performed at B3LYP/6-31G (d, p) level using Gaussian 09 programme. The mechanism for the fluorescence quenching can be explained by the ground state donor-acceptor charge transfer between TAAPP and Na^+ ion. Ground state charge transfer can occur when the HOMO of the probe is situated near the LUMO of the analyte [38]. Since the LUMO of Na^+ ion (3.5 eV) is situated near the HOMO of the probe (5.4 eV), charge transfer occurs between the probe and analyte which results in the formation of a non-fluorescent ground state complex.

Frontier molecular orbital analysis had done to determine the electronic behaviour of the probe in the presence of Na^+ ion. The optimized geometries and Frontier molecular orbital diagram of TAAPP and TAAPP- Na^+ complex are shown in Fig. 8 and Fig. 9. The HOMO in the optimized geometry of TAAPP displayed electron distribution over the

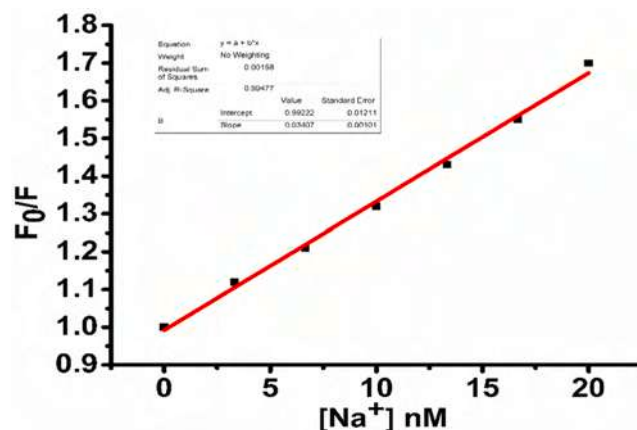


Fig. 6. The Stern-Volmer plot in terms of fluorescence measurements.

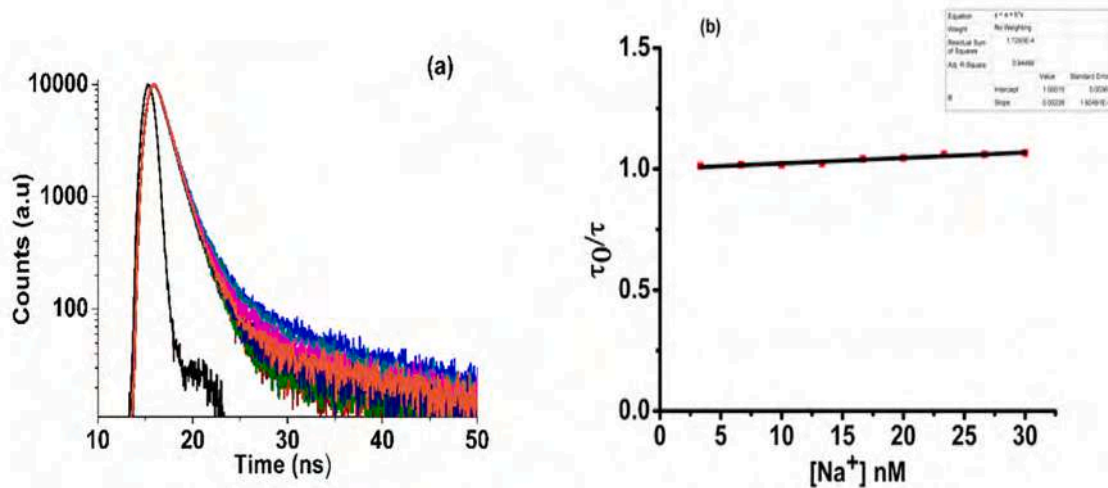


Fig. 7. (a) Time-resolved emission Spectra of TAAPP in the presence of Na^+ solution (3–30 nM) by exciting at 340 nm. (b) The Stern-Volmer plot in terms of lifetime measurement.

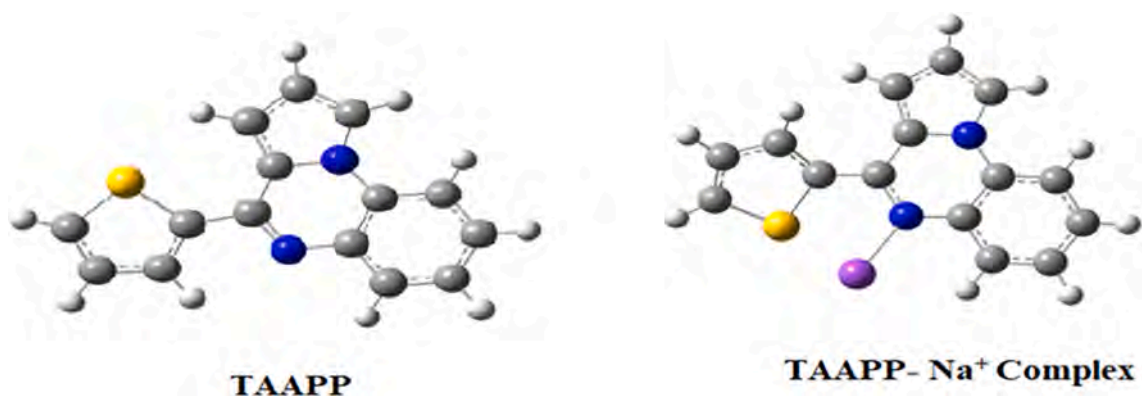


Fig. 8. Optimized geometries of TAAPP and TAAPP- Na^+ complex calculated at the DFT level using B3LYP/6-31G (d, p) basis set.

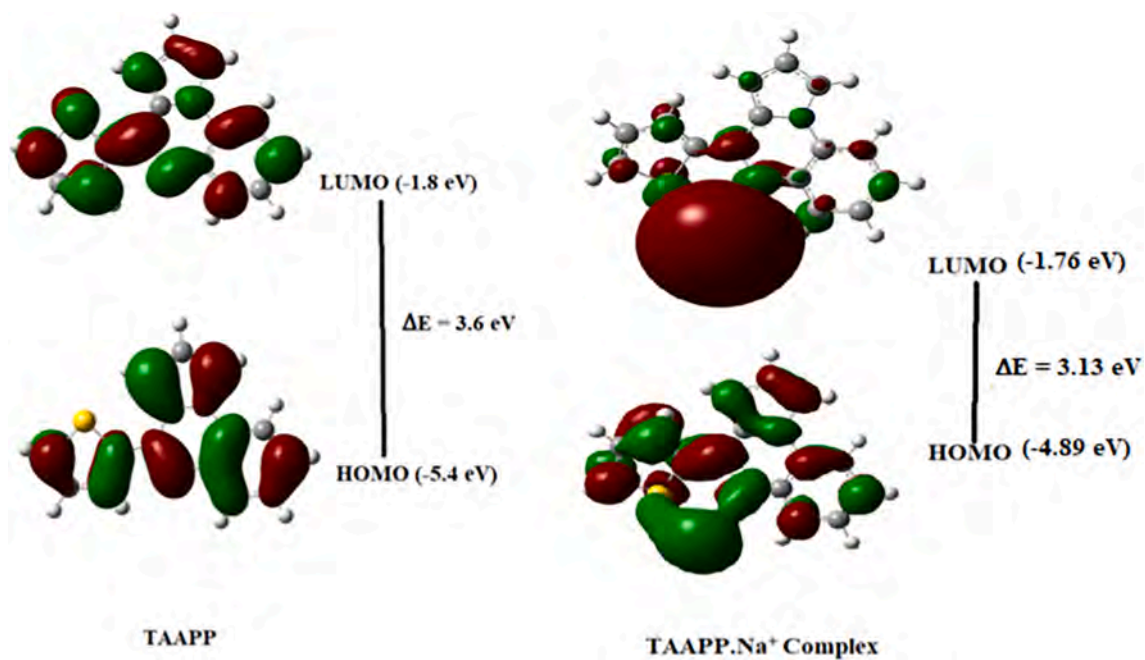


Fig. 9. Energy diagram of HOMO and LUMO orbitals of TAAPP and TAAPP. Na^+ complex calculated at the DFT level using a B3LYP/6-31G (d, p) basis set.

whole molecule. Upon the addition of Na^+ ion, the electron density of LUMO has dispersed mainly on the metal centre by considerable charge transfer. The HOMO-LUMO energy gap in the TAAPP- Na^+ complex is lower as compared to free TAAPP, which might play a significant role in the optical response of the probe upon complexation. The slight lowering of the HOMO-LUMO gap in the TAAPP- Na^+ complex validates the experimentally witnessed small redshift in the absorption spectrum.

Additionally, there is a considerable decrease in the total energy (−29526.70 eV of TAAPP to −33943.29 eV of TAAPP- Na^+ complex) of TAAPP upon complexation with Na^+ ion recommends that the complex formed is stable.

DFT analysis recommended that effective charge transfer occurs from the electron-rich nitrogen atom in the quinoxaline ring of the probe to the Na^+ centre which results in the formation of a non-fluorescent ground state complex. This accounts for the fluorescent quenching of TAAPP in the presence of Na^+ ions.

3.7. Cyclic voltammetry studies

To shed some light on the theoretical assessment of energy gaps, we have conducted the experimental determination of energy bandgap through cyclic voltammetry studies. The electrochemical behaviour of the probe in presence of Na^+ is shown in Fig. 10. In Fig. 10 (a), the TAAPP voltammogram shows the main oxidation peak at 0.95 V and the main reduction peak at −1.098 V. Upon complexation, the main oxidation peak is localized at 0.936 V and the main reduction peak at −0.881 V. The shift in the reduction potential may be due to the electron transfer process involved in complexation [39]. HOMO and LUMO energy levels of TAAPP in the presence and absence of Na^+ were obtained from the cyclic voltammogram. The empirical relation $E_{\text{HOMO}} = E_{1/2}^{\text{ox}} + 4.8 - E_{1/2, \text{Fc, Fc}^+}$ [eV] was used to calculate the HOMO energy levels of the probe, where $E_{1/2}^{\text{ox}}$ is the half-wave potential of the probe concerning Ag/AgCl (sat) reference electrode [40] and $E_{1/2, \text{Fc, Fc}^+}$ is the half-wave potential of Fc/Fc^+ redox couple which was estimated to be 0.38 V [41]. E_{HOMO} of the probe was calculated to be −4.094 eV. From the UV-visible absorption studies, the optical band gap can be calculated by following the relation E_g [eV] = $\frac{1240}{\lambda(\text{nm})}$ [40,42]. The optical band gap and LUMO energy of the probe were calculated to be 3.97 and −0.124 eV, respectively. Similarly, E_{HOMO} , E_{LUMO} and E_g of the probe in presence of Na^+ ion were calculated to be −3.883, −0.443 and 3.44 eV respectively. The energy bandgap of the probe decreases from 3.97 to 3.44 in the presence of Na^+ . The bandgap values obtained from cyclic voltammetry

measurements are in good agreement with the theoretical values. Experimentally and theoretically obtained HOMO, LUMO and bandgap values are listed in Table S1.

3.8. Applicability of the probe

The hands-on application of the proposed probe was assessed by estimating the sodium ions in tap water samples and synthetic urine by the spike recovery method [43]. The average recovery percentage obtained for the tap water sample was 97–99% with a relative standard deviation of 1.16–1.27%. Similarly, the studies were repeated with synthetic urine samples where the recovery percentage obtained was 102–104% with a relative standard deviation value of 2.26–2.57% as shown in Table S2. The accurate recovery percentage and reasonably lower RSD values point to the practicability of the developed probe for the determination of sodium in biological and environmental samples.

4. Conclusion

In brief, a simple and efficient fluorescent probe has been developed from thiophene 2-carboxaldehyde for the selective determination of sodium by a turn-off fluorescence mechanism. The structure and chemical composition of the probe were well characterized and its optical properties were studied by UV-visible absorption as well as fluorescence studies. The probe displays excellent sensing performance towards the detection of the sodium ion with a linear range of 3–30 nM and LOD of 2.219 nM. The probe encountered no interference from other competing metal ions and biomolecules and presented rapid sensing. Mechanism behind the sensing was found to be of static nature based on the observations from the UV-visible absorption spectra, fluorescence spectra and lifetime measurements. Cyclic voltammetric studies were carried out to understand the electrochemical behaviour of the probe in the presence of sodium ions. The binding mechanism of Na^+ with the probe TAAPP was further established by DFT studies. In practical application, the probe could be used to detect and quantify sodium in water and synthetic urine samples with a high degree of accuracy.

CRediT authorship contribution statement

K.M. Divya: . D.P. Savitha: Methodology. G. Anjali Krishna: . T.M. Dhanya: Visualization. P.V. Mohanan: .

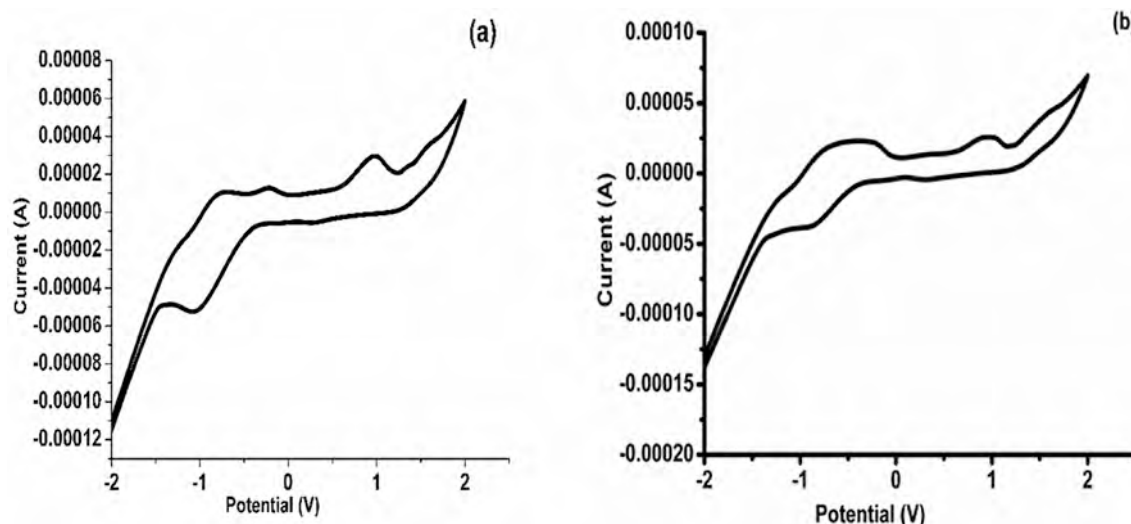


Fig. 10. (a) Cyclic voltammogram of the probe TAAPP (b) TAAPP in the presence of Na^+ .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are thankful to SAIF STIC, CUSAT for NMR and CHNS analysis data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphotochem.2022.114046>.

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Role of indirect coupling through external medium on cellular dynamics

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Received: 10 February 2022 / Accepted: 28 March 2022

Abstract: Cellular functions are regulated by intracellular networks of biochemical pathways. Every cell—single or part of a tissue or cell-collective—interacts with its environment and neighbours to sense and respond to signals for performing relevant functions. Such interactions can occur either *directly* through physical contacts/junctions between cells, and diffusion of signal molecules between the source and target cells; or, *indirectly* through the common medium in which the cells reside. To study the role of indirect interaction on cellular dynamics, model cells incorporating a simple activator–inhibitor pathway are coupled through diffusion of the pathway end product to the external medium in which the cells reside. In single isolated cells, the pathway can exhibit a wide variety of dynamics with changes in biochemical parameters. The dynamical effects of two kinds of indirect interactions— bi-directional, and threshold-dependent uni-directional diffusion— are studied in single and ensemble of cells. With intrinsic periodic dynamics of varying frequencies of the pathway end product in each cell, the results show emergence of several variations due to indirect interactions in both single and ensemble of cells. Persistence, appearance of sub-critical Hopf bifurcations, modulation of cell’s intrinsic dynamics, and threshold-dependent phase synchrony and frequency-locking are observed due to indirect coupling. This study with a model system elaborates the dynamical effects of indirect interactions through external medium, in single and coupled cell systems, describing this mode’s role in cell signalling. It also provides clues how threshold-dependent and bi-directional diffusion may be used by cellular systems as a means to modulate their functional dynamics for different types of “secrete and sense” cellular signalling in nature.

Keywords: Biochemical pathways; Cell–cell signalling; Indirect interactions; Coupled Cells

1. Introduction

Cellular functions in living organisms are performed by a network of biochemical reactions (“biochemical pathways”) involving regulatory feedback loops that incorporate higher order nonlinearities representing molecular interactions in the chemical reactions [1]. As has been shown in modelling studies with simple pathways, these nonlinearities can induce highly complex functional dynamics [2, 3]. Some of the common dynamical features exhibited by the cells are self-sustained oscillations [4], birhythmicity [5, 6], and chaos [7–9].

Even though a cell has its own self-regulatory network, it is not an isolated entity in a multicellular organism or in a cell collective [10]. Every cell interacts with other cells,

and also with its environment, to regulate its activity. Such cell–cell signalling is ubiquitous in nature. The cell–cell interactions may be either through direct physical contacts, such as the “gap junctions” in insulin secreting beta cells in islets in the pancreas and diffusion of molecules between the source and target cells in hormone signalling, or, indirectly through the common medium in which the cells reside as happens in “quorum-sensing”. Whether it is direct contacts, or diffusion of signalling molecules between cells, or interaction via a common medium in which the cells are present, in all the cases of information exchange the cells “sense” and communicate with their surrounding and modulate their activities for effective functionality. There are many biochemical pathways, known as “signalling pathways”, that are activated or modulated through the “signals” (ions, small molecules, proteins) and are “sensed” by receptors on the cell surface. Different types of signalling (between adjacent and distant

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cells) are known and their importance in cell–cell interactions and development are well-established [11].

The type of signalling that is relevant to this study on the effect of external signal concentration on cellular dynamics, is the *Autocrine signalling*, where a molecule (“signal”) released from the cell binds to the same cell through a cognate receptor, and *Paracrine signalling* where it affects the neighbouring cells present in the same external medium. In a more general sense, studies have shown that these “secrete-and-sense cells” can respond to a self-secreting molecule and to signals from neighbouring cells, thus encompassing a wider range of *autocrine cells*, generally found in animals, and neighbour-communicating *quorum sensing cells* that are mostly seen in bacteria. Several biological examples are given in literature [12, 13].

The indirect coupling of cells through an extra-cellular medium is the basic mechanism of the “quorum sensing” phenomena in bacterial populations, which provides for inter-cellular communications by fast diffusion of specific small molecules (auto-inducers) [14]. Here, the cells exist as an ensemble, often in an external medium in which various substrates are present, and the diffusion of the substrate between the cells and the medium regulates their intra- and extra-cellular concentrations, thereby indirectly responding to cell density. In many situations, the cells respond to the extra-cellular substrate only after a threshold concentration (“quorum”) is reached [15]. There have been many modelling studies on quorum-sensing (see [16] for a review).

We are specifically interested in analysing and comparing the dynamical aspects of the indirect interaction of a secreting substrate with the extra-cellular medium under different types of diffusion conditions, in single cell and in population of cells. We choose a simple model system that considers each cell to incorporate a three-step activator–inhibitor pathway, which exhibits a wide variety of complex dynamical features on changes in parameters [2]. Earlier studies on lattice of cells with direct diffusive coupling have shown that the collective dynamics of coupled cells can be different from that of the individual cell, depending on the number of cells and the coupling strength [17]. Under direct coupling (both local and long distance), the cells in the lattice also showed different types of synchronization [18].

Instead of direct cell to cell coupling, in this study we consider indirect coupling, where the end product of the pathway can diffuse to and from the surrounding medium. We consider two types of diffusion-mediated information transfer—(i) bi-directional diffusion between the cells and the medium, and (ii) uni-directional diffusion, where diffusion is restricted to one direction by a threshold condition on the concentration of the end product. The threshold condition makes the diffusion rate zero when the internal

concentration of the end product reaches a specific value, thereby stopping the end product to diffuse back to the cell from the external medium. The results of our investigations on single and ensemble of cells, for uni- and bi-directional diffusion, show different types of dynamics, such as suppression of oscillatory behaviour and reverse bifurcations from periodicity to stable states, that are significantly affected by the threshold condition. This type of indirect interaction through a common extra-cellular medium, in an ensemble of cells, leads the cells to phase synchrony and frequency-entrainment with the constituent cells oscillating with different amplitudes and dynamics. The unidirectional diffusion can also induce period doubling bifurcations in some cells in an ensemble. The dynamical variations observed in this simple model system elaborates the effects of indirect interactions in single and multi-cell systems. It also provides clues how uni- and bi-directional threshold-dependent diffusions may be used by cellular systems as a means to modulate their functional dynamics—both naturally and in engineered systems. The results are discussed in the context of different types of cell–cell signalling in nature.

2. Models

First, the model single cell incorporating the simple activator–inhibitor pathway, used in this study, is described. The type of indirect interaction that the isolated cell experiences, when interacting through the external concentration of the end product of the pathway, is described next. Finally, the model for the ensemble of cells, interacting through the external concentration of the end product of the pathway, is given. The threshold-dependent signalling phenomenon (similar to “quorum sensing”) is modelled by introducing a threshold condition for uni-directional diffusion.

2.1. An isolated cell

The highly networked biochemical pathways in cells regulate cellular functions through combinations of single, multiple, and coupled negative and positive feedback processes inherent in genetic and metabolic reactions [19]. The most common form of regulation that conserves energy in cellular economy and desensitizes the pathways to intra-cellular and environmental perturbations, are the negative feedback processes—particularly the inhibition by the end product in a multi-step pathway. They are naturally selected to have a stabilizing role and help in achieving homeostasis. The positive feedback processes, on the other hand, are potentially destabilizing and used in many biochemical processes for initiating excitable dynamics for the

amplification needed in switching and rapid-response processes. We use a model single cell that incorporates a three-step biochemical reaction sequence, where the first biochemical/substrate yields a reaction product, which acts as the second substrate to yield the end product. Each reaction is catalyzed by enzymes that are regulated by these substrates. In the activator–inhibitor pathway, the production of the first substrate is inhibited by the end product, and the end product catalyzes its own production (autocatalytic). Thus, the concentrations of the three substrates inside an isolated cell, that does not interact with its external environment through diffusion of any of these chemicals, are regulated solely by two feedback loops—a negative feedback through inhibition of the first substrate by the end product, and, a positive feedback through an allosteric enzyme-mediated autocatalytic activation of the second substrate by the end product—a motif commonly observed in real biochemical pathways (for details of the model, see [20]).

The dynamics of the model pathway can be described by the following equations

$$\begin{aligned}\frac{dx}{dt} &= F(z) - kx \\ \frac{dy}{dt} &= x - G(y, z) \\ \frac{dz}{dt} &= G(y, z) - qz\end{aligned}\quad (1)$$

where x, y, z are the normalized concentrations of the pathway substrates inside the cell, and k and q are the rates of degradation of the first substrate and the end product, respectively, which follow first-order kinetics and are non-saturated. The nonlinear functions $F(z)$ and $G(y, z)$ represent the negative and positive feedback processes, respectively. For $F(z)$ and $G(y, z)$, we consider molecular interactions that are commonly employed in the biochemical pathways in cells. The negative feedback term $F(z)$ is a function of the end product z alone regulating the production of x , and the reaction requires cooperative binding of n molecules of z for inhibiting the conversion reaction of x to y . The production of z from y is regulated by the positive feedback term $G(y, z)$, involving an auto-catalytic allosteric enzyme, which obeys the concerted transition model and follows the quasi-steady-state hypothesis for the enzymatic forms [20]. These nonlinear kinetic processes are common in mechanisms of biochemical regulation, such as gene expression and metabolic reactions, like the cell cycle, glycolysis, hormonal signalling, cAMP oscillations in cellular slime moulds, etc.

The two feedback processes in this activator–inhibitor pathway are modelled by,

$$F(z) = \frac{1}{1 + z^4} \quad (2)$$

$$G(y, z) = \frac{Ty(1 + y)(1 + z)^2}{L + (1 + y)^2(1 + z)^2} \quad (3)$$

where, $L = 10^6$ and $T = 10$ are the allosteric constant and maximum velocity of the enzyme. This system possesses only one real positive steady-state for all parameters studied. The dynamics exhibited by this pathway variables, when perturbed near the steady state, is a function of the parameters.

The function $G(y, z)$ can be approximated using the following function [21]

$$G'(y, z) = \frac{Ty(1 + y)(1 + z)^2}{L} \quad (4)$$

2.2. Single cell with diffusion of end product of the pathway

The cell exists in a medium to which the end product of the pathway z can diffuse through the cell membrane. Here, we model the effect of diffusion of z to the external medium in two situations: i) diffusion can be in either direction (bi-directional), and ii) it is restricted by a threshold condition that makes it unidirectional, i.e. the rate of diffusion is zero when the concentration of the end product inside the cell goes below a threshold concentration S . Here, also the variables x, y and z are the normalized intra-cellular concentrations of the substrates in the activator–inhibitor pathway. However, the intra-cellular concentration of z in this model cell is modulated by diffusion. The model equations of the cell are given by,

$$\begin{aligned}\frac{dx}{dt} &= \frac{1}{1 + z^4} - kx, \\ \frac{dy}{dt} &= x - \frac{Ty(1 + y)(1 + z)^2}{L}, \\ \frac{dz}{dt} &= \frac{Ty(1 + y)(1 + z)^2}{L} - qz - \eta(z - S),\end{aligned}\quad (5)$$

where, η is the diffusion constant, and S is the concentration of the end product z in the external medium. The unidirectional diffusion is represented by the threshold condition

$$\eta = 0 \quad , \quad \text{if } S > z \quad (6)$$

2.3. An ensemble of cells coupled by diffusion of end product

Here, we consider a population of N cells in a medium and the variables x, y and z represent the normalized intra-cellular concentrations of the substrates of the activator–inhibitor pathway in each of the cells. However, the intra-

cellular concentration of z in every cell in this model of an ensemble of cells is modulated by its diffusion to the surrounding, whereas the external concentration of the end product (z_e) varies as a result of diffusion from all the cells. Hence, the cells are indirectly coupled by the interaction with a common medium through diffusion. The dynamics of the i th cell, where $i = 1, 2, \dots, N$, is modelled by the following equations

$$\begin{aligned} \frac{dx_i}{dt} &= \frac{1}{1+z_i^4} - k_i x_i, \\ \frac{dy_i}{dt} &= x - \frac{T y_i (1+y_i)(1+z_i)^2}{L}, \\ \frac{dz_i}{dt} &= \frac{T y_i (1+y_i)(1+z_i)^2}{L} - q z_i - \eta(z_i - z_e), \end{aligned} \quad (7)$$

where z_e is the concentration of the end product in the extra-cellular medium, and its time evolution is given by the equation:

$$\frac{dz_e}{dt} = -m z_e + \eta_{ext} \sum_{i=1}^N (z_i - z_e) \quad (8)$$

where, η and η_{ext} are the diffusion constants inside and outside the cell, respectively, and m is the degradation rate of z in the medium. The constant η_{ext} depends on different factors, such as the surface area of cells and the total volume of the medium. Eq. 8 can be rewritten as

$$\frac{dz_e}{dt} = -m z_e + \epsilon(\bar{z} - z_e) \quad (9)$$

where, $\epsilon = N\eta_{ext}$

3. Results and discussion

In the following, we first recapitulate the variety of dynamics exhibited by the activator-inhibitor pathway in a single isolated cell (given by Eq. 1), with variation in parameters, in a bifurcation diagram [20]. We then state few analytical and numerical results for the steady state and local stability analysis of the single cell in a medium with which it interacts through diffusion of end product of the pathway. Finally, we numerically describe the dynamics of a single cell and ensemble of cells with diffusion of the end product with and without thresholds.

3.1. An isolated cell

This pathway (Eq. 1) has been shown to exhibit a variety of dynamics—stable, periodic, bi-rhythmic with fractal basin, and chaotic oscillations—depending on the values of parameters [20]. Figure 1 shows the bifurcation diagram of the pathway dynamics when the parameter k is varied for a

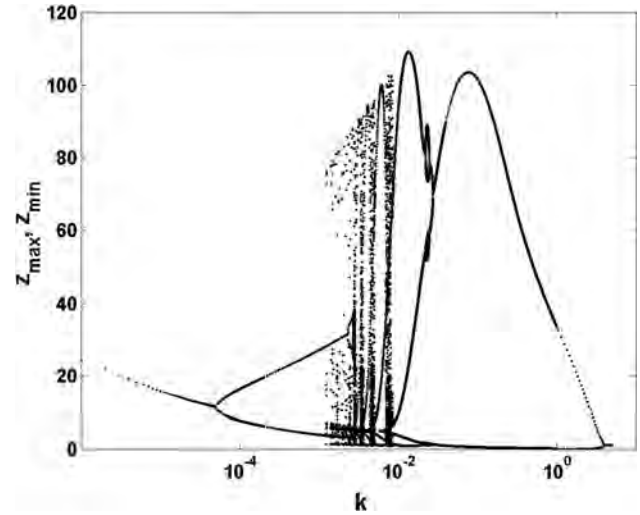


Fig. 1 Bifurcation diagram of the pathway in an isolated cell. Maxima and minima of the end product z is plotted against the parameter k . $q = 0.1$.

constant q . For smaller values of k , the system shows stable steady-state behaviour. For $k = 5 \times 10^{-5}$, it undergoes a Hopf bifurcation leading to a stable limit cycle dynamics. As k is increased, period doubling bifurcations take place, and the pathway shows various kinds of complex dynamics, such as birhythmicity and chaos. For higher values of k , the pathway follows reverse period doubling, and attains stability at $k = 3.9$.

3.2. Single cell with diffusion of end product to the external medium

To show the effect of diffusion of the end product of the pathway, z , to the external medium, we first study the positive steady state of Eq. 5, and its local stability.

3.2.1. Steady-state analysis

The condition of steady states is achieved by equating the RHS of Eq. 5 to zero.

$$\frac{dx}{dt} = 0, \quad (10)$$

$$\frac{dy}{dt} = 0, \quad (11)$$

$$\frac{dz}{dt} = 0 \quad (12)$$

Applying the condition given by Eq. 12 in Eq. 5, we get the equations of the steady states for x and z ,

$$x_0 = (q + \eta)z_0 - \eta S, \quad (13)$$

$$z_0^5 - \frac{\eta S}{q + \eta} z_0^4 + z_0 - \left(\frac{\eta S}{q + \eta} + \frac{1}{(q + \eta)k} \right) = 0 \quad (14)$$

From Descartes's Rule of Signs, it is evident that z_0 will have at most 3 positive real roots and no negative roots. Equating the second equation in Eq. 5 to 0 and substituting Eq. 13, we obtain the steady-state equation for y ,

$$y_0^2 + y_0 - \frac{L}{T(1 + z)^2} ((q + \eta)z_0 - \eta S) = 0, \quad (15)$$

which gives the steady-state solution of y as

$$y_0 = \frac{1}{2} \left(-1 \pm \sqrt{1 + \frac{4qL}{T} \frac{z_0}{(1 + z_0)^2}} \right) \quad (16)$$

We have numerically found the roots of Eqs. 13, 14 and 15 for a range of parameters (k and q). Only positive solutions are considered, since x , y and z are concentrations of biochemical substrates.

3.2.2. Local stability analysis

The local stability of steady states can be examined by linearizing Eq. 5 around the steady states. Expanding Eq. 5 in Taylor's series and applying the first-order approximation, the equations governing the evolution of any small perturbation (δx , δy , δz) around the steady state (x^* , y^* , z^*) becomes

$$\begin{pmatrix} \delta \dot{x} \\ \delta \dot{y} \\ \delta \dot{z} \end{pmatrix} = \begin{pmatrix} -k & 0 & -A \\ 1 & -B & -C \\ 0 & B & C - Q \end{pmatrix} \begin{pmatrix} \delta x \\ \delta y \\ \delta z \end{pmatrix} \quad (17)$$

where,

$$\begin{aligned} A &= \frac{\partial F}{\partial z} = \frac{4z^3}{(1 + z^4)^2}, \\ B &= \frac{\partial G}{\partial y} = \frac{T(1 + 2y)(1 + z)^2}{L}, \\ C &= \frac{\partial G}{\partial z} = \frac{2T(1 + y)(1 + z)}{L} \end{aligned} \quad (18)$$

and $Q = q + \eta$, the re-scaled rate of degradation of the end product z . Hence, the Jacobian of the system at the steady state (x^* , y^* , z^*) is given by

$$\mathbf{J} = \begin{pmatrix} -k & 0 & -A \\ 1 & -B & -C \\ 0 & B & C - Q \end{pmatrix} \quad (19)$$

The characteristic equation of the system can be obtained from

$$\begin{vmatrix} -k - \lambda & 0 & -A \\ 1 & -B - \lambda & -C \\ 0 & B & C - Q - \lambda \end{vmatrix} = 0, \quad (20)$$

where λ is the eigenvalue of $\mathbf{J}$. The characteristic equation of the linearized system is given by

$$\begin{aligned} \lambda^3 + (B - C + Q + k)\lambda^2 + [(B - C)k + BQ + Qk]\lambda \\ + (Qk + A)B = 0, \end{aligned} \quad (21)$$

The stability of the steady states is determined from the nature of the eigenvalues. The Routh–Hurwitz criteria for stability for the polynomial equation,

$$\lambda^3 + a_1\lambda^2 + a_2\lambda + a_3 = 0, \quad (22)$$

are given by the following conditions. The eigenvalue λ is stable iff,

$$a_1 > 0, \quad a_3 > 0, \text{ and } a_1a_2 - a_3 > 0$$

Here,

$$\begin{aligned} a_1 &= B - C + Q + k, \\ a_2 &= (B - C)k + BQ + Qk, \\ a_3 &= (Qk + A)B \end{aligned} \quad (23)$$

It can be shown that the functions A , B , C are always positive, and hence $a_3 > 0$. However, testing the other two conditions analytically are difficult. Therefore, we examined these conditions numerically in the (S, η) parameter space, and show the stable (dotted) and unstable (white) regimes in Fig. 2. The rectangular white space in the plot at high S for all η values represents parameters where no positive steady state exists.

Since η is the diffusion constant of the end product z , and S is the concentration of z in the extracellular medium, it is clear that the pathway shows unstable dynamics at—(i)

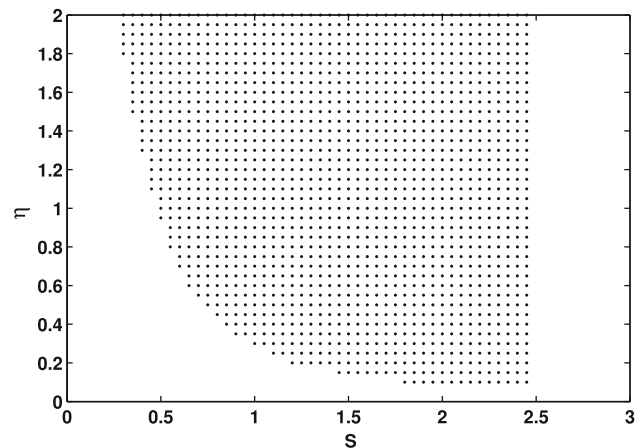


Fig. 2 Stability plot of the model (Eq. 5) in $S - \eta$ space. The dotted portion represents the stable regime in the parameter space

a large range of diffusion rates when the S is low, and (ii) for a wide range of S for low diffusion rates. In general, for the majority of medium η and S values, the pathway is stable. One may note that the local stability analysis shows that at high values of S , the system does not possess a positive steady state for any η , indicating extinction of pathway activity.

3.3. Single cell dynamics for different types of diffusion of end product

Here, we show the bifurcation diagrams and stability plots for z in cells residing in an external medium for bi-directional and uni-directional diffusion.

3.3.1. Bi-directional diffusion

Diffusion is bi-directional when the end product z can diffuse in and out of the cell. The bifurcation diagrams for increasing external concentration of the end product (S) for $\eta = 0.1$, and increasing diffusion constant η for $S = 1$ are shown in Figs. 3 and 4, respectively.

The thick solid lines show the maxima and minima of z , the thin black and grey lines show the stable and unstable fixed points. The parameters k and q are chosen as 0.1 at which the pathway shows stable periodic behaviour in absence of diffusion (see Fig. 1). In Fig. 3, the folded nature of the curves indicates that the system goes through a subcritical Hopf bifurcation with increasing S . The unstable fixed point becomes stable at $S = 1.78$. However, the stable limit cycle continues to exist till $S = 1.98$. The dynamics of the system is critically determined by the initial conditions in this range of S from 1.78 to 1.98, where

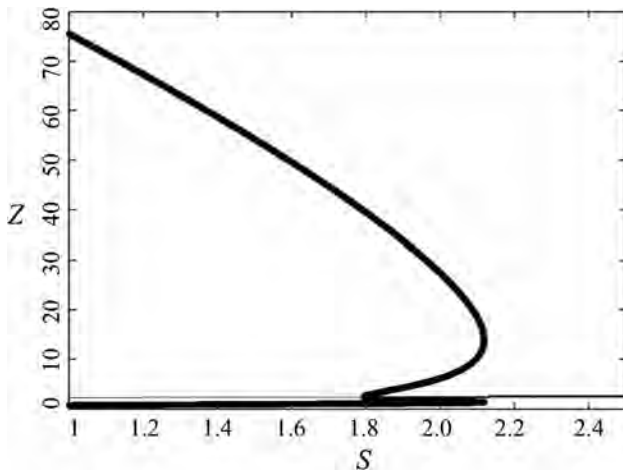


Fig. 3 Bifurcation diagram for bi-directional diffusion with increasing S . The maxima of z (z_{max}), the steady state (z^*), and the unstable limit cycle (z_c) are plotted in the y-axis against the external concentration of the end product S in x-axis. $\eta = 0.1$

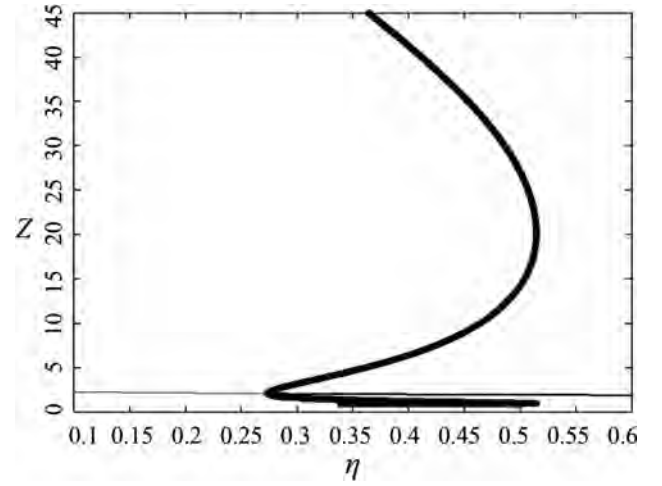


Fig. 4 Bifurcation diagram for bi-directional diffusion with increasing η . The y-axis is similar to Fig. 3 and x-axis shows the coefficient of diffusion η . $S = 1$

two stable solutions coexist - a limit cycle and the fixed point dynamics. The basins of attraction of the two attractors are separated by an unstable limit cycle that are represented by the inner lines of the folded curves.

Figure 4 shows the bifurcation diagram for increasing η when $S = 1$. The lines in the plot are the same as discussed in Fig. 3. A similar subcritical Hopf bifurcation is shown here between $\eta = 0.32 - 0.34$, where there is coexistence of two attractors—the limit cycle exists till $\eta = 0.34$, and the fixed point attractor exists from $\eta = 0.32$.

These results show the dependence of pathway dynamics on initial conditions, thereby indicating dynamic heterogeneity—oscillating and steady-state dynamics—in “self-sensing” but non-interacting cells residing in a common medium. This is a new property not observed in the pathway in an isolated cell, and arising solely due to the bi-directional diffusion of the end product.

3.3.2. Diffusion with a threshold

Here, we consider a cell, which allows diffusion of the end product as long as the internal concentration of z exceeds that of the external concentration S by gating. Such uni-directional diffusion is modelled with the threshold condition as in Eq. 5.

$$\eta = 0 \quad , \quad \text{if } S > z \quad (24)$$

Simulation results show that uni-directional diffusion through incorporation of a threshold S , affects the dynamics of the cell at different parameter regimes. Figure 5 shows the stable and unstable regimes in the parameter space (S, η). On comparing with Fig. 2, one can see that the results are similar when the steady-state value of the end product is less than the external concentration

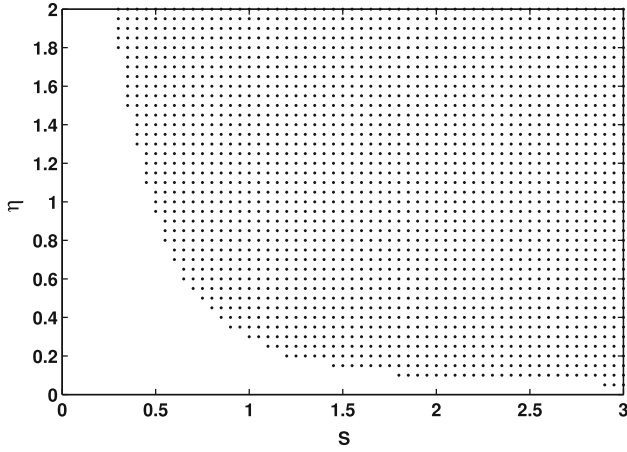


Fig. 5 Stability plot in $S - \eta$ parameter space for diffusion with a threshold. The dotted portion and the white part represent the stable and unstable regimes of the parameter space

($z^* < S$), which is the original system without diffusion ($\eta = 0$). However, for diffusion with threshold, the system shows persistence and steady-state dynamics at higher S values.

The details of the dynamics of the pathway in this cell are shown in bifurcation diagrams, Figs. 6 and 7. In contrast to the earlier case of bi-directional diffusion (Fig. 3), Fig. 6 shows two subcritical Hopf bifurcations when the threshold external concentration S is varied (for $\eta = 0.1$).— one in the forward direction, and the other in the reverse direction with increasing S . A major difference between bi- and uni-directional diffusion is that the larger limit cycle remains stable for the complete range of parameter S , and the fixed point becomes locally stable only between $S = 1.78$ and $S = 2.45$, during which there is coexistence of limit cycle and fixed point dynamics. The dotted curve gives the amplitude of the

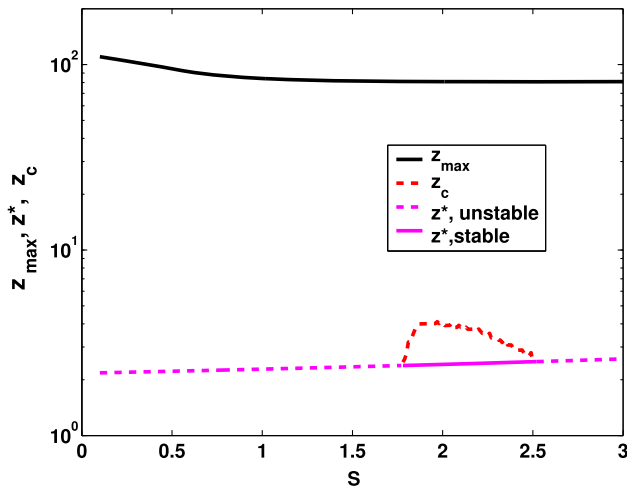


Fig. 6 Bifurcation diagram for increasing threshold concentration of the substrate, S . $\eta = 0.1$

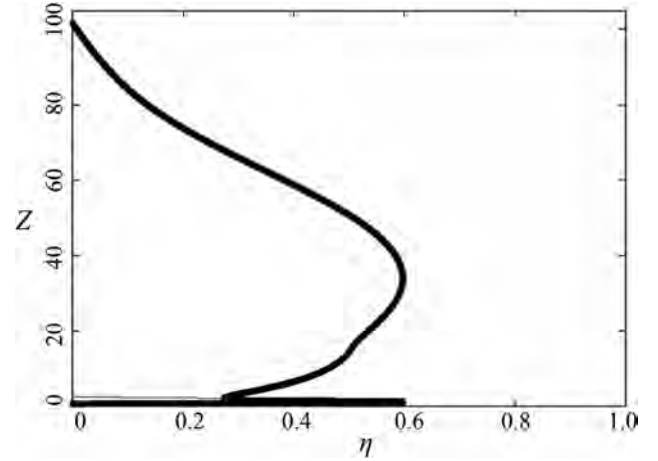


Fig. 7 Bifurcation diagram for increasing diffusion rate η . $S = 1$

unstable limit cycle that leads to fixed point dynamics, separating the basins of attractions of the stable and unstable oscillations. Thus, even though the position of the first Hopf bifurcation remains unaffected in parameter space, yet the cell can exhibit oscillatory dynamics along with stable steady-state dynamics at some parameter range.

Figure 7 shows the bifurcation behaviour for varying η keeping $S = 1$. It is clear that co-existence of oscillatory and fixed point dynamics occurs for different combinations of diffusion rate η and threshold S . In addition, comparing the two types of diffusion, the bifurcation diagrams show that the one-way nature of the diffusion can lead the cells to exhibit oscillatory behaviour at larger values of S and shows stable steady state behaviour for higher diffusion rates (η) when compared to bi-directional diffusion.

3.4. Ensemble of cells in a common medium indirectly coupled by diffusion

Here, we study the dynamical response of cells that exist in a common medium and interact indirectly through regulating diffusion of their pathway end product z depending on the concentration of it in the external medium (refer to Eq. 7, Eq. 8, and Eq. 9). In absence of diffusion (i.e. $\eta = 0$), the cells oscillate with different frequencies depending on the parameters (see Fig. 1). First, we show the results of an ensemble of cells ($N = 1000$) having all parameters the same, except k , which is *uniformly* randomly distributed in the range $k = 0.1$ to $k = 1.1$. We then state the results for the case when k is *normally* distributed in the same range of k values.

Uniformly distributed k :

In an ensemble of cells where k is uniformly randomly distributed, the frequencies of the oscillating cells are also distributed randomly [22]. In absence of diffusion, with

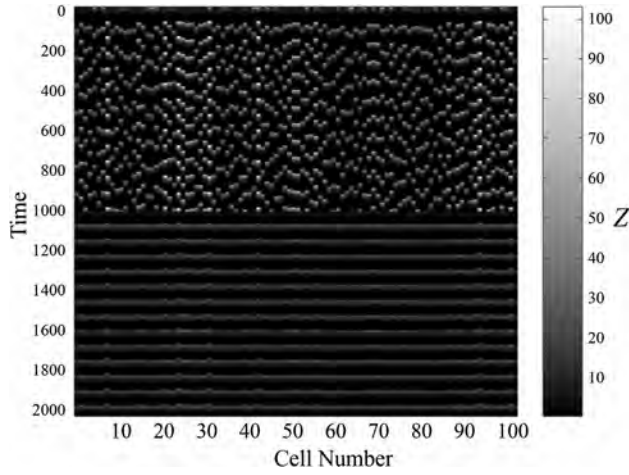


Fig. 8 Space-Time plot with uniformly distributed random k values (100 cells are shown): Diffusion is switched on after 1000 time units from zero to $\eta = 0.6$ and $\epsilon = 1$

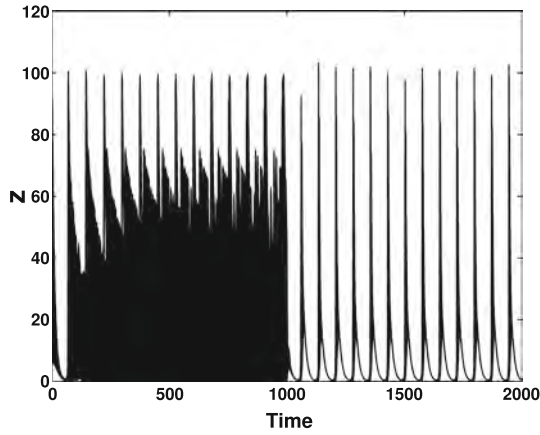


Fig. 9 Superposition of time series of z in 1000 cells with uniformly distributed random k values before and after diffusion is switched on from zero to $\eta = 0.6$ and $\epsilon = 1$

random initial values of the substrates, the phases of oscillations in the cells also vary randomly. Figures 8 and 9 show the space-time and time series plots, respectively, of such an ensemble of cells ($N = 1000$), which depicts the dynamics before and after diffusion is allowed. After an initial interval of 1000 time units, the diffusion constant is changed from $\eta = 0$ to $\eta = 0.6$ and $\epsilon = 1$. Both plots show that the cells, after they interact through diffusion to the external medium, are frequency locked, and the phases are confined to a narrow range. Interestingly, the frequency locked cells show very different amplitudes, and the shape and type of oscillations also are different from their intrinsic oscillations. Thus, even though the indirect interaction through diffusion to the common medium synchronizes the cells to oscillate with the same frequency, the temporal dynamics of each cell is different. This is shown

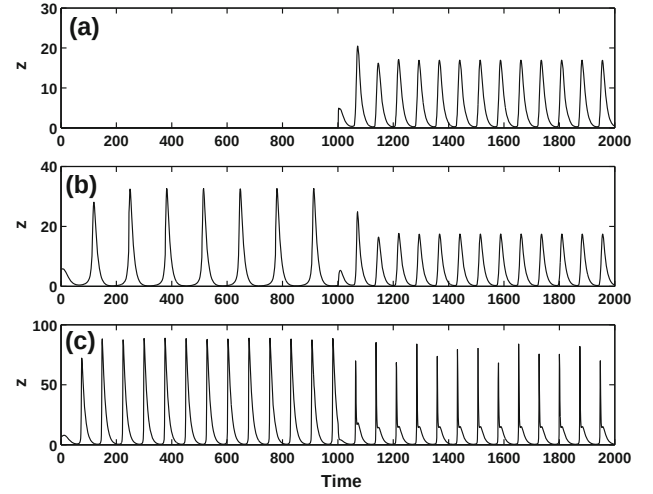


Fig. 10 Variety of oscillations generated in the frequency locked cells with uniformly distributed random k values. Time series of z in—(a) External medium, (b) and (c) in two cells. Diffusion switched on at $t = 1000$

in Fig. 10 with the time series of z in the extra-cellular medium and in two other cells in the ensemble.

Figure 10a shows that the concentration of z in the external medium, which is zero till $t = 1000$, starts oscillating periodically when diffusion starts. Figures 10b and 10c show the temporal dynamics of intracellular z in two cells in the ensemble, as examples of changes induced due to diffusion. The intrinsic periodic dynamics in the cell, as shown in Fig. 10a, is damped in amplitude, and its frequency of oscillation is faster. It is also frequency locked with the oscillations in z in the external medium and other cells. The time series of another cell in the ensemble, as shown in Fig. 10c, clearly shows frequency-locking, period doubling and quasi-periodicity after the onset of diffusion. Thus, in an ensemble, the indirect interaction of cells by diffusion through the common medium leads to collective frequency synchronization along with various types of changes in the oscillatory dynamics in individual cells.

Figure 11 gives a summary of the numerical experiments done to obtain these results in a typical ensemble of cells. The distribution of the parameter value (k_i) of cells is given in Fig. 11a. They are uniformly distributed in the range $k = 0.1$ to $k = 1.1$, where the cells show periodic oscillations (see Fig. 1) in absence of diffusion. The relation between k and frequency of oscillations in z in this range is given in Fig. 11b. It shows that the cells in the ensemble, when uncoupled (i.e. in absence of diffusion), oscillate with different frequencies (see the histogram given in Fig. 11c). When the cells are indirectly coupled through diffusion of z to the external medium, the frequencies of all cells lock into a narrow range, as is shown in the histogram in Fig. 11d.

Normally distributed k :

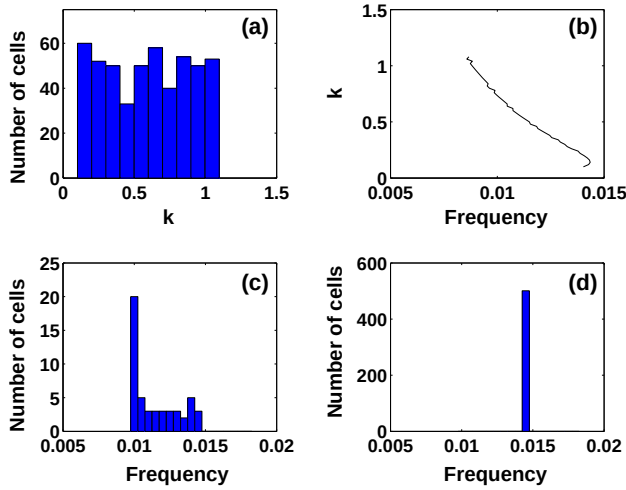


Fig. 11 Distribution of parameters and frequencies of the coupled and uncoupled cell system: (a) Histogram of k (b) the relation between k and frequency, (c) and (d) the frequency histograms of uncoupled and coupled ($\eta = 0.6$, $\epsilon = 1$) systems, respectively

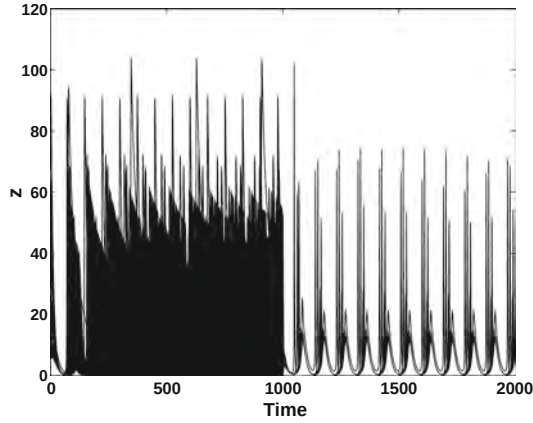


Fig. 12 Superposition of time series of z of 1000 cells with normally distributed random k values $\epsilon = \eta = 0$ for $t < 1000$ and $\epsilon = 0.6$ and $\eta = 1$ for $t \geq 1000$

Similar studies were done with cells having normally distributed random k values. Fig. 12 shows the overlapping time series of an ensemble of 1000 cells with randomly normally distributed k values, simulated with random initial conditions, where diffusion is allowed for $t \geq 1000$. Clearly, the cells show high degree of frequency synchronization when they interact through diffusion of z to the external medium. The intrinsic dynamics of the cells also change considerably in both amplitude and frequency when interacting indirectly, as is shown in Fig. 13– temporal dynamics of z in the external medium (Fig. 13a), and Fig. 13b and c for two cells in the ensemble showing various kinds of dynamics. The numerical experiment is summarized in Fig. 14. Figure 14a and b shows the k value distributions and frequency dependence in the non-interacting cells. Figure 14c and d shows the distribution of

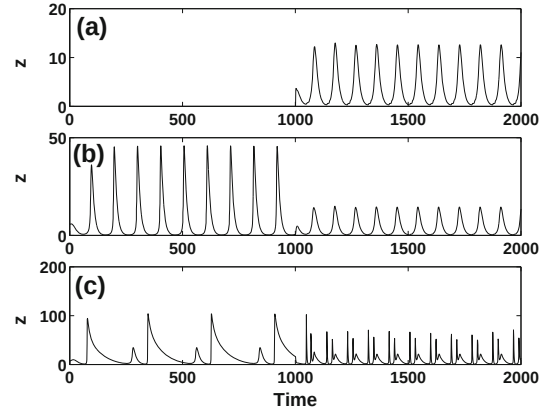


Fig. 13 Various types of oscillations generated by coupling of cells with normally distributed random k values. The time series of z in the (a) external medium, (b) and (c) two cells in the ensemble

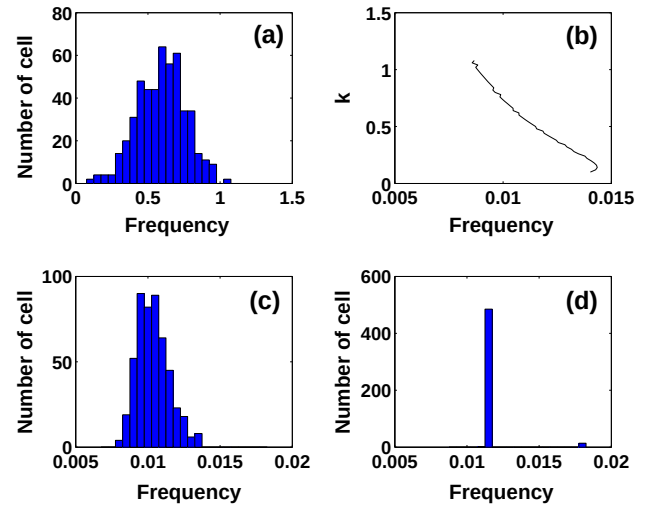


Fig. 14 Distribution of parameters and frequencies of the coupled and uncoupled cell system with normally distributed random k values: (a) Histogram of k ; (b) the relation between k and frequency, (c) and (d) the frequency histograms of uncoupled and coupled ($\eta = 0.6$, $\epsilon = 1$) systems, respectively

frequencies of oscillations in the cells without and with coupling through diffusion of the end product z , indicating frequency synchronization. The synchronized frequency is different from the uniformly distributed k values. Detailed studies (not shown here) with many random uniform and normal distributions of k values in the same range do not change the generality of the results.

4. Conclusions

There are innumerable examples in nature where cells can exist as independent single-celled organisms, or in an ensemble as part of a multi-cellular organism/collective. There are organisms, such as the cellular slime mould,

which have unicellular, aggregative, and multicellular life stages [23]. Whether single or in a group, in all situations there is signalling within and between the cells to respond to environmental cues, and to their neighbours for effective functionality. As discussed earlier, both self and neighbour communications by a secreting signalling molecule can occur through direct or indirect processes. In this theoretical study we have considered model cells (either single, or in an ensemble) where the “signal” molecule is the end product of a simple intra-cellular activator–inhibitor pathway, which can diffuse to the common medium in which these cells reside. The indirect interaction between the cells occurs through the secreting end product, which can regulate its own intra- and extra-cellular concentrations by regulating the direction of diffusion based on a threshold of the end product concentration. The bi-directional and uni-directional diffusion resonates with the ideas of “autocrine” and “quorum-sensing” types of signalling, where, in the former the cell secretes and senses the end product, and the latter involves all cells in the ensemble to respond to the end product concentration in a threshold-dependent manner.

The dynamics of this activator–inhibitor pathway, incorporating two opposing negative and positive feedbacks regulating the production of the end product (z), has been studied extensively [17, 20]. The wide array of stable and unstable dynamics observed in this model pathway in an isolated model single cell has also been studied under small random fluctuations in parameters and substrate concentrations, where it shows that the cellular dynamics can exhibit both robust and non-robust behaviour in noisy environment at different parameters [10, 22]. An analysis of the qualitative and quantitative changes in the pathway dynamics for constant bias and random external perturbations acting on the individual (locally), as well as, all steps (globally) of the pathway, had shown that random external noise induced only small quantitative variations in the substrate concentrations, but bias produced qualitative change in dynamics of the variables. It was shown that the dynamic response of this model biochemical pathway to external perturbations depends not only on their stoichiometry and network topology, but also on the pathway dynamics [24].

This study of model single cells interacting with its environment, by secreting and sensing the end product of the pathway through diffusion, elaborates the changes in the pathway dynamics due to this indirect interaction. The results show that there are similarities, as well as, differences in dynamics for bi-directional and uni-directional diffusion. Even though the dominant dynamics is oscillatory, appearance of sub-critical Hopf bifurcations in both cases leads to coexistence of stable and unstable limit cycle dynamics for a small range of parameters (η and S).

Diffusion with a threshold shows persistence and stable steady-state dynamics at higher threshold of S , and presence of forward and reverse bifurcations with increasing S . Therefore, in self-signalling single cells, we provide evidence of differences in dynamics of the pathway when it interacts with its surrounding medium through diffusion of the end product (without and with threshold), in comparison to a non-diffusive cell’s dynamics.

Synchronization of activity in coupled multi-unit systems is ubiquitous in the natural world [25]. It is not only important to understand the theoretical concepts underlying this collective mode of behaviour, it is also an active area of investigation in biological experiments, where synchronized cells are needed to study the underlying regulatory mechanisms involved in normal and diseased states [26]. Earlier theoretical work with lattices of these model cells (having different types of intrinsic dynamics as shown in Fig. 1), with direct coupling of the end product to the nearest neighbouring cells, have exhibited different types of synchronized dynamics—complete synchrony, intermittent synchrony, waves, and phase synchronization. Partially synchronized cells were shown to attain complete synchronization with a few long-distance couplings [17, 18, 27, 28]. In this study, we have elaborated the collective behaviour in ensemble of cells that are indirectly coupled through a common extra-cellular medium, to and from which diffusion of the end product of the pathway can occur. This is similar to “quorum-sensing” coupling. Such inter-cellular communication has been modelled extensively (see [16] for a review), for the “Repressilator” gene circuit under different conditions [29–32], for oscillators with different types of dynamics [33], and also for different architectures of cell-circuits [34].

The present study compares the effect of two types of diffusion (with and without threshold) on the end product dynamics in an ensemble of cells indirectly coupled through the common external medium in which the cells reside. Figure 10a–c and Fig. 13a–c are interesting results for two reasons. First, we have shown that, even in absence of direct cell–cell interactions, such indirect interactions through the common medium can frequency-synchronize the pathway dynamics in all cells of the ensemble. Thus, even while each non-interacting cell exhibits different types of intrinsic oscillatory dynamics, this type of “self-sensing” and “neighbour-sensing” interactions among cells not only synchronize their frequencies, but also modify their individual dynamics. Secondly, in such a collective of cells, each cell continues to retain individuality in their periodic dynamics (in amplitude and shape), which are different from their intrinsic dynamics. The results do not depend on the nature of the intrinsic frequency distribution (uniform or normal) of z in the cells. These features allow them to be capable of responding to

frequency-dependent processes in a coordinated manner, and also represent an interesting case of frequency, amplitude and shape coding of oscillations (as is known in calcium signalling) that can elicit different downstream gene expressions in cell collectives [35–37].

Additionally, the results also show that, in response to diffusion, the passive external medium starts responding actively to the cells' internal dynamics, and, in turn, regulates the dynamics of the end product in each cell. Our results show that the frequency of oscillation of z in the external medium is dependent on the threshold concentrations of the end product at which uni-directional diffusion occurs. This opens up the possibility of controlling signal dynamics through engineering membrane-mediated diffusion. Further theoretical investigations are required to elucidate and elaborate on the additional pathway-dependent factors (such as parameters, types of feedbacks, and the topology of the pathway) that regulate the external medium's dynamics. Such studies may point towards development or engineering of control protocols to plan ensemble dynamics in signalling cells.

Acknowledgements SS thanks the Indian National Science Academy for the INSA Honorary Scientist award. SR thanks CSIR-CCMB, Hyderabad where the work was initiated. Authors thank Suman Hait for helping with the figures.

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Effect of Seasonal and Altitude Variations in the Reactive Oxygen Species Production and Antioxidant's Concentration in *Mangifera indica* L. (var. kottukonam)

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(Received: 03 January 2023; Revised: 05 February 2023; Accepted: 06 February 2023; Published: 08 February 2023)

(Published by Research Trend)

ABSTRACT: Reactive oxygen species produced as by-product of aerobic metabolism are toxic but plays an important signaling role in plants and controlling the processes like growth and development. Their production are regulated by various biotic and abiotic stresses. Free radicals like Superoxides ($O_2^{\cdot-}$), Hydroxyl radicals (OH $\cdot$) and non-radicals such as Hydrogen Peroxide (H_2O_2) and Singlet Oxygen ($^1O_2$) are the common ROS which make serious oxidative damages to cells. Mango (*Mangifera indica* L.), a member of Anacardiaceae is perhaps the most sweetest and nutritious fruit yielding tropical tree. Kottukonam is one of the varieties over 1500 varieties of *Mangifera indica* L. found globally and are commonly cultivated in the southern parts of Kerala state of India especially in Thiruvananthapuram and Kollam districts was the study material. The leaf samples of kottukonam have been collected from three altitudes in four seasons. The major challenge of the study was to learn the dominant machineries in mango tree to alleviate the serious effects generated by ROS in relation with seasonal and altitude variations. The study revealed that changing the concentration of enzymatic (Ascorbate Peroxidase, Catalase, Glutathione Reductase, Guaiacol Peroxidase, Mono Dehydro Ascorbate Reductase, Dehydro Ascorbate Reductase, NADPH Oxidase and Super Oxide Dismutase) and non-enzymatic (Proline, Ascorbic acid and Reduced Glutathione) antioxidants with respect to the fluctuation in the concentration of ROS is the considerable mechanism to reduce the significant damages by them in cells. The concentration of ROS, enzymatic and non-enzymatic antioxidant composition is higher in pre-reproductive winter and reproductive summer seasons. The coastal area samples showed more concentration of such antioxidants and ROS due to an additional environmental stress, high salinity. Results indicated that the flowering and fruiting is also the upshot of higher production of many antioxidants along with other hormonal changes in relation with high intensity of major abiotic stresses in *Mangifera indica* L. (var. kottukonam). High antioxidant property of mango fruits is the reflection of such abiotic stresses in winter and summer seasons.

Keywords: Reactive Oxygen Species, Antioxidant compounds, *Mangifera indica*, Kottukonam, abiotic and biotic stresses.

Abbreviations: ROS, Reactive Oxygen Species; APX, Ascorbate Peroxidase; GR, Glutathione Reductase; GPX, Guaiacol Peroxidase; MDHAR, Mono DehydroAscorbate Reductase; DHAR, DehydroAscorbate Reductase; SOD, Superoxide Dismutase; $O_2^{\cdot-}$, Superoxide radicals; H_2O_2 Hydrogen Peroxide; $^1O_2$, singlet oxygen; OH $\cdot$, Hydroxyl radical; vzm, Vizhinjam; kza, Kulathupuzha; vjd, Venjaramoodu

INTRODUCTION

Antioxidants, Reactive Oxygen Species (ROS) scavengers are phytochemicals which can inhibit the oxidation of biomolecules like DNA, proteins and lipids by scavenging the free radicals and alleviating stress (Raja *et al.*, 2017; Marcioand Isabel 2013; Jeremy and Alan 2012; Jin-Jian *et al.*, 2012). ROS are produced constantly due to aerobic respiration, various biotic and abiotic stresses in the chloroplast (Dietz *et al.*, 2016), mitochondria (Sako *et al.*, 2020; Honglin *et al.*, 2019), peroxisomes (Sandalio and Romero-Puertas 2015) and also by apoplast (Jan *et al.*, 2020; Amna and Frank 2018; Aryadeep and Supratim 2012; Tana *et al.*, 2009)

which cause damages to the plant cells. But generally they are unable to cause damage, as they are being scavenged by different antioxidant mechanisms (Christine and Graham 2005). The different stress factors like drought, pathogen infection, extreme temperature, salinity, high irradiance, pollution and heavy metals may imbalance the ROS generation and ROS scavenging. Here the survival of plants depends on some other factors such as severity and duration of stress, change in growth conditions and the capability of plants to suddenly adapt to changing energy equation (Gad *et al.*, 2010).

The $^1\text{O}_2$ (singlet oxygen) (Hasanuzzaman *et al.*, 2019), O_2^- (Superoxide radicals), H_2O_2 (Hydrogen Peroxide) and $\text{OH}^\cdot$ (Hydroxyl radical) are the lethal ROS which cause damages to DNA, lipids and proteins and affects normal functioning of cells (Huang *et al.*, 2016; Christine and Graham 2005; Changbin and Martin 2000). They are formed in phototrophs as a result of photosynthesis which is important for the existence of life on earth. Singlet Oxygen ($^1\text{O}_2$) is generated by the reaction of triplet state of Chlorophylls with Oxygen in the antenna system. Stomatal closure due to various environmental stresses like drought, heavy metals and high salinity leads to insufficient CO_2 concentration in plant cells which in turn favours the formation of singlet oxygen (Sheikh *et al.*, 2022). This is an atypical ROS which can cause severe damage to Photosystem I and Photosystem II. These singlet oxygen plays a key role in regulating genes that are meant for giving protection from photo-oxidative stress (Anja *et al.*, 2008). Super oxide radical (O_2^-) is constantly generated in the chloroplast due to the oxygen partial reduction or the result of energy transfer to oxygen. It never cause severe damage to the cells by itself but its transformation into hydroxyl radical and singlet oxygen is more toxic and reactive which results in membrane lipid peroxidation (Barry 2006).

The moderately reactive ROS such as Hydrogen peroxide (H_2O_2) produced in plant cells in normal condition and also by oxidative stress due to intense light, chilling, drought, UV radiation, pathogen infection and wounding (Pallavi *et al.*, 2012) is damaging at higher concentrations in plant cells. Hydroxyl radical ($\text{OH}^\cdot$) is the most toxic and most reactive ROS capable of damaging cellular components by lipid peroxidation, membrane damage and destruction of proteins. Cellular death is the result of excess accumulation of this ROS, which is due to the absence of enzymatic system to scavenge them in plant cells. The functioning of the antioxidant machinery contains (1) antioxidant enzymes such as APX (Ascorbate Peroxidase), GPX (Guaiacol Peroxidase), CAT (Catalase), SOD (Superoxide Dismutase), GR (Glutathion Reductase), MDHAR (Monodehydroascorbate Reductase), DHAR (Dehydroascorbate Reductase) and NADPH Oxidase (Ahanger *et al.*, 2019), and (2) the non-enzymatic antioxidants like Ascorbic acid, Reduced Glutathione, α -tocopherol, Carotinoids, Phenolics, Flavonoids and Prolineto overcome the stressful conditions in plants (Naz *et al.*, 2016; Jeremy and Alan 2012; Sarvajeet *et al.*, 2010; 2011). The survival of plants depends on the counteractions of these antioxidant machineries against the ROS produced.

Mango (*Mangifera indica* L.), a member of Anacardiaceae is perhaps the most sweetest and nutritious fruit yielding tropical tree and support the economy of many developing countries. It is commonly designated in India as 'The king of fruits'. Kottukonam is one of the variety over 1500 varieties of *Mangifera indica* L. found globally and are commonly cultivated in the southern parts of Kerala state of India especially in Thiruvananthapuram and Kollam districts. Since the

variation in the quantity of ROS especially superoxide anions and hydrogen peroxide, and antioxidant compounds in relation with seasonal and altitude variations in *Mangifera indica* L. (var. kottukonam) is an underexplored area of research. The present study focused to quantify the antioxidant compounds from the leaves of plants which are collected in four seasons (summer, south west monsoon, north east monsoon and winter) from three altitudes [Vizhinjam (coastal area), Kulathupuzha (semi high range area) and Venjaramoodu (normal sea level area)].

MATERIALS AND METHODS

Planting materials and sampling. *Mangifera indica* L. (var. kottukonam) was the experimental material. The leaves of experimental plants were collected from (1) Venjaramoodu, normal sea level area (Latitude: 8.68 N, Longitude: 76.91 E, Altitude: 53.00m above sea level) (2) Vizhinjam, coastal area (Latitude: 8.38 N, Longitude: 77 E, 45.00m above sea level) and (3) Kulathupuzha, semi high range area (Latitude: 8° 54'32.46"N Longitude: 77° 3'33.57"E Altitude: 203.6m above sea level). The first two sampling areas belong to the Thiruvananthapuram district and the last one from Kollam of Kerala state in India. The samples were collected in four seasons such as summer (February – May, average temp. of 32 to 36°C and 135mm pptn.), South West Monsoon (June – September, average temp. of 19 to 30°C and 2250 to 2500mm pptn.), North East Monsoon (October – November, average temp. of 29 to 35°C and 450 to 500mm pptn.) and Winter (December – beginning of February, average temp. of 18 to 28°C and 25mm pptn.). All the mango trees selected for the study are about 50 to 55 years old.

Antioxidant compounds and enzymes. Analysis of antioxidant compounds and enzymes in the samples from three altitudes in four seasons were performed by standard protocols. Non-enzymatic antioxidants namely ascorbic acid (Jadhao *et al.*, 2016), glutathione (Nadia *et al.*, 2022) and proline (Bates *et al.*, 1973); antioxidant enzymes such as NADPH Oxidase (Evan and Federica 2018), Guaiacol peroxidase (Angela and Sabine 2003), Ascorbate peroxidase (Gad *et al.*, 2010), Catalase (Amna *et al.*, 2010), Superoxide dismutase (Chris *et al.*, 2011), Monodehydroascorbate reductase (Vincent *et al.*, 2017) and Dehydroascorbate reductase (Zhong and Daniel 2006) was studied.

Quantification of signaling ROS.

H_2O_2 . 1gm fresh tissue was homogenized with 10 ml of 10 mM KH_2PO_4 buffer with pH 7. Filtered extract were centrifuged at 10000 rpm for 10min, collected the supernatant and made up to known volume. Took 1.5 ml extract and added 1.5 ml assay reagent (500 μM ammonium ferrous sulphate, 0.05M conc. H_2SO_4 , 200 μM xylene orange and 0.2M sorbitol) and kept it for 45 min. Took the absorbance at 560 nm against the blank.

Superoxide anion. 1gm fresh tissue was homogenized with 10 ml of 50 mM Sodium acetate buffer (pH 6.5), centrifuged the filtered extract at 10000 rpm for 10 min. Collected the supernatant and made up to a known volume. Took 0.1 ml aliquots and mix with assay reagent (0.01M Potassium phosphate buffer (pH 7.8),

0.05% Nitrobluetetrazolium, and 10 mM Sodium azide). After 30 min of incubation took the OD at 580 nm. Put the mixture in a water bath at 85°C for 15 min and took the final OD at 580 nm. OD difference was taken and calculated the amount of superoxide anion in 1gm tissue.

Statistical analysis. The data obtained were analyzed statistically by one-way analysis of variance (ANOVA) and t- test ($p < 0.05$). The results were average of five replications and represented as mean $\pm$ SD.

RESULTS AND DISCUSSION

Amount of ROS (superoxide anions and hydrogen peroxide) (Table 1a& b) and antioxidant compounds present in methanol extract of *Mangifera indica* L. (var. kottukonam) from three altitudes in four seasons were quantified. The results revealed that the quantity of superoxide anions and hydrogen peroxide, enzymatic and non-enzymatic ROS scavengers varied in accordance with the variation in altitude and season (Table 2a & b). The amount of Reactive Oxygen Species such as hydrogen peroxide and superoxide anions were high in Pre- flowering winter season in all the samples followed by flowering and fruiting season samples in summer. During summer, in addition to the common abiotic stresses like high temperature, high light intensity and water scarcity, the plant again stressed due to flowering and fruiting. These stresses resulted the over production and accumulation of superoxide anions and hydrogen peroxide in mango which are counter balanced by producing various secondary metabolites, enzymatic and non-enzymatic antioxidants. Winter season is the pre-flowering season during which the plants prepared for the reproductive face, much more quantity of ROS were produced than the south west and north east monsoon seasons. Additional stress, high salinity resulted higher ROS concentration in coastal (Vizhinjam) samples of all the seasons (Table 2a and b).

Proline. Proline, an amino acid capable of mitigating the effect of abiotic and biotic stresses includes drought, high salinity, high temperature and pathogen attack. It is a major part of structural protein and suggests its role as stress amino acid in plants. The exposure of plants to abiotic stresses leads to the production of Reactive Oxygen Species (ROS) that enhances the programmed cell death (Sheikh 2022; Klaus and Heribert 2004). To defeat such a crisis the plants over produced the stress amino acid namely proline which act as an active ROS scavenger and prevent them from programmed cell death. Concentration of proline accumulation in plant cells during different seasons in the present study might be related to their responses to various abiotic and biotic stresses [S1vzm, 685.48 $\pm$ 13.48mg/gm tissue; S1kza, 455.18 $\pm$ 1.96mg/gm tissue; S1vjd, 550.30 $\pm$ 1.40mg/gm tissue; S2vzm, 469.35 $\pm$ 1.44mg/gm tissue; S2kza, 341.96 $\pm$ 0.54mg/gm tissue; S2vjd, 388.68 $\pm$ 0.86mg/gm tissue; S3vzm, 488.49 $\pm$ 0.86mg/gm tissue; S3kza, 325.57 $\pm$ 2.15mg/gm tissue; S3vjd, 377.94 $\pm$ 0.88mg/gm tissue; S4vzm, 701.14 $\pm$ 0.55mg/gm tissue; S4kza, 467.80 $\pm$ 0.56mg/gm tissue; S4vjd, 556.98 $\pm$ 1.56mg/gm

tissue] (Table 2a & b). The more stressful conditions in pre reproductive winter and reproductive summer seasons indicated the direct correlation between the production of ROS and antioxidant proline during during such seasons. The effect of additional stress called high salinity can be noticed in the result of coastal samples.

Ascorbic acid. Ascorbic acid is the most extensively studied and abundant ROS scavenging compound in plants. Ascorbate produced in plant cells by Smirnoff-Wheeler pathway is powerful antioxidant compound counteract the ROS produced as it can donate electrons to enzymatic and non-enzymatic reactions. These are also involved in retrograde signaling (Konig *et al.*, 2018). The present study also revealed that the ascorbic acid concentration varied along with the change in altitude and season. The highest content of ascorbic acid was noted in pre-flowering winter season [S4vzm, 57.10 $\pm$ 0.84mg/gm tissue; S4kza, 44.11 $\pm$ 0.62mg/gm tissue; S4vjd, 50.96 $\pm$ 0.59mg/gm tissue] and then in the flowering and fruiting summer season [S1vzm, 48.12 $\pm$ 0.85mg/gm tissue; S1kza, 41.10 $\pm$ 0.11mg/gm tissue; S1vjd, 44.90 $\pm$ 0.33mg/gm tissue] than the other two seasons (south west monsoon and north east monsoon) (Tables 2a & 2b). The major reason behind this in winter and summer seasons is due to abiotic stresses. High salinity acted an additional stress in coastal area (Vizhinjam) resulted much more Ascorbate content in the samples from there. König *et al.* (2018) and Seung and Adel (2000) reported that high light intensity and water scarcity increases the Ascorbic acid content of plant tissues.

Reduced Glutathione (GSH). Glutathione is a low molecular weight thioltripeptide. Present in the cell components like Cytoplasm, Mitochondria, Endoplasmic Reticulum, Vacuoles, Chloroplast, Peroxisomes and Apoplast. The high reductive potentiality of glutathione resulted their involvement in a wide range of processes in plants like growth, cell division, senescence, cell death, nucleotide and protein synthesis, detoxification of xenobiotics and the expression of stress responsive genes (Philip and Thomas 2005). GSH protects the plant cells from ROS like H₂O₂, ¹O₂, OH⁻, and O₂⁻ formed by various biotic and abiotic stresses due to its reducing power by the presence of a cysteine residue with nucleophilic character. Earlier studies revealed that the glutathione content varied along with seasonal variations. In *Mangifera indica* L. (var. kottukonam), glutathione is high in the winter samples [S4vzm, 56.00 $\pm$ 0.34mg/gm tissue; S4kza, 47.71 $\pm$ 0.42mg/gm tissue; S4vjd, 50.71 $\pm$ 0.40mg/gm tissue] during which the plant prepared for flowering. In addition to the preparations for flower production the plant also faced other abiotic stresses especially high light intensity and temperature in day time, water scarcity etc. also act as key reasons for the formation of ROS in higher quantity during winter. High quantity of glutathione were also observed in the samples of summer season [S1vzm, 55.19 $\pm$ 0.74mg/gm tissue; S1kza, 44.09 $\pm$ 0.17mg/gm tissue; S1vjd, 49.14 $\pm$ 0.21mg/gm tissue] during which flowering and fruiting occurs. Due to the high salinity

stress in coastal area along with other abiotic stresses, Vizhinjam samples in all the seasons showed much more glutathione content than the others (Tables 3a & b). The result of the study have a correlation to the previous work of Konig *et al.* (2018).

Antioxidant enzymes. Various abiotic and biotic stresses persuade the photosynthetic system of leaves to photo-inhibition, resulting in a light-dependent inactivation of the primary photochemistry associated with photosystem II, which often persists after re-watering (Deeba *et al.*, 2012). Photosynthesis to be affected by water deficits and high salt contents, via decreased CO₂ diffusion to the chloroplast and metabolic constraints (Pinheiro and Chaves 2011). Acclimation of plants to drought, temperature, light intensity, salinity, diseases and pathogen attack is often associated with increased levels of reactive oxygen species (ROS). During stressful conditions the production of ROS become transcend and the action of antioxidative system to scavenge ROS resulted in oxidative stress (Paul and Panneerselvam 2013). Antioxidant enzymes such as Ascorbate peroxidase, Catalase, Glutathione Reductase, Mono DehydroAscorbate Reductase, DehydroAscorbate Reductase, Guaiacol Peroxidase, NADPH Oxidase and Superoxide Dismutase protected plants from the damage caused by ROS Zeng *et al.* (2017); Ron *et al.* (2011); Ron Mittler (2002).

Ascorbate Peroxidase (APX). APX is an efficient H₂O₂ scavenger during stress periods in plants commonly located in the chloroplast, peroxisomes, mitochondria and cytoplasm (Zeng *et al.*, 2017; Pallavi and Dubey 2004). It is an unavoidable component of Ascorbate-Glutathione cycle. In the present study the highest quantity of APX noted in the most stressful seasons such as in winter (pre-flowering) and summer (flowering) in *mangifera indica* L. (var. kottukonam) than that in south west and north east monsoon seasons (S1vzm, 600.57±0.36 unit/gram tissue; S1kza, 323.60±2.20 unit/gram tissue; S1vjd, 585.66±3.08 unit/gram tissue; S2vzm, 420.81±0.79 unit/gram tissue; S2kza, 257.18±1.48 unit/gram tissue; S2vjd, 291.54±4.06 unit/gram tissue; S3vzm, 418.14±2.51 unit/gram tissue; S3kza, 247.59±0.38 unit/gram tissue; S3vjd, 215.04±3.63 unit/gram tissue; S4vzm, 678.61±0.38; S4kza, 342.09±0.20 unit/gram tissue; S4vjd, 599.69±1.47 unit/gram tissue) (Table 3a & 3b).

Catalase (CAT). Catalase, a unique antioxidant enzyme having greater affinity to H₂O₂, located in mitochondria, chloroplast, peroxisomes and cytosol. Oxidative stress lead to the over production of H₂O₂ (Sheikh, 2022; Ron Mittler, 2002) which are removed efficiently by CAT in an energy efficient way. Maximum amount of CAT resulted in *Mangifera indica* L. (var. kottukonam) during the most stressful seasons namely summer and winter (S1vzm, 2214.81±2.18 unit/gram tissue; S1kza, 1082.45±2.79 unit/gram tissue; S1vjd, 1981.54±1.23 unit/gram tissue; S4vzm, 2561.06±12.01 unit/gram tissue; S4kza, 1023.85±0.39 unit/gram tissue; S4vjd, 2086.64±2.37 unit/gram tissue) (Table 3a & 3b). Caverzan (2016); Paul and Panneerselvam (2013); Pinheiro and Chaves (2011)

reported that the strength of stresses were overcome by the plants by producing the antioxidant catalase enzyme in much more concentration.

Glutathione Reductase (GR). GR is a flavoprotein oxidoreductase used to reduce Glutathione disulfide (GSSG) to reduced Glutathione (GSH) commonly found in chloroplast and small amount in cytoplasm and mitochondria. They are the scavenger of detrimental ROS, ¹O₂ and OH[•]. The result of the present study indicated the amount of GR is highest in the samples of winter season (S4vzm, 1245.55±1.24 unit/gram tissue; S4kza, 810.94±1.01 unit/gram tissue; S4vjd, 1154.39±2.88 unit/gram tissue) and then in the summer season (S1vzm, 1117.54±0.56 unit/gram tissue; S1kza, 788.01±2.03 unit/gram tissue; 1057.42±1.18 unit/gram tissue) because of pre-flowering and flowering stresses to the tree in addition to the common environmental stresses. Caverzan (2016) reported that the stressful conditions leads to the over production of antioxidant enzymes including GR.

Monodehydro Ascorbate Reductase (MDHAR) and Dehydro Ascorbate Reductase (DHAR). By using the reducing agent, MDHAR regenerating Ascorbic acid from the short lived Monodehydro Ascorbate, resulted the restoring of Ascorbate in the Ascorbic acid pool of cells. MDHAR occurred in the peroxisomes and mitochondria along with Ascorbate peroxidase and helped for scavenging the ROS especially Hydrogen Peroxide (Sheikh, 2022; Ron, 2002). The present study revealed that the most stressful condition faced by *Mangifera indica* L. (var. kottukonam) is the winter and summer, and thus the quantity of Antioxidant enzyme MDHAR in these two seasons is also comparatively more than the other two seasons (Table 3a & b). The samples from coastal area in all seasons showed more quantity of MDHAR due to an additional stress, high salinity.

Using Reduced Glutathione as an electron donor DHAR reduces Dehydro Ascorbate to Ascorbic acid (Amin *et al.*, 2007) to enriched the Ascorbic acid pool of cells and maintaining the redox state of the cells (Zhong and Daniel 2006). This indicated that the stressful conditions led to the higher production of DHAR (S1vzm, 1252.20±2.22 unit/gram tissue; S1kza, 984.66±5.32 unit/gram tissue; S1vjd, 1176.37±1.95 unit/gram tissue; S4vzm, 1286.80±1.31 unit/gram tissue; S4kza, 901.73±5.28 unit/gram tissue; S4vjd, 1116.96±1.40 unit/gram tissue) like that of MDHAR and the present study also an agreement to this facts (Table 3a & b).

Guaiacol Peroxidase (GPX). GPX is a heme containing protein seen associated with many biosynthetic processes and defense against biotic and abiotic stresses. They oxidise aromatic electron donors at the expense of hydrogen peroxide (Pallavi *et al.*, 2012). The present study in *Mangifera indica* L. (var. kottukonam) indicated that like that of other antioxidant enzymes GPX content also more during winter and summer seasons and much more in coastal samples (S1vzm, 653.81±1.75 unit/gram tissue; S2vzm, 457.23±1.48 unit/gram tissue; S3vzm, 463.30±0.36 unit/gram tissue; S4vzm, 678.67±0.40 unit/gram tissue)

(Table 3a & b). The result also an agreement to the previous studies of Caverzan (2016); Andre *et al.* (2006); Jingxian and Khirkham (1996).

NADPH Oxidase. NADPH Oxidases in plants are commonly called Respiratory Burst Oxidase Homologues (RBOHs) which act as a catalyst in the physiological generation of short - lived superoxide radicals (David, 2004). In the present study, the concentration of NADPH Oxidases is more in winter and summer seasons especially in coastal samples due to over environmental stresses (Table 3a & 3b) and this stimulate the production of ROS. The earlier studies revealed that the over presence of this enzyme is a signal to the plants to the environmental stresses and the present study is also an agreement to that findings (Smirnoff and Arnaud 2018).

Superoxide Dismutase (SOD). Metalloenzyme common in all aerobic organisms is the first line of defense against ROS induced damages under abiotic

stresses (Boguszewska, 2010). SOD generally localised in cytoplasm, chloroplast, mitochondria and peroxisomes. In *Mangifera indica* L. (var. kottukonam), the SOD content is higher in winter season (S4vzm, 457.36±3.81 unit/gram tissue; S4kza, 287.66±3.82 unit/gram tissue; S4vjd, 433.08±0.78 unit/gram tissue) and then in flowering and fruiting summer season (S1vzm, 418.32±1.08 unit/gram tissue; S1kza, 223.66±0.43 unit/gram tissue; S1vjd, 376.30±0.51 unit/gram tissue) than other two seasons (south west monsoon and north east monsoon) due to the over stress faced by the plant from environment (Table 3a & b). The coastal samples (vzm) in all seasons showed much more content of SOD than other altitude samples in different seasons because of the additional salt stress. The present study results were also revealed that the earlier studies of Lu *et al.* (2017); Zlatev *et al.* (2006); Kukreja *et al.* (2002) was truthful.

Table 1a: Quantification of ROS, superoxide anions and hydrogen peroxide in the leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 1, summer; sample 2, south west monsoon] in mg/gm fresh wt. Values with ± SD from five independent experiments.

Reactive Oxygen Species (ROS)	Sample 1 [S1]			Sample 2 [S2]		
	Vzm	kza	vjd	vzm	kza	vjd
Superoxide Anion	19.45±0.46	9.39±0.32	13.96±0.37	4.27±0.26	6.14±0.17	8.35±0.34
Hydrogen Peroxide	5.09±0.069	3.25±0.207	3.31±0.196	3.38±0.115	1.90±0.137	2.35±0.279

Table 1b: Quantification of ROS, superoxide anions and hydrogen peroxide in the leaf samples of *Mangifera indica* L. (var. kottukonam) from the three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 3, north east monsoon; sample 4, winter] in mg/gm fresh wt. Values with ± SD from five independent experiments.

Reactive Oxygen Species (ROS)	Sample 3 [S3]			Sample 4 [S4]		
	Vzm	kza	vjd	vzm	kza	vjd
Superoxide Anion	3.81±0.209	6.30±0.368	7.81±0.726	20.44±0.563	10.71±0.653	15.35±0.743
Hydrogen Peroxide	6.40±0.334	3.60±0.234	3.75±0.209	6.05±0.088	3.57±0.329	3.60±0.365

Table 2a: Amount of Non enzymatic Antioxidant compounds in the leaves of *Mangifera indica* L. (var. kottukonam) from the three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 1, summer; sample 2, south west monsoon] [Values are with ± SD from five experiments each] (in mg/gm fresh wt.).

Antioxidant compound	Sample 1 [S1]			Sample 2 [S2]		
	vzm	kza	vjd	vzm	kza	vjd
Proline(μmol/gm fresh wt.)	685.48.±13.48	455.18±1.96	550.30±1.40	469.35±1.44	341.96±0.54	388.68±0.86
Ascorbic Acid (mg/gm fresh wt.)	48.12±0.85	41.10±0.11	44.90±0.33	42.25±0.65	38.54±0.33	41.19±0.73
Reduced Glutathione (mg/gm fresh wt.)	55.19±0.74	44.09±0.17	49.14±0.21	49.05±0.42	39.73±0.90	41.28±0.61

Table 2b: Content of Non enzymatic Antioxidant compounds in the leaves of *Mangifera indica* L. (var. kottukonam) from the three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 3, north east monsoon: sample 4, winter] [Values are with ± SD from five experiments each] (in mg/gm fresh wt.).

Antioxidant compound	Sample 3 [S3]			Sample 4 [S4]		
	vzm	kza	vjd	vzm	kza	Vjd
Proline (μmol/gm fresh wt.)	488.49±0.86	325.57±2.15	377.94±0.88	701.14±0.55	467.80±0.56	556.98±1.56
Ascorbic Acid (mg/gm fresh wt.)	44.05±0.11	37.87±1.85	44.14±0.76	57.10±0.84	44.11±0.62	50.96±0.59
Reduced Glutathione (mg/gm fresh wt.)	46.99±0.45	38.07±0.11	39.05±1.94	56.00±0.34	47.71±0.42	50.71±0.40

Table 3a: Specific activity of Antioxidant Enzymes in the leaf samples of *Mangifera indica* L. (var. kottukonam) from the three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 1, summer; sample 2, south west monsoon] [Values are with $\pm$ SD from five experiments each] (in unit/gm fresh wt.)

Antioxidant Enzymes (unit/gm tissue)	Sample 1 [S1]			Sample 2 [S2]		
	vzm	kza	vjd	vzm	kza	Vjd
Ascorbate Peroxidase	600.57 $\pm$ 0.36	323.60 $\pm$ 2.20	585.66 $\pm$ 3.08	420.81 $\pm$ 0.79	257.18 $\pm$ 1.48	291.54 $\pm$ 4.06
Catalase	2214.81 $\pm$ 2.18	1082.45 $\pm$ 2.79	1981.54 $\pm$ 1.23	1332.64 $\pm$ 10.23	951.66 $\pm$ 0.61	1118.10 $\pm$ 2.49
Glutathione Reductase	1117.54 $\pm$ 0.56	788.01 $\pm$ 2.03	1057.42 $\pm$ 1.18	884.79 $\pm$ 8.98	679.95 $\pm$ 2.96	900.69 $\pm$ 0.47
Monodehydro Ascorbate Reductase	998.46 $\pm$ 1.18	720.53 $\pm$ 0.55	980.05 $\pm$ 0.92	857.35 $\pm$ 3.56	659.01 $\pm$ 0.60	821.35 $\pm$ 1.76
Dehydro Ascorbate Reductase	1252.20 $\pm$ 2.22	984.66 $\pm$ 5.32	1176.37 $\pm$ 1.95	987.32 $\pm$ 1.43	857.79 $\pm$ 1.91	1055.88 $\pm$ 2.51
Guaicol Peroxidase	653.81 $\pm$ 1.75	400.77 $\pm$ 0.34	535.78 $\pm$ 2.55	457.23 $\pm$ 1.48	377.81 $\pm$ 0.90	490.71 $\pm$ 1.02
NADPH Oxidase	489.49 $\pm$ 0.36	330.16 $\pm$ 1.56	427.74 $\pm$ 1.47	387.24 $\pm$ 0.81	317.84 $\pm$ 0.98	353.84 $\pm$ 0.89
Super Oxide Dismutase	418.32 $\pm$ 1.08	223.66 $\pm$ 0.43	376.30 $\pm$ 0.51	370.21 $\pm$ 1.14	197.08 $\pm$ 1.65	229.005 $\pm$ 0.83

Table 3b: Specific activity of Antioxidant Enzymes in the leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam- coastal area (vzm): Kulathuppuzha- semi high range area (kza) and Venjaramoodu- normal sea level area (vjd)] in two seasons [sample 3, north east monsoon: sample 4, winter] [Values are with $\pm$ SD from five experiments each] (in unit/gm fresh wt.).

Antioxidant Enzymes (unit/gm tissue)	Sample 3 [S3]			Sample 4 [S4]		
	vzm	kza	vjd	vzm	kza	vjd
Ascorbate Peroxidase	418.14 $\pm$ 2.51	247.59 $\pm$ 0.38	215.04 $\pm$ 3.63	678.61 $\pm$ 0.38	342.09 $\pm$ 0.20	599.69 $\pm$ 1.47
Catalase	1288.71 $\pm$ 1.44	985.70 $\pm$ 2.12	1104.38 $\pm$ 3.61	2561.06 $\pm$ 12.01	1023.85 $\pm$ 0.39	2086.64 $\pm$ 2.37
Glutathione Reductase	867.66 $\pm$ 0.92	723.02 $\pm$ 1.17	908.05 $\pm$ 1.17	1245.55 $\pm$ 1.24	810.94 $\pm$ 1.01	1154.39 $\pm$ 2.88
Monodehydro Ascorbate Reductase	808.32 $\pm$ 0.87	674.79 $\pm$ 3.91	797.50 $\pm$ 5.29	1010.79 $\pm$ 0.29	740.97 $\pm$ 0.70	996.33 $\pm$ 2.46
Dehydro Ascorbate Reductase	884.74 $\pm$ 0.92	720.84 $\pm$ 2.46	998.01 $\pm$ 1.30	1286.80 $\pm$ 1.31	901.73 $\pm$ 5.28	1116.96 $\pm$ 1.40
Guaicol Peroxidase	463.30 $\pm$ 0.36	359.00 $\pm$ 1.16	591.96 $\pm$ 1.01	678.67 $\pm$ 0.40	435.08 $\pm$ 0.51	609.92 $\pm$ 0.31
NADPH Oxidase	368.78 $\pm$ 0.28	321.39 $\pm$ 0.41	375.19 $\pm$ 0.88	495.86 $\pm$ 0.48	361.25 $\pm$ 2.23	461.16 $\pm$ 0.99
Superoxide Dismutase	367.69 $\pm$ 0.87	201.11 $\pm$ 0.28	248.62 $\pm$ 0.59	457.36 $\pm$ 3.81	287.66 $\pm$ 3.82	433.08 $\pm$ 0.78

CONCLUSIONS

In relation with seasonal and altitude variations on enzymatic and enzymatic antioxidants were produced in *Mangifera indica* L. (var. kottukonam). The environmental stresses like drought, high temperature, high salinity, high oxygen concentration and high light intensity leads to the over production of various ROS especially Hydrogen peroxide and Superoxide anions, and the plant defeated these injuries by the extra production of certain non enzymatic antioxidant compounds such as Proline, Ascorbic acid and Reduced Glutathione, and antioxidant enzymes such as Ascorbate Peroxidase, Catalase, Glutathione Reductase, Mono DehydroAscorbate Reductase, Dehydro Ascorbate Reductase, Guaiacol Peroxidase, NADPH Oxidase and Superoxide Dismutase. The quantity of these antioxidants varied in relation with the production of ROS which in turn related with the intensity and type of stresses faced by the plant in a specific season and altitude. The present result concludes that these

antioxidant machinery seems to be sufficient in *Mangifera indica* L. (var. kottukonam) to fight against various environmental stresses.

FUTURE SCOPE

The leaves of *Mangifera indica* L. (var. kottukonam) was the material used in the present study and identified that the variation in the concentration of antioxidant compounds and triggering ROS in relation with seasonal and altitude variations. In addition to the fruits the leaves developed in the summer season is also a rich source of antioxidants. The present investigation suggested that the extraction of these antioxidants from leaves which are very useful to human health will be a promising one to the pharmaceutical industry.

Acknowledgements. We are thankful to the Principal, HOD and the teachers in the P G and Research department of Botany, Christian college Kattakada, Thiruvananthapuram, Kerala to provide valuable support in the research lab.

Conflict of Interest. None.

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How to cite this article: Shalaj Rasheed, Raveendran P.B. and Lubaina Abdulhadeef Shereefa (2023). Effect of Seasonal and Altitude Variations in the Reactive Oxygen Species Production and Antioxidant's Concentration in *Mangifera indica* L. (var. kottukonam). *Biological Forum – An International Journal*, 15(2): 177-184.



REVIEW ARTICLE

A review on the genus *Calophyllum* L. (Clusiaceae): a potential medicinal tree species

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ARTICLE HISTORY

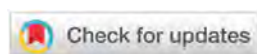
Received: 11 April 2022

Accepted: 17 October 2022

Available online

Version 1.0 : 20 April 2023

Version 2.0 : 01 July 2023



Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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CITE THIS ARTICLE

Chinthu R V, Raveendran P B, Raveendran M. A review on the genus *Calophyllum* L. (Clusiaceae): a potential medicinal tree species. Plant Science Today. 2023;10(3): 01-05. <https://doi.org/10.14719/pst.1818>

Abstract

Calophyllum L. is the genus of evergreen tropical flowering plants in the Clusiaceae family. They are predominantly located in Asia with some of its genus distributed in the Pacific Islands, Americas, Australasia and Africa. Plants of the genus are well known for their chemical properties with lots of secondary metabolites such as triterpenes, flavonoids, coumarins and xanthones. Compounds from *Calophyllum* have been reported to have cytoprotective, anti-HIV, anti-secretory, cytotoxic, antinociceptive, molluscicidal and antimicrobial properties. Some of the plants in *Calophyllum* are used in folk medicine to treat conditions like peptic ulcers, tumours, inflammation, infections and pain. *Calophyllum* genus is important with respect to their ecological as well as their medicinal properties. But in India, some species located in Western Ghats are in vulnerable stage. So, for a sustainable environment, we should focus on the conservation, restoration and rehabilitation of the genus that makes significant differences in their population. This article reviews the genus *Calophyllum* of Western Ghats as a potential medicinal tree species.

Keywords

Poon, oil, timber, xanthones, inflammation

Introduction

The genus *Calophyllum* L. (Clusiaceae) includes about 187 species. Of these, 179 are present in the Old World (mainly in the Indo-Malaysian region) and about 8 are in the New World, from Mexico and the Caribbean to Argentina. Some species are ornamental and others yield timber, commercially classified as POON and oil (1). When the Old World taxa were revised, 14 species from India and Sri Lanka were accepted. The *Calophyllum* species which occur in India, especially in Western Ghats are *Calophyllum apetalum*, *C. inophyllum*, *C. polyanthum*, *C. austroindicum* and *C. pascalianum* (2).

Materials and Methods

Available manuscripts like research articles, journals, review papers, books, e-papers, e-books, electronic thesis, dissertations and search engines like Google, Yahoo, Microsoft Bing were used for collecting data.

Results

Plants of the World Online (POWO.org) shows 185 scientific names species rank under the genus *Calophyllum*. Of these, 179 are accepted names of species.

Calophyllum apetalum Willd ('Poonspar of Travancore')

IUCN Red Data list category

Vulnerable

Ecology

A medium-sized to large, straight and cylindrical stemmed, evergreen tree with a girth up to 2.1 m, distributed in the forests of Western Ghats, at an altitude up to 1350 m often found by the sides of rivers and water course. The tree is found along the foothills of Western Ghats from Maharashtra and Goa southward through the forests of Karnataka, Tamilnadu and Kerala. It is often found growing along the rivers which flow down the Western Ghats to the sea. The tree attains large size in Kerala, particularly in the interior of evergreen forests. The tree often grows away from streams; elsewhere it is medium-sized, mostly along the streams. Species are receptive to various viruses, fungi, an insect pest that affects the leaves, roots and the fruits

Regeneration :

Natural regeneration is generally adequate under the parent tree. The seedlings thrive in the dense shade of the heavy undergrowth, found along the streams and continue to grow slowly until they emerge into the open. Afterwards the crown assumes a round shape. In Karnataka, transplanting of the species was successful in Dandeli and Telgeri-Kans division. The percentage of survival is reported to be 60–70 % (3).

Uses

The timber is great for construction. It is employed for construction of boats, oil mills, bridges and for cabinet work. It is suitable for manufacture of matchboxes and poles. The timber is suitable for plywood. It is a good fuel. Besides timber, the tree yields edible fruits. They contain a cream-coloured kernel, which is eaten; it is enclosed in a hard shell. The fruits are collected and sold. The kernels contain apetalolide and β -sitosterol- β -D-glucoside. The sun-dried kernels yield a greenish yellow fatty oil (45–56 %), having a characteristic odour and a bitter taste. The oil is used as an illuminant, lubricant and in soap making and leather-treating. The oil is used in sepsis, rheumatism, leprosy and skin infestations (3).

Related works

Three new xanthonoids, apetalinones A–C, in which A was a novel xanthone were isolated from the roots of *C. apetalum*. The bark of *C. apetalum* has a new xanthonoid, apetalinone-D (4). *In vitro* multiplication of *C. apetalum* were carried out. Out of 345 plants restored to their native habitat in the forest at three locations 293 plants survived and showed uniform growth free of morphological defects (5). The acetone extract of leaves of *C. apetalum* records the isolation and structure determination of a dipyrano-coumarin α -hydroxytomentolide-A along with known friedelin, triterpenoids, apetalolactone, calophyllol and

inophyllum C (6). Pollination biology and breeding systems of *C. apetalum* shows flowers are pollinated by both anemophily (through wind) and entomophily (through insects) gives effective pollination results and in successful fruit set. The manual cross and self-pollinations are significantly different which depicts the importance of naturally mediated cross-pollination by pollinators (7). Seed bank estimation and regeneration studies of *C. apetalum* results reports that, the seed densities are insignificant among the forest ranges and significant with the distances. The studies highly recommended that the *ex-situ* regeneration possibly give greater percentage of seed germination and so it as a best suitable method of conservation of this species (8).

Calophyllum inophyllum Linn. (Alexandrian Laurel)

IUCN Red Data list category

Least concern

Ecology

A medium to large sized, evergreen and spreading tree, height up to 20 m and girth 1.5 m distributed in the coastal regions of Maharashtra, Goa, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa and the Andamans. Often it grows wild but also reported to be naturalized in Arunachal Pradesh. The tree is a native of the 'Beach Forests' of the Arabian Sea, found all along the sea coasts above the high watermark, including coastal backwaters down to the edge of the sea from Bombay southward to Kerala on the west coast and from Cuttack to Madras through Andhra Pradesh on the east coast; it is also common in the Andaman and Nicobar Islands. The tree is reported to be growing in West Bengal.

Regeneration

The tree reproduces fairly well in nature, as the seeds are dispersed by fruit-eating bats and birds. It can also be raised either by direct sowing or transplanting. In case of direct sowing, the seeds must be from ripe and fresh fruits. It can easily be raised in sandy soils. The germinative capacity of the seeds is fair, but the thick shell delays their germination. When sown during the warmer months, 48% seeds germinate and take 50–55 days. They germinate without any difficulty, if sown soon after ripening; the removal of shell is reported to be very effective in reducing the period of germination, and increasing the germination percentage as also the vigour potentials during initial stages of growth and development. The seeds are sown in the nursery for transplanting and these seedling have been raised on the coast of Orissa, either pure or mixed, under the shade of *Casuarina equisetifolia* Linn. (3).

Uses

C. inophyllum is cultivated for its edible fruits, seed oil, timber and as an ornamental in various parts of India and rarely, as an avenue tree. The ripe fruits are red and sweet and are reported to be eaten. The ripe seeds yielded calophyllide, inophyllide and calophyllic acid which are reputed to be useful in the treatment of leprosy; whereas unripe seeds gave cinnamic, inophyllic and calophyllic acids and ponnalide. Presence of leucocyanidin is reported

in the seed coat. A considerable difference was found in the chemical constituents of the ripe and unripe seeds. The oil seed is used for burning and as a varnish for painting wood work since it contains considerable amount of resin (10–30%). It has recently recommended as a lubricant in place of castor oil. Sometimes it used for oiling up vegetable-tanned leather. The refined oil is excellent for soap making and is used in industries. It is, however unfit for human consumption. Combined with the resin of *Vateria indica* L (Dipterocarpaceae), the oil is applied for caulking boats. Heat polymerization of the oil with the fat of *Mallotus philippensis* (Lam.) Muell.Arg. (Euphorbiaceae) gives good stoving enamels. A paste of the kernels is applied to relieve painful joints. The oil is used as a stimulant embrocation in rheumatism and gout. It is antifungal and has a beneficial influence over the mucous membrane of the genito-urinary organs, therefore, used in the treatment of venereal diseases. The refined oil is intramuscularly injected to alleviate pain in leprosy. The oil cures scabies and other cutaneous diseases. The soap from the alcoholic extract of the oil also has strong bactericidal activity against the gram positive microorganisms and fungistatic properties. The camphor-mixed oil is used against ring worm. Graveyard tests on natural durability of wood showed that average life of the timber is about 67 months; preservative-treated wood is slightly more durable. The wood is used for posts, beams, furniture, railway carriages, cooperage, crane-shafts, yokes, tool handles and for cabinet-work. On the west coast and in the Andaman and Nicobar Islands, the wood is used for keels and pulley blocks of ships and for boats and dugout canoes. It is also used for railway sleepers. It peels satisfactorily and is good for commercial plywood. The tree can be used as a suitable support for pepper vine cultivation. If properly pruned, it is a useful wind break. As the flowers attract honey bees in large numbers, apiaries can be established near these trees (3).

Related works

Calophyllum inophyllum which have been widely used traditionally as a local medicine. According to the scientific literature on *C. inophyllum*, a broad spectrum of pharmacological properties has been shown by this tree as well as it is reported to be rich in several active bio-compounds (9). Different parts of *Calophyllum inophyllum* tree have some pharmacological effects: anti-inflammatory, wound healing, osteogenic, antidiabetic, anticancer, lipid-lowering, antioxidant, antifungal, neuro-pharmacological and antibacterial effects. The bioactive compounds have been observed, leading towards the development of novel potent drugs for ordinary diseases (10). Immature and mature ball nut seed meal were similar in chemical contents and could therefore serve as potential animal's feed resource due to its high energy level, amino acids profile as well as low fibre and other anti-nutritional factors present in the meals (11). *Calophyllum inophyllum* plant extracts consist compounds having antimicrobial properties that can serve as antimicrobial agents in new drugs for the therapy of infectious diseases (12). An effective protocol for *in vitro* micropropagation of *Calophyllum inophyllum*, is

through multiple shoot formation from seed explants was developed (13). From the *C. inophyllum* leaves beneficial phytochemicals were identified. It was found that all parts of *C. inophyllum* plant can be used for human needs. The information is limited to extraction and identification of mixture of phytochemical compounds that are obtained from plant extracts (14). Oil content in the seeds is higher in large and medium weight classes which can be used for oil extraction more economically (15). Genetic structure, genetic diversity and degree of gene flow were discovered for island populations of *C. inophyllum* - a plant that employs sea-drift for seed dispersal (16). *C. inophyllum* plant contains a few compounds that are capable of binding to and inhibiting the epidermal growth factor protein and thereby preventing cell proliferation in an uncontrolled manner (17). *In vitro* production of secondary metabolites from cultured cells/tissues and molecular characterization of *Calophyllum inophyllum* have promising results (18). Seeds are used as oil-seed and as biofuel (19). *In vitro* studies on the cytotoxicity and apoptosis induction by the methanol extract of the fruit of *Calophyllum inophyllum* in MCF - 7 human breast cancer cell line also showing promising results (20).

***Calophyllum polyanthum* Wall.ex.chois** (Poonspar Tree)

IUCN Red Data list category

Not Evaluated

Ecology

A medium sized to large, straight, cylindrical stemmed, evergreen tree, 45 m in height and 4.5 m in girth, distributed on the western coast and ghats from Maharashtra to Kerala, in Tamil Nadu and in the hill forests of northern Bengal, Sikkim, Bhutan, Arunachal Pradesh, Assam, Meghalaya and Mizoram, up to an elevation of 1500 m. The tree inhabits the wet evergreen and semi-evergreen forests of Western Ghats and it is one of the tallest trees found there. The tree is a shade-bearer, especially in its early stages, overhead light being essential for the rapid development of the tree. It cannot tolerate relatively dry conditions as prevalent in a moist deciduous forest, but does well in the evergreen forest. It grows well near the streams. The best sizes are reached on the moist and fertile soils of sholas and foothills which are rich in organic debris and decomposing bacteria. The coppicing-power of the tree is poor; it is sensitive to fire and is encouraged by fire protection. Flowering in February- March and fruiting during April-August. The seeds weigh 140- 180 to a kg and can be stored in gunny bags without appreciable loss in fertility. In the seedbed, several seeds often germinate only in the second year (3).

Regeneration

Germinative capacity of fresh seed is generally low, though the recorded highest is 89%. Pre-soaking the seeds in cold or hot water are advantageous. The tree regenerates in nature under dense sunshade of the evergreen forests.

The seeds germinate early in the rains, soon after

falling. Clearing the weeds improves germination and growth. Opening of the top canopy, after obtaining adequate recruitment on the ground, encourages establishment and further development of the plants. Regeneration of these trees is adequate in the localities where its distribution is low, but it is inadequate in the areas where it occurs more frequently. The tree can be raised by direct sowing or by transplanting the nursery-raised seedlings. The seedlings are quite slow growing. The direct sowings under the tree may sometimes be disappointing owing, probably to the overhead drip. Stump-planting generally yields poor results. Planting or sowing in the prepared soil seems to be advantageous. The nursery-raised plants are generally better than natural forest-seedlings (3).

The best season for planting the species in Tamil Nadu is from mid-June to early July. In very heavy rainfall areas, such as those at the head of the Western Ghats, planting after the heavy monsoon, sometime in mid-August, is advantageous. Opening the top canopy and a light thinning are reported to be advantageous. The tree is generally removed in selection-fellings and the minimum exploitable girth varies: in Kerala, it is 1.2 m; in Karnataka, 1.8 – 2.3 m and in Tamil Nadu, 2.7 m.

Uses

Timber is ideal for construction purposes and is much used for house posts, beams, ceiling – boards, floorboards, for squash courts, rafters, planking, cheap furniture, poles, masts, spars, helms, dugouts and ferries, chests, stores, railway coaches, tent poles, mathematical instruments etc. and for construction of bridges. Carefully selected and graded wood is suitable for aircraft construction. The antiseptic treated wood may be utilized for sleepers; the diffusion process after incision is recommended in preference to other processes of treatment. For poles also this processes can be considered. The timber is reported to be suitable for paper –pulp and various grades of plywood. It may be used as a substitute for Oregon Pine (*Pseudotsuga menziesii* (pinaceae) (Mirbel) Franco) (3).

***Calophyllum austroindicum* Kosterm. ex P.F. Stevens**
(Rhomb-leaved Poon)

IUCN Red Data list category

Not Evaluated

Ecology

It is a medium to large sized tree, usually 10 – 30 m in height and 3 m in girth, Canopy trees in medium and high elevation wet evergreen forests between 1100 and 1800 m. Flowering and fruiting: November-June.

***Calophyllum pascalianum* B.R. Ramesh, Ayyappan & De Franceschi**

IUCN Red Data list Category

Data Deficient

Ecology

Tree to 18 m tall. Bark yellowish, longitudinally fissured, blaze reddish, sap yellow. Young branchlets quadrangular, slightly puberulent when young, leaves opposite, fruits immature, drupaceous, one seeded. Distribution in India

at Agasthyamalai region of the Western Ghats, Tamil Nadu, in evergreen and semi-evergreen forests, along riverbanks on the Mundanthurai plateau. Flowering and fruiting: March to May (21).

Conservation status

Data Deficient (DD)

This species is named after Dr J.P. Pascal. As the species is ripi cole and found downstream of the Servalar dam, and considered that the species is endangered (22).

Conclusion

Calophyllum species are important for their timber, leaves, roots, fruits and also for the medicinal properties. Plants of the genus are also known for their chemistry, with a variety of secondary metabolites isolated, such as, triterpenes, coumarins, flavonoids, and xanthones. Compounds from the genus have been reported to have cytotoxic, anti- HIV, antisecretory, cytoprotective, antinociceptive, molluscicidal and antimicrobial properties. Many species are important for their seed oil. The regeneration rate of each species is very low. Among these species only three species were evaluated and other species even not evaluated and also data deficient. The approaches to these tree species should change because the genus consists of trees with recalcitrant type seeds also. Recalcitrant seeded plants can be conserved only through *ex situ* mode. The most studied species is *Calophyllum inophyllum* (23). Other species are poorly concerned. Based on the current environmental scenario the trees should be evaluated and make sure that the species are not prone to extinction. Most of the tree species can be propagated only through seeds. So there is an urgent need to study this genus and conserve, regenerate and restore the plants, thus make the environment sustainable.

Acknowledgements

I wish my deep sense of gratitude to Dr. Kamarudeenkunju M. (Late), Associate Professor, Dept. of Botany, University of Kerala, Kariavattom, Thiruvananthapuram for his valuable guidance during this endeavour.

Authors contributions

CRV has done the literature survey, RPB corrected the manuscript & RM contributed towards correction of the manuscript and finalization.

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Polyphenol Compounds and their Benefits of *Mangifera indica* L. (Var. Kottukonam) Grow in varied Seasons and Altitude

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(Received: 04 January 2023; Revised: 12 February 2023; Accepted: 15 February 2023; Published: 20 February 2023)

(Published by Research Trend)

ABSTRACT: Secondary metabolites are bioactive chemical compounds that play a key role in defense and adaptation mechanisms, widely distributed in all parts of plants. Among them carbon-based metabolites such as polyphenols and tannins have greater ecological and economic importance. The major challenge of the present study was to fractionate and quantify the polyphenol composition through HPLC and LC-MS/MS analysis from the leaves of *Mangifera indica* L. (var. kottukonam) collected from three altitudes in four different seasons. The results of the study revealed great variation in the type and concentration of polyphenols with respect to the season and altitude. Pre-reproductive winter and reproductive summer seasons showed higher concentration of polyphenols than south west and north east monsoon seasons, coastal Vizhinjam sample revealed higher concentration of such antioxidant phytochemicals than the other altitude samples. Among different polyphenols Gallic acid concentration is highest in almost all the samples. More stressed conditions resulted to the production of more polyphenols in kottukonam variety of mango.

Keywords: HPLC, LC-MS/MS, *Mangifera indica*, Kottukonam, Polyphenols, Gallic acid.

Abbreviations: HPLC, High Performance Liquid Chromatography; LC-MS/MS, Liquid Chromatography-Mass Spectroscopy; ppm, parts per million; Rt, Retention time.

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most economically important tropical fruit crop belongs to the family Anacardiaceae. It is an evergreen tree well known for its nutritious and sweet fruit designated as 'the King of Fruits' in India. Over 1500 varieties of mangoes were reported globally (Kshirsagar *et al.*, 2018) and their exporting greatly influences the economy of many developing countries. Kottukonam is one of the most popular variety of *Mangifera indica* L. cultivated and restricted only to the southern parts of Kerala especially in the Thiruvananthapuram and Kollam Districts. The fruits of Kottukonam are so sweet, nutritious and more solid after ripening than other common varieties available in our state and is very rich in fibre content also.

Secondary metabolites are bioactive chemical compounds that play a key role in defense and adaptation mechanisms, widely distributed in all parts of plants. Among them carbon-based metabolites such as polyphenols and tannins have greater ecological and economic importance. Polyphenols are omnipresent secondary metabolites present in 5 to 50 gm/kg dry weight of plants and were synthesized via phenyl propanoid pathway. They provide protection to the plants against pathogens, herbivores (Skarpe and Hester 2008) and UV radiations (Tharayil *et al.*, 2011). These compounds are directly related to their pharmacological properties and have the greatest potential of being

valuable to human health (Podsdek and Anna 2007). Epidermological evidence supported the role of polyphenols in diet which in turn play an important role in the prevention of cancers and cardiovascular diseases (Pandey *et al.*, 2009). They also have antioxidant and anti-inflammatory properties could have therapeutic and prophylactic effects for neurodegenerative diseases and these health benefits makes the phenolic compounds become the most researched secondary metabolic compounds (Cory *et al.*, 2018).

Polyphenols having biogenetic precursor are categorised in to; benzoic acid derivatives, hydroxycinnamic acid derivatives and depside. Ferulic, p-Caumaric, simple Coumarins, Caffeic, and Sinapic acids are simple and common phenylpropanoids and are formed through methylation, hydroxylation and dehydration reactions of cinnamic acid. The glycosides such as vanillic, p-hydroxy benzoic acid, protocatechuic etc. are benzoic acid derivatives with antibiotic, analgesic, antipyretic, sedative and cholagogic properties. Flavonoids are a class of polyphenols in plants having the general structure of a 15-carbon skeleton with two phenyl rings and a heterocyclic ring. Fisetin, quersetin, myricetin and kaempferol are the most studied flavonoids. They have a broad spectrum of biological activities in plants including cell signalling, auxin transport and pigmentation. Nutraceutical, medicinal, pharmaceutical and cosmetic applications makes the flavonoids an indispensable part in human life. The other biomolecules such as tannins, alkaloids, saponins and steroids also

plays key role in plants in the protection from predation and are having immense biological potentialities. The availability and concentration of phytochemicals in plants are affected by seasonal variations (Uma *et al.*, 2015; Mervat *et al.*, 2009; Yao *et al.*, 2005; Mayer *et al.*, 1995). The change in amount of secondary metabolites in relation with seasonal and altitude variations in *Mangifera indica* L. (var. Kottukonam) is an underexplored area of research. In this scenario, the present study focused to fractionate and quantify the polyphenol composition through chromatographic techniques such as LC-MS/MS and HPLC analysis from the leaves of *Mangifera indica* L. (var. kottukonam) collected from three altitudes in four seasons.

MATERIALS AND METHODS

Plant and Sample Collection Area. The leaves from *Mangifera indica* L. (var. Kottukonam) with 50 to 55 years old is the study material collected from (1) Venjaramoodu (normal sea level area with Latitude: 8.68 N, Longitude: 76.91 E, Altitude: 53.00m above sea level) (2) Vizhinjam (Latitude: 8.38 N, Longitude: 77 E, 45.00m above sea level) and (3) Kulathupuzha (Latitude: 8° 54'32.46"N Longitude: 77° 3'33.57"E Altitude: 203.6m above sea level). The first two sampling areas belongs to the Thiruvananthapuram district and last one to Kollam of Kerala state in India. Kulathupuzha and Vizhinjam are semi high range and coastal areas respectively. The samples were collected in four seasons such as summer (February – May, average temp. of 32 to 36°C and 135mm pptn. – sample 1), South West Monsoon (June – September, average temp. of 19 to 30°C and 2250 to 2500mm pptn. – sample 2), North East Monsoon (October – November, average temp. of 29 to 35°C and 450 to 500mm pptn. – sample 3) and Winter (December – beginning of February, average temp. of 18 to 28°C and 25mm pptn. – sample 4).

Soxhlet hot continuous extraction. Finely hewed 100g of plant leaves are dried in shade at room temperature, powdered and successively extracted with 100 ml methanol for eight hours using Soxhlet hot continuous extraction method. The filtered extract was concentrated using rotary evaporator (50°C).

Preliminary qualitative screening of polyphenols. The soxhlet extract of samples were screened for the presence of different polyphenolic compounds based on the method of Khandelwal (2007).

Test for tannins. Ferric chloride test: Add a few drops of ferric chloride solution in a small amount of extract which is diluted with distilled water in the ratio 1:4. The presence of tannin were indicated by the appearance of blue or green colour.

Test for phenols. Ferric chloride test: The extract was treated with a few drops of 5% neutral ferric chloride solution, appearance of an intense blue colour resulted the presence of phenols.

Test for flavonoids. Aqueous Sodium hydroxide test: The extract was treated with 1N aqueous NaOH, resulted yellow orange colour revealed the presence of flavonoids.

Test for alkaloids. Wagner's test: The addition of wagner's reagent (1.27g of iodine and 2g of potassium

iodide dissolved in 5ml of water and made up to 100ml with distilled water) to a fraction of extract, appearance of reddish-brown precipitate indicates the presence of alkaloids

Test for saponins. Foam test: A small amount of plant extract was vigorously shaken, formation of persistent foam indicates the presence of saponins.

Test for terpenoids. Liebermann-Burchard test: A few drops of acetic anhydride followed by a little drops of conc. H₂SO₄ were added to the extract (1ml) dissolved in chloroform producing dark green colour confirms the presence of terpenoids.

Test for steroids. To the extracts evaporated to dryness, add a little drops of conc. H₂SO₄ and acetic anhydride; an array of colour changes from yellow, green, brown to black detects the presence of steroids.

Quantification of phenols and flavonoids. Total phenols content were estimated by the method of Mayer *et al.*, 1995. An aliquot of the extracts of four samples in 80% methanol were pipetted out separately and made up to 3 ml with the solvent. Add 0.5 ml Folin-ciocalteu reagent in to it and kept for 3 minutes. Boiling the mixture in a water bath for 1 minute after adding 2 ml of 20% Na₂CO₃. Remove the white precipitate by centrifuging it for few min and the absorbance of the clear light blue solution was recorded at 650 nm against the reagent blank containing 80% methanol (3ml), 0.5 ml Folin's reagent and 2 ml of 20% Na₂CO₃. Blue complex formation is due to the reaction between phenols and an oxidizing agent phosphomolybdate in Folin-ciocalteu reagent. A standard graph of phenols was constructed with pyro catechol by taking absorbance against concentration. The total phenols/gm tissue was determined from the standard graph.

AlCl₃ method with slight modification done the quantification of total flavonoid content in the methanol extract. Concisely 100 µl of extract was mixed with 100 µl of 20% AlCl₃ and add 2 drops of glacial acetic acid. After diluting the mixture with methanol to 3 ml incubated for 45 minutes. Then read the OD at 415 nm against the blank having extract, methanol and glacial acetic acid. Construct the standard curve by using quercetin (50-250 µg/ml) in methanol under the same condition. Total flavonoid were indicated as mg quercetin equivalent/gm of weight (Absorbance = 4.9747 mg quercetin, R² = 0.9846).

Quantification by high performance liquid chromatography (HPLC). The methanol extract of four samples from four seasons and 13 reference compounds (1mg/mL) were filtered through 0.45µm PTFE filter; 10µL was injected into the HPLC system. The analysis was performed on a prominence UFLC system Shimadzu, Japan) containing LC-20AD system controller, phenomenex Gemini C18 column (250 × 4.6mm, 5µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 10µl volume and a diode array detector (SPD-M20A). The mobile phase used was, solvent A: methanol- acetic acid – water (10:2:88, v/v) and solvent B; methanol – acetic acid – water (90:2:8, v/v) with gradient program 0-15min 15% B, 16 -20min 50% B, 21 -35min 70% B, 36-50min 100% B and finally the column was regenerated in 10min. The

injection volume was 10 μ L, and the flow rate was kept at 1 mL/min. The column was maintained at room temperature and eluted fractions were monitored at 280 nm, each calibration solution was analyzed. Sample peaks were identified by comparing with retention times of standard peaks. The results are represented as polyphenols in parts per million. LC Lab solution software was used for data acquisition and analysis.

Quantification using liquid chromatography and mass spectroscopy (LC-MS/MS). The extracts in 80% methanol and 28 reference compounds (catechol, catechin, quinine, naringenin, tocopherol, gallic acid, chlorogenic acid, epicatechin, syringic acid, vanillic acid, caffeic acid, epigallocatechin, ferulic acid, myricetin, quercetin, p-Coumaric acid, luteolin, apigenin, kaempferol, rutin, diadzein, hesperetin, shikimic acid, elagic acid, morin, genistein, cinnamic acid and chrysin were injected to LC-MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) - HPLC (Nexera LC-30AD) equipped with an autosampler (SIL-30AC), temperature-controlled column oven (CTO-20AC) and prominence diode array detector (SPD-M20A) coupled to triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). Working standards were prepared by diluting the stock solution with water concentration ranging from 0.01- 1 μ g/mL. The quantification of all the polyphenols was carried out on Shimadzu Shim-pack GISS C18 column (150 $\times$ 2.1 mm i.d, 1.9 μ m) that used water/ formic acid (100/0.1%) mobile phase for solvent A and 100% methanol for solvent B. Polyphenols were eluted with a linear gradient system as follows: 0.5 – 1.9 min 5% of solvent B, 2.0 – 10.0 min 98% of solvent B, 10.1 – 15 min 98% of solvent B and 15.1 – 17 min 5% of solvent B, a flow rate of 0.3 mL/min, the injection volume was 10 μ L and oven temperature of 40 $^{\circ}$ C. Negative and positive modes of multiple reaction-monitoring (MRM) mode were operated during LC-MS/MS with electrospray ionization (ESI). LC-MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). An interface temperature of 400 $^{\circ}$ C was conditioned for ionization, desolvation line temperature of 300 $^{\circ}$ C, heat block temperature of 400 $^{\circ}$ C, nebulizing gas flow (nitrogen) at 3 L/min and drying gas flow (nitrogen) at 10 L/min. Each calibration solution was analyzed. The results obtained are represented as polyphenols in parts per million.

Statistical Analysis. The data obtained were analyzed statistically by one-way analysis of variance (ANOVA) and t- test ($p < 0.05$). The results were average of five replications and represented as mean $\pm$ SD.

RESULTS AND DISCUSSION

The phytochemical extraction in methanol and their profiling through HPLC and LC-MS/ MS provides an understanding of polyphenols in *Mangifera indica* L. (var. Kottukonam) from three altitudes in four seasons. The qualitative screening of various phytochemicals in 12 samples revealed the presence of phenols, alkaloids, tannins, steroids, flavonoids, terpenoids and saponins (Table 1). The quantification of Carbon based phenolic

compounds such as phenols and flavonoids showed their remarkable variations in these 12 samples (Table 2a & 2b). Phenol (sample 1 Vzm, 47.45 $\pm$ 0.23 mg/gm tissue: sample 1 Kza, 34.87 $\pm$ 0.27 mg/gm tissue: sample 1 Vjd, 28.439 $\pm$ 0.30 mg/gm tissue), (sample 4 Vzm, 45.41 $\pm$ 0.22 mg/gm tissue: sample 4 Kza, 32.23 $\pm$ 0.11 mg/gm tissue: sample 4 Vjd, 25.82 $\pm$ 0.26 mg/gm tissue) and flavonoid (sample 1 Vzm, 56.52 $\pm$ 0.27 mg/gm tissue: sample 1 Kza, 47.36 $\pm$ 0.55 mg/gm tissue: sample 1 Vjd, 41.472 $\pm$ 0.22 mg/gm tissue), (sample 4 Vzm, 58.36 $\pm$ 0.21 mg/gm tissue: sample 4 Kza, 48.32 $\pm$ 0.32 mg/gm tissue: sample 4 Vjd, 38.554 $\pm$ 0.92 mg/gm tissue) concentration were relatively much more in the samples of summer (sample 1) and winter (sample 4) seasons than that are in the south west monsoon and north east monsoon seasons. The additional stress, salinity in coastal region influenced the mango tree and the higher concentration of phenolic compounds in them is the result than the samples of other altitudes in all the four seasons. The result of the present study also an agreement to the previous work (Kale 2010). HPLC and LC-MS/MS analysis of the 12 samples gave more concrete informations about the concentration of different polyphenols in different altitude and seasons.

Thirteen standard phenolic compounds (1 mg/mL) namely (1) Gallic Acid (2) Chlorogenic Acid (3) Syringic Acid (4) p-Coumaric Acid (5) Kaempferol (6) Catechol (7) Apigenin (8) Caffeic Acid (9) Ferulic Acid (10) Cinnamic Acid (11) Quercetin (12) Myricetin and (13) Elagic Acid were analysed by HPLC, their retention time (Rt.) in minutes were 4.27, 23.31, 24.42, 25.98, 34.11, 11.74, 34.72, 23.84, 26.42, 30.39, 31.27, 28.81, and 28.35 respectively (Table 3) & (Fig. 1). Quantification and identification were done by comparing the Retention time and spectra of samples with that of standards.

Fractionation of polyphenols by HPLC in the leaves collected during summer season (sample 1) revealed the presence of gallic acid (Vzm, 5686.518: Kza, 34.294 : Vjd, 4064.68 ppm), chlorogenic acid (Vzm, 658.242 ppm: Kza, 990.934 ppm: Vjd, 888.382 ppm), Syringic acid (Vzm, 24.995 ppm: Kza, 143.623 ppm: Vjd, 11.100 ppm), p-coumaric acid (Kza, 0.604 ppm: vjd, 9.570 ppm), ferulic acid (Kza, 237.534 ppm: Vjd, 38.784 ppm), cinnamic acid (Vzm, 1.404 ppm: Vjd, 1.404 ppm) and quercetin (Vzm, 1.585 ppm) whereas leaves from South West Monsoon (sample 2) indicated the presence of gallic acid (Vzm, 473.831 ppm: Kza, 35.432 ppm: Vjd, 4346.023 ppm), chlorogenic acid (Vzm, 832.826 ppm: Kza, 477.516 ppm: Vjd, 258.802 ppm), syringic acid (Vzm, 67.671 ppm: Kza, 55.769 ppm: Vjd, 18.223 ppm), p-coumaric acid (Vzm, 84.365 ppm: Kza, 39.31 ppm) and ferulic acid (Vzm, 107.403 ppm: Kza, 65.428 ppm) (Table 4a) (Fig. 2a).

Polyphenol profiling in the leaves of *Mangifera indica* L. (var. kottukonam) in North East Monsoon (sample 3) by HPLC revealed the presence of four phenolic compounds namely gallic acid (Vzm, 736.608 ppm: Kza, 4.751 ppm: Vjd, 4499.064 ppm), chlorogenic acid (Kza, 54.213 ppm: Vjd, 84.0797 ppm), syringic acid (Vzm, 266.592 ppm: Kza, 157.05 ppm) and ferulic acid (Vzm, 296.349 ppm: Kza, 186.80 ppm), but the samples

collected during Winter season (sample 4) showed gallic acid (Vzm, 5393.412 ppm: Kza, 8.443 ppm: Vjd, 4334.636ppm), chlorogenic acid (Kza, 495.188 ppm: Vjd, 66.828ppm), syringic acid (Vzm, 26.198 ppm: Kza, 78.807 ppm: Vjd, 2.156 ppm), ferulic acid (Kza, 97.246 ppm: Vjd, 1.308 ppm), cinnamic acid (Vjd, 1.956ppm) and quercetin (Vjd, 1.708ppm) (Table 4b) & (Fig. 2b) From the 12 samples of *Mangifera indica* L. (var. kottukonam), summer (Vzm, 5: Kza, 5: Vjd, 6) and winter (Vzm, 2: Kza, 4: Vjd, 6) samples showed more forms of polyphenols. Gallic acid, a powerful antioxidant found in highest quantity almost in all the samples, followed by Chlorogenic acid (Table 4A and 4B) but which is absent in north east and winter season samples from coastal Vizhinjam region. p-Coumaric acid is totally absent in the samples from coastal area but ferulic acid present in them only in north east monsoon season. Cinnamic acid revealed in the summer samples from Vizhinjam (coastal) and Venjaramoodu (sea level), and Quercetin present only in coastal (Vizhinjam) sample in summer season and winter sample from normal sea level region (venjaramoodu). The diversity in the quantity and forms of phenolic compounds in various seasons and altitudes revealed the influence of environmental factors and is agreed with the result of previous researchers (Mpofu *et al.*, 2006; Yao *et al.*, 2005; Chinnusamy 2003).

To facilitates specific separation, identification and quantification of polyphenols in the 12 leaf samples of *Mangifera indica* L. (var. kottukonam) were subjected to the LC-MS/MS analysis against 28 standard polyphenolic compounds namely (1) Catechol, (2) Catechin (3) Quinine, (4) Naringenin, (5) Tocopherol, (6) Gallic acid, (7) Chlorogenic acid, (8) Epicatechin, (9) Syringic acid, (10) Vanilic acid, (11) Caffeic acid, (12) Epigallocatechin, (13) Ferulic acid, (14) Myricetin, (15) Quercetin, (16) p-Coumaric acid, (17) Luteolin, (18) Apigenin, (19) Kaempferol, (20) Rutin, (21) Diadzein, (22) Hesperetin, (23) Shikimic acid, (24) Elagic acid, (25) Morin, (26) Genistein, (27) Cinnamic acid and (28) Chrysin. The results obtained were represented in Table 5 (Fig. 3).

Polyphenol profiling by LC-MS/MS of sample 1 (summer season) revealed the presence of 24, 20, and 22 different phenolic compounds in Vizhinjam (coastal area), Kulathuppuzha (semi high range area) and Venjaramoodu (normal sea level area) respectively whereas their number in south west monsoon samples (sample 2) are 19 (Vzm), 18 (Kza) and 21 (Vjd) (Table 6a) & (Fig. 4a). Gallic acid and apigenin showed in highest and the lowest quantities respectively in sample 1 and sample 2.

LC-MS/MS analysis of the samples in north east monsoon (sample 3) indicated 22 (vizhinjam), 17 (kulathuppuzha) and 22 (venjaramoodu) phenolic compounds. 20 (vizhinjam), 17 (kulathuppuzha) and 23 (venjaramoodu) are the number in the forms of polyphenols in the leaf sample of winter season (sample 4) (Table 6b) & (Fig. 4b). Chrysin and apigenin showed least quantities and gallic acid revealed the highest concentration in almost all the samples in these two seasons.

LC-MS/MS analysis of the methanol extracts of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes in four seasons revealed the influence of different abiotic stresses in the quantity and types of polyphenols. Gallic acid found in highest amount in almost all the samples, and apigenin and chrysin are in lowest amount. Gallic acid is a natural antioxidant helps to strengthen veins (Singh *et al.*, 2017) and to inhibit the formation of amyloid fibrils- the principal causes of Alzheimer's diseases (Mair 2000). Cysin and apigenin showed almost similar quantity in the samples (Table 6a & 6b) but other polyphenols exhibited greater variations from sample to samples. Variation in polyphenol concentration is a mechanism that helps the plants to overcome or cooperate with various stressful environmental conditions.

Catechin- a natural polyphenol and effective for the treatment of neurodegenerative diseases (Pervin *et al.*, 2018) is found in all the samples except sample in north east monsoon season from coastal area. Chlorogenic acid were absent in all the semi high range area samples in all the four seasons, but present in the samples from coastal Vizhinjam region (except in winter season) and also in sea level region (Venjaramoodu) samples except in south west monsoon season have anti-oxidant and anti-inflammatory properties (Sasaki *et al.*, 2010). Caffeic acid is a well-known antioxidant which boosts immunity and controls lipid levels in blood also showed its presence in all the samples. Flavonoids are phenolic compounds isolated from plants and are valuable for their immense biological potentialities. A flavonoid namely quercetin found in all the 12 samples displayed anti-histamine, anti-cancer and anti-inflammatory activities and have also been found to reduce serum levels of low-density lipoproteins (LDL) and increase high-density lipoproteins (HDL) in addition to the reduction of triglycerides and free fatty acids, attributes to its cardiovascular disease prevention (Semwal *et al.*, 2016; Nabavi *et al.*, 2015). Myricetin another polyphenol found in all samples known to interact with a range of DNA and RNA polymerases, reverse transcriptases, telomerases, kinases and helicases due to its iron-chelating, antioxidant, anti-inflammatory and anti-carcinogenic properties. Reverse transcriptase inhibition activities exposed it as a possible antiviral drug against Rauscher murine Leukemia Virus (RLV) and Human Immunodeficiency Virus (HIV) infections (Crozier *et al.*, 1997). Syringic acid present in all samples except in the semi high range area sample in north east monsoon and winter seasons were well known for its anti-cancer, anti-proliferative, sedative, decongestant and hepato-protective actions (Ramachandran and Raja 2010) and p-coumaric acid found in S1 Vzm, S1 Vjd and S4 Vjd were well documented for its antioxidant behavior reducing the formation of carcinogenic nitrosamines in the stomach. Ferulic acid is well-known for its physiological functions such as anti-microbial, anti-inflammatory and anti-cancer activities were noticed in all samples in summer season, sea level region (venjaramoodu) sample in south west monsoon, coastal and sea level samples in north east monsoon and only in the sea level region sample in

winter season. Ferulic acid acts as a natural protector against UV radiation (Rasouli *et al.*, 2017) and lowers cholesterol level in serum and increases sperm viability.

Kaempferol revealed its presence in all the 12 samples were specific to its potent pharmacological and nutraceutical activities. The consumption of plants containing kaempferol thereby conferring innumerable health benefits in the form of reducing scourge of cardio vascular diseases, cancer and arteriosclerosis due to its antioxidant properties (Saw *et al.*, 2014). It also modulate crucial component in cellular signal transduction pathways associated with apoptosis, angiogenesis, metastasis and inflammation (Bradley and David 2005). Naringenin found in all the samples is anti-inflammatory, anti-oxidant flavonoids and is effective in controlling sepsis, acute hepatitis, fibrosis and cancer (Jin *et al.*, 2017; Qin *et al.*, 2011; Du *et al.*, 2009) and another polyphenol called epigallocatechin possesses many biological activities including angiogenesis (Kondo *et al.*, 2002) also present in all the samples. Tocopherol, a natural antioxidant was absent only in S1 Vjd. Epicatechin -a flavone having direct anti-oxidant property as the scavenger of free radicals or indirectly as a modulator of glutathione peroxidase and superoxidedismutase (Simos *et al.*, 2012) were noticed in all the samples. Hesperetin -another flavonon present in all the twelve samples have anti-oxidant, anti-inflammatory and neuroprotective effect in different models of neurodegeneration (Ikram *et al.*, 2019;

Muhammad *et al.*, 2019). Shikimic acid were commonly utilized as an initial material for industrial synthesis of an antiviral drug, found highest amount in S1 Vjd but absent in the samples of Kulathuppuzha in all the four seasons. Elagic acid with anti-mutagenic and anti-cancer properties are used to reduce the elevated hepatic contents of phospholipids, cholesterol, triglycerides and free fatty acids were present in all samples whereas Morin, a polyphenol found in all samples have anti-cancer (Hyun *et al.*, 2015; Park *et al.*, 2014; Gupta *et al.*, 2013; Manna *et al.*, 2007) anti-diabetic (Sendrayaperumal *et al.*, 2014; Paoli *et al.*, 2013) anti-inflammatory (Dilshara *et al.*, 2016; Dhanasekar *et al.*, 2015; Ola *et al.*, 2014; Qureshi *et al.*, 2012) anti-oxidant (Komirishetty *et al.*, 2016; Saw *et al.*, 2014; Mohammad and Elham 2013; Kim *et al.*, 2010) effects. Genistein found in all samples is an isoflavons used against bladder, breast and prostate cancers (Cory *et al.*, 2018; Arts *et al.*, 2005; Mills *et al.*, 1989) and Chrysin is an effective inhibitor of tumor cell-induced angiogenesis (Fu *et al.*, 2007) were found in all samples of summer season, only in the venjaramoodu sample in south west monsoon season, coastal and sea level region samples of north east monsoon and winter seasons in very few quantities. The presence of several phenolic acids and flavonoids in *Mangifera indica* L. (var. kottukonam) having high biological and pharmacological potentialities related with their antioxidant property against seasonal and altitude stresses.

Table 1: Preliminary polyphenol analysis using methanol extracts of *Mangifera indica* L. (var. kottukonam) leaves from three altitudes [Vizhinjam (Vzm): Kulathuppuzha (Kza): Venjaramoodu (Vjd) in four seasons [sample 1, summer: sample 2, south west monsoon: sample 3, north east monsoon: sample 4, winter] (+++; Strong positive, ++; Moderately positive, +; Low positive, -; Negative].

Phytochemical	Sample 1			Sample 2			Sample 3			Sample 4		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Phenols	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Flavonoids	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Tannins	++	++	+	++	++	+	++	++	+	++	++	+
Alkaloids	+++	++	++	+++	+++	+++	+++	++	++	+++	++	++
Terpenoids	+++	++	++	+++	++	++	+++	++	++	+++	+++	+++
Steroids	+++	++	++	+++	++	++	+++	++	++	+++	+++	+++
Saponins	+	-	++	+	+	++	+	+	++	+	-	++

Table 2a: Total Phenols and Flavonoids from methanol extract of the leaves of *Mangifera indica* L. (var. Kottukonam) in summer (sample 1) and south west monsoon (sample 2) seasons in mg/ gm tissue [Values are mean $\pm$ SD of five independent replications].

Phytochemical	Sample 1			Sample 2		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Phenols	47.45 $\pm$ 0.23	34.87 $\pm$ 0.27	28.439 $\pm$ 0.30	39.28 $\pm$ 0.21	29.11 $\pm$ 0.33	20.355 $\pm$ 0.21
Flavonoids	56.52 $\pm$ 0.27	47.36 $\pm$ 0.55	41.472 $\pm$ 0.22	49.77 $\pm$ 0.25	46.24 $\pm$ 0.26	20.927 $\pm$ 0.18

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 2b: Total Phenols and Flavonoids from methanol extract of the leaves of *Mangifera indica* L. (var. Kottukonam) in north east monsoon (sample 3) and winter (sample 4) seasons in mg/ gm tissue [Values are mean $\pm$ SD of five independent replications].

Phyto Chemical	Sample 3			Sample 4		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Phenols	38.31 $\pm$ 0.33	29.34 $\pm$ 0.51	20.259 $\pm$ 0.99	45.41 $\pm$ 0.22	32.23 $\pm$ 0.11	25.82 $\pm$ 0.26
Flavonoids	47.24 $\pm$ 0.32	43.42 $\pm$ 0.32	18.897 $\pm$ 0.91	58.36 $\pm$ 0.21	48.32 $\pm$ 0.32	38.554 $\pm$ 0.92

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 3: The Retention time (Rt) of standard mixture of polyphenols.

Sr. No.	Polyphenols	Retention Time (in min.)
1.	Gallic Acid	4.27
2.	Chlorogenic Acid	23.31
3.	Syringic Acid	24.42
4.	p-Coumaric Acid	25.98
5.	Kaempferol	34.11
6.	Catechol	11.74
7.	Apigenin	34.72
8.	Caffeic Acid	23.84
9.	Ferulic Acid	26.42
10.	Cinnamic Acid	30.39
11.	Quercetin	31.27
12.	Myricetin	28.81
13.	Elagic Acid	28.35

Table 4a: Quantification of polyphenols in six leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam, Kulathupuzha and Venjaramoodu] in two seasons [Sample 1, summer and sample 2, south west monsoon] by HPLC analysis.

Polyphenols (in ppm)	Sample 1			Sample 2		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Gallic acid	5686.518	34.294	4064.68	473.831	35.432	4346.023
Chlorogenic acid	658.242	990.934	888.382	832.826	477.516	258.802
Syringic acid	24.995	143.623	11.100	67.671	55.769	18.223
p-coumaric acid	-	0.604	9.570	84.365	39.31	-
Ferulic acid	-	237.534	38.784	107.403	65.428	-
Cinnamic acid	1.404	-	1.404	-	-	-
Quercetin	1.585	-	-	-	-	-

Vzm: Vizhinjam (coastal area), Kza: Kulathupuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 4b: Quantification of polyphenols in six leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam, Kulathupuzha and Venjaramoodu] in two seasons [Sample 3, north east monsoon and sample 4, winter] by HPLC analysis.

Polyphenols (in ppm)	Sample 3			Sample 4		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Gallic acid	736.608	4.751	4499.064	5393.412	8.443	4334.636
Chlorogenic acid	-	54.213	84.0797	-	495.188	66.828
Syringic acid	266.592	157.05	-	26.198	78.807	2.156
p-coumaric acid	-	-	-	-	-	-
Ferulic acid	296.349	186.80	-	-	97.246	1.308
Cinnamic acid	-	-	-	-	-	1.956
Quercetin	-	-	-	-	-	1.708

Vzm: Vizhinjam (coastal area), Kza: Kulathupuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 5: The LC-MS/MS analysis of 28 standard polyphenols.

Sr. No.	Polyphenols	Rt (min)	Parent ion (m/z)	Molecular Ion (m/z)	Ion Mode	CE (V)	r ²	Linear Range (µg/L)	DL/QL (µg/L)
1.	Catechol	1.87	111.20	78.95/6410	+ve	-9/-25	0.986	5-50	0.07/0.23
2.	Catechin	6.75	291.20	139.10/165.05	+ve	-15/-13	0.993	5-150	2.20/6.68
3.	Quinine	6.88	325.20	307.10/184.05	+ve	-24/-28	0.996	5-150	0.27/0.81
4.	Naringenin	7.28	273.2	153.05/147.15	+ve	-17/-21	0.992	5-150	0.78/2.37
5.	Tocopherol	12.87	429.50	163.15/205.05	+ve	-22/-23	0.993	5-150	2.31/7.01
6.	Gallic acid	1.91	169.20	125.05/81	-ve	17/17	0.992	5-150	3.20/9.70
7.	Chlorogenic acid	6.81	353.00	191.20/92.90	-ve	16/43	0.997	5-150	1.27/3.85
8.	Epicatechin	6.77	289.00	245.20/205.20	-ve	14/16	0.993	5-150	7.08/21.46
9.	Syringic acid	7.20	197.20	182.20/123.05	-ve	14/24	0.996	5-150	10.44/31.65
10.	Vanillic acid	6.79	167.20	152.10/108.10	-ve	17/19	0.991	5-150	14.35/43.48
11.	Caffeic acid	6.91	179.20	135.15/134.10	-ve	16/30	0.995	5-150	0.49/1.49
12.	Epigallocatechin	2.01	456.90	169.15/125.05	-ve	17/40	0.987	5-50	2.90/8.79
13.	Ferulic acid	7.36	193.20	134.00/178.00	-ve	15/10	0.992	5-50	4.37/13.23
14.	Myricetin	7.65	317.00	151.20/179.20	-ve	25/19	0.995	5-150	0.25/0.75
15.	Quercetin	7.92	301.20	151.05/179.00	-ve	22/18	0.991	5-150	0.62/1.88
16.	p-Coumaric acid	7.34	163.00	119.15/93.10	-ve	15/33	0.992	5-150	2.13/6.46
17.	Luteolin	7.92	285.20	151.10/175.05	-ve	25/25	0.991	5-150	0.27/0.83
18.	Apigenin	8.18	269.20	149.05/151.00	-ve	23/25	0.993	5-150	0.48/1.45
19.	Kaempferol	7.83	285.20	159.15/187.05	-ve	31/27	0.993	5-150	1.44/4.36
20.	Rutin	7.34	609.20	300.00/301.15	-ve	38/34	0.995	5-150	0.08/0.23
21.	Diadzein	7.91	252.90	208.20/224.15	-ve	29/25	0.981	5-150	0.71/2.16
22.	Hesperetin	7.86	301.20	164.10/286.05	-ve	25/18	0.993	5-150	0.57/1.73
23.	Shikimic acid	1.76	172.90	111.20	-ve	11	0.969	5-150	5.79/17.55
24.	Ellagic acid	7.56	300.90	185.10/145.20	-ve	31/40	0.992	5-150	2.28/6.92
25.	Morin	7.74	301.20	151.00/149.15	-ve	20/25	0.994	5-150	0.59/1.79
26.	Genistein	7.81	269.20	133.20/132.05	-ve	31/46	0.990	5-150	0.59/1.79
27.	Cinnamic acid	7.93	147.00	103.05	-ve	13	0.993	5-150	6.60/20.01
28.	Chrysin	8.39	252.90	62.95/143.20	-ve	32/27	0.996	5-150	0.41/1.26

Table 6a: LC-MS/MS identification and quantification of phenolic compounds in *Mangifera indica* L. (Var. kottukonam) leaves collected in two seasons [Sample 1: summer and Sample 2: South West Monsoon] from three altitudes [vizhinjam (coastal), kulathupputha (semi high range) and venjaramoodu (sea level)].

Sr. No.	Phenolic Compound (in ppm)	Sample 1			Sample 2		
		Vzm	Kza	Vjd	Vzm	Kza	Vjd
1.	Catechin	0.473	0.036	0.468	0.043	0.035	0.314
2.	Naringenin	0.045	0.031	0.078	0.05	0.039	0.062
3.	Tocopherol	0.022	0.026	-	0.026	0.028	0.023
4.	Gallic acid	739.055	7.826	269.119	111.832	121.587	318.372
5.	Chlorogenic acid	0.0133	-	0.016	-	-	-
6.	Epicatechin	0.28	0.284	0.615	0.217	0.362	0.647
7.	Syringic acid	0.086	0.002	1.234	0.104	0.154	0.179
8.	Vanilic acid	0.095	0.479	0.749	0.174	0.273	0.152
9.	Caffeic acid	0.003	0.003	0.008	0.0035	0.008	0.014
10.	Epigallocatechin	0.123	0.017	0.07	0.056	0.034	0.036
11.	Ferulic acid	0.12	0.005	0.484	-	-	0.046
12.	Myricetin	1.043	0.06	0.994	0.061	0.032	0.956
13.	Quercetin	1.899	0.277	0.841	0.036	0.003	0.486
14.	p-Coumaric acid	0.001	-	0.023	-	-	-
15.	Luteolin	0.083	0.017	0.018	0.007	0.001	0.029
16.	Apigenin	0.008	0.005	0.006	0.005	0.005	0.005
17.	Kaempferol	0.858	0.179	0.286	0.037	0.011	0.147
18.	Hesperetin	0.202	0.039	0.06	0.012	0.008	0.045
19.	Shikimic acid	11.544	-	79.761	14.196	-	1.452
20.	Elagic acid	33.067	13.13	9.868	4.598	21.126	8.316
21.	Morin	4.523	0.994	2.051	0.126	0.029	1.097
22.	Genistein	0.047	0.013	0.02	0.005	0.006	0.035
23.	Cinnamic acid	0.102	-	-	-	-	-
24.	Chrysin	0.009	0.008	0.009	-	-	0.009

Vzm: Vizhinjam (coastal area), Kza: Kulathupputha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 6b: LC-MS/MS identification and quantification of phenolic compounds in *Mangifera indica* L. (Var. kottukonam) leaves collected in two seasons [S3: north east monsoon and S4: winter] from three altitudes [vizhinjam (coastal), kulathupputha (semi high range) and venjaramoodu (sea level)].

Sr. No.	Phenolic Compound (in ppm)	Sample 3			Sample 4		
		VZM	KZA	VJD	VZM	KZA	VJD
1.	Catechin	-	0.027	0.362	0.042	0.028	0.445
2.	Naringenin	0.037	0.026	0.071	0.062	0.03	0.04
3.	Tocopherol	0.024	0.023	0.023	0.032	0.031	0.023
4.	Gallic acid	266.454	8.926	234.689	1971.493	9.061	437.567
5.	Chlorogenic acid	0.03	-	0.015	-	-	0.024
6.	Epicatechin	0.202	0.481	0.512	0.084	0.471	0.652
7.	Syringic acid	0.444	-	0.527	0.0087	-	1.207
8.	Vanilic acid	0.478	0.003	0.528	0.368	0.405	1.207
9.	Caffeic acid	0.014	0.007	0.014	0.005	0.006	0.021
10.	Epigallocatechin	0.352	0.02	0.079	0.058	0.014	0.074
11.	Ferulic acid	0.116	-	0.111	-	-	0.883
12.	Myricetin	0.63	0.043	0.652	0.127	0.033	1.337
13.	Quercetin	1.044	0.029	0.868	0.64	0.0328	1.298
14.	p-Coumaric acid	-	-	-	-	-	0.257
15.	Luteolin	0.013	0.003	0.023	0.018	0.009	0.027
16.	Apigenin	0.005	0.005	0.006	0.005	0.005	0.005
17.	Kaempferol	0.323	0.04	0.293	0.044	0.0408	0.554
18.	Hesperetin	0.023	0.011	0.075	0.08	0.009	0.123
19.	Shikimic acid	14.61	-	2.006	5.71	-	28.891
20.	Elagic acid	9.759	4.442	11.432	5.633	4.52	3.432
21.	Morin	2.189	0.116	2.041	2.048	0.105	2.825
22.	Genistein	0.035	0.013	0.031	0.034	0.009	0.012
23.	Cinnamic acid	0.001	-	-	-	-	-
24.	Chrysin	0.009	-	0.009	0.008	-	0.009

Vzm: Vizhinjam (coastal area), Kza: Kulathupputha (semi high range area), Vjd: Venjaramoodu (sea level area)

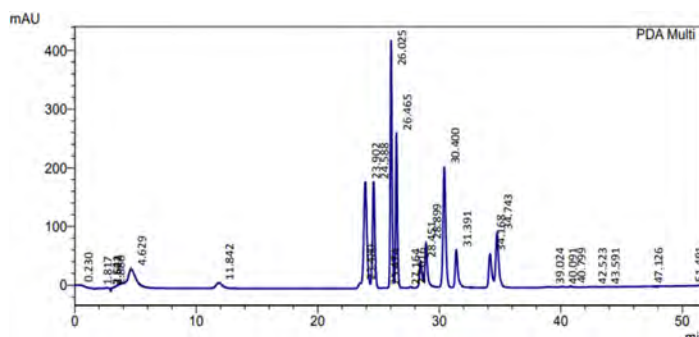


Fig. 1. HPLC chromatogram of standard mixture of polyphenols contains Gallic acid (4.27), Catechol (11.74), Chlorogenic acid (23.31), Caffeic acid (23.84), Syringic acid (24.42), p-Coumaric acid (25.98), Ferulic acid (26.42), Elagic acid (28.35), Myricetin (28.81), Cinnamic acid (30.39), Quercetin (31.27), Apigenin (34.72) with Rt.

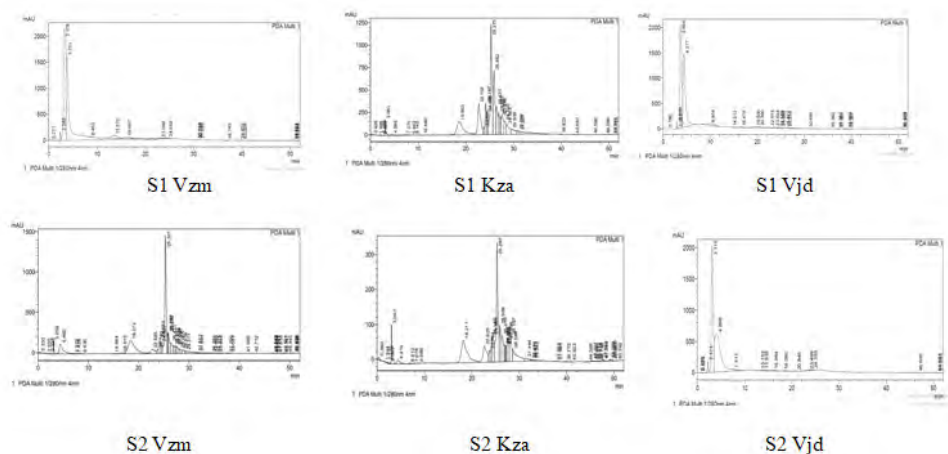


Fig. 2a. HPLC Chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes (Vjm: Vizhinjam, Kza: Kulathuppuzha, Vjd: Venjaramoodu) in two seasons (S1: Summer, S2: South West monsoon).

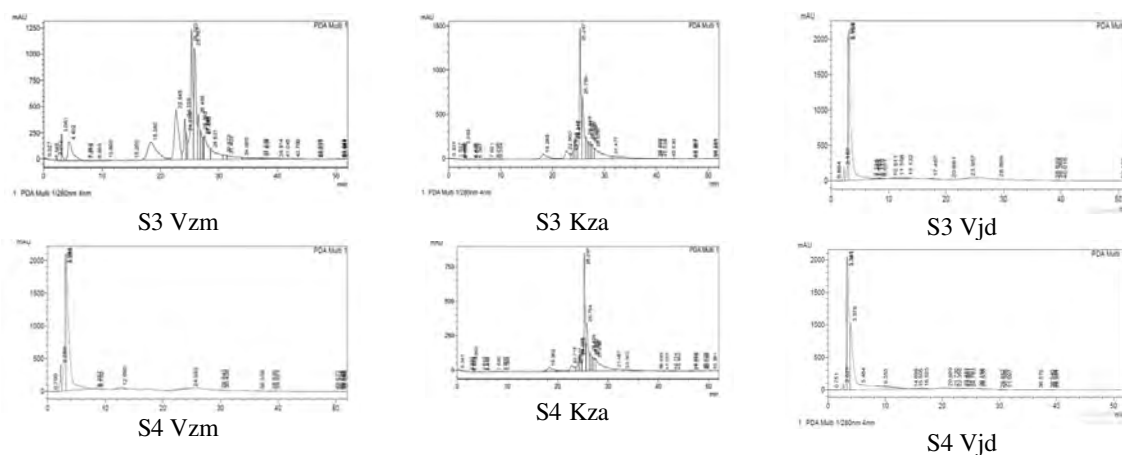
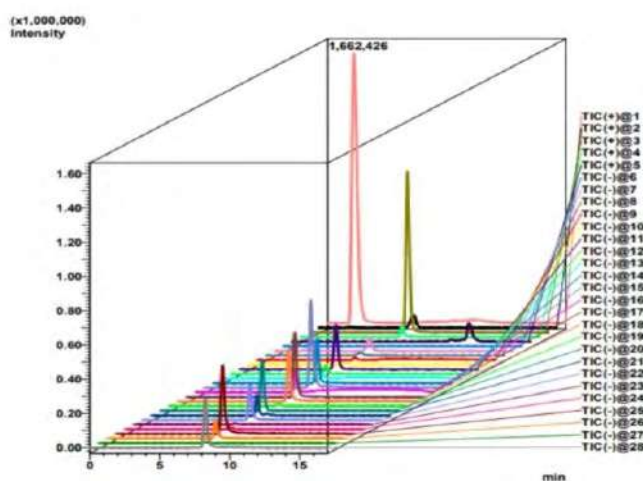


Fig. 2b. HPLC Chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes (Vjm: Vizhinjam, Kza: Kulathuppuzha, Vjd: Venjaramoodu) in two seasons (S3: North East monsoon, S4: Winter)

Fig. 3. LC-MS/MS Chromatogram of 28 standard polyphenols.



1. Catechol, 2. Catechin, 3. Quinine, 4. Naringinin, 5. Tocopherol, 6. Gallic acid, 7. Chlorogenic acid, 8. Epicatechin, 9. Syringic acid, 10. Vannilic acid, 11. Caffeic acid, 12. Epigallocatechin, 13. Ferulic acid, 14. Myricetin, 15. Quercetin, 16. p-Coumaric acid, 17. Luteolein, 18. Apigenin, 19. Kaempferol, 20. Rutin, 21. Diadzein, 22. Hesperitin, 23. Shikimic acid, 24. Elagic acid, 25. Morin, 26. Genistein, 27. Cinnamic acid and 28. Chrysin

Fig. 3. LC-MS/MS Chromatogram of 28 standard polyphenols.

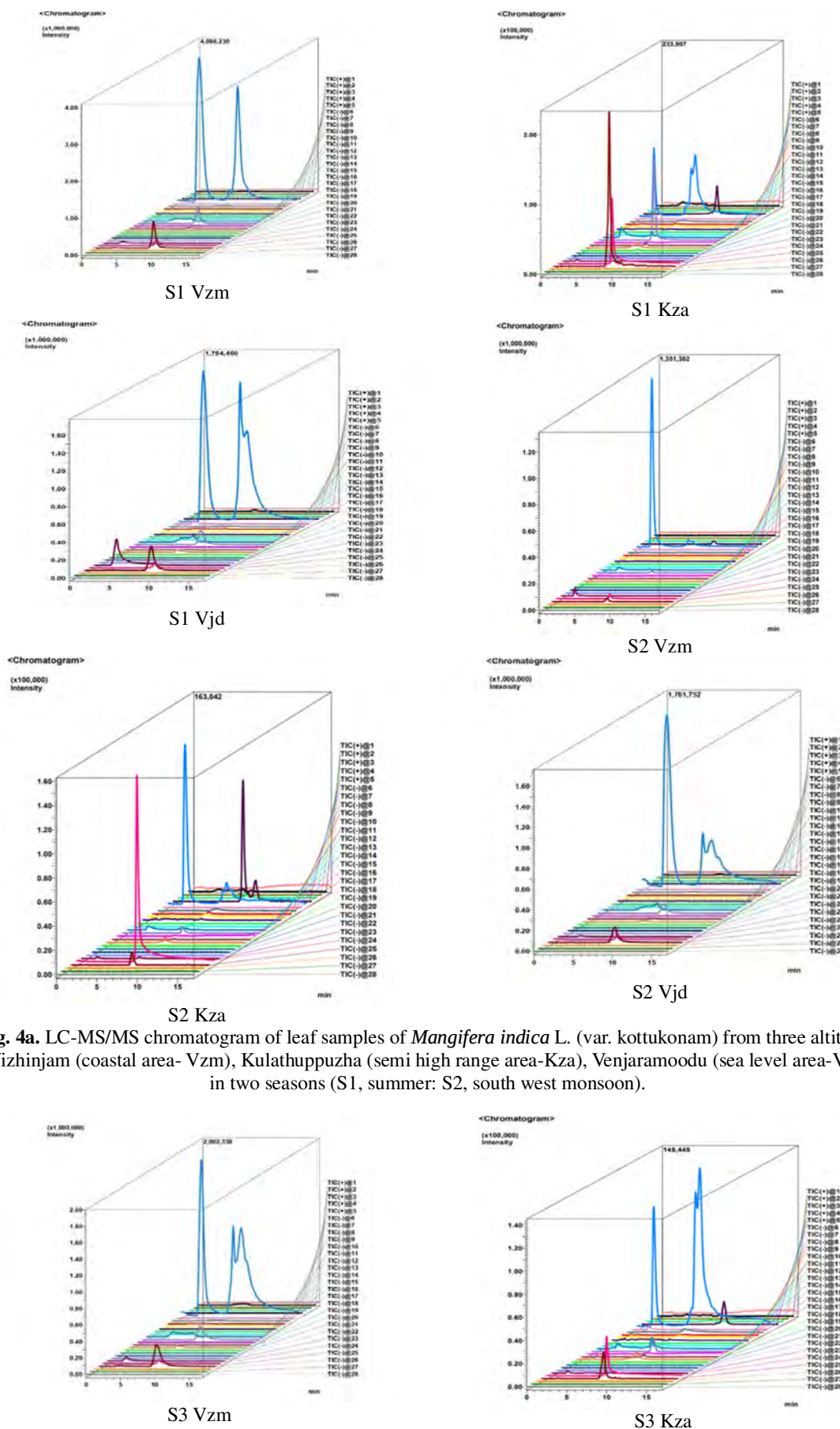


Fig. 4a. LC-MS/MS chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam (coastal area- Vzm), Kulathupuzha (semi high range area-Kza), Venjaramoodu (sea level area-Vjd)] in two seasons (S1, summer: S2, south west monsoon).

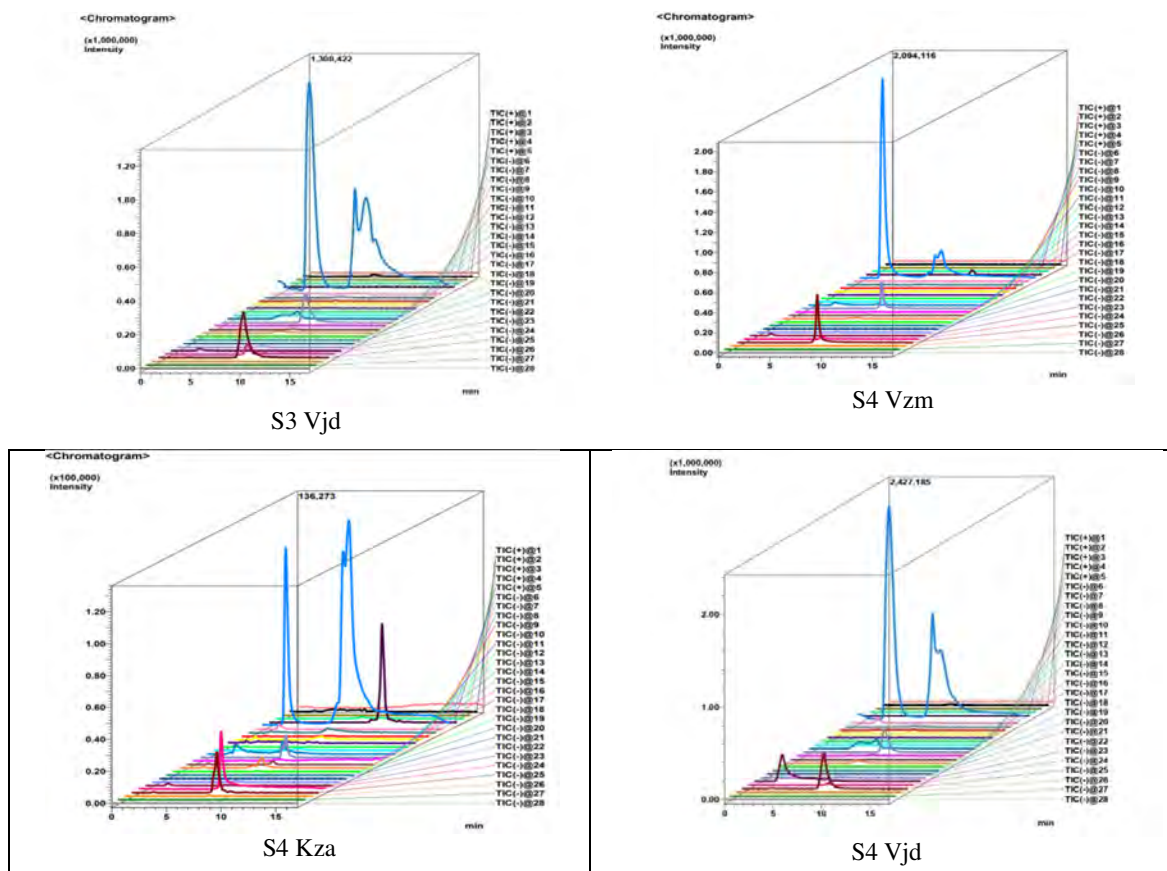


Fig. 4b. LC-MS/MS chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam (coastal area- Vzm), Kulathupuzha (semi high range area- Kza), Venjaramoodu (sea level area- Vjd)] in two seasons (S3, north east monsoon: S4, winter).

CONCLUSIONS

Efforts have been made in the identification, isolation and quantification of bioactive phytochemicals in the last few decades because of their immense therapeutical effects to human life. The results of the study disclosed the presence of an array of phytochemicals such as phenols, flavonoids, tannins, saponins, steroids, terpenoids and alkaloids in *Mangifera indica* L. (var. kottukonam). The major phytochemicals, polyphenols especially phenols and flavonoids showed noticeable level in all the twelve samples of *Mangifera indica* L. (var. kottukonam) and are identified through their specific quantitative assay methods. The LC-MS/MS and HPLC analysis of the methanol leaf extracts of the study material in four seasons from three altitudes against the standard polyphenols confirmed the presence of different phenolic compounds having antioxidant property and these results would serve as a benchmark for future comparative studies in the biochemical changes *Mangifera indica* L. (var. kottukonam) during flowering (reproductive stage) and non-flowering seasons. The quantitative analysis of the actual phenolic compounds present in the samples from summer, south west monsoon, north east monsoon and winter seasons would be facilitated by means of comparison with standard chromatogram, empowering identification and confirmation of polyphenols. The results of the study recommends that more stressed conditions were overcome by the tree by producing different polyphenols (antioxidant phytochemicals) which also will resulted to

the flowering and fruiting.

FUTURE SCOPE

Polyphenols are secondary metabolites with immense biological potentialities. They are very useful to human health and has many medicinal properties. Significant variations in the concentration of different forms of phenolic compounds with respect to season and altitude was noticed in the leaves of kottukonam variety of *Mangifera indica* L. Further studies are needed to extract the phenolic compounds from the leaves of kottukonam in commercial manner and which will support the pharmaceutical industry.

Acknowledgement. Authors are thankful to Dr. Nisha p, Scientist, Mr. Billu, Abraham and Mrs. Heeba S Research Scholars, NIIST, Regional Centre, Pappanamcode, Thiruvananthapuram, Kerala, India for giving facilities and valuable advices in HPLC and LC-MS/MS analysis.

Conflict of Interest. None.

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How to cite this article: Shalaj Rasheed, Raveendran P.B. and Lubaina Abdulhadeef Shereefa (2023). Polyphenol Compounds and their Benefits of *Mangifera indica* L. (Var. Kottukonam) Grow in varied Seasons and Altitude. *Biological Forum – An International Journal*, 15(2): 784-795.

Group Fuzzy Languages and its Generalizations

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Abstract

In fuzzy language theory, every monoid is the syntactic monoid of some fuzzy language. By using this result the properties of fuzzy language can be studied by the algebraic properties of the syntactic monoids. There are so many methods for studying fuzzy languages. We adopt the above method to analyze different class of fuzzy languages and also characterize certain varieties of fuzzy languages. In this paper we give the variety description of group fuzzy languages and also provide the Eilenberg variety theorem for the class of group fuzzy languages. Moreover we described a fuzzy language whose syntactic monoid is isomorphic to the group $\mathbb{Z}_n$.

Keywords: syntactic congruence; group fuzzy language; pseudovariety.

2020 AMS subject classifications: 20M35; 20M07; 68Q45.¹

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¹Received on January 22, 2023. Accepted on August 20, 2023. Published on September 3, 2023. DOI: 10.23755/rm.v48i0.1095. ISSN: 1592-7415. eISSN: 2282-8214. ©Archana V. P. et al. This paper is published under the CC-BY licence agreement.

1 Introduction

The formal study of semigroups has started in the early 20th century. The most relevant and intensively studied classes of semigroups are finite semigroups, regular semigroups and inverse semigroups. The theory of finite semigroups has great importance in the theoretical computer science because of its natural link with finite automata; in the sense that semigroups can arise as syntactic semigroup of regular languages and as transition semigroups of finite automata. This concept is mainly useful to give characterization for regular languages by using the algebraic properties of their corresponding syntactic semigroups. Eilenberg [7] put forward the idea of theory of varieties of rational languages. He establishes a one to one correspondence between the varieties of formal languages and pseudo varieties of finite semigroups through his well known variety theorem.

The notion of fuzzy language was introduced by Lee and Zadeh [9]. It is a generalization of crisp language in the sense that the characteristic function has been replaced by membership function. Since the characteristic function χ_L of a language L is a fuzzy language, every language is a fuzzy language.

The variety of fuzzy languages were introduced by T. Petkovic[11]. A class F of fuzzy language over an alphabet A is called a variety if it is closed under union, intersection, complements, multiplication by constants, quotients, inverse homomorphic images and c -cuts. He proved that there exists a one to one correspondence between variety of fuzzy languages and pseudo variety of monoids. This result is known as Eilenberg variety theorem for fuzzy languages. He also proved that there exists a mutually inverse lattice isomorphism between the lattices of all varieties of fuzzy languages and all varieties of crisp languages. Using this result and Eilenberg variety theorem, he has established a one-to-one correspondence between the lattice of all varieties of fuzzy languages and all pseudo varieties of finite monoids. One of the major result in fuzzy language theory has been considered as every monoid is the syntactic monoid of some fuzzy language[11]. Using this result we study certain classes of fuzzy languages ([2], [3], [4], [5], [6]). The aim of this paper is to provide a variety structure of group fuzzy languages. Also we give the Eilenberg variety theorem for the class of group fuzzy languages. Moreover we described a fuzzy language whose syntactic monoid is isomorphic to the group $\mathbb{Z}_n$.

2 Preliminaries

Here we recall the basic definitions and notations that will be used in the sequel. All undefined terms are as in [10]. A non-empty set S with an associative binary operation is called a semigroup. If there is an element $1 \in S$ with

$1s = s = s1$ for all $s \in S$, then S is called a monoid (semigroup with identity).

Definition 2.1 (cf. [7]). A pseudovariety of monoids (resp. semigroups) is a class $\mathbf{V}$ of finite monoids (resp. semigroups) that is closed under finite direct product, morphic image and subobject. That is,

- (i) If $S, T \in \mathbf{V}$, then $S \times T \in \mathbf{V}$;
- (ii) If $S \in \mathbf{V}$ and $\varphi : S \rightarrow S'$ is a homomorphism, then $\varphi(S) \in \mathbf{V}$;
- (iii) If $S \in \mathbf{V}$ and S' is a submonoid (resp. subsemigroup) of S , then $S' \in \mathbf{V}$.

Let A be a non-empty finite set called an alphabet. Elements of A are called letters. A word is a finite sequence of letters from A . The length of a word is the number of letters in it. A word of length zero is called the empty word, it is denoted by 1. A^+ denote the set of all non-empty words. Then, $A^* = A^+ \cup \{1\}$ together with the binary operation concatenation is called a free monoid(semigroup) on A .

A fuzzy language λ in $A^*(A^+)$ is a fuzzy subset of $A^*(A^+)$. To each fuzzy language λ we associate a congruence P_λ called syntactic congruence, as follows:

For $u, v \in A^*(A^+)$, $uP_\lambda v$ if and only if $\lambda(xuy) = \lambda(xvy)$ for all $x, y \in A^*(A^+)$. The quotient monoid(semigroup), $\text{Syn}(\lambda) = A^*/P_\lambda$ ($\text{Syn}\lambda = A^+/P_\lambda$) is called the syntactic monoid (semigroup) of λ .

A fuzzy language λ over an alphabet A is recognizable by a monoid (semigroup) S if there is a homomorphism $\phi : A^* \rightarrow S$ ($\phi : A^+ \rightarrow S$) and a fuzzy subset π of S such that $\lambda = \pi\phi^{-1}$, where $\pi\phi^{-1}(u) = \pi(\phi(u))$.

For fuzzy languages $\lambda, \lambda_1, \lambda_2$ over an alphabet A , complement, union and intersection are defined respectively by

$$\begin{aligned}\bar{\lambda}(u) &= 1 - \lambda(u), \\ (\lambda_1 \vee \lambda_2)(u) &= \lambda_1(u) \vee \lambda_2(u), \\ (\lambda_1 \wedge \lambda_2)(u) &= \lambda_1(u) \wedge \lambda_2(u).\end{aligned}$$

Further left and right quotients are defined respectively by;

$$\begin{aligned}(\lambda_1^{-1}\lambda_2)(u) &= \bigvee_{v \in A^*} (\lambda_2(vu) \wedge \lambda_1(v)), \\ (\lambda_2\lambda_1^{-1})(u) &= \bigvee_{v \in A^*} (\lambda_2(uv) \wedge \lambda_1(v)).\end{aligned}$$

Let $c \in [0, 1]$ be arbitrary. Then the fuzzy language $c\lambda$ defined by $(c\lambda)(u) = c \cdot \lambda(u)$ is called multiplication by a constant c .

Let A, B be finite alphabets, $\phi : A^* \rightarrow B^*$ ($\phi : A^+ \rightarrow B^+$) be a homomorphism and ψ a fuzzy language in $B^*(B^+)$, then the inverse image of ψ (under ϕ) is a fuzzy language $\psi\phi^{-1}$ over A defined by $(\psi\phi^{-1})(u) = \psi(\phi u)$.

For a fuzzy language λ by a c -cut, $c \in [0, 1]$, we mean the crisp language λ_c defined by $\lambda_c = \{u \in A^* : \lambda(u) \geq c\}$.

The following theorem gives a characterization for regular fuzzy languages.

Theorem 2.1. [10] *A fuzzy language λ is regular if and only if $Im(\lambda)$ is finite and language λ_c is regular for every $c \in [0, 1]$.*

Unless otherwise specified all the fuzzy languages considered here are regular.

Definition 2.2. *A family $\mathcal{F} = \mathcal{F}(A)$ of regular fuzzy languages is a variety of fuzzy languages in $A^*(A^+)$ if it is closed under unions, intersections, complements, multiplication by constants, quotients, inverse homomorphic images and cuts.*

Theorem 2.2. *Let λ be a fuzzy language over an alphabet A . Then we have the following:*

- (i) $Syn(\lambda)$ recognizes λ ;
- (ii) If M is any other monoid (resp. semigroup) recognizing λ , then $Syn(\lambda)$ is a homomorphic image of M .

Proof. (i) Clearly $\eta_\lambda : A^* \rightarrow Syn(\lambda)$ (resp. $A^+ \rightarrow Syn(\lambda)$) is an onto homomorphism. Define $\pi : Syn(\lambda) \rightarrow [0, 1]$ by $\pi(\eta_\lambda(u)) = \lambda(u)$. Then $\lambda = \pi\eta_\lambda^{-1}$ and so $Syn(\lambda)$ recognizes λ .

(ii) Since M recognizes λ , there is an onto homomorphism $\varphi : A^* \rightarrow M$ ($A^+ \rightarrow M$) and a function $\pi' : M \rightarrow [0, 1]$ such that $\lambda = \pi'\varphi^{-1}$. Define $\psi : M \rightarrow Syn(\lambda)$ by $\psi(\varphi(u)) = \eta_\lambda(u)$. Then ψ is an onto homomorphism, since η_λ is an onto homomorphism. Thus $Syn(\lambda)$ is a homomorphic image of M . $\square$

Theorem 2.3. *If the fuzzy languages λ_1 and λ_2 are recognizable by monoids (semigroups), then $\lambda_1 \vee \lambda_2$, $\lambda_1 \wedge \lambda_2$ are recognizable by monoids (semigroups).*

Proof. Let M_1, M_2 be monoids (semigroups), $\varphi_i : A^* \rightarrow M_i$ ($A^+ \rightarrow M_i$), $i = 1, 2$ be onto homomorphisms and $\pi_i : M_i \rightarrow [0, 1]$, $i = 1, 2$ be mappings such that $\lambda_i = \pi_i\varphi_i^{-1}$, $i = 1, 2$. Define $\varphi : A^* \rightarrow M_1 \times M_2$ ($A^+ \rightarrow M_1 \times M_2$) by

$$\varphi(u) = (\varphi_1(u), \varphi_2(u))$$

and $\pi : M_1 \times M_2 \rightarrow [0, 1]$ by

$$\pi(\varphi_1(u), \varphi_2(u)) = \pi_1(\varphi_1(u)) \vee \pi_2(\varphi_2(u)).$$

Then φ and π are well defined mappings and φ is a homomorphism.

For all $u \in A^*$ (resp. A^+), we have

$$\begin{aligned}\pi\varphi^{-1}(u) = \pi(\varphi(u)) &= \pi(\varphi_1(u), \varphi_2(u)) \\ &= \pi_1(\varphi_1(u)) \vee \pi_2(\varphi_2(u)) \\ &= \pi_1\varphi_1^{-1}(u) \vee \pi_2\varphi_2^{-1}(u) \\ &= \lambda_1(u) \vee \lambda_2(u) \\ &= (\lambda_1 \vee \lambda_2)(u).\end{aligned}$$

Thus, $\lambda_1 \vee \lambda_2 = \pi\varphi^{-1}$, and hence $\lambda_1 \vee \lambda_2$ is recognized by $M_1 \times M_2$.
Now define $\pi' : M_1 \times M_2 \rightarrow [0, 1]$ by

$$\pi'(\varphi_1(u), \varphi_2(u)) = \pi_1(\varphi_1(u)) \wedge \pi_2(\varphi_2(u)).$$

Then π' is a well defined mapping. For all $u \in A^*$ (resp. A^+), we have

$$\begin{aligned}\pi'\varphi^{-1}(u) = \pi'(\varphi(u)) &= \pi'(\varphi_1(u), \varphi_2(u)) \\ &= \pi_1(\varphi_1(u)) \wedge \pi_2(\varphi_2(u)) \\ &= \pi_1\varphi_1^{-1}(u) \wedge \pi_2\varphi_2^{-1}(u) \\ &= \lambda_1(u) \wedge \lambda_2(u) \\ &= (\lambda_1 \wedge \lambda_2)(u).\end{aligned}$$

Thus $\lambda_1 \wedge \lambda_2 = \pi'\varphi^{-1}$ and hence $\lambda_1 \wedge \lambda_2$ is recognized by $M_1 \times M_2$. $\square$

Theorem 2.4. *Let λ_1, λ_2 be fuzzy languages over an alphabet A . If a monoid M recognizes λ_1 , then $(\lambda_2^{-1}\lambda_1)$ and $(\lambda_1\lambda_2^{-1})$ are recognized by M .*

Proof. Since M recognizes λ , there exists an onto homomorphism $\varphi : A^* \rightarrow M$ and a function $\pi : M \rightarrow [0, 1]$ such that $\lambda_1(u) = \pi(\varphi(u))$ for all $u \in A^*$. Define $\pi_1 : M \rightarrow [0, 1]$ by

$$\pi_1(m) = \bigvee_{v \in A^*} (\pi(\varphi(vu)) \wedge \lambda_2(v)),$$

where $m = \varphi(u)$. Then $\pi_1(\varphi(u)) = \bigvee_{v \in A^*} (\lambda_1(vu) \wedge \lambda_2(v)) = \lambda_2^{-1}\lambda_1(u)$. So M recognizes $\lambda_2^{-1}\lambda_1$. Similarly $\lambda_1\lambda_2^{-1}$ is recognized by M . $\square$

Lemma 2.1 (cf. [11], Lemma 2). *Let A and B be alphabets, $\lambda, \lambda_1, \lambda_2$ be fuzzy languages over A , τ be a fuzzy language over B , $\varphi : A^* \rightarrow B^*$ be a homomorphism and $c \in [0, 1]$ be a constant. Then*

$$(i) \ P_{\lambda} = P_{c\lambda} = P_{\lambda}, P_{\lambda_1 \vee \lambda_2}, P_{\lambda_1 \wedge \lambda_2} \supseteq P_{\lambda_1} \cap P_{\lambda_2};$$

$$(ii) P_{\lambda_1^{-1}\lambda_2}, P_{\lambda_2\lambda_1^{-1}} \supseteq P_{\lambda_2};$$

(iii) $\varphi \circ P_\tau \circ \varphi^{-1} \subseteq P_\tau \varphi^{-1}$, where $\varphi \circ P_\tau \circ \varphi^{-1}$ is a congruence on A^* defined by $(u, v) \in \varphi \circ P_\tau \circ \varphi^{-1} \Leftrightarrow (u\varphi, v\varphi) \in P_\tau$.

Corolary 2.1. *Let λ be a fuzzy language over A . Then*

$$(i) \text{Syn}(\bar{\lambda}) = \text{Syn}(\lambda) \text{ and}$$

$$(ii) \text{Syn}(c\lambda) = \text{Syn}(\lambda) \text{ for all } c \in [0, 1].$$

Proof. (i) By Lemma 2.1(i), we have $P_{\bar{\lambda}} = P_\lambda$. So $\text{Syn}(\bar{\lambda}) = \text{Syn}(\lambda)$.

(ii) Again by Lemma 2.1(i), $P_\lambda = P_{c\lambda}$ and hence $\text{Syn}(\lambda) = \text{Syn}(c\lambda)$ for all $c \in [0, 1]$. $\square$

For a variety of fuzzy languages $\mathcal{F}$, let $\mathcal{F}^s$ be the family of finite monoids defined by $\mathcal{F}^s = \{\text{Syn}(\lambda) : \lambda \in \mathcal{F}(A), \text{ for some } A\}$. For a variety of finite monoids $\mathcal{S}$, let $\mathcal{S}^f = \mathcal{S}^f(A)$ be the family of fuzzy languages defined by $\mathcal{S}^f(A) = \{\lambda : \lambda \text{ is a fuzzy language over } A : \text{Syn}(\lambda) \in \mathcal{S}\}$.

Theorem 2.5. (cf.[11],Theorem 7) *The mappings $\mathcal{F} \rightarrow \mathcal{F}^s$ and $\mathcal{S} \rightarrow \mathcal{S}^f$ are mutually inverse lattice isomorphisms between the lattices of all varieties of fuzzy languages and all varieties of finite monoids.*

3 Group Fuzzy Languages

A fuzzy language $\lambda \in A^*$ is called group fuzzy language if its syntactic monoid ($\text{Syn}(\lambda)$) is a group. Also if it is regular, then we call as regular group fuzzy language.

Example 3.1 (cf. [8]). *Let G be a finite group and $\varphi : A^* \rightarrow G$ be an onto homomorphism. If $L = \varphi^{-1}(e)$, then χ_L is a group fuzzy language.*

The class of regular group fuzzy languages in A^* is denoted by **GFL**. By the above example **GFL** $\neq \emptyset$.

The following lemma shows that **GFL** is closed under the boolean operations.

Lemma 3.1. *Let $\lambda, \lambda_1, \lambda_2 \in \mathbf{GFL}$. Then $\bar{\lambda} \in \mathbf{GFL}$, $\lambda_1 \vee \lambda_2 \in \mathbf{GFL}$ and $\lambda_1 \wedge \lambda_2 \in \mathbf{GFL}$.*

Proof. Since $\text{Syn}(\lambda) = \text{Syn}(\bar{\lambda})$ (Corollary 2.1 (i)) and $\text{Syn}(\lambda)$ is a group, it follows that $\bar{\lambda} \in \mathbf{GFL}$. Since $\text{Syn}(\lambda_1), \text{Syn}(\lambda_2)$ are groups and $\lambda_1 \vee \lambda_2$ and $\lambda_1 \wedge \lambda_2$ are recognized by $\text{Syn}(\lambda_1) \times \text{Syn}(\lambda_2)$ (Theorem 2.3), we see that $\lambda_1 \vee \lambda_2$ and $\lambda_1 \wedge \lambda_2$ are in **GFL**. $\square$

Next result shows that **GFL** is closed under the inverse homomorphic images.

Lemma 3.2. *Let A be finite alphabet, λ be a fuzzy language on A^* and $\lambda \in \mathbf{GFL}$. If $\varphi : X^* \rightarrow A^*$ is a homomorphism, then $\lambda\varphi^{-1} \in \mathbf{GFL}$, where $\lambda\varphi^{-1}(u) = \lambda(\varphi(u))$ for all $u \in X^*$.*

Proof. Since $\text{Syn}(\lambda)$ recognizes λ , there is a homomorphism $\psi : A^* \rightarrow \text{Syn}(\lambda)$ and a mapping $\eta : \text{Syn}(\lambda) \rightarrow [0, 1]$ such that $\lambda = \eta\psi^{-1}$, where $\eta\psi^{-1}(u) = \eta(\psi(u))$. So $\psi \circ \varphi$ is a homomorphism from X^* into the group $\text{Syn}(\lambda)$ and

$$\begin{aligned} \eta((\psi \circ \varphi)^{-1})(u) &= \eta((\psi \circ \varphi)(u)) \\ &= \eta(\psi(\varphi(u))) \\ &= \eta\psi^{-1}(\varphi(u)) \\ &= \lambda(\varphi(u)). \end{aligned}$$

So $\lambda\varphi^{-1}$ is recognized by $\text{Syn}(\lambda)$. Since $\text{Syn}(\lambda\varphi^{-1})$ is a homomorphic image of $\text{Syn}(\lambda)$, we have $\lambda\varphi^{-1} \in \mathbf{GFL}$. $\square$

Lemma 3.3. *Let $\lambda_1, \lambda_2 \in \mathbf{GFL}$. Then $\lambda_2^{-1}\lambda_1$ and $\lambda_1\lambda_2^{-1}$ are in $\mathbf{GFL}$.*

Proof. By Theorems 2.2 and 2.4, $\lambda_2^{-1}\lambda_1$ and $\lambda_1\lambda_2^{-1}$ are recognized by $\text{Syn}(\lambda_1)$. Since $\text{Syn}(\lambda_1)$ is a group and $\text{Syn}(\lambda_2^{-1}\lambda_1)$, $\text{Syn}(\lambda_1\lambda_2^{-1})$ are homomorphic images of $\text{Syn}(\lambda_1)$, we have $\lambda_2^{-1}\lambda_1$ and $\lambda_1\lambda_2^{-1}$ are in $\mathbf{GFL}$. Hence $\mathbf{GFL}$ is closed under the quotients. $\square$

Lemma 3.4. *Let $\lambda \in \mathbf{GFL}$ and $c \in [0, 1]$. Then $c\lambda \in \mathbf{GFL}$.*

Proof. By Corollary 2.1(i), we have $\text{Syn}(c\lambda) = \text{Syn}(\lambda)$. Since $\text{Syn}(\lambda)$ is a group, we see that $c\lambda \in \mathbf{GFL}$. $\square$

Next result shows that $\mathbf{GFL}$ is closed in c -cuts.

Lemma 3.5. *Let $\lambda \in \mathbf{GFL}$ and $c \in [0, 1]$. Then the syntactic monoid of the language $\lambda_c = \{u \in A^* : \lambda(u) \geq c\}$ is a group.*

Proof. Since $G = \text{Syn}(\lambda)$. Then there is an onto homomorphism $\varphi : A^* \rightarrow G$ and a function $\pi : G \rightarrow [0, 1]$ such that $\lambda = \pi\varphi^{-1}$. Let $P = \{m \in G : \pi(m) \geq c\}$. Then

$$\begin{aligned} \varphi^{-1}(P) &= \{u \in A^* : \varphi(u) \in P\} \\ &= \{u \in A^* : \pi(\varphi(u)) \geq c\} \\ &= \{u \in A^* : \pi\varphi^{-1}(u) \geq c\} \\ &= \{u \in A^* : \lambda(u) \geq c\} \\ &= \lambda_c. \end{aligned}$$

So G recognizes λ_c . Since the syntactic monoid of λ_c ($M(\lambda_c)$) is a homomorphic image of G , $M(\lambda_c)$ is a group. $\square$

Theorem 3.1. (i) **GFL** is a variety of fuzzy languages;

(ii) There exists a one-one correspondence between **GFL** and the pseudovariety **G** of finite groups.

Proof. (i) Follows from Lemmas 3.1, 3.2, 3.3, 3.4 and 3.5.

(ii) Let $\lambda \in \mathbf{GFL}$. Then by definition $\text{Syn}(\lambda) \in \mathbf{G}$.

Conversely, assume that $G \in \mathbf{G}$. Since every monoid is a syntactic monoid of some fuzzy language (cf. [11], Theorem 4), there is a fuzzy language λ such that $\text{Syn}(\lambda) = G$. So $\lambda \in \mathbf{GFL}$. $\square$

Next theorem gives an example of a fuzzy language whose syntactic monoid is a group.

Theorem 3.2. Let $t \geq 1$ and $n > 0$. For $u \in A^*$, define $\lambda_n : A^* \rightarrow [0, 1]$ by

$$\lambda_n(u) = \frac{k}{k+t} \text{ if } |u| \equiv k \pmod{n}.$$

Then $\text{Syn}(\lambda_n)$ is isomorphic to the group $\mathbb{Z}_n$ (under addition modulo n).

Proof. Let $u, v \in A^*$ and let $uP_{\lambda_n}v$. Then $\lambda_n(xuy) = \lambda_n(xvy)$ for all $u, v \in A^*$. If $|xuy| \equiv k_1 \pmod{n}$ and $|xvy| \equiv k_2 \pmod{n}$, then by the definition of λ_n , we have $\frac{k_1}{k_1+t} = \frac{k_2}{k_2+t}$. So $k_1(k_2+t) = k_2(k_1+t)$. That is, $(k_1 - k_2)t = 0$. Since $t \geq 1$, we have $k_1 = k_2$ and so $|xuy| \equiv |xvy| \pmod{n}$. Since $|xuy| = |x| + |u| + |y|$ and $|xvy| = |x| + |v| + |y|$, we see that $||u| - |v|| \equiv 0 \pmod{n}$. Thus $[u]_{\lambda_n} = \{v \in A^* \mid ||u| - |v|| \equiv 0 \pmod{n}\}$. If $w_0 = \epsilon$ and w_i is a word of length i , then

$$[w_0] = \{\epsilon\} \bigcup \{u \in A^+ : |u| = kn, k = 1, 2, \dots\}$$

and for $i = 1, 2, \dots, n-1$

$$[w_i] = \{u \in A^+ : |u| = i + kn, k = 1, 2, \dots\}.$$

So $\text{Syn}(\lambda_n) = \{[w_0], [w_1], \dots, [w_{n-1}]\}$. Clearly $\text{Syn}(\lambda_n)$ is a monoid. Since $[w_0][w_i] = [w_i] = [w_i][w_0]$ for all i , it follows that $[w_0]$ is the identity element in $\text{Syn}(\lambda_n)$. If $i + j \equiv 0 \pmod{n}$, then $[w_i][w_j] = [w_iw_j] = [w_0] = [w_j][w_i]$. So $[w_j]$ is the inverse of $[w_i]$. Thus $\text{Syn}(\lambda_n)$ is a group. Define $\varphi : \text{Syn}(\lambda_n) \rightarrow \mathbb{Z}_n$ by $\varphi([w_i]) = |w_i|$ for all i . Then φ is an isomorphism. $\square$

4 Conclusion

In fuzzy language theory, every monoid is the syntactic monoid of some fuzzy language. With this concept we study different types of fuzzy languages with the algebraic properties of its syntactic monoids. In this paper we give the variety structure of group fuzzy language and also provide the Eleinberg variety theorem for group fuzzy language. We can extend this work into a distributive lattice L , since L -fuzzy languages are generalization of fuzzy languages[1].

Acknowledgments

The authors are thankful to the referees for their valuable suggestions and comments.

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NEOTYPIFICATION OF THE NAMES *TARENNA CANARICA* AND *T. NILAGIRICA* (RUBIACEAE) FROM THE WESTERN GHATS

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Tarenna Gaertner (1788: 139) is one of the largest genera in the Pavetteae tribe of Rubiaceae family (Hooker 1880, Davis *et al.* 2009). It is represented by around 203 species (POWO 2022, Davis *et al.* 2009) with distribution in Asia, Africa and Australia (De Block & Robbrecht 1998, Reynolds & Forster 2005). In India, 15 species are identified till now (Hooker 1880, Gamble & Fischer 1921, <https://efloraindia.bsi.gov.in>), out of that, 8 are reported from southern Western Ghats with 6 endemics (Maya *et al.* 2022).

On the basis of the taxonomic issues in the genus *Tarenna* with *Pavetta* Linnaeus (1753: 110) and *Ixora* Linnaeus (1753: 110), there was a finding and designation of the types of the names of the species. The plants were described during the colonial era without any designation of types. During revisionary studies on *Tarenna* in south India, it has been found that, the original material of *Tarenna canarica* (Beddome) Bremekamp (1934: 189) and *T. nilagirica* (Beddome) Bremekamp (1934: 200) is not extant. During this study, the original material and additional specimens from CAL, MH, DD and from the online databases of K, BM, BR, E, CAL, G, MH, L, US and P were surveyed. Hence, neotypes are designated to both the names following Art. 9.8 of the International Code of Nomenclature (hereafter the ICN, Turland *et al.* 2018).

Typification

Tarenna canarica (Beddome (1872: 134/8) Bremekamp (1934: 189).

≡ *Pavetta canarica* Beddome (Beddome 1872: 134/8); *Webera canarica* (Beddome) Hooker (1882: 106); *Ixora canarica* (Beddome) Kuntze (1891: 278); *Stylocoryne canarica* (Beddome) Gamble & Fischer (1921: 448).

Type: Karnataka, South Canara Ghats (not traceable). **Neotype (here designated):**—INDIA, Kerala, Thiruvananthapuram district, Merchiston, 2000 feet, 10 April 1895, *T.F. Bourdillon* 594 (barcode L2954713, digital image). Figure 1.

Pavetta canarica Beddome (1872: 134/8) was later transferred to the genus *Tarenna* by Bremekamp (1934). In the protologue, Beddome has provided diagnostic features of the species as “glabrous throughout, the flowers pentamerous, fewer and rather larger but with a shorter thicker tube than in typical *siphonanthus*, the style stout hairy in the lower half, not much exerted (Corolla tube 1 inch, style 1 ¾ inch long)” with distribution locality as “*this I have found only on the South Canara ghats*”. However, failed to provide collection details, or illustrations. The collections by R.H. Beddome was housed in BM, K and CAL. Our search at these herbaria were in vain and we were unable to find any specimens associated with *Pavetta canarica*, and therefore no original material for *T. canarica* is known to exist. For this reason, a neotype may be designated as per the Art. 9.8 (Turland *et al.* 2018). A specimen housed at L, collected by T.F. Bourdillon from Thiruvananthapuram, Kerala on 1895 is a well-preserved specimen with flowers and buds; it is congruent with the description mentioned in the protologue and is also consistent with the current application of the name *T. canarica*. Hence it is selected here as the neotype of *T. canarica*.

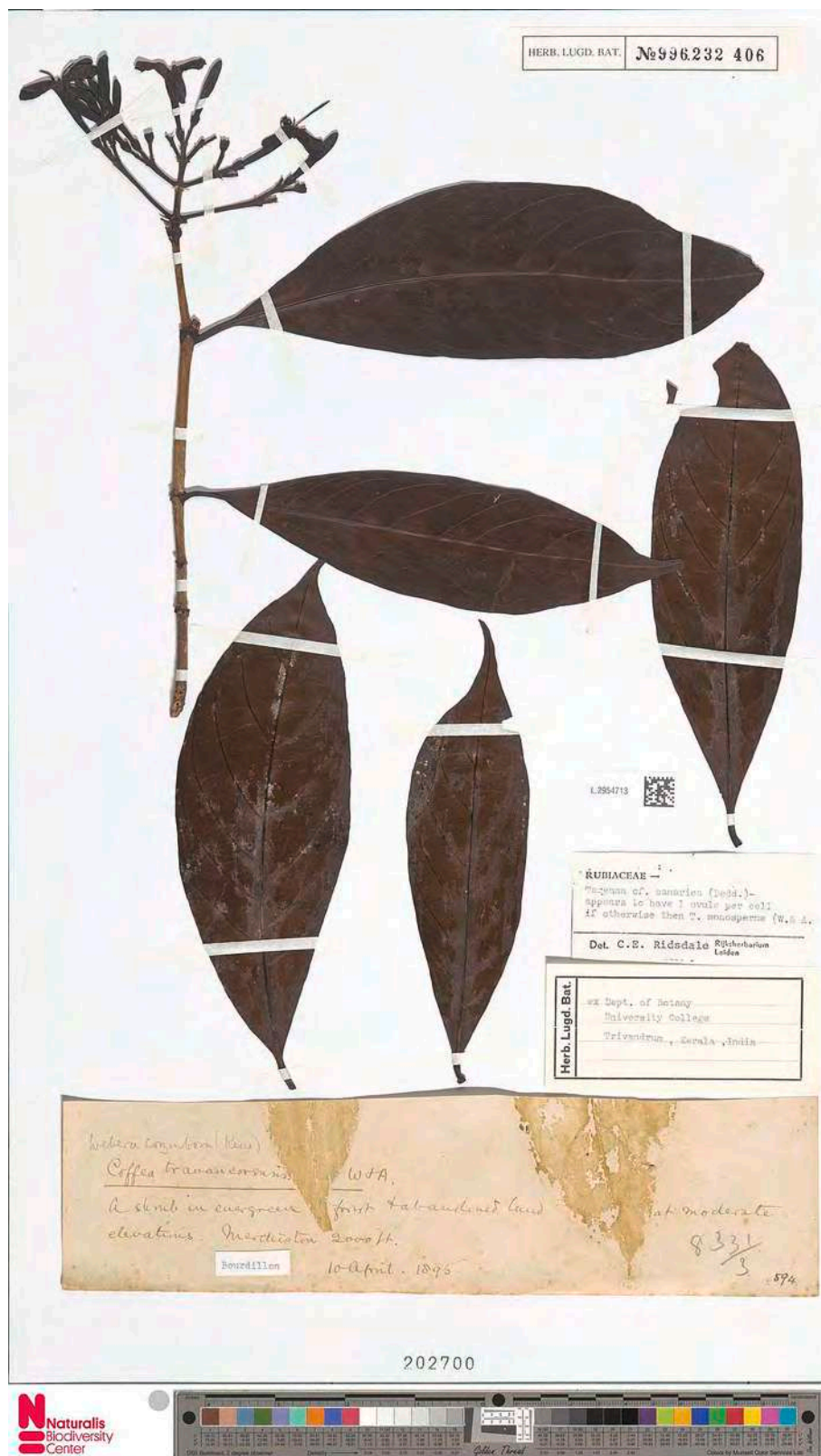


FIGURE 1. Digital image of neotype housed at L (barcode L2954713).

Tarenna nilagirica (Beddome (1872, 134/8) Bremekamp (1934: 200)

≡ *Pavetta nilagirica* Beddome (1872: 134/8); *Webera nilagirica* (Beddome) Hooker (1882: 106); *Stylocoryne nilagirica* (Hooker) Gamble (1921: 448).

Type: Tamil Nadu, Oucherlony Valley and Sispara Ghat of Nilgiri; Kerala Peria peak of Waynnad (not traceable). **Neotype**

(here designated):—INDIA, Kerala, Wayanad, Periya Peak, 1600 ft, 05 May 2022, Maya S. Nair KUBH 10592 (KUBH, barcode 11173, digital image). Figure 2.



FIGURE 2. Digital image of neotype housed at KUBH (barcode 11173).

Likewise, the proceeding species of Beddome described as *Pavetta nilagirica* (Beddome 1872: 134/8), which is now part of *Tarenna*, with the diagnostic features such as “young stem pilose almost scabrous, the leaves pilose beneath, pentamerous flowers and the style included, and glabrous”. Beddome has provided heterogenous collections as “Oucherlony Valley and Sispara Ghat of Nilgiri and Peria peak of Wayanad” without any collection details of original material. All our efforts to trace out original material in any of the herbaria are in vain and it is confirmed that, no original material referred to *Pavetta nilagirica* is extant at present and it warrants the need of neotypification. Hence, one of our collections from the Periya peak of Wayanad (Maya S. Nair 10592) of *T. nilagirica*, which bears flowers and perfectly matching with the description like the pilose young stem, the abaxial surface of the leaves are pilose, flowers are pentamerous and the style is included in the corolla tube as in the protologue, is chosen as neotype (Art. 9.8 of the ICN) and duplicate of the same as isoneotype. The choice is also congruent with the provenance indicated in the protologue.

Acknowledgement

MSN and SKT express sincere thanks to the Principal and PG & Research Department of Botany, Sanatana Dharma College, Alappuzha, Kerala, India and Kerala University, Kerala, India for the support. KMP express his gratitude to the Director, CSIR-National Botanical Research Institute, Lucknow, India for the support. Authors express sincere gratitude to the Curators and staff of CAL, MH, DD, K, BM, BR, E, CAL, G, MH, L, US, P and KUBH herbaria for their support during herbarium reference and also permitting to access their digital images to do this research. The authors further thank the authorities of Kerala Forest Department for permitting access to the forest.

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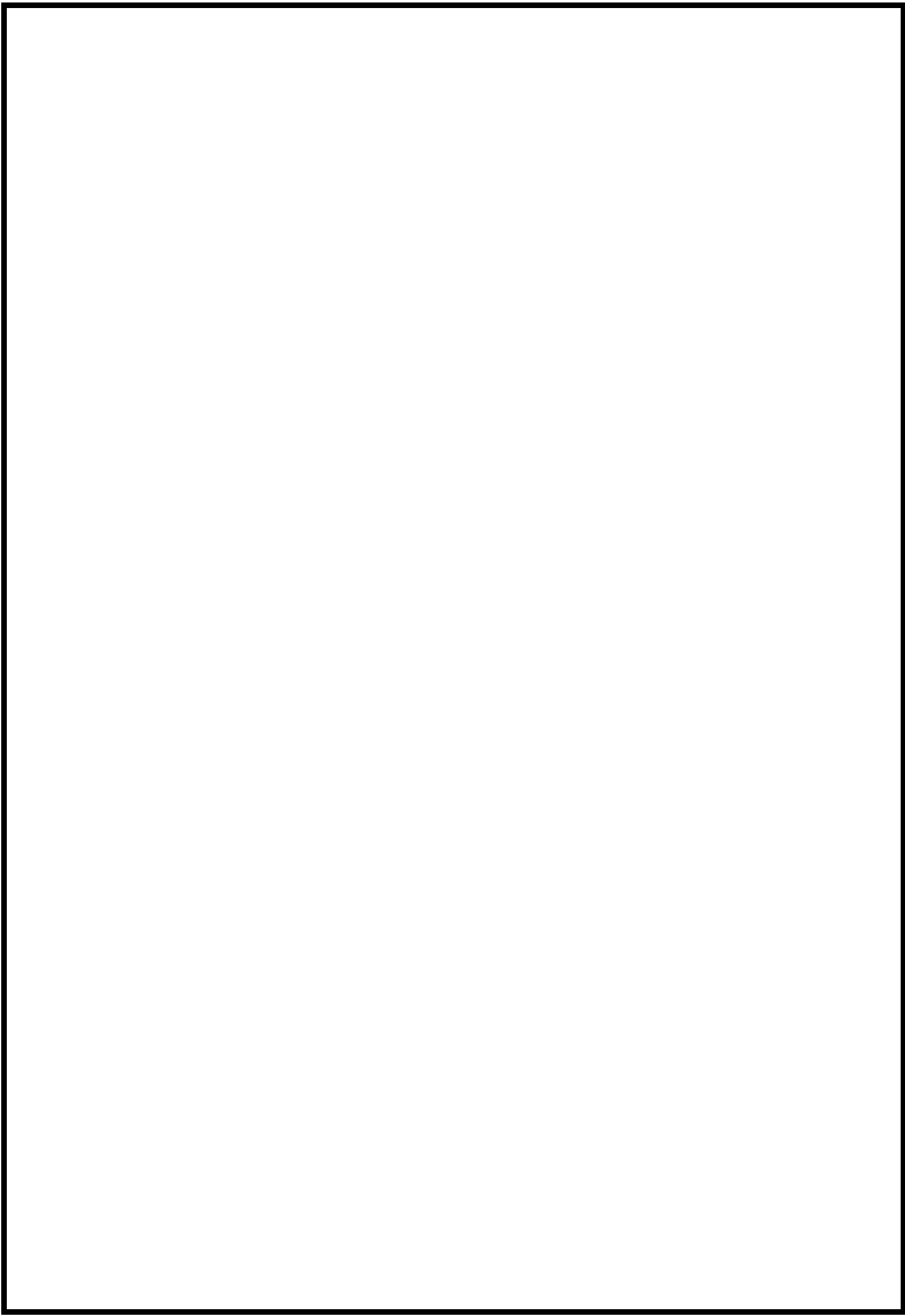
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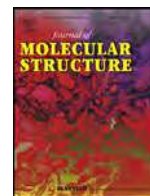
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Crystal structure, DFT studies, Hirshfeld surface and energy framework analysis of 4-(5-nitro-thiophen-2-yl)-pyrrolo [1, 2-a] quinoxaline: A potential SARS-CoV-2 main protease inhibitor

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ARTICLE INFO

Article history:

Received 3 August 2021

Revised 7 October 2021

Accepted 12 November 2021

Available online 15 November 2021

Keywords:

Pyrrolo[1,2-a] quinoxaline

Crystal structure

Hirshfeld surface

DFT

ABSTRACT

The title compound 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) was obtained by a straightforward catalyst-free reaction of 5-nitro-2-thiophene carboxaldehyde and 1-(2-aminophenyl) pyrrole in methanol and was structurally characterized by FT IR, UV-Vis, NMR spectroscopic techniques and elemental analysis. The structure of the compound has been confirmed by the single-crystal X-ray diffraction technique. The compound crystallizes in a monoclinic crystal system with space group P2₁/c. Unit cell dimensions: $a = 12.2009(17)$ Å, $b = 8.3544(9)$ Å, $c = 13.9179(17)$ Å and $\beta = 104.980(5)^\circ$. Hirshfeld surface analysis was carried out to understand the different intermolecular interactions. The two-dimensional fingerprint plot revealed the most prominent interactions in the compound. Theoretical calculations were executed using Density functional theory (DFT) by Gaussian09 software to develop optimized geometry and frontier molecular orbital analysis. Molecular docking studies revealed that the title compound is a potent inhibitor of Main protease 3CL^{pro} with PDB ID: 6LU7, the viral protease which is responsible for the new Corona Virus Disease (COVID-19).

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1. Introduction

SARS-CoV-2 outbreak poses a serious threat to humanity all over the world today. It was declared as a pandemic by World Health Organization on 11 March 2020 [1]. Currently, there are no effective drugs developed for the treatment of this disease. Researches are going on for developing vaccines, druggable molecules, monoclonal antibodies, and cell-based therapies [2]. The chymotrypsin-like cysteine protease also known as Main protease 3CL^{pro} plays an important role in viral replication of SARS-CoV-2 [3]. 3CL^{pro} is the key target in anti-COVID-19 drug design due to its non-similarity with human proteins [3,4].

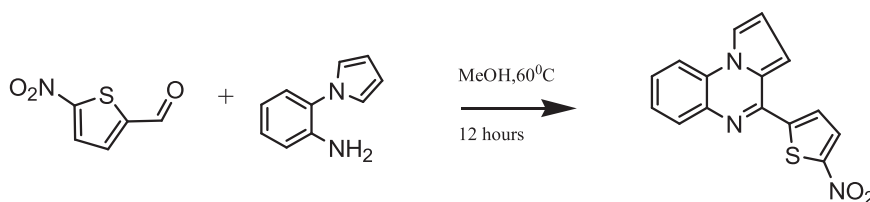
Compounds with quinoxaline ring systems are extensively studied for the last two decades due to their wide range of biological properties. Several quinoxaline derivatives have been reported to possess remarkable pharmacological properties which fight against several diseases with few side effects. They have been described as anti-inflammatory [5], antimalarial [6], antidepressants [7], antiviral [8], antimicrobial [9,10] as antifungal and antibacterial agents.

Pyrrolo [1, 2-a] quinoxalines, an important heterocyclic compound bearing quinoxaline moiety is characterized by a broad range of biological properties [11]. Derivatives of these tricyclic systems have received a great deal of attention for their miscellaneous use in the medicinal field as antileukemic [12], anti-tuberculosis [13], antimalarial [14], anti-Leishmania [15] antidiabetic [16,17] and antibacterial [18] agents. Also, well-established evidence showed that several pyrrolo [1,2-a] quinoxaline-based compounds act as inhibitors against human protein kinase CK2, AKT kinase [19]. Furthermore, suitably functionalized quinoxaline derivatives can act as promising antiviral drugs [8]. At present, research on the synthesis, characterization, and development of new candidates which can effectively bind the Covid 19 Main protease is flying up [4]. In this context, we have been enthused to screen, *in silico*, the interaction between the main protease (6LU7) active site with a pyrrolo [1, 2-a] quinoxaline-based compound.

Methods to synthesize pyrrolo [1,2-a] quinoxalines often involve the use of functionalized aniline precursors [20], transition metal catalysts [21–23], and harsh reaction conditions. We aimed to develop a simple method that avoids the use of expensive transition metal catalysts and dangerous oxidizers. In this paper, we have described the synthesis of a new pyrrolo [1, 2-a] quinoxaline compound, 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) in good yield through the simple coupling of

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Scheme 1. Synthesis of 5NO₂TAAPP

1-(2-aminophenyl) pyrrole and 5-nitro-2-thiophene carboxaldehyde. We also report the single-crystal X-ray structure, spectral characterization, Hirshfeld Surface analysis, and from the docking studies, predicted the binding affinity of this compound to the active site of 6LU7.

2. Experimental

2.1. General characterization techniques

1-(2-aminophenyl) pyrrole and 5-nitro-2-thiophene carboxaldehyde were purchased from Sigma Aldrich. All the chemicals and reagents were of analytical grade and used without further purification. Electronic spectra of the compound were recorded in DMF on a Thermo electron Nicolet evolution 300 UV-Vis spectrophotometer. FT-IR spectra of the compound were recorded as KBr pellets with a JASCO-8000 FT-IR spectrophotometer in the 400–4000 cm⁻¹ range. Elemental analyzes of the compound were done using an Elementar Vario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India. ¹H and ¹³C NMR spectra were recorded in CDCl₃ on a Burkert Advance DRX 300 FT-NMR spectrometer with TMS as the internal standard.

2.2. Synthesis of 5NO₂TAAPP

A methanolic solution (10 mL) of 5-nitro-2-thiophene carboxaldehyde (2 mmol) and 1-(2-amino phenyl) pyrrole (2 mmol) was refluxed in an oil bath at 60 °C for 12 h. On completion of the reaction as observed using TLC (20:80; Ethyl acetate: Hexane), the solvent was evaporated. The crystalline precipitate formed was collected through filtration using a vacuum pump, washed with cold methanol, dried, and recrystallized from methanol. The light-yellow single crystals were collected. The yield and melting point of the product were determined. Yield 80%; m. p 125–127 °C; IR (KBr, cm⁻¹): 3070(CH), 1607(C=N), 1495(NO₂); UV Vis (λ, nm): 228, 315; ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 7.9(1H, *J* = 4.4 Hz, d), 7.4(1H, *J* = 12 Hz, d), 7.1(1H, *J* = 4 Hz, d), 6.7–6.9(4H, m), 6.2(1H, *J* = 3.2 Hz, t), 6.01(1H, *J* = 2.8 Hz, d); ¹³C NMR (DMSO-d₆, δ ppm): 157.9, 149.9, 145.1, 134.8, 130.4, 126.8, 125.5, 125.2, 124.6, 119.4, 116.2, 115.8, 115.2, 110.7, 106.1; Anal. Calcd. For: C₁₅H₉N₃O₂S (295.04) C, 61.01; H, 3.07; N, 14.23; S, 10.86. Found: C, 60.80; H, 3.12; N, 14.16; S, 10.52.

2.3. Crystal structure determination and refinement

Single crystal of the compound 5NO₂TAAPP, suitable for X-ray structure analysis was obtained by slow evaporation at room temperature from its methanolic solution over 24 h. The single-crystal X-ray diffraction studies were carried out using a Bruker AXS Kappa Apex 2 CCD diffractometer, with graphite monochromator Mo Kα radiation (*k* = 0.71073 Å). The unit cell dimensions and intensity data were recorded at 296 K. The structure was solved with the direct method using SIR 92 [24] and refinement was carried out by full-matrix least-squares on F² using SHELXL-97

[25]. The program SAINT/XPREF was used for data reduction and APEX2/ SAINT for cell refinement [26]. Software used for computing molecular graphics ORTEP 3 [27] and Mercury [28]. Software used to prepare material for publication SHELXL-97.

2.4. DFT and Hirshfeld surface analysis

The density functional theory calculations were performed with Gaussian 09 package [29] with the B3LYP exchange-correlation functional and the 6–31 G (d, p) basis set. For the 5NO₂TAAPP optimized structure, Frontier molecular orbitals (HOMO, LUMO) and Mulliken atomic charges were generated using GaussView05 [30]. Hirshfeld surfaces were created using the Crystal Explorer 17 program [31]. The energy framework and interaction energies of the 5NO₂TAAPP molecule were calculated using the TONTO program [32], which is inherent in Crystal Explorer 17 software. The initial geometries were taken from the X-ray data CIF file of 5NO₂TAAPP and used as input files for DFT and Hirshfeld surface analysis.

2.5. Molecular docking

The structure of 3CL^{pro} protein having PDB ID: 6LU7 was retrieved from RCSB Protein Data Bank [33]. An online server, ExPASy protparam which provides all information regarding the protein was used for its characterization. Molecular docking was performed using Autodock 4.2.6 [34]. Both protein and ligand were prepared in pdbqt format. Polar hydrogens and Gasteiger charges were added to the receptor. Grid centered at −19.173, 20.969, 68.039 Å⁰ along x, y, z axes was prepared with 60 X 60 X 60 Å^{0 3} with spacing 0.375 Å⁰. The genetic algorithm was employed as a search parameter with 50 runs, 300 population size and 27,000 number of generations, respectively.

3. Results and discussion

3.1. Characterization

The title compound 4-(5-nitro-thiophen-2-yl)-pyrrolo [1, 2-a] quinoxaline (5NO₂TAAPP) was synthesized (Scheme 1) and characterized. The synthesized compound was crystalline, non-hygroscopic, insoluble in water but soluble in methanol, DMF and DMSO. The compound was characterized by elemental analysis, IR, UV-Visible spectra and NMR analysis. UV-Visible, IR and NMR (¹H and ¹³C) spectra of the title compound are shown in Figs. S1, S2, S3, S4, respectively in the supporting information file.

3.2. Crystal structure of 5NO₂TAAPP

The compound could be crystallized from the slow evaporation of its methanolic solution at room temperature. ORTEP diagram of the compound 5NO₂TAAPP with atom numbering scheme is given in Fig. 1(a). Crystal data and refinement parameters of the compound are given in Table 1. X-ray crystallographic analysis revealed that the compound crystallizes in a monoclinic crystal system with space group P2₁/c. Unit cell dimensions of the crystal are *a* = 12.2009(17) Å, *b* = 8.3544(9) Å, *c* = 13.9179(17) Å

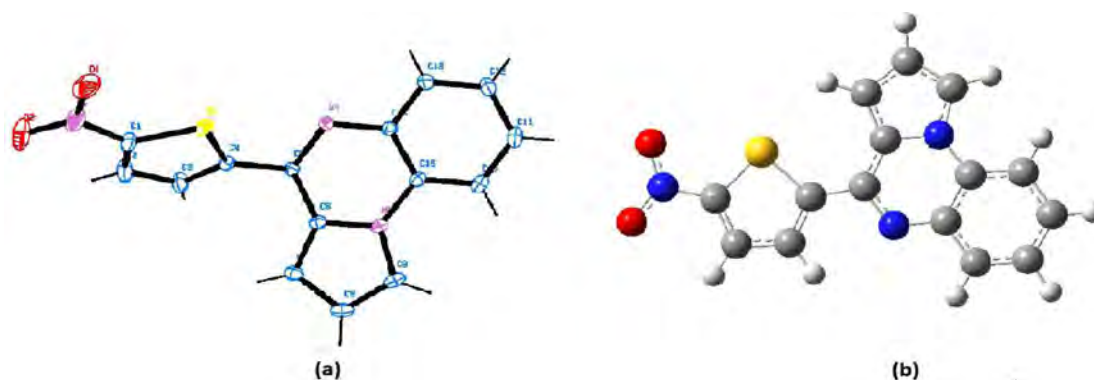


Fig. 1. (a) ORTEP diagram of the compound with thermal ellipsoids drawn at 50% probability level (b) The optimized structure of the compound

Table 1
Crystal data and details of the structure refinement for the title compound.

Parameters	Compound
CCDC number	2,093,318
Empirical formula	C ₁₅ H ₉ N ₃ O ₂ S
Formula weight	295.32
Temperature (K)	296(2)
Crystal system	Monoclinic
Space group	P 2 ₁ /c
a (Å)	12.2009(17)
b (Å)	8.3544(9)
c (Å)	13.9179(17)
Volume (Å ³)	1370.5(3)
Z	4
Calculated density (mg/cm ³)	1.431
μ (mm ⁻¹)	0.244
F(000)	608
Crystal size (mm ³)	0.300 × 0.200 × 0.200
Θ range for data collection (°)	1.728 to 28.420 deg
Index ranges	−16 ≤ h ≤ 11, −10 ≤ k ≤ 10, 18 ≤ l ≤ 18
T _{min} , T _{max}	0.931, 0.953
Reflections collected/unique	11,100 / 3405 [R (int) = 0.0335]
Completeness to theta	25.242, 99.9%
Data/restraints/parameters	3405 / 0 / 190
Goodness-of-fit on F ²	1.130
Final R indexes [I ≥ 2σ (I)]	R1 = 0.0576, wR2 = 0.1683
Final R indexes [all data]	R1 = 0.0855, wR2 = 0.1924
Largest diff. peak/hole (e Å ⁻³)	0.631 and −0.360

Table 2
Selected bond lengths (Å) and angles (°) of the crystal structure of the compound.

Bond	Length (Å)	Bond	Angle (°)
C(1)–S(1)	1.701(3)	N(3)–C(5)–C(4)	108.88(17)
C(4)–C(5)	1.502(3)	N(3)–C(5)–C(6)	108.40(17)
C(4)–S(1)	1.711(2)	C(6)–C(5)–C(4)	111.32(18)
C(5)–N(3)	1.474(3)	C(5)–C(4)–S(1)	120.24(16)
C(6)–N(2)	1.369(3)	C(3)–C(4)–S(1)	112.24(19)
C(9)–N(2)	1.375(3)	C(14)–N(3)–C(5)	116.96(17)
C(14)–N(3)	1.398(3)	C(6)–N(2)–C(15)	122.75(19)
C(15)–N(2)	1.406(3)	C(1)–S(1)–C(4)	89.55(13)
N(1)–O(1)	1.221(4)	O(1)–N(1)–C(1)	117.2(3)
N(1)–O(2)	1.217(4)	O(2)–N(1)–C(1)	118.5(4)

and $\beta = 104.980(5)^\circ$. Bond lengths and bond angles obtained from crystal data are summarized in Table 2.

In the crystal structure of the compound, all C–C and C–H bond lengths in the benzene ring are in the normal range and bond angles are approximately 120° . The bond lengths and bond angles in the pyrrole and thiophene ring are comparable to other previously reported crystal structures [35]. The angle between the benzene ring and quinoxaline ring C(13)–C(14)–N(3) and C(10)–C(15)–N(2)

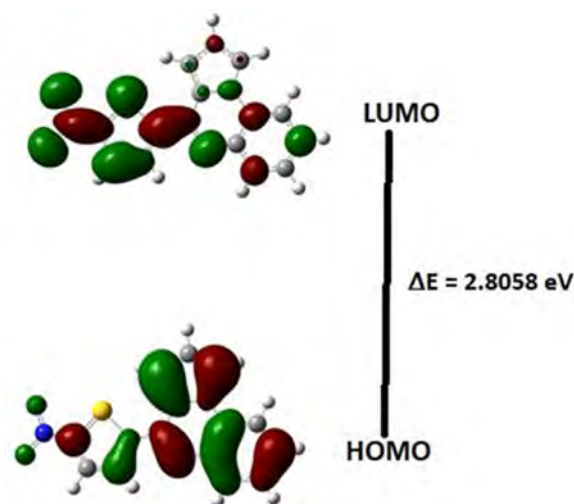


Fig. 2. HOMO-LUMO and energy gap of the title compound

are 120.6° and 122.7° , respectively. The thiophene ring joined to the quinoxaline ring through the bonds N(3)–C(5)–C(4), C(6)–C(5)–C(4) at an angle of 108.88° , 111.32° , respectively. The torsion angle between the quinoxaline ring and the thiophene ring is -25.3° and -85.1° for the atoms S(1)–C(4)–C(5)–N(3) and C(3)–C(4)–C(5)–C(6), respectively. CCDC deposition number 2,093,318 contains supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

3.3. Geometry optimization, frontier molecular orbitals and mulliken charges distribution

The optimized geometry of the compound is given in Fig. 1(b). Frontier molecular orbital analysis was carried out which provide an insight into its biological potential and chemical reactivity [36,37]. The highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energies were calculated to be $E_{\text{HOMO}} = -6.0504$ eV and $E_{\text{LUMO}} = -3.2446$ eV. The HOMO – LUMO energy gap is 2.8058 eV. By using frontier molecular orbital energy values, the global reactivity descriptors such as hardness (η), chemical potential (μ), softness (S), electronegativity (χ) and electrophilicity index (ω) have been defined [38, 39]. The frontier molecular orbital and their energy gap for the compound is shown in Fig. 2. Table 3 lists the energy gap and the other global reactivity descriptors for the title compound.

Mulliken charge distribution within the molecule is very significant because it affects the overall stability, reactivity, electronic

Table 3
HOMO –LUMO and global reactivity descriptors of the title compound.

Parameter	Value
E_{HOMO}	−6.0504 (eV)
E_{LUMO}	−3.2446 (eV)
Energy gap (ΔE)	2.8058 (eV)
Ionization potential (I)	6.0504 (eV)
Electron Affinity (A)	3.2446 (eV)
Chemical Hardness (η)	1.4029 (eV)
Global softness (σ)	0.3564 (eV ^{−1})
Electronegativity (X)	4.6475 (eV)
Chemical potential (μ)	−4.6475 (eV)
Electrophilicity (ω)	7.698 (eV)

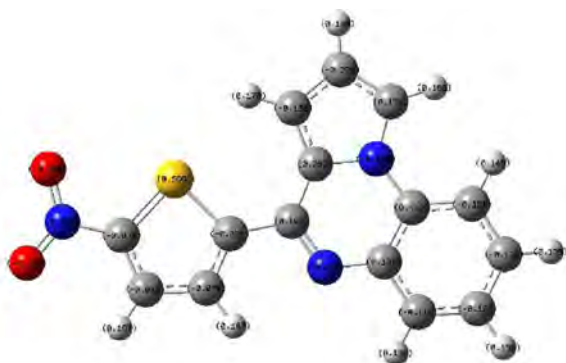


Fig. 3. Mulliken charge distribution for the optimized compound

structure and more properties of the molecular system [40]. The net atomic charges 5NO₂TAAPP molecule obtained by using Mulliken population analysis, are plotted in Fig. 3. The result indicates that the S atom has the highest positive charge, with the corresponding value of charge +0.599. The two nitrogen atoms in the quinoxaline ring have the highest electronegativity and the lowest net atomic charge values (−0.848 e and −0.477 e). Therefore, it is anticipated that the quinoxaline ring as a whole or the individual nitrogen atoms within the ring can interact through hydrogen bonds or other intermolecular forces with the suitably positioned amino acid residue in the target protein. In addition, the carbon atoms in the thiophene ring (−0.321, −0.039, −0.074, −0.079 e) and oxygen atoms of the nitro group (−0.302, −0.295 e) also have relatively low atomic charges, may also be involved in prolific binding interactions with the enzymes.

3.4. Hirshfeld surface analysis

Hirshfeld surface analysis enables us to understand the packing modes, the surface of the molecular system and intermolecular interactions in crystals. Hirshfeld surface volume and surface area of the compound are 335.91 Å³ and 312.47 Å², respectively. The globularity and the asphericity for the shape are 0.748 and 0.213, respectively. In the Hirshfeld surface mapped over normalized contact distance d_{norm} (Fig. 4), the white surface area indicates the contact with distances equal to the sum of van der Waals radii, and the red and blue colours indicate the distances shorter (in close contact) or longer (distinct contact) than the van der Waals radii, respectively [41].

The 2D fingerprint plot permit us to calculate the percentage contribution of each type of contacts for the total Hirshfeld surface area. The H . . . H contacts are responsible for the largest contribution (30.6%) to the Hirshfeld surface. Beside these contacts, H . . . O/O . . . H (24.8%), H . . . C/C . . . H (14.4%), H . . . S/S . . . H (7.3%), H . . . N/N . . . H (5.6%), C . . . C (5.5%), N . . . C/C . . . N (4.6%) and S . . . C/C . . . S (3.1%) interactions contribute considerably to the total Hirshfeld

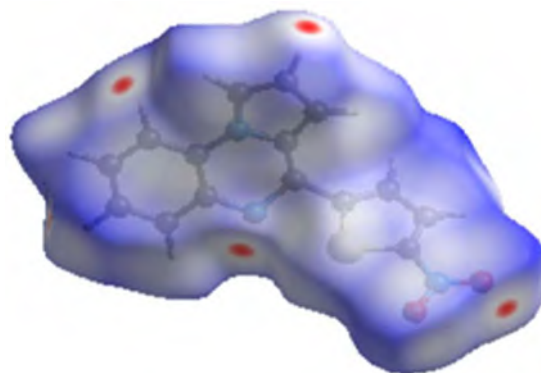


Fig. 4. Three-dimensional Hirshfeld surface of the title compound plotted over d_{norm} in the range −0.1039 to 1.4949 a. u.

surface area. The 2D fingerprint plot for all major contacts are depicted in Fig. 5. The contributions of other contacts are only minor and add to N . . . N (0.1%), S . . . O/O . . . S (0.1%) and S . . . N/N . . . S (0.1%).

Hirshfeld surface plotted over shape index, curvedness and electrostatic potential map (Fig. 6.) give more details about the shape and molecular packing in crystals. The shape index map (Fig. 6a.) of the title compound was generated in the range of −1 to 1 Å². The surface around the acceptor atoms are indicated by the blue colour regions and the surface around the donor atoms are indicated by red colour regions in the shape index map. The curvedness map (Fig. 6b.) of the title compound was generated in the range −4 to 4 Å². The large green regions indicate a planar surface area, while the blue regions reveal the areas of curvature. The presence of π – π stacking interactions is also evident as the flat regions around the thiophene and benzene ring on the Hirshfeld surface plotted over curvedness. In the electrostatic potential map (Fig. 6c.) over the Hirshfeld surface, the electropositive regions (around hydrogen bond donor) are indicated by blue colour; whereas red colour regions are electronegative regions (around hydrogen bond acceptor) [42].

3.5. Energy framework analysis

The intermolecular interaction energies for the title compound was calculated using CE-B3LYP/6–31 G(d, p) energy model available in crystal explorer with scale factors $k_{\text{ele}} = 1.057$, $k_{\text{pol}} = 0.740$, $k_{\text{disp}} = 0.871$, $k_{\text{rep}} = 0.618$, respectively [31]. The different interaction energies, coulombic interaction energy (red), dispersion energy (green), total interaction energy (blue) of the compound are depicted in Fig. 7. The magnitude of the interaction energy is proportional to the radii of the corresponding cylinder. Table 4 list the result of different interaction energies of the title compound, rotational symmetry operations to the reference molecule (Symop), the centroid to centroid distance between the reference molecule and the interacting molecule (R) and the number of pairs of the interacting molecule to the reference molecule (N).

The calculated electrostatic, polarization, dispersion, repulsion energies are −45.0 KJ/mol, −22.3 KJ/mol, −179.1 KJ/mol and 82.7 KJ/mol, respectively. The calculated total energy of the molecule is −154.7 KJ/mol. The result revealed that dispersion energy is predominant over the other interaction energies and have a key role in the total forces in the crystal packing.

3.6. Docking analysis

The purpose of this docking analysis was to assess the binding affinity of the amino acids within the 3CLP^{pro} active site to the target ligand 5NO₂TAAPP. The Auto Dock tool was used to perform the

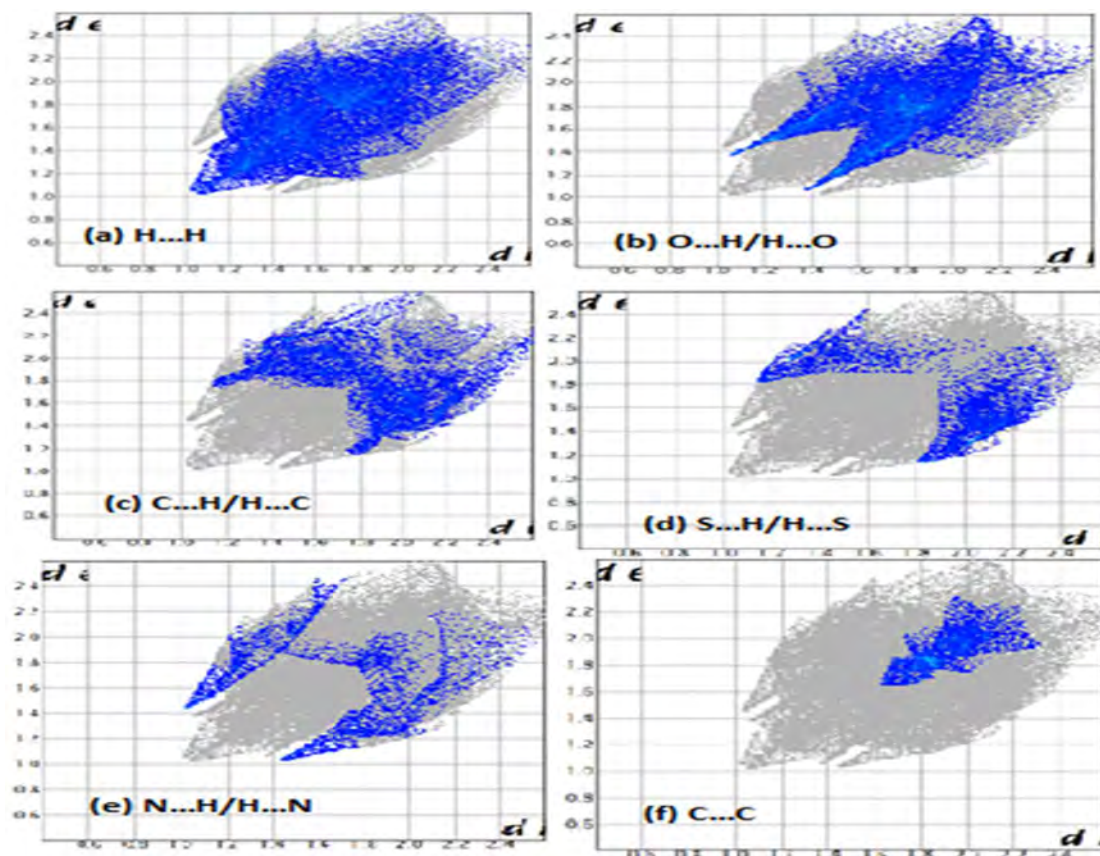


Fig. 5. 2D fingerprint plots for all major contacts on Hirshfeld surface. d_i and d_e denote the closest internal and external distances in Å from a point on the surface.

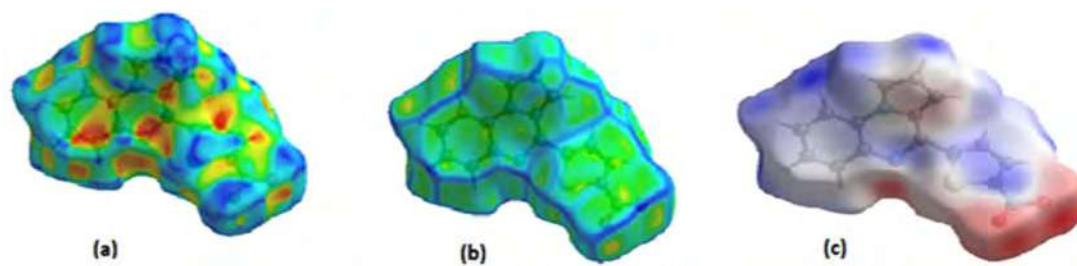


Fig. 6. Hirshfeld surface mapped over (a) shape index (b) curvedness (c) electrostatic potential

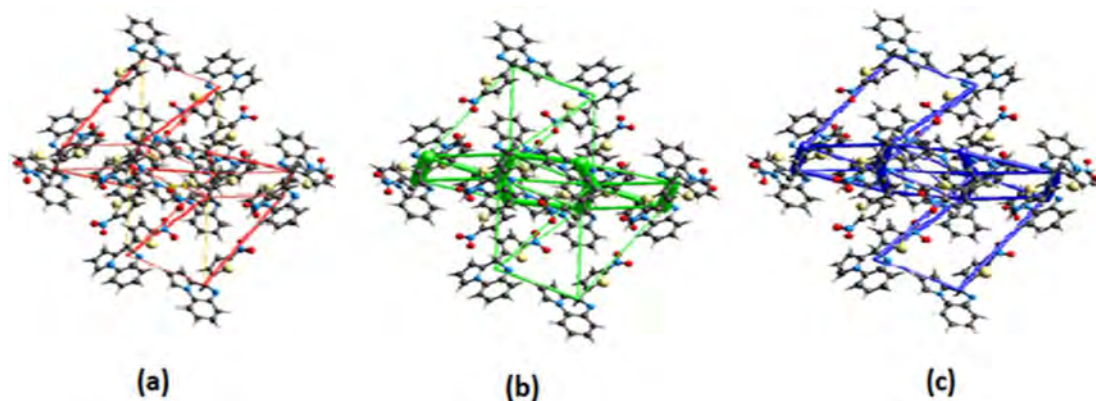


Fig. 7. Graphical representation of electrostatic interactions (a) coulomb interaction energy (b) dispersion energy (c) total energy of the title compound

Table 4
Different interaction energies of compound in KJ/mol.

N	Symop	R (Å ⁰)	Eele	Epole	Edis	E rep	Etot
1	-x, -y, -z	13.42	2.1	-0.5	-3.1	0.0	-0.9
2	x, -y + 1/2, z + 1/2	12.65	-15.6	-3.7	-8.3	5.3	-21.4
2	x, y, z	12.20	5.4	-2.4	-10.1	5.7	-0.6
2	-x, y + 1/2, -z + 1/2	13.80	0.5	-0.2	-2.0	0.0	-1.4
1	-x, -y, -z	7.21	-12.4	-2.9	-15.8	5.3	-24.5
2	-x, y + 1/2, -z + 1/2	4.29	-8.7	-4.0	-71.2	34.5	-47.6
1	-x, -y, -z	7.20	1.0	-4.0	-36.7	20.5	-18.0
2	x, -y + 1/2, z + 1/2	7.43	-6.1	-2.1	-18.8	9.8	-16.7
1	-x, -y, -z	11.56	-7.9	-2.2	-9.6	1.5	-16.9
1	-x, -y, -z	9.76	-3.3	-0.3	-3.5	0.1	-6.7

**Fig. 8.** The pictorial representation of the compound 5NO₂TAAPP bound inside the pocket of SARS-CoV-2 main protease 3CL^{pro}.**Table 5**
Characterization of protein.

Properties	Values
molecular weight	333,797.64 KDa
energy	-16,473.465 kJ mol ⁻¹
Number of amino acids	306
Theoretical pI	5.95
Instability index	27.65
Aliphatic index	82.12
GRAVY	-0.019
Resolution	2.16 Å

docking of the ligand into the catalytic binding site of SARS-CoV-2 3CL^{pro} with PDB ID: 6LU7 (Fig. 8). The selected protein was characterized by primary and secondary structure analysis as shown in Table 5.

The isoelectric point (pI value) 5.95 indicates that the protein is slightly acidic. The relative volume of a protein occupied by its aliphatic side chains is termed as the aliphatic index (AI). The aliphatic index plays role in protein thermal stability. A high

**Fig. 9.** Interaction of 5NO₂TAAPP with SARS-CoV-2 main protease.

aliphatic index specifies that the protein is thermally stable over a wide temperature range. Aliphatic index in the range of 66.5 to 84.33 indicate high thermal stability and hydrophobicity of protein, which help them for biological membrane perturbation [43]. The Grand average of hydropathy (GRAVY) value is a measure of the hydrophilic nature of protein [44]. The value obtained for GRAVY is -0.019, which is close to zero, indicate the hydrophobic nature of the protein. The instability index is an assessment of the stability of a protein experimentally. A protein whose instability index is smaller than 40 is predicted as stable [45]. These results indicated that 6LU7 is a stable, hydrophobic protein.

The binding affinity of the inhibitor (ligand or drug) is an important parameter, which determines the strength of its binding interaction with the target protein or the biomolecule. These interactions can be of many kinds such as hydrogen bonding, electrostatic interactions, hydrophobic and van der Waals forces. The 2D interaction diagram of the compound with the target 6LU7 is shown in Fig. 9. Compound forms two hydrogen bonds with GLY 143 and CYS 145 through an oxygen atom of the nitro group; van der Waals interactions with GLU 166, HIS 164, GLN 189, ASP 187, ARG 188, PRO 52, TYR 54, HIS 41, SER 144, ASN 142; π -sulphur interaction by Sulphur atom of CYS 44 with the π electron cloud of benzene ring; and a π -donor hydrogen bond interaction with the -SH group of CYS 145. MET 165 and MET 49 form π -alkyl bonds with the compound.

The binding affinity of a ligand with a protein is measured in terms of binding energy. The negative value of binding energy indicates a release of energy while forming a protein-ligand docked complex, which imparts stability. The more negative the binding energy, the higher will be the stability and binding affinity [46]. The binding energy obtained for the compound 5NO₂TAAPP

with 6LU7 is $-7.95 \text{ Kcal mol}^{-1}$. Hydroxychloroquine and remdesivir, which were approved drugs as inhibitors to SARS-CoV-2 having binding affinity values -6.06 and $-4.96 \text{ Kcal mol}^{-1}$, respectively [47]. In general, molecular docking results reveals that there should be effective hydrogen bonding and other hydrophobic interactions between the compound and the target protein. These interactions together with the predicted binding affinity, indicating that the compound 5NO₂TAAPP should show significant SARS-Cov-2 inhibitory activity.

4. Conclusion

The new biologically active compound, 4-(5-nitro-thiophen-2-yl)-pyrrolo[1,2-a] quinoxaline (5NO₂TAAPP) was synthesized and structurally characterized by FT IR, UV-Vis, NMR and elemental analysis techniques. Single crystal X-ray diffraction analysis was used to confirm the three-dimensional conformation of the compound. Hirshfeld surface analysis revealed the important intermolecular interactions and contacts within the crystal structure. Using DFT calculations, its molecular structure was optimized, frontier molecular orbitals were deduced. Molecular docking experiments disclosed that the compound formed important binding interactions with the amino acid residues within the active site of 3CL^{pro}, the main protease of SARS-CoV-2. Hydrogen bonding and many other hydrophobic interactions are responsible for the binding affinity of the compound with the target protein. The result revealed that the studied compound has a comparable binding affinity for 3CL^{pro} to that of approved drugs for COVID-19 such as remdesivir and favipiravir. This suggests that the compound can be chosen for further studies as a potential therapeutic candidate for COVID-19.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

CRediT authorship contribution statement

K.M. Divya: Writing – original draft, Formal analysis, Visualization. **D.P. Savitha:** Formal analysis, Writing – review & editing. **G. Anjali Krishna:** Formal analysis, Visualization. **T.M. Dhanya:** Formal analysis. **P.V. Mohanan:** Project administration, Writing – review & editing.

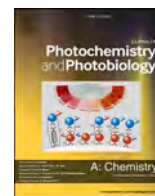
Acknowledgments

The author wishes to thank SAIF-STIC, CUSAT, Kerala for their help in single-crystal XRD analysis.

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A thiophene based pyrrolo [1, 2-a] quinoxaline fluorescent probe as a “turn-off” sensor for the selective nanomolar detection of sodium ion

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ARTICLE INFO

Keywords:

Thiophene based fluorescent sensor
Fluorescence turn-off
Nanomolar
Sodium ions

ABSTRACT

A novel thiophene based pyrrolo [1, 2-a] quinoxaline (TAAPP) fluorescent sensor, suitable for the nanomolar detection of sodium ions was successfully developed and characterized by various spectroscopic techniques. The fluorescent sensor showed excellent selectivity for sodium ions over other biologically and environmentally important metal ions/biomolecules by a remarkable quenching of fluorescence. Stern-Volmer studies reveal that static quenching predominates over dynamic quenching. The detection limit was found to be 2.219 nM within the linear range of 3 to 30 nM. DFT studies were performed to validate the results. The applicability of the probe can be extended to quantify sodium ions in real samples.

1. Introduction

Sodium is the primary cation in the extracellular fluids of animals and humans, which play a substantial role in many physiological processes acting to control blood volume, blood pressure, osmotic equilibrium and pH [1]. The concentration of sodium in the blood is around 130–150 mM [2], while that in intracellular fluid is only 5–30 mM [3]. Any irregularities in its concentration may enhance the risk of strokes, heart failure, diabetes, hyponatremia and kidney problems [4]. The salinity of water and soil is mainly due to the excessive concentration of sodium salts [5]. A high concentration of sodium in plant cells can disturb the water structure, inhibit enzymes and create nutritional imbalance [6]. So the development of a selective chemosensor for the quantification of sodium plays a vital role in biological and environmental monitoring.

Recognition of sodium ions can be achieved by using various techniques such as colourimetry, electrochemical methods, flame photometry, and inductively coupled plasma-atomic emission spectrometry [7–13]. In contrast to these techniques, fluorescence spectroscopy received greater attention in this field due to its simplicity of operation, high sensitivity, high selectivity and rapid response [14]. Various types of fluorescent sensors for sodium-based on small molecule chelators [15–17], nanoparticles [18,19], DNazymes [20] and DNA aptamer [21] have been developed.

Fluorescent thiophene based materials have been explored as sensing

materials for various metal ions [22–24], anions [25,26] and small molecules [27–29]. The ease of synthesis of pyrrolo[1,2-a] quinoxalines from thiophene 2- carboxaldehyde by a one-pot bicomponent cyclo condensation reaction and the fluorescent nature of the obtained product inspired us to explore this scaffold as a fluorescent probe. The present report describes an efficient and highly sensitive thiophene based probe, which offers an excellent fluorescent turn-off sensing scheme for the selective monitoring of sodium ions. The practical utility of the probe can be expanded to the estimation of sodium ions in biological and environmental samples.

2. Experimental details

2.1. Materials and methods

1-(2-amino phenyl) pyrrole and thiophene-2-carboxaldehyde were purchased from Sigma-Aldrich. Methanol, acetonitrile and DMF were purchased from Spectrochem and used without further purification.

Fluorescence and lifetime measurements were acquired on Horiba Fluorolog 3 (FL-1057). UV-visible spectra of the compounds were recorded in DMF on a Thermo Scientific™ Evolution 201 UV-Vis spectrophotometer. FT-IR spectra of the compounds were recorded as KBr pellets with a JASCO-8000 FT-IR spectrophotometer in the 400–4000 cm⁻¹ range. ¹H and ¹³C NMR spectra were measured on a Burkert Advance DRX 300 FT-NMR spectrometer with chemical shifts reported

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<https://doi.org/10.1016/j.jphotochem.2022.114046>

Received 26 February 2022; Received in revised form 17 May 2022; Accepted 25 May 2022

Available online 28 May 2022

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in ppm (in DMSO- d_6 ; TMS as the internal standard). Elemental analysis of the synthesized compound was done using an Elementar Vario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India. LC-MS spectrum was recorded on Waters 3100 Mass Detector utilizing ESI-MS procedure at Cochin University of Science and Technology, Kochi, India. Cyclic voltammetry studies were carried out using a CH analyser.

2.2. Synthesis of thiophene based pyrrolo [1, 2-a] quinoxaline (TAAPP)

Compound TAAPP was synthesized according to our previously reported synthetic procedure with slight modification [30]. Detailed synthetic procedure and scheme is outlined in [supporting information](#).

3. Results and discussion

3.1. Characterization and optical properties of TAAPP

The ligand was characterized by ^1H and ^{13}C NMR, LC-MS and IR spectra (see the ESI, [Fig. S1-S4](#)). The optical and photophysical properties of the ligand were also explored. The UV-Visible spectrum of TAAPP (1 mM) in DMF shows an absorption peak at 312 nm, which may be due to intramolecular charge transfer (ICT). The ligand showed a distinct fluorescence emission band at 470 nm upon excitation at 340 nm in DMF. The absorption and emission spectra of TAAPP in DMF are shown in [Fig. S5](#). Quantum yield (QY) is an important parameter which describes how proficiently a fluorophore transforms the excitation light into fluorescence. The quantum yield of the compound was determined to be 75% (see [Fig. S6](#)).

3.2. Fluorescent detection for Na^+

Using UV-visible and fluorescence spectroscopy, we studied the interaction of various metal chlorides (Na^+ , Li^+ , K^+ , Fe^{2+} , Fe^{3+} , Mn^{2+} , Co^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} and Al^{3+} , 60 nM) with the ligand to assess the effective interaction between the ligand and the metal ions. The study shows that the ligand selectively identifies Na^+ ions. Upon the addition of Na^+ ions, the UV-Visible spectrum of TAAPP changes considerably and absorption maxima shifted to 360 nm ([Fig. 1](#)). The shift in absorption band may be due to the formation of a ground-state complex by charge transfer from the ligand to the analyte.

The emission intensity of the ligand is not affected by the addition of various metal ions except Na^+ ions. The fluorescence emission intensity of TAAPP underwent significant quenching in presence of Na^+ ions ([Fig. 2a](#)). It may be due to the high binding affinity of the nitrogen atom (present in the quinoxaline ring) to Na^+ ions than the other metal ions

through a ground state donor- acceptor charge transfer process.

Fluorescence titrations were carried out at room temperature to understand the coordination behaviour of TAAPP. The progressive addition of Na^+ (0–60 nM, DMF) to the probe TAAPP (0.6 nM, DMF) caused a significant decrease in emission intensity at 470 nm, indicating the “turn-off” behaviour of the sensor ([Fig. 2b](#)). A significant linear relationship is attained over a range of 3–30 nM and the detection limit is found to be 2.219 nM ($R^2 = 0.9909$) from the detection limit calculation ([Fig. 2c](#)).

3.3. Selectivity and stability studies

The selectivity and sensitivity of the probe towards Na^+ ions in the presence of other metal ions (Li^+ , K^+ , Fe^{2+} , Fe^{3+} , Mn^{2+} , Co^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} and Al^{3+}) was confirmed through the competition experiment. Accordingly, solutions of various metal ions (60 nM, DMF) were added to TAAPP solution (0.6 nM, DMF) in the presence of 60 nM Na^+ ion solution under the same experimental conditions, and their fluorescence intensity was recorded at 470 nm. Fluorescent quenching is obtained for Na^+ without significant interference from the other metal ions ([Fig. 3](#)).

To extend the analytical application of the probe in the biological environment, the influence of the various co-existing biomolecule on the emission of the probe-analyte mixture has been investigated. The biomolecules such as aspartic acid, ascorbic acid, urea, glutamic acid, glucose, creatinine and ammonia (60 nM, DMF/ H_2O ; 9:1 v/v) did not interfere in the analysis. Thus, TAAPP act as an excellent fluorescent detection probe which shows distinct selectivity toward Na^+ ion in the competitive environment ([Fig. S7](#)).

After selectivity studies of the probe, the effect of counter ions was studied by using different salts of sodium such as sodium chloride, sodium fluoride, sodium acetate, sodium nitrite, sodium sulphite, sodium carbonate, and sodium hypochlorite. The emission intensity of the probe was quenched effectively with all these salts of sodium indicating that the counter ions did not affect the selectivity of the probe ([Fig. S8](#)).

The emission profile of the probe was recorded in the presence and absence of the analyte for 8 h to explore its stability. No substantial change was observed in the emission profile of the probe which proved stability ([Fig. S9](#)). The sensing performance of the probe to Na^+ under different pH was studied by varying the pH values from 2 to 10. Emission intensity is maximum at a pH of 7.0 and then decreases gradually in alkaline pH ([Fig. S10](#)). The result suggests that the probe could be effectively used in physiological pH for biological applications.

3.4. Nature of binding and stoichiometry

The binding stoichiometry of the probe with Na^+ was examined by Job's continuous variation method [31]. The binding isotherm ([Fig. 4](#)) indicates a maximum value of 0.5 which correspond to a 1:1 stoichiometry between TAAPP and Na^+ . The association constant of TAAPP with Na^+ was determined as $1.51 \times 10^3 \text{ M}^{-1}$ using the Benesi-Hildebrand equation [32] ([Fig. 5](#)).

3.5. Mechanism of fluorescent quenching

Fluorescent quenching can occur mainly either by static or dynamic processes. Dynamic quenching is caused by the diffusive encounters between the fluorophore and the quencher during the lifetime of the excited state [33]. Since the dynamic quenching affects only the excited state, the absorption spectrum of the fluorophore is not expected to change [33,34]. Static quenching takes place through the formation of the nonfluorescent ground-state complex between the fluorophore and the quencher and hence absorption spectrum of the fluorophore is expected to change in the presence of the quencher [33].

To understand the fluorescence quenching mechanism, emission quenching results were quantitatively subjected to the Stern-Volmer

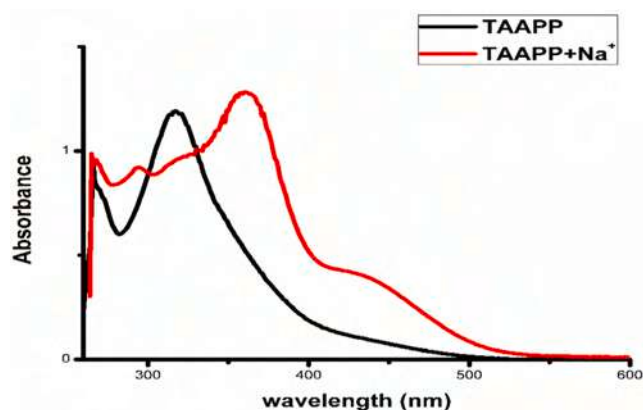


Fig. 1. UV-visible spectra of TAAPP (black line) and TAAPP + Na^+ (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

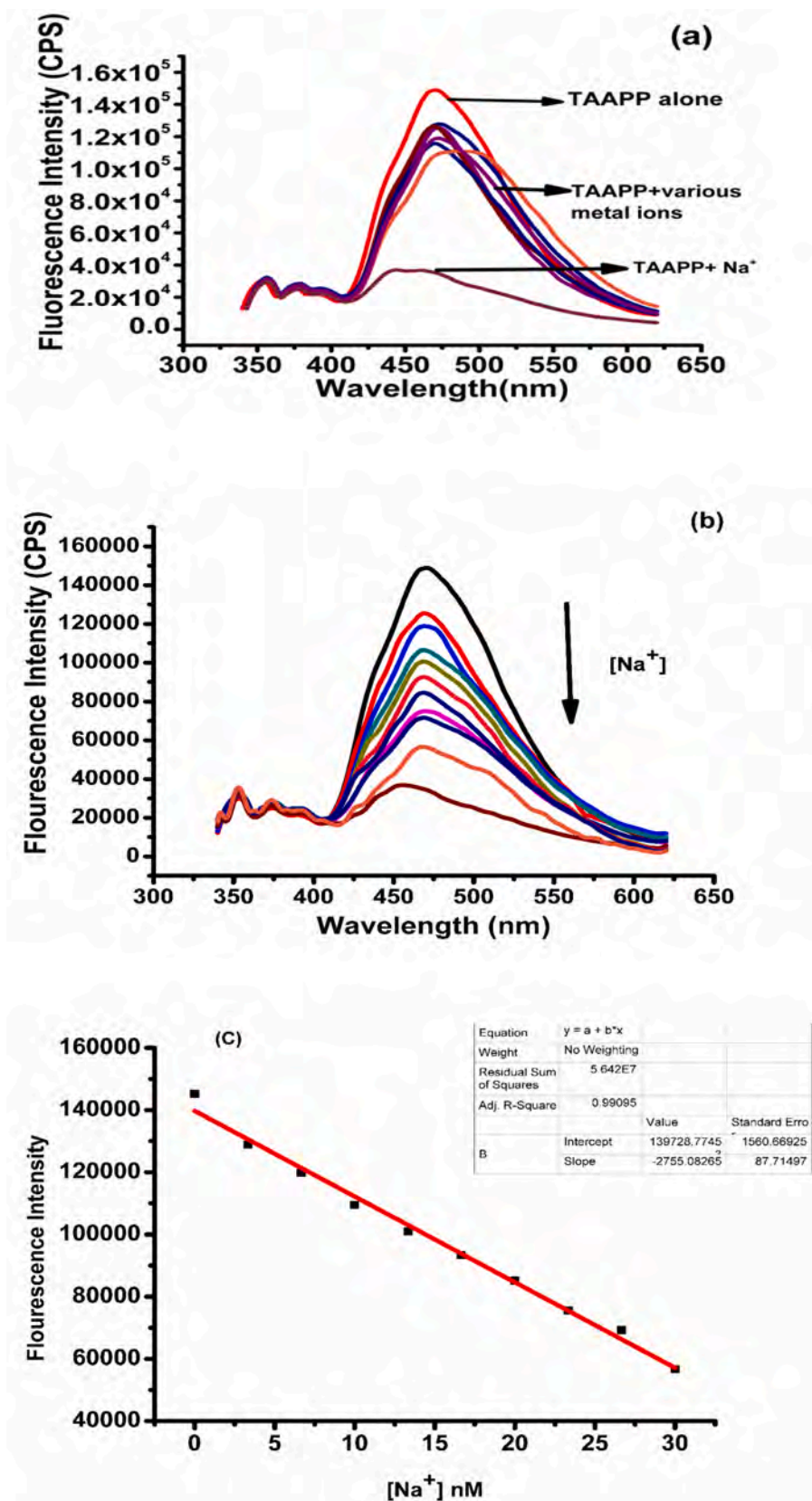


Fig. 2. (a) Fluorescence spectral change of TAAPP (0.6 nM) in the presence of various metal ions (Li⁺, K⁺, Fe²⁺, Fe³⁺, Mn²⁺, Co²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Al³⁺, 60 nM) in DMF (b) Fluorescence spectral change of TAAPP (0.6 nM) upon the addition of various concentrations of Na⁺ ion (0–30 nM) in DMF (c) The Linear relationship of fluorescence intensity at 470 nm and Na⁺ concentration (TAAPP: 0.6 nM; λ_{ex} : 340 nm).

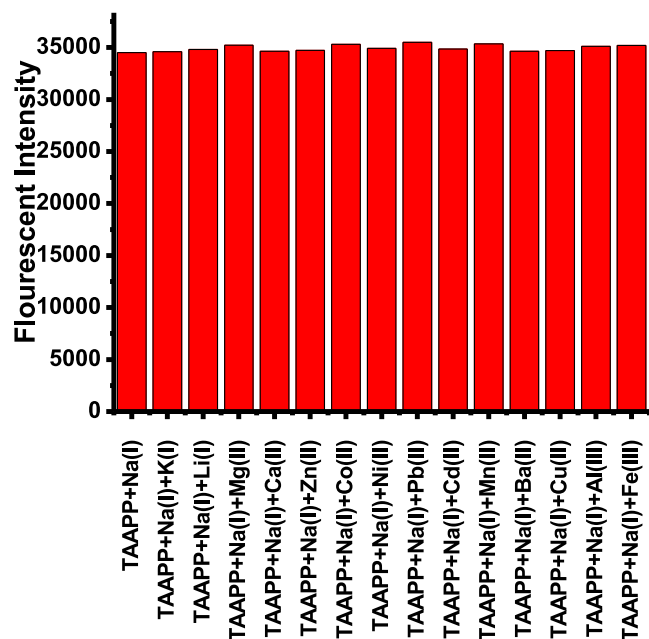


Fig. 3. Fluorescence response of TAAPP in the presence of Na^+ with various metal ions in DMF (TAAPP: 0.6 nM; λ_{ex} : 340 nm).

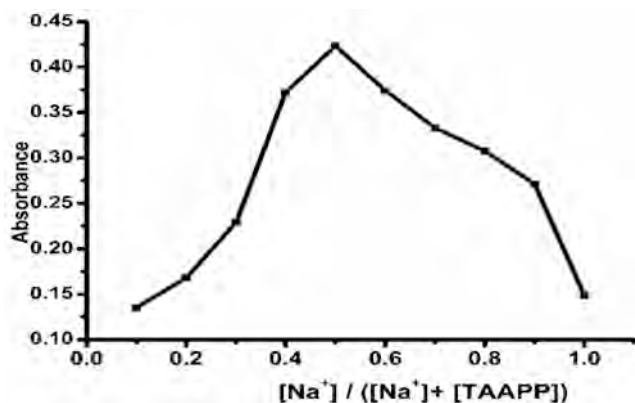


Fig. 4. Job's plot for the determination of binding stoichiometry of TAAPP with Na^+ obtained from absorbance data.

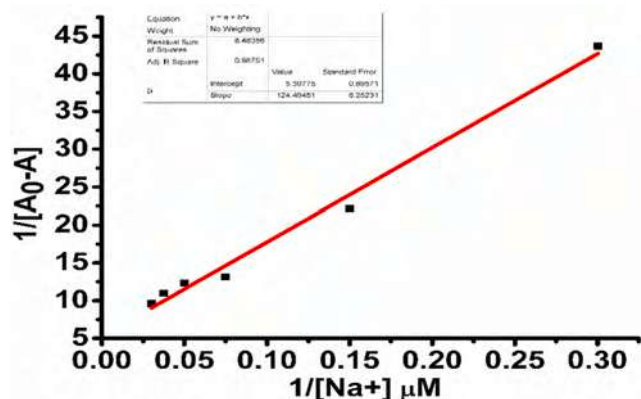


Fig. 5. B-H plot of TAAPP assuming 1:1 stoichiometry for the association between TAAPP and Na^+ .

equation [33].

$\frac{F_0}{F} = 1 + K_{\text{sv}}[Q]$, Where F and F_0 indicate the emission intensities with and without the addition of Na^+ ions, $[Q]$ is the concentration of Na^+ ions and K_{sv} is the Stern-Volmer quenching constant. The Stern-Volmer plot of emission quenching of TAAPP versus concentration of Na^+ ions is given in Fig. 6. The linear SV plot indicates a static quenching mechanism in which emission quenching of TAAPP is directly related to the quantity of Na^+ ions. From the slope of the SV plot, the Stern-Volmer quenching constant is found to be $3.407 \times 10^7 \text{ mol}^{-1} \text{ dm}^3$, which discloses a strong emission quenching of TAAPP with Na^+ ions.

Fluorescent lifetime analysis is the most conclusive method to understand the mechanism of quenching. For static quenching $\tau_0/\tau = 1$ and dynamic quenching $F_0/F = \tau_0/\tau$ [33]. To get a clear understanding of the fluorescent quenching mechanism, the fluorescent lifetime decay analysis of TAAPP before and after the addition of Na^+ ion was examined. The time-resolved fluorescence spectra of TAAPP in the presence of Na^+ (3–30 nM) solution (Fig. 7a) and Stern-Volmer plot of TAAPP in terms of lifetime measurements (Fig. 7b) are shown below.

The fluorescence lifetime of TAAPP alone is 1.52 ns. Soon after the addition of Na^+ ions (30 nM) to TAAPP, the average fluorescence lifetime of TAAPP with Na^+ ion is slightly reduced from 1.52 ns to 1.36 ns. The small change in the average lifetime value in the presence of Na^+ ions suggests a static quenching process rather than a collisional quenching process [35]. The quenching constant k_q is calculated to be $2.505 \times 10^{16} \text{ M}^{-1} \text{ s}^{-1}$ by substituting the value of τ in the equation, $k_q = \frac{K_{\text{sv}}}{\tau}$, where K_{sv} is the Stern-Volmer quenching constant [36]. The static process behind the quenching is confirmed, since the obtained quenching constant value is higher than the maximum scattering collision quenching constant ($2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [36,37].

3.6. Theoretical studies

To get an idea about the proposed binding mode and the optical response of the probe TAAPP upon the addition of sodium ion, DFT studies were performed at B3LYP/6-31G (d, p) level using Gaussian 09 programme. The mechanism for the fluorescence quenching can be explained by the ground state donor-acceptor charge transfer between TAAPP and Na^+ ion. Ground state charge transfer can occur when the HOMO of the probe is situated near the LUMO of the analyte [38]. Since the LUMO of Na^+ ion (3.5 eV) is situated near the HOMO of the probe (5.4 eV), charge transfer occurs between the probe and analyte which results in the formation of a non-fluorescent ground state complex.

Frontier molecular orbital analysis had done to determine the electronic behaviour of the probe in the presence of Na^+ ion. The optimized geometries and Frontier molecular orbital diagram of TAAPP and TAAPP- Na^+ complex are shown in Fig. 8 and Fig. 9. The HOMO in the optimized geometry of TAAPP displayed electron distribution over the

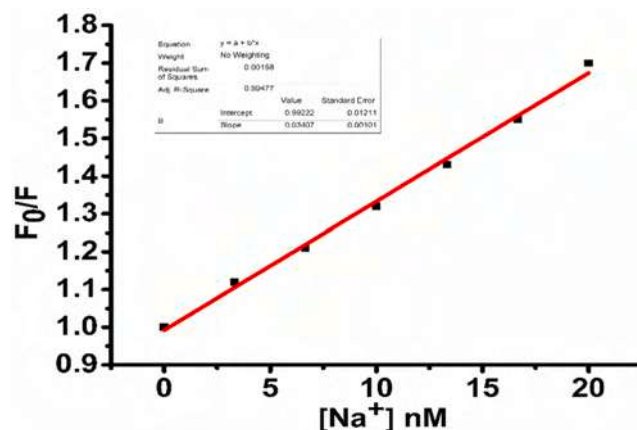


Fig. 6. The Stern-Volmer plot in terms of fluorescence measurements.

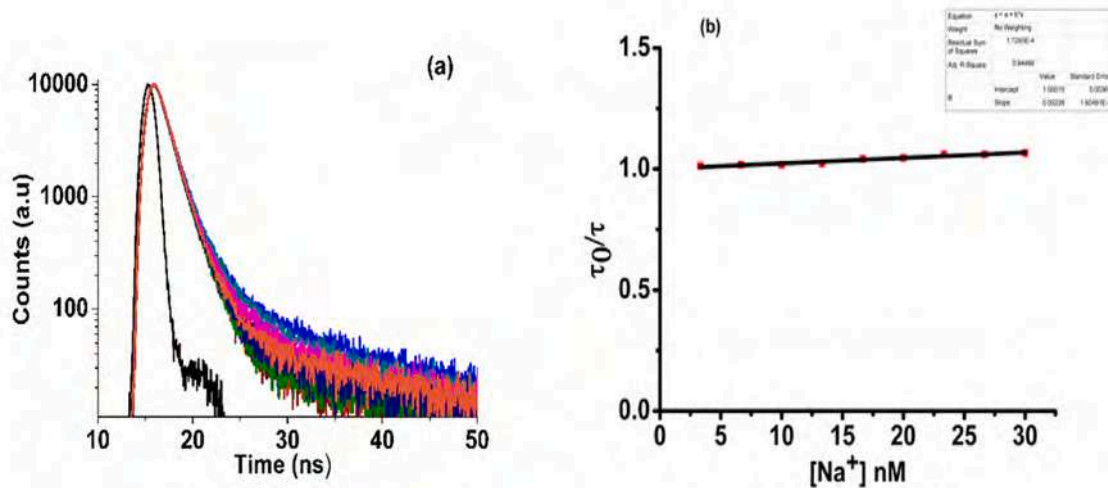


Fig. 7. (a) Time-resolved emission Spectra of TAAPP in the presence of Na^+ solution (3–30 nM) by exciting at 340 nm. (b) The Stern-Volmer plot in terms of lifetime measurement.

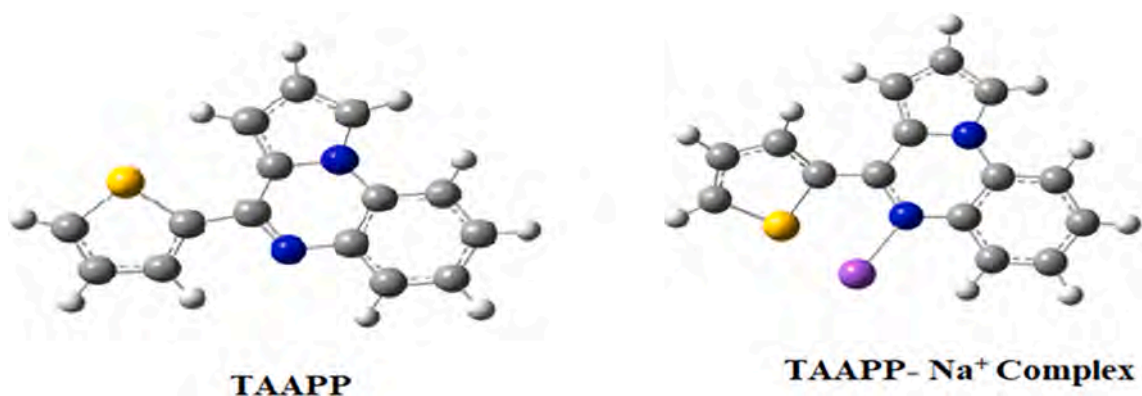


Fig. 8. Optimized geometries of TAAPP and TAAPP- Na^+ complex calculated at the DFT level using B3LYP/6-31G (d, p) basis set.

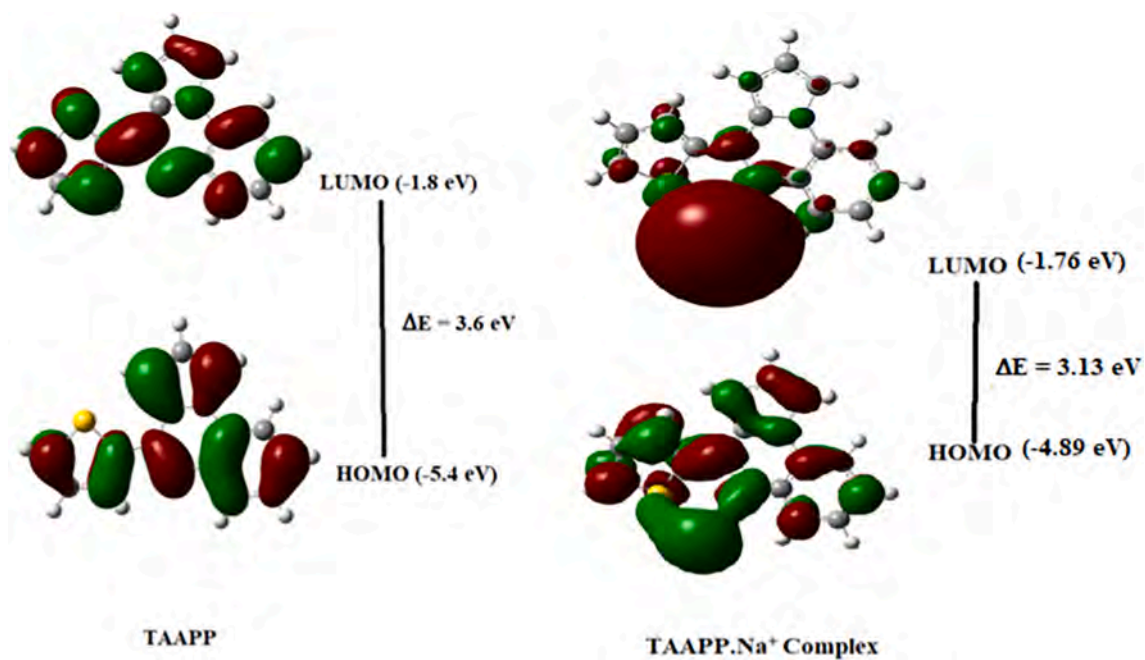


Fig. 9. Energy diagram of HOMO and LUMO orbitals of TAAPP and TAAPP. Na^+ complex calculated at the DFT level using a B3LYP/6-31G (d, p) basis set.

whole molecule. Upon the addition of Na^+ ion, the electron density of LUMO has dispersed mainly on the metal centre by considerable charge transfer. The HOMO-LUMO energy gap in the TAAPP- Na^+ complex is lower as compared to free TAAPP, which might play a significant role in the optical response of the probe upon complexation. The slight lowering of the HOMO-LUMO gap in the TAAPP- Na^+ complex validates the experimentally witnessed small redshift in the absorption spectrum.

Additionally, there is a considerable decrease in the total energy (−29526.70 eV of TAAPP to −33943.29 eV of TAAPP- Na^+ complex) of TAAPP upon complexation with Na^+ ion recommends that the complex formed is stable.

DFT analysis recommended that effective charge transfer occurs from the electron-rich nitrogen atom in the quinoxaline ring of the probe to the Na^+ centre which results in the formation of a non-fluorescent ground state complex. This accounts for the fluorescent quenching of TAAPP in the presence of Na^+ ions.

3.7. Cyclic voltammetry studies

To shed some light on the theoretical assessment of energy gaps, we have conducted the experimental determination of energy bandgap through cyclic voltammetry studies. The electrochemical behaviour of the probe in presence of Na^+ is shown in Fig. 10. In Fig. 10 (a), the TAAPP voltammogram shows the main oxidation peak at 0.95 V and the main reduction peak at −1.098 V. Upon complexation, the main oxidation peak is localized at 0.936 V and the main reduction peak at −0.881 V. The shift in the reduction potential may be due to the electron transfer process involved in complexation [39]. HOMO and LUMO energy levels of TAAPP in the presence and absence of Na^+ were obtained from the cyclic voltammogram. The empirical relation $E_{\text{HOMO}} = E_{1/2}^{\text{ox}} + 4.8 \cdot E_{1/2, \text{Fc, Fc}^+}$ [eV] was used to calculate the HOMO energy levels of the probe, where $E_{1/2}^{\text{ox}}$ is the half-wave potential of the probe concerning Ag/AgCl (sat) reference electrode [40] and $E_{1/2, \text{Fc, Fc}^+}$ is the half-wave potential of Fc/Fc^+ redox couple which was estimated to be 0.38 V [41]. E_{HOMO} of the probe was calculated to be −4.094 eV. From the UV-visible absorption studies, the optical band gap can be calculated by following the relation E_g [eV] = $\frac{1240}{\lambda(\text{nm})}$ [40,42]. The optical band gap and LUMO energy of the probe were calculated to be 3.97 and −0.124 eV, respectively. Similarly, E_{HOMO} , E_{LUMO} and E_g of the probe in presence of Na^+ ion were calculated to be −3.883, −0.443 and 3.44 eV respectively. The energy bandgap of the probe decreases from 3.97 to 3.44 in the presence of Na^+ . The bandgap values obtained from cyclic voltammetry

measurements are in good agreement with the theoretical values. Experimentally and theoretically obtained HOMO, LUMO and bandgap values are listed in Table S1.

3.8. Applicability of the probe

The hands-on application of the proposed probe was assessed by estimating the sodium ions in tap water samples and synthetic urine by the spike recovery method [43]. The average recovery percentage obtained for the tap water sample was 97–99% with a relative standard deviation of 1.16–1.27%. Similarly, the studies were repeated with synthetic urine samples where the recovery percentage obtained was 102–104% with a relative standard deviation value of 2.26–2.57% as shown in Table S2. The accurate recovery percentage and reasonably lower RSD values point to the practicability of the developed probe for the determination of sodium in biological and environmental samples.

4. Conclusion

In brief, a simple and efficient fluorescent probe has been developed from thiophene 2-carboxaldehyde for the selective determination of sodium by a turn-off fluorescence mechanism. The structure and chemical composition of the probe were well characterized and its optical properties were studied by UV-visible absorption as well as fluorescence studies. The probe displays excellent sensing performance towards the detection of the sodium ion with a linear range of 3–30 nM and LOD of 2.219 nM. The probe encountered no interference from other competing metal ions and biomolecules and presented rapid sensing. Mechanism behind the sensing was found to be of static nature based on the observations from the UV-visible absorption spectra, fluorescence spectra and lifetime measurements. Cyclic voltammetric studies were carried out to understand the electrochemical behaviour of the probe in the presence of sodium ions. The binding mechanism of Na^+ with the probe TAAPP was further established by DFT studies. In practical application, the probe could be used to detect and quantify sodium in water and synthetic urine samples with a high degree of accuracy.

CRediT authorship contribution statement

K.M. Divya: . D.P. Savitha: Methodology. G. Anjali Krishna: . T.M. Dhanya: Visualization. P.V. Mohanan: .

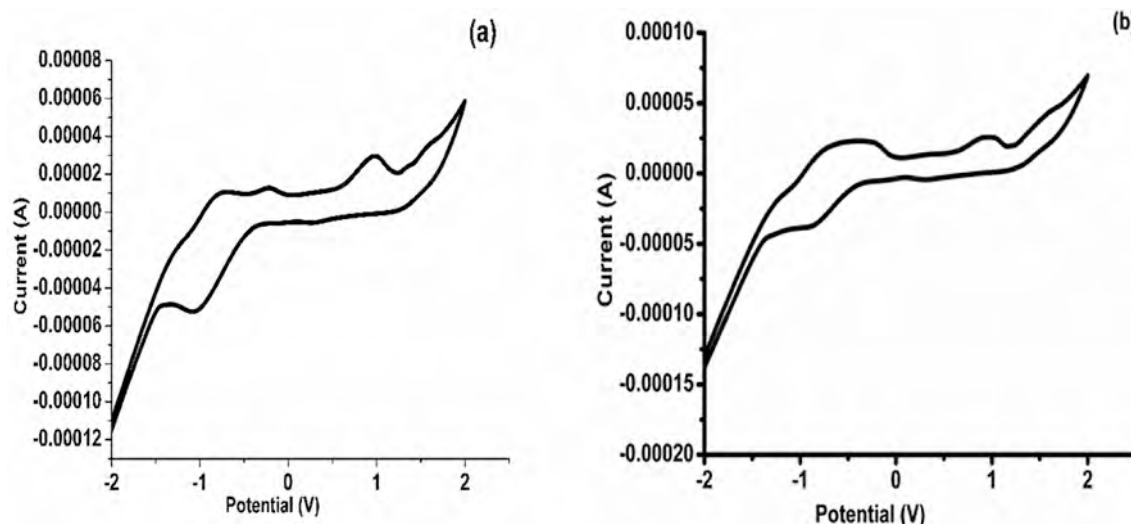


Fig. 10. (a) Cyclic voltammogram of the probe TAAPP (b) TAAPP in the presence of Na^+ .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are thankful to SAIF STIC, CUSAT for NMR and CHNS analysis data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphotochem.2022.114046>.

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Received on 01 June 2020; received in revised form, 07 January 2021; accepted, 19 May 2021; published 01 August 2021

SECONDARY METABOLITES AS LEAD MOLECULES FOR ANTICANCER THERAPIES: A REVIEW

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Keywords:

Cancer, Therapy, Phytochemicals, Malignant cells, Apoptosis, Cell cycle, Caspase proteins

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ABSTRACT: Cancer initiates from the transformation of normal living cells into malignant cells via a multistage event that usually advances from a pre-cancerous phase to a malignant tumour. Anti-metastatic drugs or anti-neoplastic drugs prevent and regulate the growth of onco cells. Based on the mode of drugs, the universally anticancer drugs market is categorized into cytotoxic, targeted, and hormonal drugs. The targeted drugs class is further divided into monoclonal antibodies, tyrosine kinase inhibitors, and others. The worldwide anticancer drugs market falls into five regions: North America, Europe, Asia Pacific, Latin America, and the Middle East and Africa. Cancer is considered the second largest cause of death globally and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 out of 6 deaths is due to cancer. Approximately 72% of deaths from cancer occur in developed and developing countries. Synthetic drugs and other modes of treatment are pre-existing. Chemotherapy treatments have its own limitations due to their toxic effects on non-targeted tissues and it further leads to health problems. Therefore, the demands for alternative treatments from naturally derived or plant-based anticancer agents are need of the day. Many plant-based anticancer compounds are effective inhibitors against many cancer cell lines, which make them in high demand. The Discovery of anticancer cancer compounds having specificity towards cancer cell lines via apoptotic cell death or derailment of the cell cycle in malignant cells can be considered for clinical trials. This review provides some glimpses of naturally-derived compounds from plants and their mode of action against selected cancer cell lines.

INTRODUCTION: From ancient period onwards, herbal medicines were used by the native people and are still the primary source of medicine in most parts of developing and underdeveloped countries.

The Oldest known document of plants based drugs has been recorded on a Sumerian clay slab, nearly 5000 years old, consists of 12 recipes referring to 250 diverse plant species, like poppy, henbane, and mandrake, etc.¹

There was evidence for the use of plant products in the treatment of various diseases and for revitalizing body systems from Indian, Chinese, Egyptian, Greek, and Roman civilizations. According to WHO, plant-based treatment forms the main source of medicine. India has about 4.5

QUICK RESPONSE CODE 	DOI: 10.13040/IJPSR.0975-8232.12(8).4042-59 The article can be accessed online on www.ijpsr.com DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(8).4042-59

million plant species, and among them, only 250,000-500,000 plant species are screened phytochemically for its biological or pharmacological activities. The bioactive constituents of plants extracts were used for treatment of various diseases and further, the formulation for the novel drugs in pharmaceutical industries. Researches are intended in searching the biological properties of plant extracts as potential drugs for curing many diseases including cancer ².

There are considerable economic benefits in validating the indigenous knowledge and in the use of herbals for the treatment of many diseases. Outstanding contributions to modern therapeutics were provided by medicinal plants. Hundreds of novel plants based drugs were introduced in the USA drug market during 1950-70, which includes vinblastine, vincristine, deserpine, and reserpine, while during 1991-95 about 2% of drugs (paclitaxel, topotecan, gemtuzumab, irinotecan etc.) were introduced ³. In the middle of the 20th century, the use of medicinal plants was reduced because of the discovery of synthetic chemicals for curing diseases.

Plants produce vast and diverse types of compounds, and many of them were not directly involved in the growth and development of the species. These substances are referred to as secondary metabolites. In recent years, the commercial importance of secondary metabolites is increased. Plant secondary metabolites are usually classified according to their biosynthetic pathways. The phenyl propanoid pathway is involved in the secondary metabolite synthesis among the majority of species. Search for novel molecules of therapeutic properties within unexplored parts of biological diversity is a challenge. For the past few decades, a large number of herbals used by the tribes as ailments were exploited and experimentally evaluated. Its usage ranged from surface applications for preventing infections to internal consumption against relief from many illnesses and inflammations.

Cancer or malignant tumours represent the second-largest common disease. Evidence for cancer is increasing on a global scale. There is more than one plausible explanation for cancer initiation. Due to this, there is no effective cancer therapy available to date, and the disease can keep expanding on a

global scale ⁴. Cancer is a major public health issue both in developed and developing countries. It is a group of diseases characterized by unregulated division and spread of cells and also a complex genetic disease that is caused primarily by environmental factors. The carcinogens can be present in food, water, air, chemicals, and sunlight that people are exposed. More significantly, unhealthy lifestyles and the adoption of many features of the modern Western diet will increase cancer incidence.

In terms of behaviour, tumours are either „benign“ or „malignant“. Benign tumours are slow-growing, expansive masses that compress and invade surrounding tissues. Malignant tumours are usually rapidly growing, invading surrounding tissue and most significantly colonizing in distant organs. The ability of tumour cells to detach from the primary tumour and set up a metastasis *i.e.*, secondary tumour, discontinuous with the primary is the proof of malignancy. The suffix „oma“ usually denotes a benign tumour. Sometimes, the cancerous cells may occur in liquids (like leukemia). However most of the tumors occur as solids, originally appear in various tissues and parts of the body.

Different types of Cancers such as in Blood and lymphatic systems (Waldenstrom's disease, Hodgkin's disease, Lymphomas, Multiple myeloma, Leukemia's), digestive Systems (esophageal cancer, stomach cancer, liver cancer, cancer of pancreas, rectal and colon cancer, anal cancer), skin cancers (malignant melanoma), urinary system (kidney cancer, bladder cancer, testis cancer, prostate cancer), reproductive organs in women (ovarian cancer, breast cancer, choriocarcinoma, gynecological cancer), miscellaneous types (bone cancer, brain cancer, chondrosarcoma, soft tissue cancer, nasopharyngeal cancer, retroperitoneal sarcomas, thyroid cancer). Breast cancer is one of the highest forms of cancer worldwide ⁵.

Factors like smoking, diet, and infectious diseases as well as chemicals and radiation, along with trace levels of pollutants in food, drinking water, and air are found reasons for cancer. Some other factors which are more likely to affect are tobacco use, unhealthy diet, not enough physical activity, pollutants concentration, intensity, and exposure. The cancer risk becomes highly increased where

workers are exposed to ionizing radiation, carcinomas chemicals, certain metals, and some other specific substances or even exposed at low levels. Passive tobacco smoking increases the risk in a large population who do not smoke but exposed to exhaled smoke of smokers ⁶.

Over 60% anticancer agents are derived from natural resources like plants, marine organisms, and microorganisms ⁷. The national cancer institute has screened around 114,000 extracts for anticancer activity ⁸. Compounds that are identified and extracted from plants for their anticancer properties mainly include polyphenols. This review provides a recent outline of the ethnic plants used by the tribes, isolation of the compounds, and its validation towards anticancer potentialities.

Cancer Therapy: Cancer treatment is a combination of number of different processes, which depend on the stage and type of cancer, which includes: surgery, radiation therapy, chemotherapy, biological therapy, and hormone therapy. If the tumour is amenable to surgery, then surgery is the most effective tool for treatment. Targeted radiotherapy together with combinations of anticancer drugs is another method of treatment. Usually, conventional anticancer drugs are designed with deoxyribonucleic acid (DNA) synthesis as their target. Tumour cells are not only seen as proliferating cells in the body; they are also present in the alimentary tract, bone marrow cells, cells to fight infection, and epidermal cells, including those that generate hairs, all are highly proliferative. So the patients with cancer undergoing chemotherapy commonly suffer unwanted hair loss and sometimes potentially life-threatening anaemia and proneness to infections (side effects that limit the treatment). Localized tumors can be successfully removed by surgery or irradiation with high survival rates ⁹. Complementary or alternative therapies, which do not use known cancer drugs or use approaches not common in the medical community, commonly use methods to control symptoms. It is important to research and understand the risks and benefits of these therapies.

The step towards the development of cancer involves alterations of epigenetic processes and their deregulation. So the control of hypermethylation of tumor-suppressor genes is

deregulated in cancer cells. This results in gene silencing and inactivation of tumor-suppressor genes. Drugs that can inhibit or reverse these epigenetic alterations have been in development over recent years ¹⁰. There is many forms of cancer in the human population but share similar characteristics or genotypes. Plant-derived compounds have demonstrated properties to inhibit cancer cell activities like inhibiting the proliferation of cancer cells and inducing apoptotic cell death.

In developing countries, utilization of all plant parts, including the stem, leaf, root, and bark are included in the treatment. There are huge demands for medicinal plants in developing countries, and attention is being drawn towards food with medicinal properties like cruciferous vegetables and fruit berries ¹¹. Raw by-products from industries are also utilized to extract anticancer agents. For example, rape seed extract is often added in ingredients of food products due to its human health benefits. In the wine industry, grape stems are raw by-products after making wine. Its high polyphenolic content makes it advantageous for anticancer drug development and makes a profitable scheme to reduce environmental issues. Grape stem extracts have been demonstrated to have antioxidant properties, prevent DNA damage and show anti-carcinogenic potentiality against an array of cancer cell lines from cervical cancer to thyroid cancer ¹².

Many studies have reported that inhibition of enzymes that stop tumor growth is mainly performed in human cell lines. Many plants play anticancer roles through their different classes of secondary metabolites. However, the study of these plants should not limit the study of a plethora of anticancer plants, some of which are still unexplored. Studies are needed to highlight the mechanism of anticancer action of many already explored and unexplored plants.

Secondary Metabolites vs. Anticancer Properties: Secondary metabolites (SMs) synthesized in plants mostly function as scavengers of free radicals/ reactive oxygen species, which raises the possibility of their application in food and pharmaceutical fields. The knowledge gained at the cellular and molecular levels and biological activities of SMs would be useful in planning for

future epidemiological studies and human cancer prevention trials, especially when a high dosage is not the option to deliver the active compounds into many tissues. It is well documented that cell cycle alteration by regulatory gene expression is frequently found in tumour tissues or cancer cell lines. Studies have suggested that SMs are potential targets as anticancer drugs *via* cell cycle regulations.

From time immemorial, plants have been used as a source of medicines to regulate lifestyle disorders. Many molecules were under clinical studies like flavone - favopiridol, derived from the alkaloid rohitukine was isolated from *Dysoxylum binectariferum*, was currently under phase I and phase II clinical trials, which shows broad activity against tumors, leukemia, lymphomas, and solid tumors¹³. Similarly, Olomucine from *Raphanus sativus* was designed into a synthetic agent, roscovitine, and in Europe, it is in Phase II and III clinical trials¹⁴.

Betulinic acid (pentacyclic triterpenoid) was isolated from *Zizyphus* species, and they exhibited selective cytotoxic activity against human melanoma cell lines¹⁵. Silvesterol was isolated from the fruits of *Aglaia sylvestre*, exhibits cytotoxicity against breast and lung cancer cells. Different studies are attempted to determine the exact mechanism of action for silvesterol and other bioactive molecules^{16,17}. Phytochemical studies on roots of *Erythroxylum prvillei* has resulted in the isolation of pervilleine-A. Studies were conducted on multidrug-resistant oral epidermoid cancer cell lines using pervilleine-A (cytotoxic) along with vinblastine as preventive drugs¹⁸. Combretastatin was isolated from the bark of *Combretum caffrum* and has been found effective against lung and colon cancers leukemia and we presumed this was the most cytotoxic phytomolecule isolated so far¹⁹.

Nowadays, plant-derived drugs are preferred mainly for anticancer treatment as they are natural, easily available, and can be readily administered orally as part of patient's dietary intake²⁰. Being natural, the phytochemicals from plants are non-toxic to normal human cells²¹. However, there are some exceptions like lectins, saponins, lignans, cyanogenetic glycosides, and some taxanes. If plant-derived drugs are non-toxic to normal cell lines and show cytotoxicity in cancer cell lines,

these drugs can be lead to clinical trials for further therapeutic developments. Epothilones are bacterial macrolides with potent antiproliferative activity, which have served as successful lead structures for the discovery of several clinical candidates for cancer treatment²². Major secondary metabolites from plants that have been used by the native people for anticancer therapies are listed below:

Polyphenols: Flavonoids, resveratrol tannins, curcumin, and gallacatechins are major polyphenols having anticancer potential²³. Resveratrol was prevalent in food items like peanuts, grapes, and red wine, while gallacatechins in green tea. The cytotoxicity of polyphenols on a range of cancer cells has been demonstrated with their antioxidant properties²⁴. Fractionation of methanolic extracts of *Leucobrym bowringii* by RP-HPLC revealed a pool of phenolic acids, and further studies revealed a role in human breast cancer cell lines²⁵. Polyphenols are reported to have apoptosis-inducing properties. The mechanism of action by polyphenols is mostly via apoptosis initiation by regulating the mobilization of copper ions which are bound to chromatin leads to DNA fragmentation. Another mode of action is by interfering with proteins which promotes cell growth. The structural feature of polyphenol facilitates the action via acetylation, methylation, or phosphorylation processes²⁶.

Tannins: These are naturally occurring water-soluble polyphenols having varying molecular weights and are the most abundant polyphenolic compounds with the capacity to precipitate proteins from solutions. In-plant cells, the tannins are located away from the proteins and the enzymes of cytoplasm, but when the tissues are damaged, tannins may react with proteins. Tannin-containing plants are used in ethnic medicine from ancient times onwards. *Quercus robur* is rich in tannins (gallotannins, ellagitannins, monomeric and dimeric catechins, and leucocyanidins), and has been used for its medicinal properties. Tannins may be procarcinogenic, anticarcinogenic, mutagenic, or antimutagenic. However, there is no evidence that tannins are procarcinogenic in humans²⁷. Sakagami *et al.*,²⁸ proved that ellagitannins exhibit higher cytotoxicity against human oral squamous cell carcinoma and salivary gland tumor cell lines than against normal gingival fibroblasts.

Catechins: Catechins are a group of polyphenolic compounds, (epigallocatechin-3-O-gallate (EGCG), epigallocatechin (EGC), epicatechin-3O-gallate (ECG) and epicatechin (EC), which are abundantly present in vegetables, plant-derived beverages, and foods²⁹. Chemically, they are polyhydroxylated flavonoids, which exhibit water-soluble characteristics. Being important micro-constituents of the human diet, catechins need to be taken into account when the relationship between diet and chronic diseases are to be investigated³⁰.

Studies indicated that EGCG can preferentially induce apoptosis in T lymphocytes of leukemia patients or cultured cancer cell lines³¹. According to them, when compared to normal controls, the transformed NIH-pATMras fibroblasts were more sensitive to EGCG at a 5 μ M concentration. Anticancer activity of the catechins tested was found to be effective, and therefore they have therapeutic values.

Flavonoids: Flavonoids constitute one of the large family of plant secondary metabolites with 10,000 known structures (anthocyanins, flavones, flavonols, chalcones, and many more). They are physiologically active due to their health benefits and become high scientific demand in various industries¹¹. Various plants have been investigated for their flavonoid content with their mode of action in cancer cells³². Cao *et al.*,³³ showed that purified flavonoids with anticancer activities against human cancer cell lines such as hepatoma (Hep-G2), cervical carcinoma (Hela), and breast cancer (MCF-7).

Apigenin, quercetin, and luteolin were the major flavonoids identified by RPHPLC- PAD analysis of *Marchantia linearis*, a bryophyte³⁴. Many studies have showed that flavonoid extracts from fern species, even at low concentrations they demonstrate a high percentage of anticancer activity³⁵. As mentioned earlier polyphenols inhibit or alter the regulation of proteins and other agents, which may be contributing to the survival of cancer cells. Signal transducer and activator of transcription (STAT) proteins are anti-apoptotic and contribute to cancer cell growth³⁶. Flavonoids also inhibit the expression of NF- κ B, which is essential for cancer cell survival, angiogenesis, and proliferation.

Brassinosteroids: Koncz *et al.*,³⁷ reported brassinosteroids (BRs) as natural compounds occurring in plants that play key roles such as hormone signaling to regulate growth and differentiation of cells, resistance or tolerance against disease and stress. BRs are also used for the regulation of plant senescence. A characteristic feature of cancer cells is that they do not naturally undergo apoptosis, but they proliferate indefinitely. BRs induce responses necessary for growth inhibition and induce apoptosis by interacting with cell cycle³⁸. Two natural BRs have been used in cancerous cell lines -28homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL). BRs are used to treat a range of cancer cell lines which includes: lymphoblastic leukemia multiple myeloma RPMI 8226, CEM, cervical carcinoma, HeLa, lung carcinoma A-549, osteosarcoma HOS cell lines, breast cancer cell, and prostate cancer cells.

Alkaloids: Alkaloids have a wide distribution in the plant kingdom and mainly exist in higher plants. Several alkaloids were isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both under *in-vitro* and *in-vivo* conditions. Camptothecin and vinblastine were alkaloids that have been successfully developed into anticancer drugs.

The first agents introduced in clinical use were Vinca alkaloids - vinblastine (VLB) and vincristine (VCR) isolated from the *Catharanthus roseus*. During experimentation, it was noted that these plant extracts reduce the white blood cell counts significantly and also caused bone marrow depression in rats. The plant extract also prolongs the life of mice bearing transplantable lymphocytic leukemia.

Further extraction and fractionation led to the isolation of two active alkaloids, namely vincristine and vinblastine. Jayakumar and Murugan³⁹ reported the presence of caulophyllumine A and solasodine by the fractionation of crude alkaloids from *Solanum mauritianum* having significant antiproliferation activity against cancer cell lines. Alkaloids obtained from natural herbs seem to have many targets to realize their multiple pharmacological effects.

Camptothecin was first isolated from the Chinese ornamental tree, *Camptotheca acuminata*. The extract of *C. acuminata* was showed efficacy in anti- tumor activity, and the active constituents isolated was identified as camptothecin. Researches from several organizations identified effective camptothecin derivatives and topotecan (Hycamtin) (SmithvKline Beecham) were now in clinical trials. Topotecan was used for the treatment of ovarian and small cell lung cancers, while irinotecan was used for the treatment of colorectal cancers. In addition, 15 other camptothecin derivatives were in preclinical developmental trials ⁴⁰.

Anthocyanin: Anthocyanin is a colouring pigment that belongs to polyphenols. They have shown significant potentiality as drugs for the treatment of diverse human diseases. Currently, attention has been focused on the antimetastatic potentials of anthocyanins. The anticarcinogenic effect of anthocyanins may be through redox status modification or interference with basic cellular functions. Many *in-vivo* and *in-vitro* studies in cancer cell lines were carried to study the antitumor efficiency of plant anthocyanins. The chemoprotective activity of anthocyanin-rich plant extracts from grapes, bilberry and chokeberry substantiate that the extracts reduce the colonic aberrant crypt foci formation in male rats treated with a colon carcinogen azoxymethane ⁴¹.

The cell suspension culture of *Bridelia retusa*, anthocyanin was extracted and purified by Aswathy et al., ⁴², which revealed 07 fractions comprising acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. Further, the cytotoxicity efficacy of anthocyanin extracts was studied in human oral squamous cell carcinoma (SCC4, SCC9, and SCC25) cells using cell adhesion and cell viability assay. Significant apoptosis was observed in the tested cell lines.

Similarly, anthocyanin was extracted, purified, fractionated by liquid chromatography-mass spectrometry (LC-MS/MS) from the *in-vitro* culture of teak and proved its potent antioxidant activity ⁴³. Reddivari et al., ⁴⁴ isolated anthocyanin fraction from potato extracts and showed its cytotoxic potency against prostate cancer cells through activation of caspase-dependent and independent pathways.

Saponins: These are glycosidic compounds of steroids or triterpenoids (sapogenin nucleus with one or more side chains of carbohydrates). Many leguminous plants of temperate areas are rich in saponins and exhibit positive and negative biological effects. Due to its amphiphilic nature and surface-active potentialities, the molecules are excellent foaming agents. The biological values of saponins are closely related to their chemical structure.

Despite the antinutritional attributes of saponins, they are known for their hypocholesterolemic, anticarcinogenic and immunostimulatory properties ⁴⁵. *Panax ginseng*, *P. quiquefolius*, *P. japonicus*, *P. pseudoginseng* and *Eleutherococcus senticosus* are widely employed in Chinese medicine (0.5-3%) as a tonic or adaptogenic and also for the treatment of cancer, diabetes, and hepatic and cardiovascular diseases ⁴⁶. Some of the steroidal saponins showed remarkable cytotoxicity against human oral squamous carcinoma cell lines when compared to normal human gingival fibroblasts. The tumor specificity of saponins has been reported higher than that of tannins and flavonoids, suggesting that its oxidation-mediated mechanism was not involved in the cytotoxicity activity ⁴⁶.

Podophyllotoxin Derivatives: Podophyllotoxin is another plant-derived product with derivatives such as etoposide and teniposide used in therapies like cancers and venereal wart. Its structure is closely related to the aryltetralin lactone lignans that have antineoplastic and antiviral activities. These cytotoxic therapeutic constituents were first isolated in 1880. *Podophyllum peltatum* of Podophyllaceae was used by the Native Americans for the treatment of cancer. Other closely related podophyllotoxins were also isolated during 1950's and are now introduced into clinical trials ⁴⁷.

Homoharringtonine: Homoharringtonine was isolated from the *Cephalotaxus harringtonia* var. *Drupacea*. In China, to combat acute myelogenous leukemia and chronic myelogenous leukemia, a racemic mixture of harringtonine and homoharringtonine (HHT) was used successfully. Purified homoharringtonine has shown efficacy against various leukemias. In France, elliptinium is marketed for the treatment of breast cancer ⁴⁸.

Phytoestrogens: These are natural phyto-metabolites comprising wide number of compounds such as isoflavones, lignans, coumestans and resorcylic acid lactones known for their estrogenic activity and were structurally or functionally similar to mammalian estradiol. Many members of Leguminosae and Poaceae are the major sources of dietary PEs. Daidzin and genistin are major isoflavonoids of soybeans⁴⁹. Antioxidant species act *in-vivo* to prevent oxidative damage of DNA, proteins, and lipids, thus reducing the risk of coronary heart disease and cancers. Research outputs on the health-related and clinical benefits of PEs have attracted much attention from the pharmaceutical industries. Consumption of soy products with PE levels in the diet (as much as 200 mg/day), lowers the risk of hormone-dependent cancers and other chronic diseases⁵⁰. Higher consumption of PEs was inversely correlated with the incidence and mortality rate of prostate cancer. Studies have shown that genistein inhibits prostate cancer cell growth under *in-vitro* and *in-vivo* conditions via regulating its secretion and intracellular levels of the androgen-related protein prostate-specific antigen⁴⁶.

Terpenoids: Terpenoids are secondary metabolites ubiquitously found in the plant kingdom and structurally composed of isoprenoid units. Based on structure, terpenoids are divided into monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, and polyterpenes. The diverse array of terpenoids has increased their demand in pharmaceutical industries due to their antioxidative, anti-inflammatory, and anticancer properties. Many terpenoids have been found to exhibit anticancer property on different stages of tumor development through suppression of angiogenesis, invasion, and metastasis by regulating various intracellular signalling pathways⁵¹. Triterpene acids show various pharmacological activities like anti-inflammatory, antimicrobial, cytotoxic, antiviral, and cardiovascular protection⁵². Esters and their derivatives can be obtained from many natural sources. This class of compounds exhibits a variety of structural types, including aliphatic and aromatic moieties leading to broad range of biological activities like antibacterial, antifungal, inhibition of testosterone secretion, estrogenic, antiestrogenic, relaxant effect, anti-inflammatory, immunological, cytotoxic, iono-

phoretic properties, vasodilatory effect and antagonist of calcium⁵³. Terpenoids were extracted, purified, and fractionated by GC-MS from *Hypnea musciformis* in to hexadecane, 2, 6, 10, 14 tetramethyl, Pentadecanal, phytol isomer, 4, 8, 12, 16- tetramethylheptadecan-4-olide etc.⁵⁴ Phytochemical screening of *Brachytheceium buehneri* revealed terpenoids with many pharmacological activities like anti-inflammatory, anti-malarial, anticancer, inhibition of cholesterol synthesis, anti-viral and bactericidal activities⁵⁵.

Taxanes are another class of diterpenes proven as anticancer drug that binds to tubulins/microtubules which have a key role in mitosis. Suppression of microtubule dynamics results in the blockade of cell mitosis, leading to apoptosis. Leaves of *Taxus brevifolia*, was used in the Ayurveda. Paclitaxel was isolated from the species and was used for the treatment of a wide variety of cancers, including breast, ovarian and non-small-cell lung cancer, and also against Kaposi sarcoma. Docetaxel, a semisynthetic derivative, was isolated and used in the treatment of breast cancer⁵⁶.

Essential Oils: Essential oils are proven for their therapeutic potentiality. Recently, essential oils (EOs) have been under study for their use in cancer therapy. EOs has been shown to possess anticancer properties through various cancer preventative mechanisms on the various tumor cell lines. Eos with therapeutic potential can act in two ways-chemoprevention and cancer suppression. EOs demonstrated to change expression levels of Bcl-2 and Bax genes, leading to the release of cytochrome C into cytosol in human oral epidermoid carcinoma cells⁵⁷. Similarly, a study on human melanoma cells reported that EOs induces DNA damage in cancer cells which is an indicator of apoptosis⁵⁸. Frank *et al.*,⁵⁹ studied the action of *Boswellia carteri* EO (Frankincense oil) in bladder cancer cells and observed modulation of CDKN1A, DEDD2, IER3, IL6, SGK, TNFAIP3 GAD45B, and NUDT2 genes involved in apoptosis. Chen *et al.*,⁶⁰ reported EO of *Curcuma zedoaria* has *in-vitro* and *in-vivo* anti-angiogenic effects, exhibited anti-proliferative activity against various cancer cell lines, and also suppressed melanoma growth and lung metastasis in mice. Girola *et al.*,⁶¹ tested the antitumor efficacy of camphene isolated from the EO of *Piper cernuum*

in melanoma cells. By GC -FID and GC-MS analysis of essential oil from *Pogostemon benghalensis* 41 volatile compounds were reported by Pradeep and Murugan ⁶². These oils mainly contained sesquiterpene and oxygenated sesquiterpene hydrocarbons having anti-cancer potentialities. Another study validated the antimetastatic mechanism of action of carvacrol, a phenolic mono terpenoid abundant in the EOs of oregano and thyme ⁶³.

Other Compounds: Compounds including sulforaphane, isoflavones, isothiocyanates, and pomiferin are considered to inhibit histone deacetylases (HDAC), which inhibit the activity of carcinogenic proteins. According to Davidson *et al.*, ⁶⁴ sulforaphane has been shown to inhibit important targets in breast cancer proliferation, *i.e.*, decreased expression of ER, EGFR, and HER-2 by HDAC inhibition in breast cancer cell lines. Plant-derived compounds which show inhibition of HDAC can enhance chemotherapeutic sensitivity in human cancers ⁶⁵. The investigations showed that the plant extracts with a combination of anticancer compounds were more reliable against cancer cells and showed no activity on normal human lymphocytes and fibroblasts. This makes plant extracts more desirable as therapeutic agents than chemically-derived compounds, which cause toxic complications in cancer treatment ⁶⁶. Discovery of anticancer cancer agents which show specificity towards cancer cells and can induce cell death and inhibit the growth of tumors may be considered for clinical trials.

With advancements and discoveries in naturally derived drugs, new technologies are emerging for

the application of anticancer compounds. Administrations of new drugs are an effective alternative to current treatments like chemotherapy. The field of nanotechnology and the use of nano particles (NPs), as a delivery system for drugs to reach target sites, are developing. Some compounds that have demonstrated anticancer activities are limited in their clinical development due to the need for high dosages. Bromelain, isolated from *Ananas comosus* was showed more effective as an anticancer agent in formulation with NPs than free bromelain ⁶⁷. The drug's ability to inhibit the growth of tumours and reduce unwanted side effects on other tissues is much demanded in the pharmaceutical fields.

Validation of Ethnic Plants used by the Native People for Cancer Treatment: The effort of the National Cancer Institute to develop systemic and topical formulations of drugs for potential clinical trials is an ongoing event. There are many herbal databases, which provide information on anticancer compounds in plants. The WHO estimates that approximately 80% of the people rely on traditional medicine for their primary health care ⁶⁸. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened their extracts for anticancer activity ⁶⁹. Traditional types of anti-cancer plants reported were zedoary (*Curcuma zedoaria*), marijuana (*Cannabis sativa*), turmeric (*Curcuma longa*), bamboo grass (*Loathatreum gracies*), garlic (*Allium sativum*), sunflower (*Helianthus annus*), leunca (*Solanum nigrum*), and bamboo rope (*Asparagus cochinchinensis*) ⁷⁰.

TABLE 1: SOME ETHNIC PLANTS USED BY TRIBES OF KERALA FOR PREVENTION AND TREATMENT OF CANCER

S. no.	Name of the species	Tribal / Native community	Phytochemical	Reference
1	<i>Hypnea musciformis</i> , <i>Kappaphycus alvarezii</i> & <i>Gracilaria dura</i>	Mukkuva	Terpenoids	Sumayya <i>et al.</i> , ⁷¹
2	<i>Marchantia linearis</i>	Kanni	Flavonoids	Remya Krishnan and Murugan ⁷² Manoj <i>et al.</i> , ²⁵
3	<i>Leucobryum bowringii</i>	Kanni	Secondary metabolites	
4	<i>Brachythecium buchananii</i>	Kanni	Terpenoids	Greeshma and Murugan ⁵⁵ Aswathy <i>et al.</i> , ⁴²
5	<i>Bridelia retusa</i>	Koragas	Anthocyanins	
6	<i>Pogostemon benghalensis</i> & <i>P. cablin</i>	Marattis and Malavettuvass	Essential oil	Pradeep and Murugan ⁶²
7	<i>S. mauritianum</i>	Mavilan	Alkaloids	Jayakumar and Murugan ³⁹

Ethnic plants in Kerala used by the tribals or native locals for the prevention and treatment of cancer were given below and validated scientifically **Table 1**. The data also gives information regarding the active anticancer components of the plants.

Sea Weed Terpenoids: Shelar *et al.*,⁷³ documented the ethnic knowledge of native Mukkuva community regarding the usage of selected sea weeds as tumour protective. This information was validated by Sumayya *et al.*,⁷¹. Seaweeds are rich in bioactive compounds that had a broad range of biological activities, including anticancer potential. *Hypnea musciformis*, *Kappaphycus alvarezii* and *Gracilaria dura* are marine algae. Anticancer potentiality of these sea weeds against three human cancer cell lines (A549, HeLa, and HepG2) with purified terpenoid extracts was analyzed by Sumayya *et al.*⁷¹ The cytotoxicity studies were carried out using an MTT assay. All the purified terpenoid extracts revealed antiproliferative potential against the three cancer cell lines in a dose and duration-dependent manner. *G. dura* terpenoids fraction showed significant inhibition when compared to the other red algae.

The phase-contrast microscopic visuals showed significant morphological anomalies on the treated cell lines. The extracts were subjected to column chromatography followed by GC-MS analysis revealed the presence of 17 peaks of terpenoids in *H. musciformis* (hexadecane, eicosane, pentadecanal, octadecane, tetradecanoic acid, heneicosane, 2-pentadecanone, 6,10,14-trimethyl-2-pentadecanol, phytol isomer, hexadecanoic acid methyl ester, isophytol, n-hexadecanoic acid, behenic alcohol, octadecanoic acid methyl ester, 4, 8, 12, 16-tetramethylheptadecan-4-olide, 3, 4, 8, trimethyl-2-nonenal and 9,19 cyclolanostan-3-ol-24-methylene), 10 peaks in *K. alvarezii* (hexadecane, octadecane, tricosane, nonadecane, hexadecanoic acid methyl ester, eicosane, heneicosane, stigmast-5-en-3-ol, gamma sitosterol and β - amyryl) and 4 major peaks in *G. dura* (hexadecanoic acid, n-hexadecanoic acid methyl ester, octadecanoic acid methyl ester and phytol).

The cytotoxic effect was further validated by lactic acid dehydrogenase assay, apoptosis, cell cycle arrest, and caspase activities. *Capsosiphon fulvescens* (Cf) is a green marine alga used as sea-food as well as a potential drug. Cf glycoprotein

(Cf GP) exhibits antitumor activity against several cancer cells via inducing different pathways. Analysis of Frizzled receptor, Wnt 1 signaling proteins Axin, LRP, β catenin, APC and GSK-3 beta reports the suppression of Wnt 1 signaling, β catenin, and transcription factors in AGS cells by Cf-GP. RT-PCR and Western blotting experiments of transcription factor Tcf/LEF also proved the decrease level of Wnt-1 signaling pathway and arrested the G0/G1 phase of AGS cells by Cf-GP⁷⁴. According to Kim *et al.*,⁷⁵ Cf-GP treatment not only stimulated the release of cytochrome C and apoptotic protease activating factor-1 from mitochondria to the cytosol but also inhibited the growth of AGS cells through induction of sub-G1 phase arrest, which decrease in the expression of cyclin D, E, Cdk2, Cdk4, and Cdk6 and an increase in the protein levels of p21 and p27.

Chlorella is an important genus among green algae. *Chlorella marina* has been reported to exhibit potential anti-proliferative and apoptotic effect on prostate cancer cell lines. Exposure of PC-3 and DU-145 cell lines to algal lycopene (AL) at a dose of 20 and 50 μ M significantly down regulated the growth and colonisation of cancer cell lines, and the percentage of inhibition was higher than total lycopene (TL)-treated groups. Stronger apoptosis signal was induced at higher concentrations (50 μ M) of algal lycopene. Increased DNA damage was observed in AL- and TL-treated cells which were confirmed in the comet assay. Flow cytometry results indicated that AL treatment caused PC-3 cells to accumulate in the G0/G1 phase and to undergo apoptosis. Thus, it can be summarized that algal lycopene showed significant anti-proliferative and apoptotic effect in human prostate cancer cell lines⁷⁶.

Sargassum heterophyllum has anti-proliferative activity against MDA-MB-231 breast cancer cells. Sargaquinoic acid (SQA) induces apoptosis, while polihalogenated monoterpene RU015 induce necrosis in breast cancer cells. To investigate the *in-vitro* cytotoxic activity, methanol extract of *S. swartzii* were used against HT-29, Ca co-2, T47D, MDA-MB468 and /NIH 313 cell lines and resulted selective cytotoxicity against proliferation of Caco-2 cell⁷⁷. Alcoholic extract of *S. ilicifilium* was tested against human cancer cell lines MCF-7, MDA-MB-231, HeLa, HepG2, and HT-29; the

extract showed an anti-proliferative effect against all the five cancer cell line in a dose-dependent manner⁷⁸.

Bryophytes: Remesh and Manju⁷⁹ reported the ethnobryological information from the Western Ghats, India, related to bryophytes therapeutic usage. Remya Krishnan and Murugan⁷²; Manoj et al.,²⁵ and Greeshma and Murugan⁵⁵ analyzed scientifically in selected species regarding its antimetastatic potentials.

Plagiochila beddomei, *Leucobryum bowringii* and *Octoblepharum albidum* were screened for anticancer study as per the ethnic knowledge of Kanni tribes. Bryophytes are a group of plants devoid of true vascular tissues. Being small and insignificant, bryophytes have been neglected in scientific investigations. With the advent of modern techniques and methods, it has been possible to isolate and structurally elucidate bioactive molecules in bryophytes.

Crude methanolic extracts isolated from the liverworts and mosses (*Plagiochila beddomei* Steph. (Plagiochilaceae), *Leucobryum bowringii* Mitt. and *Octoblepharum albidum* Hedw. (Leucobryaceae)) showed the presence of rutin, quercetin, and kaempferol (flavonoids) and a pool of phenolic acids. *P. beddomei* revealed the presence of phenolic acids such as gallate (199.4 µg/g), vanilate (75.3 µg/g), chlorogenate (200 µg/g), cinnamate (212.2 µg/g), protocatechol (16121.7 µg/g), coumarate (289.7 µg/g), ferulate (232.7 µg/g), sinapic (222 µg/g) and caffeate (322.4 µg/g). Fractionation of phenols in *L. bowringii* revealed the presence of phenolic acids such as gallate (10.4 µg/g), vanilate (11.3 µg/g), chlorogenate (11.2 µg/g), cinnamate (11.1 µg/g), protocatechol (13.4 µg/g), coumarate (114 µg/g), ferulate (34.8 µg/g), sinapic (87.4 µg/g) and hydroxyl benzoate (0.65 µg/g). Similarly, the total phenols in *O. albidum* revealed the presence of phenolic acids such as gallate (11.5 µg/g), vanilate (12.4 µg/g), chlorogenate (12.3 µg/g), caffeate (48.6 µg/g), protocatechol (14.7 µg/g), and ferulate (19.8 µg/g). *Plagiochila beddomei*, *Leucobryum bowringii* and *Octoblepharum albidum* were screened for their cytotoxic activity on cancer cell lines such as pharyngeal squamous carcinoma (KB), leukemia HL-60, K562, MDR K562/ A02,

breast ductal carcinoma (MDA-MB-435), P-388 murine leukemia tumor, glioma A172 cells, T98G, U87 glioma, liver hepatoblastoma (HEP-G2), lung carcinoma (A549), colon adenocarcinoma (LOVO) cell lines, osteosarcoma U2OS and MCF-7 breast cancer cell lines (Abhijit and Anuradha, 2015). MTT assay revealed cytotoxicity only in *Leucobryum bowringii* as compared to others. Further, the mode of action was confirmed in terms of apoptosis and necrosis induction such as DNA fragmentation, nuclear condensation, proteolysis of poly (ADP-ribose) polymerase (PARP), activation of caspases, inhibition of antiapoptotic nuclear transcriptional factor-kappa B and activation of p38 (mitogen-activated protein kinase). Hoechst 33342 staining assay reveals massive chromatin condensation and subsequent cleavage of structural components of the nucleus. The results indicate that methanol extracts inhibit the growth of human breast cancer cells partially through the inhibition of metallo proteinases MMP-2 and MMP-9 activities⁸⁰. *Leucobryum bowringii* alone confirmed the knowledge of the tribal people.

Marchantia linearis Lehm & Lindenb., a bryophyte, was commonly used in folklore medicine by tribals for curing many diseases. *In-vitro* cell suspension culture of *M. linearis* yielded remarkable levels of flavonoids. RPHPLC- PAD fractionation of flavonoids revealed the presence of apigenin, quercetin, and luteolin as major constituents. The study of anticancer potentialities against SW 480 colon cancer cell lines reflected significant anti-proliferation activities and was substantiated by cell apoptosis, cell cycle, and growth kinetics. Activities of quinone reductase, glutathione-S- transferase, and cytochrome P450 were correlated with reduced glutathione content and lipid peroxidation. The results of MTT assay showed that 50 µg/mL of flavonoid remarkably reduced the growth kinetics of cells coupled with induced cell apoptosis. The xenobiotic metabolic enzyme CYP 2A1 showed a marginal decrease whereas, QR and GST revealed increased activities. Caspases such as 3/7, 2, 6, 8, and 9 showed a significant level of expression⁷².

Brachythecium buchananii, yellowish-green coloured moss, grows as dense mats belong to Brachytheciaceae was used by the native people for curing tumours. The initial phytochemical analysis

of *B. buchananii* confirmed the presence of alkaloids, tannins, flavonoids, terpenoids, triterpenoids, and cardiac glycosides in varying levels. The moss contains essential amino acids such as isoleucine, phenylalanine, and tyrosine. Terpenoid showed a significant level compared to other phytochemicals. Subsequently, the terpenoids was purified with column chromatography and eluted with different combinations of PE: EA solvent system. The 90:10 (PE: EA) purified fraction revealed the presence of terpenoid-related components such as dodecanal, hexadecane, tetradecanal, heptadecane, and 6, 4, 10-trimethyl-2-pentadecanone (sesquiterpenoid). Cytotoxicity of purified terpenoid of *B. buchananii* against the HeLa, MDA-MB-231 and MG 63 cell lines (0, 6.25, 12.5, 25, 50, and 100 µg/ml) vs. different time spans (24 and 48 h) revealed a concentration and duration-dependent impact. Cancer cells exhibited significant morphological anomalies at 50 and 100 µg/ml terpenoid extracts. Significant inhibition of MG 63 invasion in a dose-dependent manner was observed. The mode of action was *via* apoptosis, cell cycle arrest, and DNA fragmentation⁵⁵.

Similarly, the 100% PE eluted fraction of *Thuidium tamariscellum* revealed 15 compounds in GC-MS analysis such as α -cubebene, caryophyllene (sesquiterpenoids), dodecanal, 1-tetradecene, tetradecane, pentadecane, heptadecane, hexadecane, octadecane, eicosane, nonadecane, heneicosane, pentacosane, docosane, and hexacosane. Further, the antimetastatic analysis of purified terpenoid from *T. tamariscellum* displayed the efficacy against HeLa cell lines only.

Spjut *et al.*,⁸¹ screened species of bryophytes as antitumor agents; interestingly 43 species were found to be active, and 75 species were toxic to mice. The most significant activities was noted in the following families like Thuidiaceae, Brachytheciaceae, Hypnaceae, Grimmiaceae, Mniaceae, Dicranaceae, Neckeraceae, and Polytrichaceae. Petroleum ether, ethyl acetate, and n-butanol leaf extracts of *Marchantia convoluta* showed cytotoxic effects to lung carcinoma (H1299) and liver carcinoma (HepG2) and it was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Crude extracts of the *Mastigophora dicladus* and *Frullania* species showed cytotoxic activity against HL-60 and KB cell lines⁸².

Ferns are one of the oldest divisions of the Pteridophyta and comprise over 12000 species⁸³. The biomedical system and Ayurvedic systems of medicine suggested the use of some ferns in the Samhita Texts. Pteridophytes were also used by physicians in the Unani system of medicine. Recently, ethnobotanical and pharmacological studies have been carried out on ferns and their allies by several investigators. According to Lee *et al.*,⁸⁴ *Asplenium polyodon*, *Asplenium nidus*, *A. capillus-veneris*, *Ophioglossum gramineum*, and *H. arifolia* have anti-cancer, antidiabetic, and antiviral activity. Yang *et al.*,⁸⁵ reported inhibition of stomach cancer, prostate cancer, malignant melanoma, and mouse fibroblasts by crude extracts and several isolated compounds of *Cyrtomium fortunei*.

Pteris semipinnata showed *in-vivo* and *in-vitro* anticancer activity in human liver adenocarcinoma cell line (HePG II), human lung adenocarcinoma cell line (SPC-A-1), human gastric adenocarcinoma cell line (MGC-803) and human nasopharyngeal carcinoma cells by MTT assay⁸⁶. Uma and Pravin⁸⁷ reported *in-vitro* cytotoxic activity of *Marsilea quadrifolia* on MCF-7 cancer cell lines. *In-vitro* MTT assay of *Blechnum orientale* extracts against human colonic adenocarcinoma HT-29, human colonic carcinoma HCT-116, human breast adenocarcinoma MCF-7 and human leukemia K562 cancer cells were carried out by Lai and Lim⁸⁸. *Dicranopteris linearis* showed significant anti-metastatic potentiality under *in-vitro* MTT assay on MCF-7, HeLa, HT-29, HL-60, K-562, and MDA-MB-231 cell lines⁸⁶.

Angiosperms: Nisha and Sivadasan⁸⁹; Raji and Raveendran⁹⁰ and Udayan *et al.*,⁹¹ documented the ethno medicinally significant plants used by traditional healers of Wayanad District, Kerala and Namakkal district, Tamil Nadu. In this part, we analyzed such species connected with cancer therapy.

In 2018, Aswathy *et al.*,⁴² isolated, purified, fractionated and analysed the anti-metastatic potentiality of anthocyanin from *Bridelia retusa* against oral squamous carcinoma cells. Anthocyanin production at pH 4.4 – 4.6, HCl-ethanol extraction for 90 min yielded the maximum anthocyanin content, and fractionation of anthocyanin using

HPLC coupled with mass spectrometry revealed 07 fractions such as acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. The cytotoxicity effect of *B. retusa* anthocyanin extracts on human oral squamous cell carcinoma (SCC4, SCC9, and SCC25) cells using cell adhesion and cell viability assay was analyzed. The morphological alterations in SCCs cells after treatment with *B. retusa* anthocyanin and apoptotic cells as revealed by Hoechst staining. Flow cytometry revealed arresting of SCC25 cells mostly in the G0/G1 and S-G2/M stages with a concomitant up-regulation of sub-G1 fraction, indicating cell death by apoptosis. Apoptosis was further substantiated by the activation of caspase-3 expression in the SCC25 cells treated with *B. retusa* anthocyanin. *In-vitro* cytotoxic screening of *Glycyrrhiza glabra* belonging to Fabaceae showed glycyrrhetic acid in the chloroform extract and is a potential natural anticancer component. The study was carried using three different extracts (chloroform, methanol, and water) by the MTT method. Cell viability of glycyrrhetic acid in the three different extracts was also determined by trypan blue method using two different cell lines MCF7-cancerous and Vero-normal cell lines. The percentage viability of two different cell lines was 45.71% for the Vero-normal cell line and 78.78% for MCF7cancerous cell line⁹².

Oroxylum indicum is a member of Bignoniaceae and was widely used by the Keralites for the treatment of various ailments. The decoction of the bark was taken for curing gastric ulcers and the paste of the bark to mouth cancer, scabies, tonsil pain and other diseases⁹³. Cytotoxic assay of a leaf extract from *Annona muricata* on WRL-68 (normal human hepatic cells), MDA-MB-435S (human breast carcinoma cells) and HaCaT (human immortalized keratinocyte cells) lines by MTT assay, revealed that the extract at its lower doses exhibited a significant cytotoxicity on MDA-MB-435S and HaCaT cells with the IC₅₀ values of 29.2 and 30.1 µg respectively. Meanwhile, it exhibited only a moderate cytotoxicity towards WRL-68 with a comparatively higher IC₅₀ value i.e., 52.4 µg, indicating the differential cytotoxicity dosage levels among the cells⁹⁴. Essential oils from medicinal plants are proven for their therapeutic potential. Anti-proliferative effects of of EOs from *Pogostemon benghalensis* and *P. cablin* on human

colon cancer cell lines (SW 480) and human breast cancer (MCF-7) was studied by Pradeep and Murugan⁹⁵. The essential oil was extracted from the fresh leaves of the plants using the Clevenger apparatus and the components were fractionated by gas chromatography-mass spectroscopy. GC-MS analysis revealed 41 and 36 volatile compounds from the essential oil of *Pogostemon benghalensis* and *P. cablin* respectively. Cytotoxic and viability of the oil against SW 480 and MCF-7 tumor cell lines were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Trypan blue dye exclusion method. *P. beghalensis* showed cytotoxicity of 58.42% and 59.22% against SW480, MCF 7 respectively at concentration of 100 µg, while 57.47 and 58.77% cytotoxic effect was noticed with the oil from *P. cablin*. Significant reduction of the viability among SW 480 and MCF-7 cells was also noticed in a concentration-dependent manner.

Achyranthes aspera, a common weed along the road sides throughout India. The methanol extract of *A. aspera*, possess alkaloid, non-alkaloid, and saponins. A crude dose of 100 µg exhibited significant inhibitory effects on the Epstein-Barr virus antigen activation induced by the carcinogen 12-O-tetradecanoylphorbol-13-acetate in Raji cells (ATCC number CCL-86) under *in-vitro* conditions⁹⁶. *Solanum* is known for its alkaloid contents. *Solanum mauritianum* is commonly known as bugs weed. Tribes use the plant to cure skin-prone diseases. Anti-proliferative effect of *S. mauritianum* alkaloids against MCF 7 breast cancer cell lines was reported by Jayakumar and Murugan³⁹. Purification and fractionation of alkaloids from *S. mauritianum* yielded blue-colored caulophyllumine A and yellow-colored solasodine, both of which inhibited proliferation of MCF 7 cancer cell lines. The results were further substantiated by flow cytometry and V-FITC/ PI analysis. 25 and 50 µg/ml solasodine displayed an increase in the G1 phase and decreased S phase. V-FITC/ PI analysis revealed a concentration and time-dependent increase in the percentage of apoptotic and necrotic cells³⁹.

Fagonia indica, known as dhamasa is a flowering plant belongs to Zygophyllaceae. Members of *Fagonia* re-known for their use in traditional medicine and are found effective in curing many

skin problems⁹⁷. The aqueous extracts of *F. indica* have been found effective against different types of cancer cell lines. Waheed *et al.*,⁹⁸ reported bioactivity-guided fractionation to isolate the active and potent compounds of the *F. indica* extract. The activity assessed against three cancer cell lines: MCF-7 breast cancer, MDA-MB-468 estrogen-independent breast cancer, and Caco-2 colon cancer cells related to apoptosis.

The fruit extract of *Garcenia indica* was used by Hong *et al.*,⁹⁹ for the isolation of garcinol. The garcinol showed positive activities in the experimental HT-29 and HCT-116 colon cancer cells along with normal immortalized intestinal cells (IEC-6 and INT-407). The garcinol at IC₅₀ values (3.2–21.4 μ M) for 72 h treatment showed strong inhibitory properties in all the intestinal cells. The anticancer properties were higher in the cancer cells as compared to normal immortalized cells. Liao *et al.*,¹⁰⁰ also observed a high tumor-inhibiting activity of *G. indica* in the human colorectal cancer cell line (HT-29).

Morus alba (white mulberry), is cultivated throughout the world. Extracts from *M. alba* are traditionally used to cure cough, edema, insomnia, bronchitis, asthma, nose bleeding, wound healing, eye infections, and diabetes. Devi *et al.*,¹⁰¹ reported that the plant contains many pharmaceutically important compounds like moranoline, morusin, calystegin kuwanol, hydroxymoricin, albufuran and albanol. The leaves contain some active compounds like quercetin, rutin, apigenin, 1-deoxynojirimycin. Chon *et al.*,¹⁰² analysed the anti-proliferative power of methanolic extracts on different human cell lines like pulmonary carcinoma (Calu-6), colon carcinoma (HCT-116) and breast adenocarcinoma (MCF-7). Kikuchi *et al.*,¹⁰³ reported apoptosis-inducing, cytotoxic activity (IC₅₀ = 1.7 μ M) in HL-60 cell line. It induced topoisomerase II (IC₅₀ = 22.8 μ M) and clearly reduced the levels of pro-caspases^{3, 8, and 9}. *Wedelia Chinensis* is an indigenous species of India, South-East Asia, and China. It is one of the important anticancer plants belonging to Asteraceae and was rich in secondary metabolites like phenol, flavonoids, and tannin.

The essential oils of *W. chinensis* showed a positive effect on lung cancer cells under an *in-vitro* study. The GC-MS analysis recorded the

presence of two important compounds carvacrol and trans-caryophyllene. The study of B16F-10 melanoma metastatic cell line showed regulation of antioxidant enzymes (including catalase, superoxide dismutase, and glutathione peroxidase) in the treated cells. The amount of glutathione also increased, while the lipid peroxidation and nitric oxide levels decreased. The histopathology studies further corroborated with analytical results¹⁰⁴.

Schischkinnin and montamine were alkaloids isolated from the seeds of *Centaurea schischkinii*, and *Centaurea montana* belongs to the family Asteraceae. Both alkaloids exhibited significant cytotoxicity against human colon cancer cell lines⁸. According to Ayurveda *Digitalis lanata* and *Digitalis purpurea* of Plantaginaceae have anticancer activity¹⁰⁵.

Brucea fruit extract is used for the treatment of carcinomas of the liver, breast, thyroid, uterine cervix, rectum, anus, oesophagus, lung, skin and colon when topically applied⁷⁰. Psoralea fruit extract is used for the treatment of heart failure, coronary heart disease, leukoderma, urine bladder atonia, prostatitis and night bed-wetting. Seeds of this herb contain psoralen which is a photosensitizer, which damages membrane of normal cells and tumor cells. It also binds to DNA of cells and induces apoptosis and death of normal cells, diseased cells and tumor cells. Glossy Privet fruit is also used for treatment of cancer *i.e.*, 20 to 30% anti-cancer efficacies in patients with leukemia or lymphoma and also caused some adverse effects. Similarly, when mixed with Milkvetch root herb it attains 90% anti-cancer efficacy and brings about 10% or minimal side-effects¹⁰⁶.

Artemisia capillaries, a medicinal plant found in Korea. The methanol extracts of *A. capillaries* were used for the evaluation of DPPH (2,2-diphenyl-1-picrylhydrazyl), hydroxyl radical (OH) scavenging, reducing power assay connected with antioxidant activity and also trailed the anticancer activities using MTT assay. The extracts exhibited potential antioxidant activity and anticancer activity under *in-vitro* condition¹⁰⁷. *Rosa roxburghii* is another beneficial species used for improving immune responses, enhancing digestive ability, and demonstrating anti-aging effects. Evidence showed that herbal medicine soups containing extracts of

this plant shows efficacy in malignant tumors. The studies were undertaken to evaluate anticancer potential against three carcinoma cell lines like human esophageal squamous carcinoma CaEs-17, human gastric carcinoma SGC-7901, and pulmonary carcinoma A549 by MTT assay and flow cytometry. The combination of *Rosa roxburghii* and *Fagopyrum cymosum* showed significant inhibition of cell growth and increased apoptosis. The mRNA and protein expression levels of Ki-67 and Bcl-2 were regulated by the extracts¹⁰⁸.

Goniiothalamus macrophyllus of Annonaceae showed the presence of goniiothalamine, a natural compound. Apoptosis induction by goniiothalamine in the Hela cervical cancer cell line was observed by MTT assay method¹⁰⁹. The ethanolic extract of *Derris scandens* showed potent radiosensitizer potency against human colon cancer HT29 cells. *D. scandens* extract in combination with gamma irradiation synergistically sensitizes HT-29 cells to cell lethality by apoptosis and mitotic catastrophe¹¹⁰. *Atractylis lancea* a medicinal herb in Asia, has been reported to have anti-tumor effects on cancer cells. *In-vitro* studies on the proliferation of the Hep-G2 liver cancer cell line by MTT test revealed effective inhibition in a concentration- and time-dependent manner¹¹¹.

Phyllanthus emblica is known to have various pharmacological properties. Cytotoxicity to human fibrosarcoma cells, HT1080, was assessed by viability assay using the 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyltetrazolium bromide. The results showed that *P. emblica* extract reduces cell proliferation, migration, invasion, and adhesion in both dose- and time dependent manners, especially growth arrest with low IC₅₀ value¹¹². Ethanolic extract of *Boerhaavia diffusa* (Punarnava) showed cytotoxic effect against Hela cell lines and inhibited the S-phase of the cell cycle. It also inhibits the growth of cancer cells in DMBA-induced cancer carcinogenesis in mice through free radical scavenging¹¹³. *In-vivo* methanolic extract of *Cucurbita maxima* was studied for anticancer activity against Ehrlich ascites carcinoma cells in mice. Oral administration of the methanolic extract of the *C. maxima* at a dose of 200 mg/kg and 400 mg/kg showed the cytotoxicity in mice in a dose-dependent manner¹¹⁴.

Calotropine, is a glycoside present in *Calotropis procera*, has shown anti-tumor activity against human epidermoid carcinoma cells of rhinopharynx under *in-vivo* conditions. They also inhibited the growth of cells and mitotic activity in a dose-dependent manner¹¹⁵. The methanolic and acetone extract of the flowers of *Parthenium hysterphorus* were showed *in-vitro* anticancer activity against the A-549 (Lung adenocarcinoma) cell lines by MTT and Trypan blue exclusion assay¹¹⁶. The methanolic extract of the leaves of the *Citrus maxima* displayed anticancer activity (at a dose 200-400 mg/kg), against *Ehrlich Ascites* carcinoma cell lines in Swiss albino mice in a dose-dependent manner¹¹⁷. *Azadirachta indica*, *Boesenbergia pandurata*, and *Coscinium fenestratum* were tested for their cytotoxic effects. The results showed that the three plants have high cytotoxic potentiality against the Hep2 cell lines with a minimum concentration of 0.05%^{118 & 119}.

CONCLUSION: There are studies on the *in-vivo* and *in-vitro* analysis of anticancer drugs and plants. It was generally established that the drugs, including the anticancer compounds, require clinical research trials for marketing permissions. Except for some exceptional circumstances, all the drugs need to go through all the phases of trials according to the guidelines of international agencies like the FDA and EMA. Plant-based compounds have shown less toxicity than conventional synthetic compounds. Insufficient data available regarding the quality, safety, and efficacy of herbal drugs lead to side effects, which is a major problem nowadays. The process of oncological drug development and marketing is regulated through the involvement of experts and advisory authorities. There are several regulatory framework models for prescribing such drugs, and there is a need for harmony among regulating. It is evident from studies that plants of the same species grown in different areas vary in their medicinal compound profile, which calls for the need to focus on the production of uniform and high-quality plants with a uniform metabolite profile and it is achieved through the advancement of biotechnology and bioinformatics. The current review comprehensively highlights the mechanism of antitumor action of important plants. It generates awareness regarding the usage of these herbs and exploring their properties.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: The authors doesn't have any conflict of interest.

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How to cite this article:

Krishnan R, Manoj GS, Pradeep DP, Sumayya SS, Greeshma GM, Jayakumar K and Murugan K: Secondary metabolites as lead molecules for anticancer therapies: a review. *Int J Pharm Sci & Res* 2021; 12(8): 4042-59. doi: 10.13040/IJPSR.0975-8232.12(8).4042-59.

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A Novel Imidazole Bound Schiff Base as Highly Selective “Turn-on” Fluorescence Sensor for Zn^{2+} and Colorimetric Kit for Co^{2+}

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Received: 30 August 2021 / Accepted: 18 October 2021

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Abstract

An imidazole based Schiff base (2-[(1H-imidazole-2-ylmethylene)-amino]-4-methyl-phenol) (IMP), with an imine unit, has been designed and characterized by various standard methods. The evaluation of the probe as a fluorogenic sensor for Zn^{2+} and a chromogenic sensor for Co^{2+} has been rationalized in terms of the PET mechanism. In the presence of Zn^{2+} , a light yellow colored solution of IMP with maximum absorption of 364 nm becomes bright yellow with maximum absorption of 410 nm and a measurable fluorescent signal at 612 nm with bathochromic enhancement. The sensitivity of the fluorescent based assay (6.78×10^{-9} M) for Zn^{2+} is far below the limit in the World Health Organization (WHO) guidelines for drinking water (7.6×10^{-5} M) and therefore it is capable of being a practical system for the monitoring of Zn^{2+} concentrations in aqueous samples. Moreover, IMP showed a highly selective colorimetric response to Co^{2+} by displayed an obvious pink color upon addition of metal solution immediately without any interference from other ions. These results provide a new approach for selectively recognizing the two most important trace elements in the human body simultaneously, for Zn^{2+} by emission spectra and Co^{2+} by the naked eye.

Keywords Imidazole · Colorimetric · Fluorogenic · Zn^{2+} · Co^{2+}

Introduction

In recent years, fluorescent molecular sensors for the detection of various metal ions have been an extensive area of research due to several advantages over other techniques [1–3]. A large portion of the metal ions assuming a

significant role in an assortment of industrial tasks like food, drug, paper, material, water treatment, assembling of batteries, and electronic field [4–6]. Fluorescence technology was broadly improved from the year 1960s and there has been sensational development in the utilization of fluorescence for cell and atomic imaging that can be seen today in various disciplines. In particular, Schiff bases have excellent coordination environment, which makes them more convenient to be used as a fluorescent probe for the detection of metal ions [7–9]. Schiff base subsidiaries containing nitrogen–oxygen-rich coordination as a receptor site pave an effective medium for fluorescent detection with observable color change [10–12].

Zinc is perhaps the most flexible and essential metal with diverse biological functions like cistron transcription, brain performance, pathology, immune perform, and cell replica [13–15]. Thus, hereditary irregularities prompting the misregulation in the activities of zinc metal may cause various sicknesses like cerebral ischemia, amyotrophic sidelong sclerosis (ALS), Parkinson's illness, and epilepsy [16]. Because of the absence of spectroscopic

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marks of Zn^{2+} , it involves incredible significance to create productive recognizing and observing techniques for Zn^{2+} [17]. Fluorescence based recognition is an amazing asset to identify Zn^{2+} due to its high affectability and quick reaction. To date, the number of chemosensors for Zn^{2+} has been developed, but a few of them actually experience issues in distinctive Zn^{2+} from other metals. Therefore, it is desirable to develop a method for monitoring and detecting Zn^{2+} , which would offer a promising approach to study its actions in biological, medical, and environmental circumstances [18].

Cobalt is an essential trace element that is actively involved in the development of vitamin B12 which is also found in specific minerals of the earth's crust [19, 20]. Although a variety of methods, such as atomic absorption spectroscopy [21, 22] chemiluminescence [23, 24], and ICP mass spectrometry [25–27], are widely used to detect cobalt ions, they have disadvantages because of high-cost technic. Hence, the interest for techniques that are straightforward and particular to distinguish cobalt particles is persistently expanding. Among these recognition strategies, a colorimetric technique for the location of metal particles is very much needful. With recent development in technology, the invention of a new small molecular systems as a probe in different forms are developing as an important colorimetric receptors for metal ions [28, 29]. Also, the visual sensing of Co^{2+} over a wide range of transition metal ions using imidazole cored Schiff base is an important method of detection which allows a direct analysis of the substrate [30].

At hand, for these reasons, we report the design, synthesis and spectroscopic characterizations of a novel imidazole bound Schiff base based chemosensor, IMP for selective fluorometric and colorimetric detection of Zn^{2+} and Co^{2+} respectively. The unique combination of imidazole and aromatic phenolic moiety with imine as a bridge results in the excellent coordination to metal ions by providing the dual property of the sensor. Until now, there are no reports on imidazole bound Schiff base sensor can successfully distinguish Zn^{2+} and Co^{2+} by turn-on fluorescence and visual effect. Moreover, it would quantify Zn^{2+} in the water sample appreciated the efficacy of the probe.

Experimental

Reagents and Equipment's

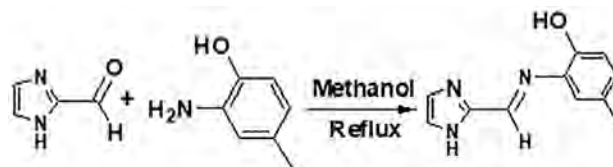
All the solvents (analytical and spectroscopic grade) were retrieved commercially. All the needed chemicals were purchased from Sigma-Aldrich. The stock solutions of various metal ions were prepared from $\text{Zn}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, NaCl , KCl , $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, InCl_3 , MnCl_2 , $\text{Cu}(\text{OAc})_2$, $\text{Co}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, $\text{Hg}(\text{OAc})_2$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{Pb}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, FeCl_3 ,

$\text{Mg}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ and $\text{Cd}(\text{OAc})_2 \cdot \text{H}_2\text{O}$. The melting point of the synthesized probe was analyzed by an electrical heating method using capillary tubes.

Absorption spectra were recorded at room temperature using Thermo Scientific™ Evolution 201 UV–visible Spectrophotometer. FT-IR spectrum was worked on JASCO-8000 FT-IR spectrophotometer using KBr pellets in the range of $400\text{--}4000\text{ cm}^{-1}$. Elemental analysis of probe was done using an ElementarVario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in DMSO-d_6 as the solvent on a Burkert Advance DRX 300 FT-NMR spectrometer with TMS as the internal standard. Liquid chromatography–mass spectrometry (LC–MS) was worked on a Waters 3100 Mass Detector using ESI–MS technique at Cochin University of Science and Technology, Kochi, India. Fluorescence measurements were performed on a Horiba Fluorolog3 FL-1057 model fluorescence spectrometer.

Synthesis of IMP

(2-[(1H-imidazole-2-ylmethylene)-amino]-4-methyl-phenol) A solution of Imidazole-2-carboxaldehyde (0.20 g, 2 mmol) in methanol was added slowly to a solution of 2-amino-p-cresol (0.25 g, 2 mmol) in methanol. The reaction mixture was blended for 8 h at $60\text{ }^\circ\text{C}$. The resulting light yellow colored solution was allowed to evaporate at room temperature. Anhydrous ether was added to deposit a yellowish precipitate. The precipitate obtained was washed several times with methanol which was then recrystallized from DMF and then dried in vacuo over anhydrous CaCl_2 . The purity of the compound was tested with TLC (Hexane:Methanol 10:1). The yield: 0.29 g (81%), m.p. $115\text{ }^\circ\text{C}$ (Scheme 1). UV–vis: λ_{max} 294, 362 nm. FT-IR: 3300 cm^{-1} (ν_{OH}), 3020 cm^{-1} (ν_{NH}), 1624 cm^{-1} ($\nu_{\text{C=N}}$ of imine), 1589 cm^{-1} ($\nu_{\text{C=N}}$ of imidazole). ^1H NMR (400 MHz, DMSO-d_6 , $25\text{ }^\circ\text{C}$): δ = 12.62 (s, 1H), 9.78 (s, 1H), 7.80, 7.72 (dd, 2H), 7.26 (s, 1H), 6.84, 6.75 (d, 1H), 6.51, 6.49 (d, 1H), 2.50 (s, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-d_6 , $25\text{ }^\circ\text{C}$): δ = 162.88, 148.20, 138.25, 136.46, 131.80, 128.38, 124.51, 122.48, 122.10, 117.81, 20.98 ppm. LC–MS: m/z calcd for $\text{C}_{11}\text{H}_{11}\text{N}_3\text{O} - \text{H}^+$: 200.40; found 200.04. CHN analysis calcd



Scheme 1 Synthesis of IMP

(%) for $C_{11}H_{11}N_3O$ (201.09): C, 64.98; H, 5.01; N, 21.23; O, 7.35; found: C, 64.78; H, 5.012; N, 20.98; O, 7.15.

Analytical Procedure

Fluorescence Titration of IMP with Zn^{2+}

Probe IMP (96 mg, 1 mmol) was dissolved in 1:9, v/v 0.01 M H_2O -DMF, pH 7 (5 mL). $Zn(OAc)_2 \cdot H_2O$ (1 mmol) was dissolved in 1:9, v/v 0.01 M H_2O -DMF, pH 7 (5 mL). Taking 2 mL from each solution and mixing them for a few seconds, fluorescence spectra were taken at room temperature.

UV-visible Titration of IMP with Zn^{2+}

Probe IMP (96 mg, 1 mmol) and $Zn(OAc)_2 \cdot H_2O$ (1 mmol) were dissolved in DMF solution (5 mL). 0.5–5 μ L of the prepared solution was carried for took UV-vis absorption spectra at room temperature.

Job's Plot Measurement of Zn^{2+}

Probe IMP (1 mmol) was dissolved in 1:9, v/v 0.01 M H_2O -DMF, pH 7 (5 mL). Prepared $Zn(OAc)_2 \cdot H_2O$ (1 mmol) solution in 1:9, v/v 0.01 M H_2O -DMF, pH 7 (5 mL). 30–170 μ L of the analyte solutions were taken and added probe solutions were until it contains 200 μ L in each vial. Each vial was then diluted with DMF to make a total volume of 3 mL. Shaken well the solutions for a few minutes, fluorescence spectra were taken at room temperature.

Detection of Zn^{2+} with IMP—Competition Experiment.

Stock solutions for various metal ions (Cu^{2+} , Fe^{2+} , Fe^{3+} , Ni^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} , Cd^{2+} , Na^+ , K^+ , In^{3+} , Sr^{2+} , Mn^{2+} , Hg^{2+} and Pb^{2+} were prepared with a 1.0×10^{-3} M concentration using metal anions in deionized water, and the pH was 6.45. The solution of the probe IMP was prepared with a concentration of 1.0×10^{-3} M in DMF, and the pH of the solution was nearly 7.

Quantum Yield Measurement

Quantum yield (QY) is defined as the efficiency of converting absorbed light into emitted light, which can be in the form of fluorescence. The QY of IMP and IMP- Zn^{2+} was measured by a comparative fluorescence method using Rhodamine B in 1×10^{-3} M DMF as the standard (std) solution (QY_{std} = 0.97 at an excitation wavelength of 414 nm, n_{std} = 1.36). The QY of probe and complex were calculated by Eq. (1).

$$QY_{Sample} = QY_{reference} \times \frac{ms}{mr} \times \left[\frac{\eta_s}{\eta_r} \right]^2 \quad (1)$$

Where ms , mr , η_s , and η_r represent slope and refractive index of the solvent of sample and reference which is measured by fluorescence spectrophotometer, respectively.

Calculation of the Binding Association Constant for IMP- Zn^{2+} Complex

The association constant (K_a) was calculated using the Benesi-Hildebrand plot (or a double-reciprocal plot) as shown in Eq. (2).

$$\frac{1}{I - I_0} = \frac{1}{K_a} x (I_{max} - I_0) x [Zn^{2+}] + \frac{1}{I_{max} - I_0} \quad (2)$$

where; I is the experimentally measured fluorescence intensity, I_0 is the fluorescence intensity of probe IMP alone, and I_{max} is the saturated fluorescence intensity of the IMP- Zn^{2+} complex.

Quantification of Zn^{2+} in Real Samples

The application study of probe IMP has been done in two different kinds of water samples collected from our home. The fluorescent analysis was carried out by adding 50 μ L (1 mmol) of probe IMP to a 3 mL real sample solution having Zn^{2+} . Solutions were blended for about 10 min at 25 °C. Their fluorescence spectra were obtained.

Colorimetric Chemosensor

Naked Eye and UV-vis Detection of Co^{2+}

Receptor IMP (96 mg, 1 mmol) was dissolved in a set of polar and non-polar solvents (2 mL) and 20 μ L of the receptor IMP was mixed with 20 μ L of $Co(OAc)_2 \cdot H_2O$ solution in DMF. After few seconds, UV-visible absorption spectra were taken at room temperature.

Job's Plot Measurement of Co^{2+}

Receptor IMP (96 mg, 1 mmol) was dissolved in DMF (2 mL). Added 30–170 μ L of the analyte solutions into receptor solutions taken in each cuvet by made up to a total of 200 μ L volume. DMF was poured to mark up to 3 mL volume of the total solution. After shaking the solutions, UV-visible absorption spectra were recorded at room temperature.

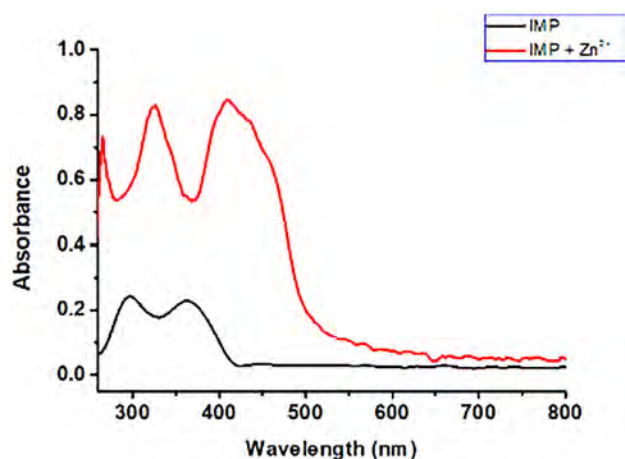


Fig. 1 A comparative UV-visible spectra of IMP and IMP-Zn²⁺

Detection of Co²⁺ with IMP—Competition Experiment.

For the color change analysis, UV-visible spectra were recorded. The colorimetric detection of various metal ions was carried out at room temperature using 1 mL volume of each metal ion solution of a 1.0×10^{-3} M concentration and 1 mL of the Schiff base ligand (1.0×10^{-3} M), and the solution mixture was diluted to 10 mL by adding the DMF solvent. The pH of the solution mixture was 7.25. After mixing properly, the UV-visible spectra of the mixtures were recorded.

Calculation of the binding association constant for IMP-Co²⁺ complex

The association constant (K_a) was calculated using the Benesi-Hildebrand plot (or a double-reciprocal plot) as shown in Eq. (3).

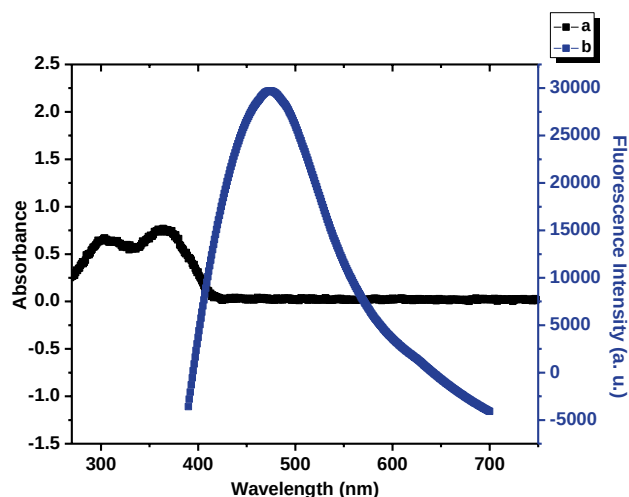


Fig. 2 Combined spectra of IMP, a UV-visible spectrum of IMP, b Fluorescence spectrum of IMP

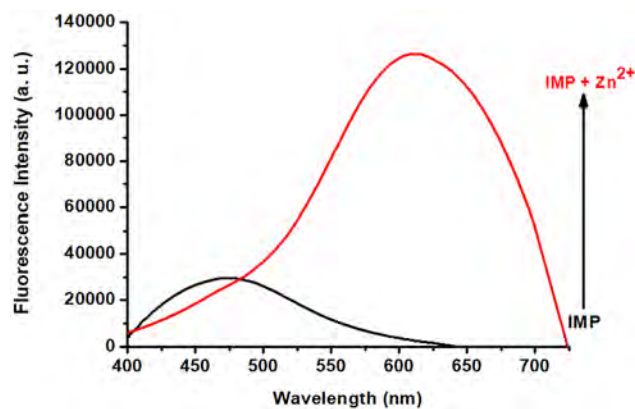


Fig. 3 Combined fluorescence spectra of ligand IMP and IMP + Zn²⁺

$$\frac{1}{A - A_0} = \frac{1}{K_a} x (A_{max} - A_0) x [Co^{2+}] + \frac{1}{A_{max} - A_0} \quad (3)$$

Where; A is the experimentally measured absorbance intensity, A₀ is the absorbance intensity of probe IMP alone, and A_{max} is the saturated absorbance intensity of the IMP-Co²⁺ complex.

Results and Discussion

We synthesized the new imidazole bound system as a sensor molecule IMP by following the general route of bonding a hetero aromatic aldehyde to an aromatic cresol and characterized by CHN analysis, UV-visible, FT-IR, elemental analysis, LC-MS and ¹H and ¹³C NMR techniques.

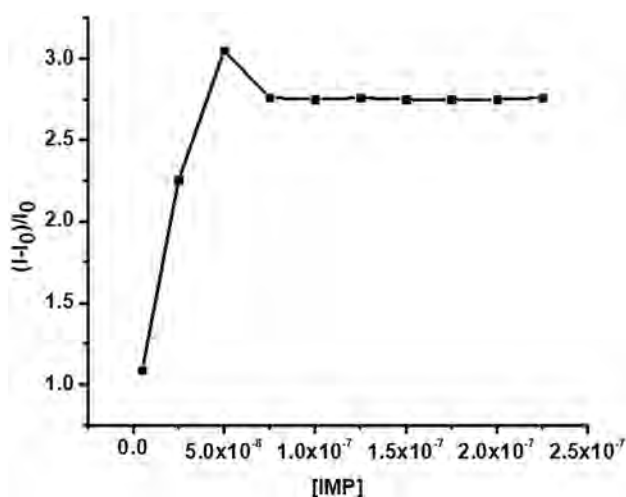


Fig. 4 Effect of concentration of IMP on fluorescence change of the system

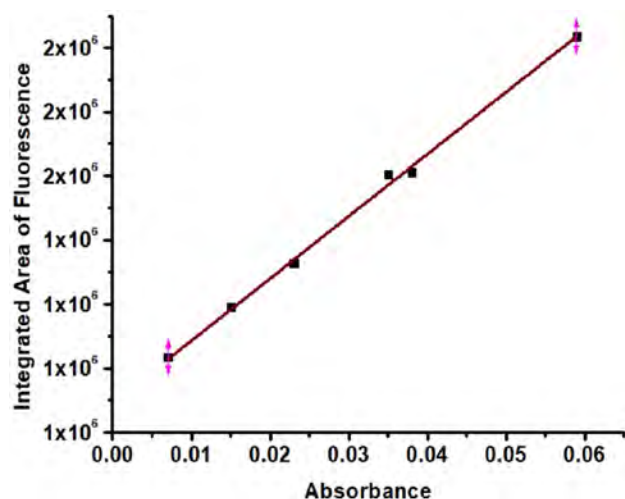
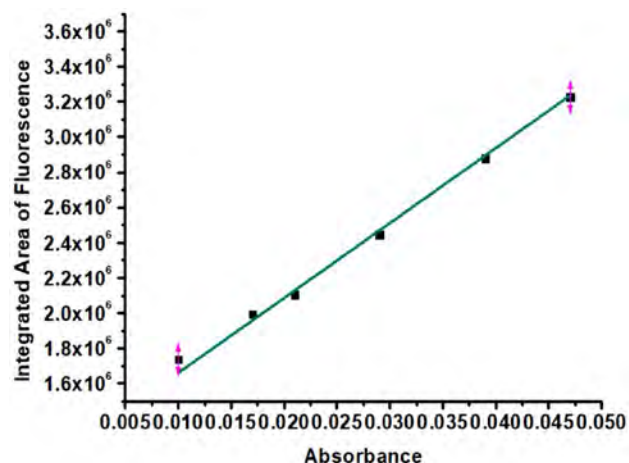
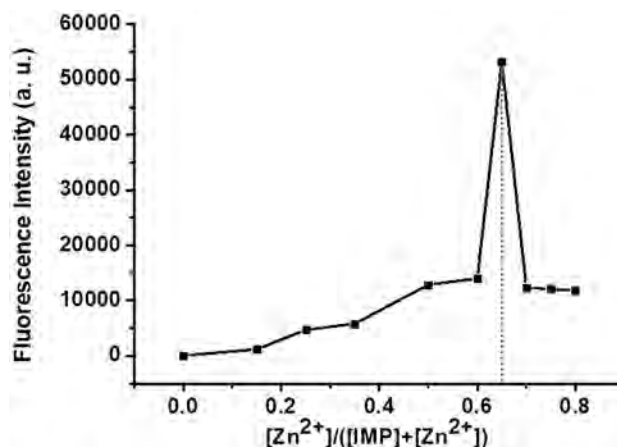


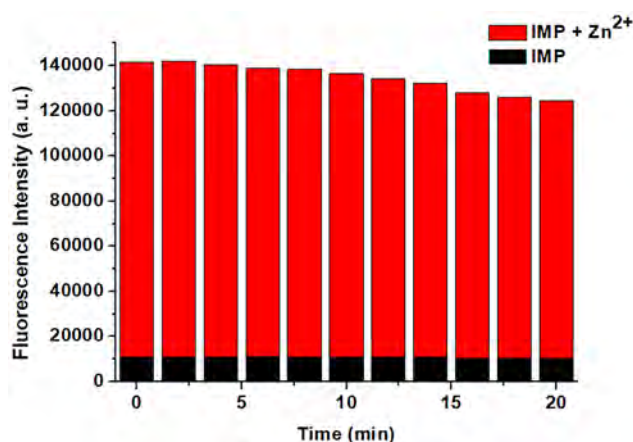
Fig. 5 QY graph of ligand IMP

Spectral Characterizations

The elemental concentrations of carbon, hydrogen, and nitrogen in the synthesized ligand were determined using CHN analysis. The results indicate that the synthesized compound is analytically pure and is in good agreement with the proposed chemical formula. The electronic spectral investigations were carried out in 1.0×10^{-5} M DMF solution of ligand in the range of 200–800 nm. The spectral assignments for electronic transitions in ligand and peaks attributed to starting materials lying in the region of 200–260 nm and 200–300 nm for aldehyde and amine respectively. As per the spectra, a new peak is formed in the region of 350–370 nm indicated the presence of the imine group. The two absorption maxima corresponding to two intra ligand transitions: a weak peak and a strong

Fig. 6 QY graph of IMP + Zn²⁺Fig. 7 Job's plot of IMP and Zn²⁺

peak at 294 nm and 362 nm. Low energy band is due to $n \rightarrow \pi^*$ on $-\text{HC}=\text{N}-$ group which is characteristic of the Schiff base [31]. The high energy band can be attributed to the $\pi \rightarrow \pi^*$ transitions on imidazole and phenol aromatic rings [32]. The important bands observed in the IR spectrum of the ligand along with their functional group assignments as per the literature data of similar compounds and the four characteristic peaks around 3300 cm^{-1} , 3020 cm^{-1} , 1624 cm^{-1} and 1589 cm^{-1} were registered as vibrational modes of O–H stretching, N–H stretching, C=N of newly formed imine and C=N of aromatic imidazole ring [33, 34]. Mass spectrometry was performed on the IMP ligand to determine its molecular weight and fragmentation pattern. The molecular ion peak $[\text{M}-1]^+$ was observed at m/e 200.4, confirming the formula weight (F.W.) which is the same as the calculated M^+ value. ^1H NMR and ^{13}C NMR spectrum of the IMP indicated the proof for ligand formation. Singlet in the region of 2.50 ppm is due to the presence

Fig. 8 Effect of time on IMP and IMP-Zn²⁺

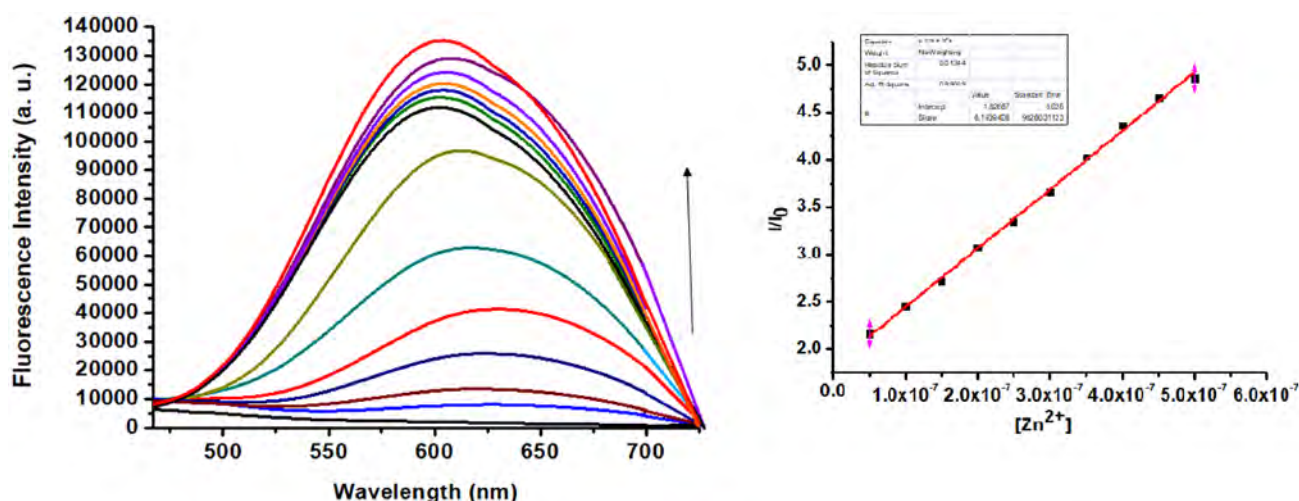


Fig. 9 Effect of concentration of Zn^{2+} on the fluorescence intensity of IMP and inset represents linear calibration graph between the ratio of fluorescence intensities and concentrations of Zn^{2+}

of methyl protons. The presence of singlet at 9.78 ppm is caused due to the presence of imine H. Another downfield singlet discernible at around δ 12.63 was allotted to NH proton. The fairly higher downfield effect on NH proton owed to strong intramolecular H bonding. The remaining protons of aromatic moieties were present in the form of singlet and doublets at δ 6.49–7.80 ppm [35]. The proton decoupled ^{13}C NMR spectrum provides direct information about the carbon skeleton of the molecule. Signals around 122 and 136 ppm corresponds to carbons of the imidazole ring. Aromatic carbons of phenol ring resonate between 117–140 ppm. A high value near 162 ppm is due to the presence of imine carbon. Carbon attached to OH resonates at 148 ppm. The methyl group in phenol resonates at 20 ppm [36].

Optical Response of IMP Towards Zn^{2+}

The ligand IMP contains imidazole moiety is frequently found as an indispensable piece of a fluorophore which gives oxygen to metal coordination and an acidic proton which could be lost open metal chelation to frame a monoanionic compound. Also, the lone pairs on imine nitrogen and solitary sets on imidazole nitrogen have been adding to develop a steady metal complex by clung to the empty d orbital of metals. The underlying visibility and substance properties are anticipated to prompt metal particle holding qualities not quite the same as the noticed for the beginning materials. Then, we inspected the UV–visible titration for IMP by adding traces of Zn^{2+} metal solution in DMF. At the point when the sensor IMP was titrated with Zn^{2+} , there seen a bathochromic shift in the frequency range, 410–430 nm with higher absorption maxima even at lower concentrations of

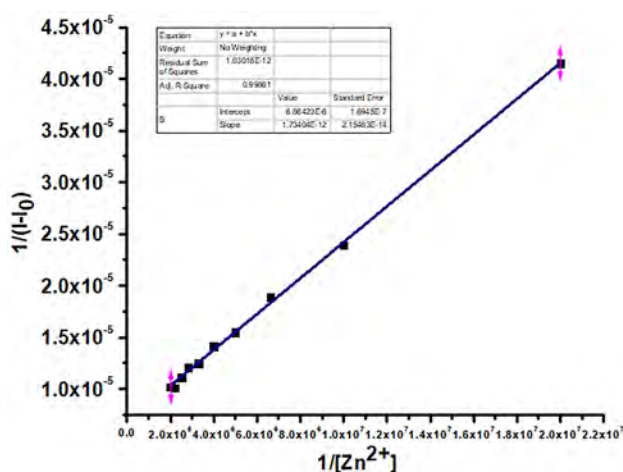


Fig. 10 Linear representation of binding constant for IMP- Zn^{2+} complex

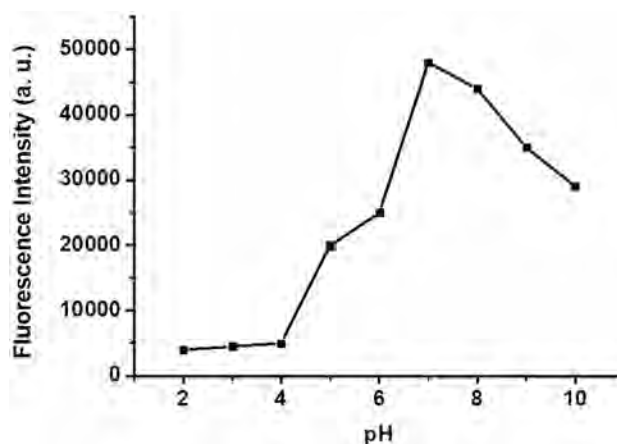
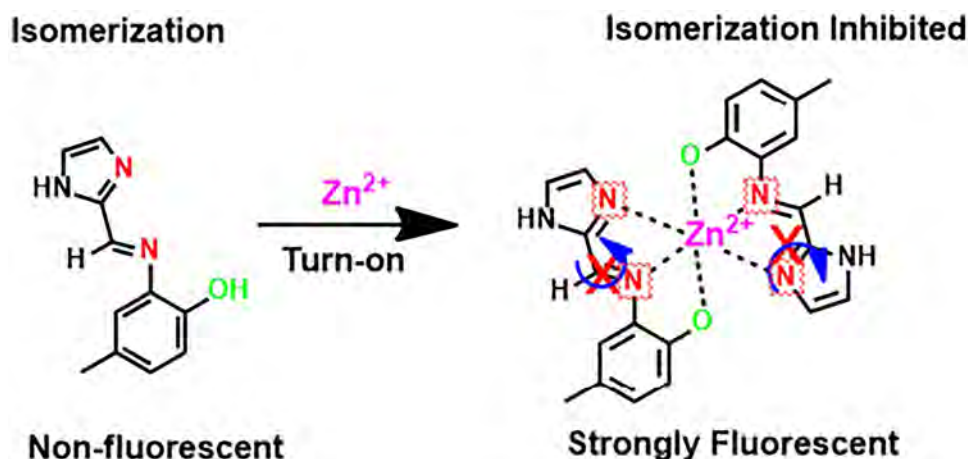


Fig. 11 Effect of pH on IMP

Scheme 2 Proposed binding mechanism of IMP with Zn^{2+} 

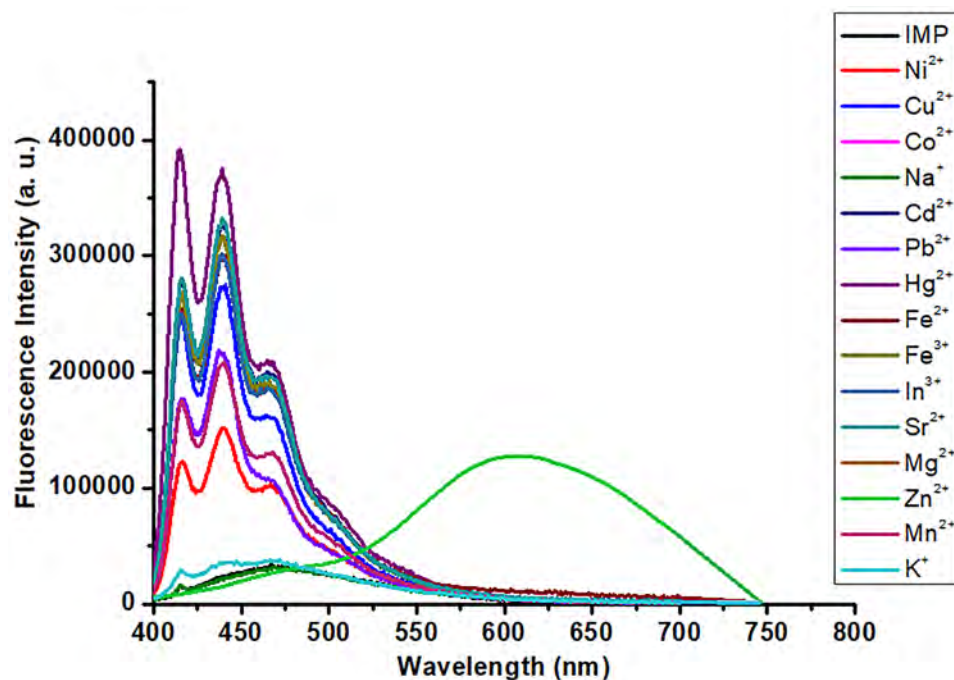
the analyte. Additionally, the peaks at 294 nm and 364 nm for the IMP ligand were dense together to form a new peak in the area of 340–350 nm for the metal complex (Fig. 1). The new peak formed thus confirmed the clean conversion of IMP to IMP-Zn^{2+} complex.

The photophysical properties and sensing behavior of the ligand IMP were investigated using UV absorption and fluorescence spectroscopy. First, the UV–vis spectrum of the ligand IMP (1×10^{-3} M) was recorded in DMF. IMP exhibited two absorbance peaks at 294 and 364 nm corresponding to the $\pi - \pi^*$ and $n - \pi^*$ transitions, respectively. Also, the fluorescence emission spectra of IMP were measured in DMF (1×10^{-3} M) and the emission peak was observed at 474 nm for IMP. The ligand exhibited excellent absorption and fluorescent characteristics are shown in Fig. 2.

Upon the addition of Zn^{2+} ions to IMP in DMF, attractive change has been noticed (Fig. 3), where the fluorescent intensity peaks for ligand would disappear and a new peak with enormous fluorescent enhancement is observed at 612 nm. Since the ligand is non-fluorescent showed a fluorescent intensive peak around the region 474 nm. The complete disappearance of peak obtained for IMP alone in IMP-Zn^{2+} complex confirmed the chelation power of metal towards the active site of the ligand.

We have investigated the concentration of ligand IMP to attain sensitivity. A graph was plotted on the concentration of IMP with a difference in fluorescence intensity, $(I - I_0)/I_0$, where I_0 is the fluorescence intensity of probe alone and I is the fluorescence intensity of probe with an analyte. In Fig. 4, there was a sharp increase in intensity on increasing

Fig. 12 Fluorescent intensity of IMP in DMF due to 1 equi. of metal ion. The fluorescence due to Zn^{2+} is shown as a new peak and fluorescence due to other metal ions and IMP are the baseline curves



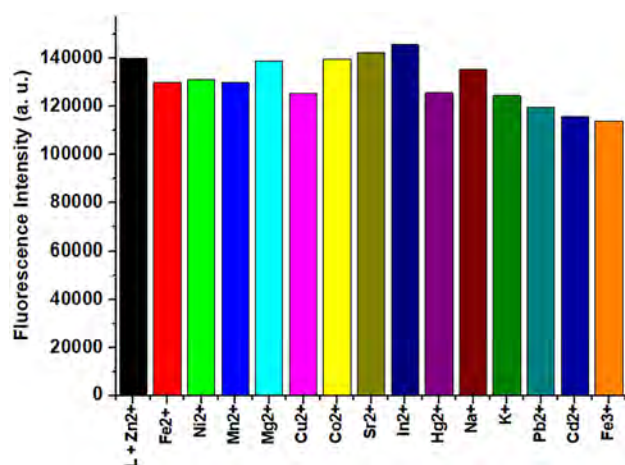


Fig. 13 Interference fluorescence of IMP- Zn^{2+} complex in the presence of various metal ions

the concentration of probe but reached a maximum value at 5×10^{-8} M and becomes constant. Hence we used the same for further studies.

The QY graphs are plotted against absorbance versus fluorescence intensity for IMP alone (Fig. 5) and IMP- Zn^{2+} (Fig. 6). From the QY calculations (Eq. 1), the fluorescence quantum yields of probe IMP in the free and Zn^{2+} bound state were found to be 0.0093 and 0.7873 respectively. However, probe with Zn^{2+} with quantum yields higher than 0.1 is still considered quite fluorescent and IMP with quantum yield lesser than 0.1 is considered as non-fluorescent [37].

A job's plot was drawn by taking the concentration of analytes on x-axis and fluorescence intensity corresponds to the analyte in y-axis showed the 1:2 binding between analyte Zn^{2+} and the probe IMP (Fig. 7). Further characterization of IMP- Zn^{2+} complex was done by CHN analysis. calcd (%): C, 57.89; H, 5.67; N, 16.88; O, 6.43. This might be a piece

of evidence for the 1:2 molar complexation of the probe with an analyte.

Change in fluorescence intensity with time was studied by illuminating the probe solution in the absence and presence of Zn^{2+} respectively. The IMP solution gave a stable fluorescence signal up to 21 min. Upon addition of Zn^{2+} , the fluorescence intensity of the probe enhanced with much more photostability which eliminates the time-consuming incubation step disposes by many of the existing methods for real samples (Fig. 8).

Execution of the Sensor

Effect of Concentration

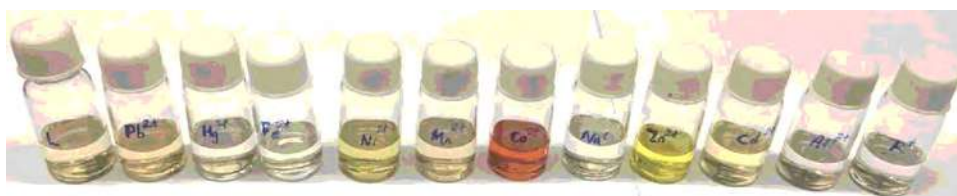
On expanding the centralization of Zn^{2+} , a consistent expansion in the intensity just as an adjustment of the shape of the fluorescence signal was noticed (Fig. 9). A calibration graph was plotted with a concentration of Zn^{2+} against I/I_0 , and it was found to be linear in the range 5×10^{-8} to 5×10^{-7} M (inset of Fig. 9). The limit of detection and limit of quantification was calculated using equations '3S/m' and '10S/m', where 'S' was the standard deviation and 'm' was the slope of the calibration graph. The limit of detection and limit of quantification was found to be 6.78×10^{-9} M and 2.26×10^{-8} M respectively. In the presence of Zn^{2+} , the probe IMP shows selective chelation enhanced fluorescence (CHEF) [38, 39]. The upgrade in fluorescence spectra in this manner force inflexibility and consequently decline the nonradiative decay of the energized state. In out range focus, the intensity of IMP isn't change due to the zinc particle uncomplexed.

The binding association constant (K_a) was graphically evaluated by plotting $1/(I-I_0)$ versus $1/[\text{Zn}^{2+}]$, as shown in Fig. 10. From the linear relationship graph, the K_a value calculated from Eq. (2) was $3.96 \times 10^6 \text{ M}^{-1}$.

Table 1 Comparison of the characteristics of the IMP sensor with those of the previously reported Zn^{2+} sensor

Sensor	Dynamic Range (M)	Detection Limit (M)	Interfering Ions	Ref
4-chloro-2-(5-phenyl-1-(pyridin-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol	0 to 7.0×10^{-6}	1.2×10^{-7}	Ni^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+}	[38]
Tris(3-(2-hydroxyacetophenone)propyl)amine	1.6×10^{-7} to 5.0×10^{-5}	8.8×10^{-8}	Cu^{2+}	[41]
(E)-3-((4-(diethylamino)-2-hydroxybenzylidene)amino)-2,3-dihydrothiophene-2-carboxamide	0 to 8×10^{-5}	2.55×10^{-6}	No interference	[42]
2-[(2-Hydroxy-benzyl)-pyridin-2-ylmethyl-amino]-N-quinolin-8-yl-acetamide	-	66×10^{-9}	Ni^{2+} , Fe^{2+} , Co^{2+} , Cu^{2+}	[37]
N,N phenylenebis salicylideaminato	1.6×10^{-7} to 1.0×10^{-5}	1.5×10^{-7}	Co^{2+}	[43]
(E)-(2-((2-nitrobenzyl)(2-((E)-(2-hydroxynaphthalen-1-yl)methyleneamino)ethyl)amino)ethylimino)methyl)naphthalen-2-ol	-	1.0×10^{-7}	-	[39]
2-[(1H-imidazole-2-ylmethylene)-amino]-4-methyl-phenol	5×10^{-8} to 5×10^{-7}	6.78×10^{-9}	No interference	This work

Fig. 14 Color of IMP upon addition of various metal ions



Effect of pH

To find out whether the probe IMP is stable at acidic or basic pH conditions, we need to take the fluorescence spectra in the pH range 1–10 (Fig. 11). From which, we have remarked that the highest fluorescent intensity was observed at pH 7 indicated the stability of probe in basis buffer. Hence, we did further experiments in the same buffer solution.

Mechanism of Zn^{2+} Detection

The probe, IMP alone could act as a non-fluorescent system and becomes highly fluorescent upon binding with Zn^{2+} ions due to the formation of a rigid system. We have been attempted to be rationalized on the basis of PET mechanism, which is a commonly used strategy for metal sensing fluorosensors showing intensity enhancement in the presence of suitable metal ions. The enhanced fluorescence in our system is a consequence of combined effects of chelation-enhanced fluorescence (CHEF), C=N isomerization, and inhibition of photo-induced electron transfer (PET). In order to substantiate it, the quantum yield values for IMP before and after binding have been undertaken [40]. Scheme 2 Proposed binding mechanism of IMP with Zn^{2+} + Scheme 2 Proposed binding mechanism of IMP with Zn^{2+} + Scheme 2 Proposed binding mechanism of IMP with Zn^{2+}

Selectivity and Interference Studies

Perhaps the main property of a sensor are its selectivity. To contemplate the selectivity of the test, various species (1×10^{-5} M) that are either primarily comparable or coinciding or the same grouped metal ions were taken and their effect on fluorescence intensity of IMP was noticed. The fluorescent enhancement in the spectra is not seen for other metal ions such as Cu^{2+} , Fe^{2+} , Fe^{3+} , Ni^{2+} , Co^{2+} , Mg^{2+} , Cd^{2+} , Na^{+} , K^{+} , In^{3+} , Sr^{2+} , Mn^{2+} , Hg^{2+} , and Pb^{2+} . The selectivity of the probe towards Zn^{2+} is shown in Fig. 12. Hence the sensor should be an ideal probe for zinc-containing samples while the sample has metals of a similar group. Noting the selective fluorescence enhancement due to Zn^{2+} , metal ions were tested for their ability to inhibit the fluorescence of the IMP- Zn^{2+} complex. The metal ions selected for interference studies were Cu^{2+} , Fe^{2+} , Fe^{3+} , Ni^{2+} , Co^{2+} , Mg^{2+} , Cd^{2+} , Na^{+} , K^{+} , In^{3+} , Sr^{2+} , Mn^{2+} , Hg^{2+} , and Pb^{2+} . The intensity of the peak of IMP with Zn^{2+} had no effect upon addition with metal ions except for Fe^{3+} , Hg^{2+} , Cu^{2+} , and Cd^{2+} (Fig. 13). The later metal ions might interfere with the peak intensity with a signal change of 5%. Meanwhile, upon adding more equivalents of metal ions (1:5, 1:10, etc.), the fluorescent intensity peaks get lowered with significant interference.

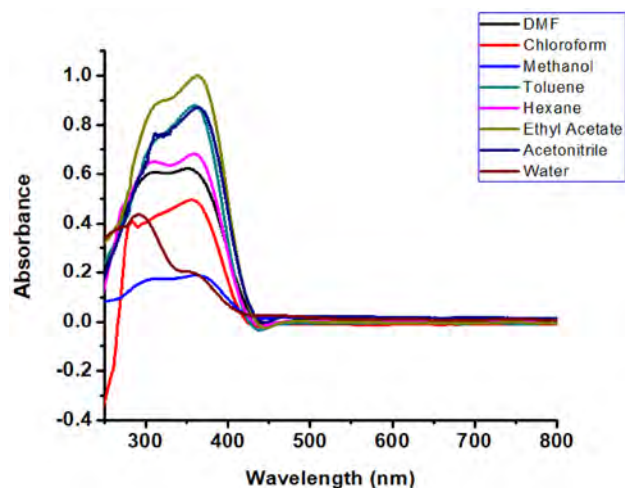


Fig. 15 UV–visible spectrum of IMP in various solvents

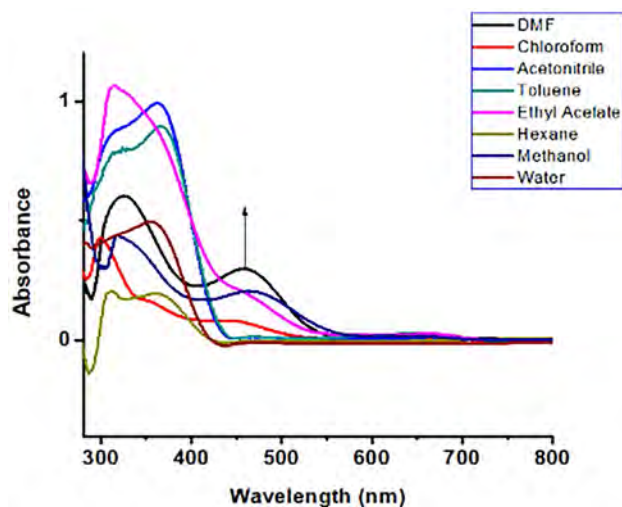


Fig. 16 UV–visible spectrum of IMP- Co^{2+} in various solvents

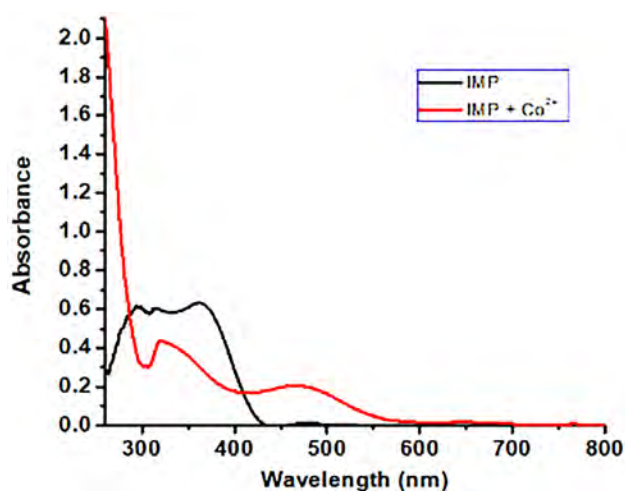


Fig. 17 A comparative UV-visible spectra of IMP and IMP + Co^{2+}

It was found that the probe IMP exhibits an off-on-type mode with high selectivity in the presence of Zn^{2+} ions. The fluorescence spectra results revealed that the fluorescence emission intensity of probe IMP decreased dramatically on complex formation and the Zn^{2+} complexes showed high emission maxima. In addition, the examination of the characteristics of the IMP sensor with those of the recently announced Zn^{2+} sensor is organized in Table 1.

Colorimetric Chemosensor

During the fluorescence investigation of detecting Zn^{2+} in DMF, we noticed the color changes of IMP within the sight of Co^{2+} ion instead of some other metals. Consequently, we inspected the chromogenic detecting capacity of IMP within

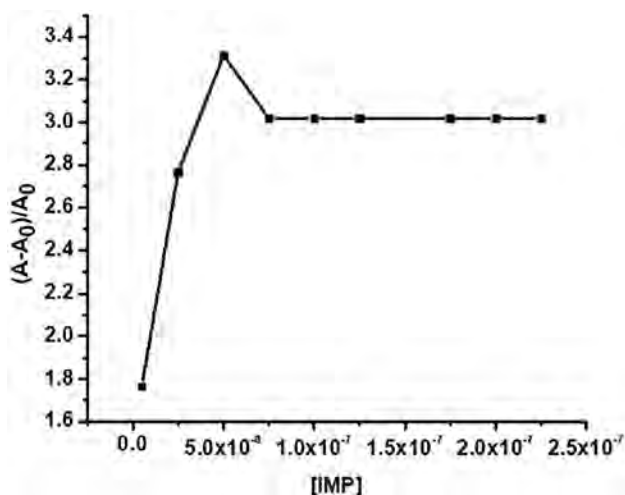


Fig. 18 Effect of concentration of IMP on absorbance change of the system

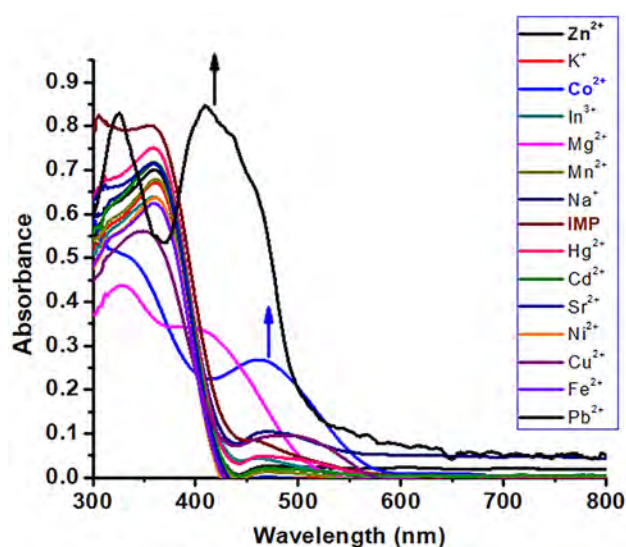


Fig. 19 UV-visible absorption spectra of IMP in the presence of different metal ions (1:1)

the sight of an assortment of metal particles like Cu^{2+} , Fe^{2+} , Ni^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} , Cd^{2+} , Na^{+} , K^{+} , In^{3+} , Sr^{2+} , Mn^{2+} , Hg^{2+} , and Pb^{2+} in DMF. The receptor IMP showed prompt color changes from dull to pink in the presence of Co^{2+} and a very low yellow intense in presence of Zn^{2+} which was purposefully omitted. While different metals caused no adjustment of color (Fig. 14).

However, the receptor IMP was miscible completely in DMF and other organic solvents. Even there seen partial miscibility in presence of highly polar water solvent with low-intensity peak assigned for $n \rightarrow \pi^*$ transition which was inferred from the UV-visible spectrum (Fig. 15). All other

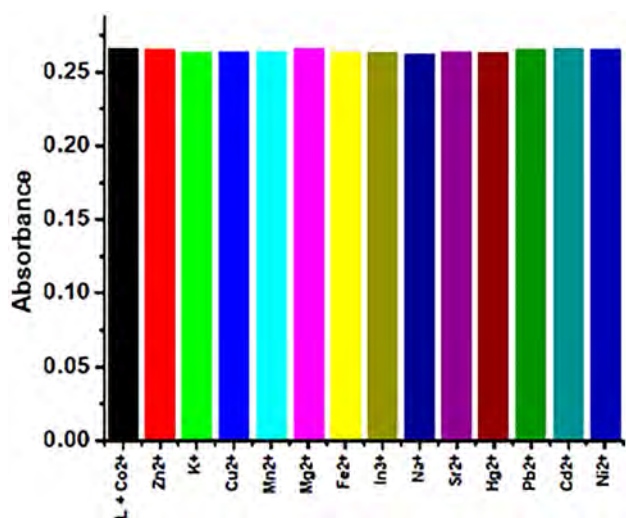


Fig. 20 UV-visible absorption spectra of IMP- Co^{2+} in the presence of different metal ions (1:1)

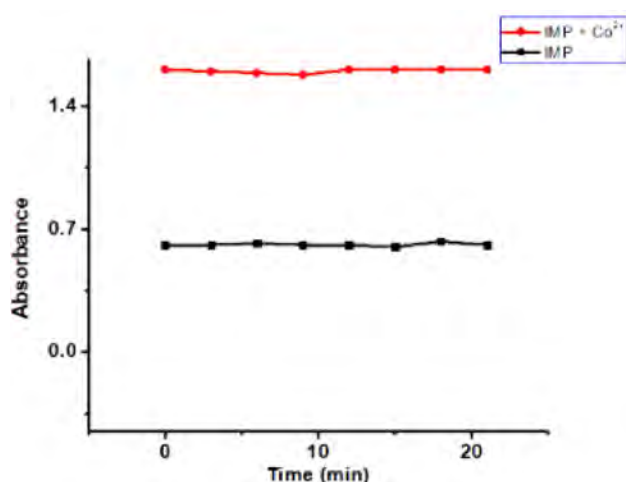


Fig. 21 Relative effect of time on IMP and IMP-Co²⁺

solvents do not cause any change in the absorption spectrum of IMP since it has two peaks in the region of 312 nm and 358 nm as weak and strong peaks respectively. So the need for solvent effect in the UV–visible spectra of IMP with Co²⁺ is mandatory. Upon adding Co²⁺, a new peak was formed at 474 nm indicated the transfer of free ligand into the metal complex. Even though, DMF is the best solvent for the complexation of receptor IMP with Co²⁺ ion with an enhancement in the absorption intensity at 460 nm was cleared from Fig. 16. Hence we continued the studies in DMF and not with buffer solutions. The UV–visible spectra changes of IMP and IMP-Co²⁺ was shown in Fig. 17.

The sensitivity of receptor IMP towards Co²⁺ was determined. The concentration of receptor IMP was obtained by plotting [IMP] against (A-A₀)/A₀ (Fig. 18) and it was found

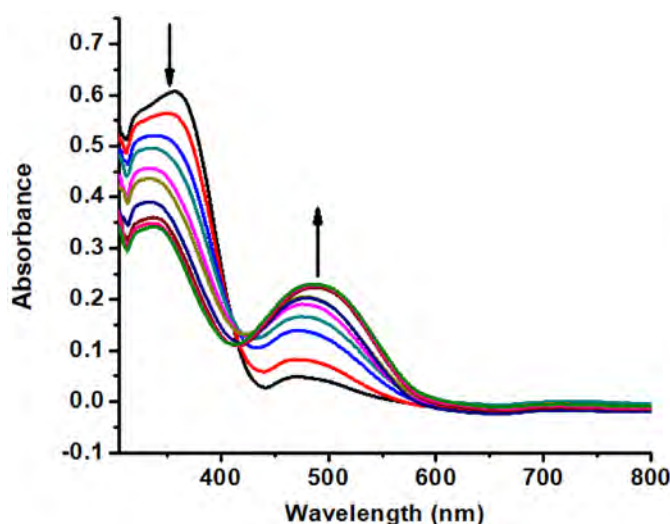


Fig. 22 Effect of concentration of Co²⁺ on the UV–visible absorption spectra of IMP. 10 to 100 μ L of (10⁻⁵ M) Co²⁺ solutions were added to receptor IMP and inset represents linear calibration graph between the ratio of UV–visible absorptions and concentrations of Co²⁺

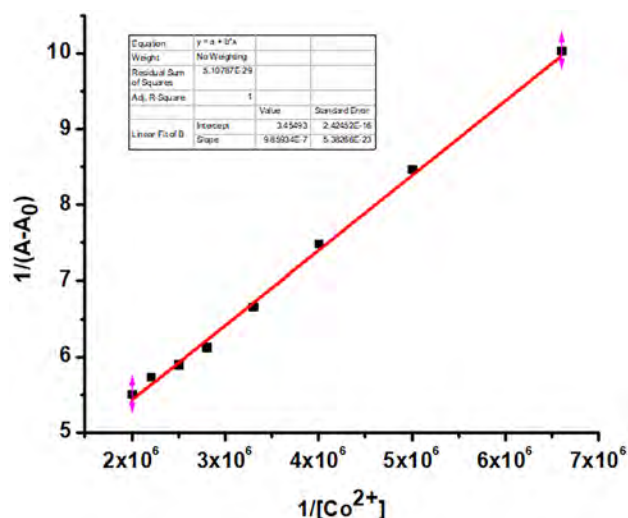
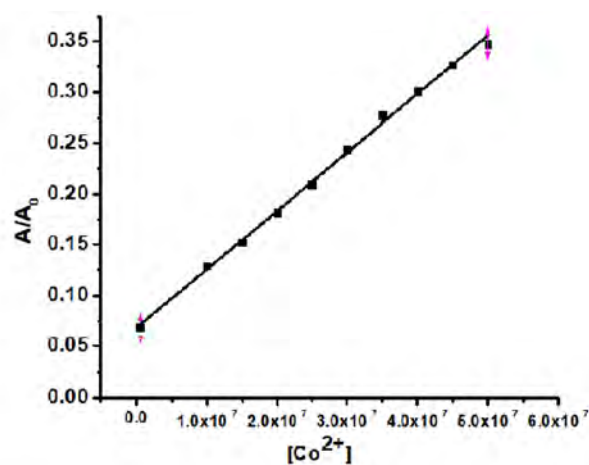


Fig. 23 Linear representation of binding constant for IMP-Co²⁺ complex

to be 5×10^{-8} M where A₀ is 0.051 nm. The selectivity of receptor IMP towards various metal ions (Fig. 19) suggested the future scope of the sensor with rapid determination and efficacy. No other metals shown any color change and characteristic peak in the region of 450–475 nm. The new peaks formed in the IMP-Co²⁺ complex might be attributed to metal to ligand charge transfer (MLCT), which is responsible for the pink color of the solution. Moreover, it is qualified to specify that the receptor IMP could be utilized as a test for the separation of Co²⁺ from Zn²⁺ by the naked eye.

To examine the interference of IMP-Co²⁺ complex in presence of other competing metal ions such as like Cu²⁺, Fe²⁺, Ni²⁺, Mg²⁺, Zn²⁺, Cd²⁺, Na⁺, K⁺, In³⁺, Sr²⁺, Mn²⁺,



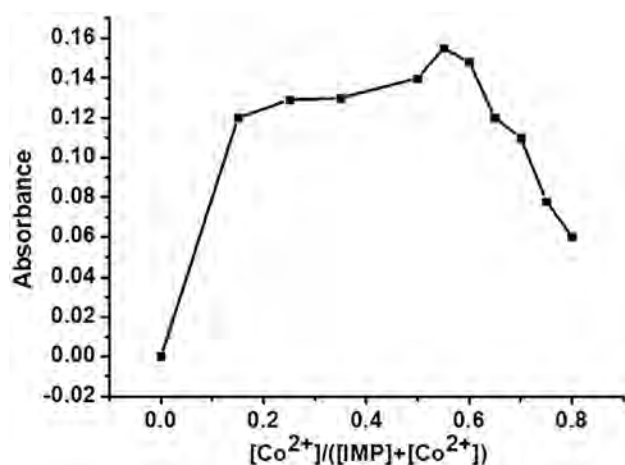


Fig. 24 Job's plot of IMP and Co^{2+} in DMF. Total concentrations of IMP and Co^{2+} were 200 μL

Hg^{2+} , and Pb^{2+} , the UV–visible competitive studies were graphically implemented (Fig. 20). Background of most competing metal ions did not interfere with the naked-eye detection of Co^{2+} even in one equivalences (1:1) and multiple equivalence (1:10 and 1:50) of other metal ion solutions.

Next, we need to investigate the time stability of receptor IMP alone and IMP- Co^{2+} complex in a period of 0–21 min at room temperature (Fig. 21). From the spectra, there should be no change in the characteristic peaks for IMP and IMP- Co^{2+} as time consumes. Thus it was kept for a long period of time without any reduction in the intensity of spectra, which will lead to the application studies in biological samples in future.

On comparing the incorporation of Co^{2+} , a gradual expansion in the intensity of absorption spectra with some sort of the change in the peaks maxima was identified (Fig. 22). A linear calibration graph was plotted with a concentration of Co^{2+} against A/A_0 , where A & A_0 are the absorptions of analyte and probe respectively. It was found to be linear in the range 5×10^{-8} to 5×10^{-7} M (inset of Fig. 22). The limit of detection and limit of quantification was found to

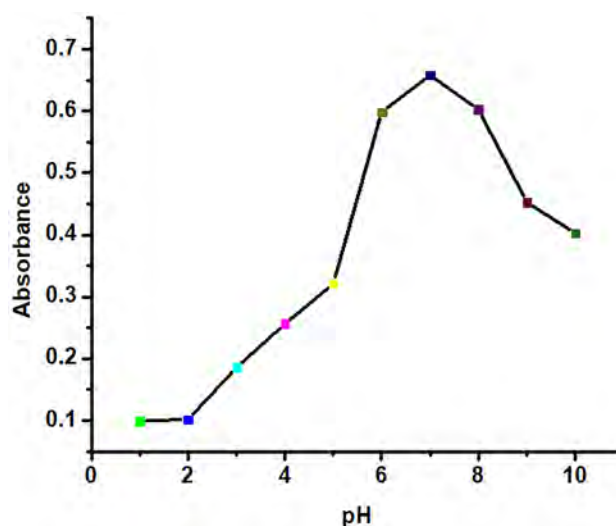


Fig. 25 Effect of pH of IMP on absorbance spectra

be 6.74×10^{-9} M and 2.22×10^{-8} M respectively. Imidazole bound sensor system is still unaware for the researchers and our proposed sensor with better detection limit value would definitely detect the Co^{2+} at the commercial level.

The binding association constant (K_a) for IMP- Co^{2+} was graphically evaluated by plotting $1/(A-A_0)$ versus $1/[\text{Co}^{2+}]$, as shown in Fig. 23. From the linear relationship graph, the K_a value calculated from Eq. (3) was $3.50 \times 10^6 \text{ M}^{-1}$.

Stoichiometry of the IMP- Co^{2+} complex was determined by Job's plot which revealed 1:2 ratio (Fig. 24) might be in good agreement with the analyte Zn^{2+} discussed above. There is a smooth transformation of receptor IMP into the cobalt complex by effective binding with possible donor atoms in the IMP or supported by the PET mechanism. In short, the comparison of IMP and other reported sensors with analyte Co^{2+} is presented in Table 2.

We took the absorbance spectra of receptor IMP at various pH conditions (Fig. 25). It is highly stable at alkaline pH conditions and thus, we did further experiments in the same buffer condition.

Table 2 Comparison of the characteristics of the IMP sensor with those of the previously reported Co^{2+} sensor

Sensor	Dynamic Range (M)	Detection Limit (M)	Ref
2,6-bis(iminomethyl)pyridine	5×10^{-4} to 5×10^{-3}	-	[44]
DISN	0 to 10×10^{-6}	1.24×10^{-7}	[45]
BNHNTA	0.1 to 1.2×10^{-6}	0.05×10^{-6}	[46]
Methylene blue, Amino thiophenol and Copper nitrate complex	-	0.04×10^{-3}	[47]
2,2':6',2''-terpyridine and 2,2'-dihydroxyazobenzene	-	0.45×10^{-6}	[48]
2-Chloro-N-(quinolin-8-yl) acetamide and 2-(((pyridin-2-yl) methylamino) methyl)phenol	-	0.85×10^{-6}	[49, 50]
IMP	5×10^{-8} to 5×10^{-7}	6.74×10^{-9}	This work

Table 3 Determination of Zn^{2+} ion in water samples with probe IMP

Sample	Zn^{2+} Added ($\mu\text{mol/L}$)	Zn^{2+} Found ($\mu\text{mol/L}$)	Recovery (%)	R.S.D (n=3) (%)
Drinking Water	15	14.2 ± 0.4	97.3 ± 1.0	4.01 ± 0.8
Tap Water	15	14.8 ± 0.6	98.5 ± 1.2	1.21 ± 1.3

*Results are based on three measurements

Analytical Application of Detection of Zn^{2+} in Water Samples

To get clear knowledge on the selectivity conduct, its higher selectivity for zinc discovery in this technique, displayed by the mentioned sensor, makes it fantastic for observing zinc particles at following fixations in various water samples. Drinking water and tap water were taken and diluted with a buffered solution in a 25.0 mL volumetric flask. Various measures of Zn^{2+} ions were added to water tests. The proposed sensor was utilized in request to decide the Zn^{2+} content thus the calibration technique was applied. The results obtained with the sensor are mentioned in Table 3. It was also found that the accuracy of Zn^{2+} detection in different water samples gives out way to biological sample quantification [41].

Conclusion

A new versatile, simple, selective, and efficient imidazole bound Schiff base has been synthesized and characterized. It exhibited a remarkable selectivity and sensitivity towards Zn^{2+} by fluorescence spectra and for Co^{2+} by UV–visible spectra over competing for other relevant metal ions. The detection limit of the probe with Zn^{2+} and Co^{2+} are far below as compared with other reported sensors. The binding ratios of the probe to M^{2+} ($\text{M}^{2+} = \text{Zn}^{2+}$, Co^{2+}) are 2:1 and the metal association constants are in the 10^6 M^{-1} range. The probe binds to Zn^{2+} through four nitrogen atoms and does so through the phenolic oxygen. Thus the results reported here provide a novel approach for the simultaneous selective identification of Zn^{2+} and Co^{2+} among various metal ions. The attractive properties of this method such as selectivity and rapidness would help us to extend its applications for the determination of Zn^{2+} ions in water samples.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10895-021-02839-5>.

Acknowledgements The authors would like to express their gratitude to CUSAT, Kerala, India for financial assistance and STIC, CUSAT for the real time analyses.

Authors' contributions Anjali Krishna Gopalakrishnan has written the manuscript and all the data's were interpreted according with the

obtained results. Shanty Antony Angamaly helped in the interpretation of mechanism of detection. Savitha Devaswamparambil Pradeep helped in doing application studies. Dhanya Thaikatt Madhusudhanan contributed in fluorescence studies. Divya Kizhakkeppurath Manoharan contributed in analyzing data. Puzhavorparambil Velayudhan Mohanan helped in interpretation of data.

Funding NA.

Data Availability All data analysed in this study are included in this article. If more [supporting information](#) is needed it can be available on request from the corresponding author (Mohan P.V.).

Code Availability NA.

Declarations

Ethics Approval NA.

Consent to Participate NA.

Consent for Publication NA.

Conflict of Interest The authors declare that they have no conflict of interest.

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Investigations on different efficient strategies for the selective synthesis of jasminaldehyde over $\text{HRhCO}(\text{PPh}_3)_3$ –hexagonal mesoporous silica and chitosan catalysts

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Received: 30 December 2021 / Accepted: 1 March 2022 / Published online: 13 March 2022
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Abstract

Various strategies are investigated for the synthesis of jasminaldehyde over heterogeneous catalysts. The jasminaldehyde synthesis via conventional cross-aldol condensation of heptanal with benzaldehyde using heterogeneous catalyst with a heptanal:benzaldehyde ratio of 1:4 was performed. Even with 100% conversion of heptanal with 100% selectivity, only 25% benzaldehyde reacts and 75% of benzaldehyde has to remain unreacted as the heptanal:benzaldehyde ratio is 1:4. So here, a strategy was applied in which, addition of heptanal in regular intervals to the reaction mixture was performed by keeping the ratio of heptanal:benzaldehyde as ~1:4. This could afford to convert 62% of benzaldehyde to jasminaldehyde with 69% selectivity in 20 h. Heptanal can be obtained by hydroformylation of 1-hexene. Hence in the next strategy, the investigations had been performed for the synthesis of jasminaldehyde via a tandem reaction by individually carrying out hydroformylation and aldol condensation. Hydroformylation of 1-hexene to heptanal and further condensation of this product mixture with benzaldehyde to jasminaldehyde, using heterogeneous catalysts $\text{HRh}(\text{CO})(\text{PPh}_3)_3$ encapsulated HMS (HF-1) and amino functionalized chitosan (CH-1) respectively were studied. The study was also extended to perform a single-pot hydroformylation and aldol condensation using heterogeneous catalysts (HF-1) and (CH-1) together. All the strategies were found to be effective for selective synthesis for jasminaldehyde, however the performance for

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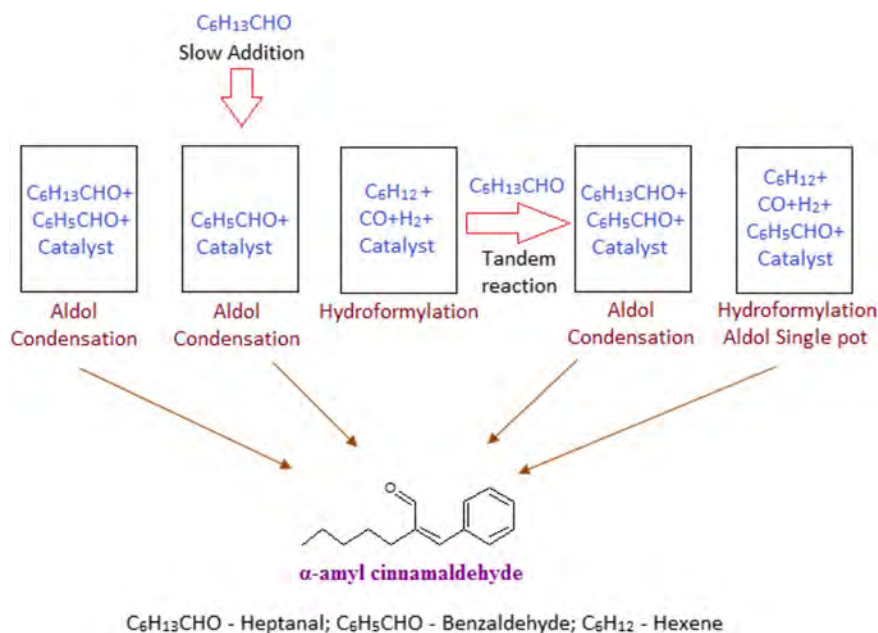
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addition of heptanal in regular intervals to the reaction mixture was remarkable due to being capable to consume 62% benzaldehyde.

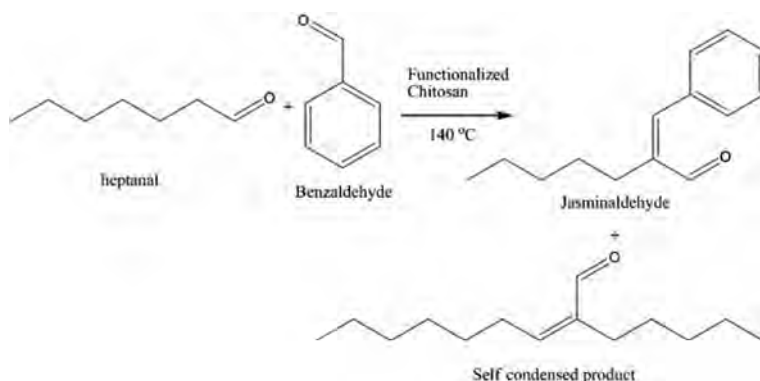
Graphical abstract



Keywords Jasminaldehyde · 1-hexene · Aldol condensation · Hydroformylation · Chitosan · Single pot reaction

Introduction

The perfumery fine chemical jasminaldehyde used as an aroma compound in perfumes possesses the characteristic scent of jasmine. Jasminaldehyde or α -amyl cinnamaldehyde is synthesized by condensation of heptanal with benzaldehyde using acid or base catalyst (Scheme 1) [1, 2]. Generally, this condensation is done in the presence of liquid alkali as a catalyst taken in more than stoichiometric amounts and adding heptanal slowly into benzaldehyde at moderate reaction temperature [3, 4]. This homogenous catalytic route using liquid alkalis has drawbacks of recyclability and disposal of alkali used. This process is associated with the problems like handling of strong liquid NaOH or KOH, separation and disposal of spent KOH or NaOH which require post-synthesis work-up and corrosion of reaction vessel. The process is not environment friendly also. A huge amount of spent liquid base gets generated during the formation of the product from aldol condensation in homogeneous conditions. It is estimated that approximately, 10–15 percent of spent base



Scheme 1 Synthesis of jasminaldehyde by aldol condensation

solutions are generated during the product formation in homogeneous aldol condensation [5]. Heterogeneous catalysts offer the potential advantage of easy separation, recycling of the catalysts and easy purification of the products. Hence heterogeneous catalysis is one of the emerging research areas to overcome the drawbacks of recyclability associated with homogeneous catalysis for many industrially required organic transformations for the production of bulk and fine chemicals. We are actively engaged since last 15 years in our wide research programme to develop heterogeneous catalytic systems, for the production of bulk chemicals like aldehydes [6–18], acids [19–22] and fine perfumery chemical jasminaldehyde [2, 23–25].

Many solid base as well as acid heterogeneous catalysts are developed to overcome the drawbacks of homogeneous catalysts for the synthesis of jasminaldehyde. Solid acid catalysts like Al-MCM-41, aluminosilicates, ALPOs, acidic zeolites and PTSA-MCM-41 were used for this condensation reaction [1, 3, 26, 27].

Modified chitosan [23], reconstructed hydrotalcites [28] mesoporous cubic Ia3d materials (Al-KIT-6) [29] Mg–Al mixed oxide supported on hexagonal mesoporous silica [30], reusable NaOH-CTAB micellar system [31], Mg–Al mixed oxides [32, 33], potassium modified alumina [34], amino modified MOF [35], modified zirconia [36], silica immobilized piperazine [37], various solid supported cesium and potassium catalysts [38] are some of the recent results on heterogeneous/reusable base catalytic systems for jasminaldehyde synthesis. Acid–base bifunctional catalysts were also found to be active for jasminaldehyde synthesis [39]. The reaction kinetics of the jasminaldehyde synthesis over heterogeneous catalysts are also studied which gave insight into the mechanism of reaction [2, 40].

Most of the reported condensation process for jasminaldehyde discusses the conventional reaction setup and conditions. In view of above and our focus to develop heterogeneous catalysts, and since jasminaldehyde is a fine chemical and produced industrially, a pressing need is realised to perform strategic investigations aiming solid base chitosan catalysed heterogeneous aldol condensation for the synthesis of jasminaldehyde. Apart from that, various synthetic strategies are discussed and compared here in the present study for jasminaldehyde synthesis. In present work, amino functionalized chitosan catalyst (CH-1) [24] is used as a base catalyst for

aldol condensation and $\text{HRh}(\text{CO})(\text{PPh}_3)_3$ encapsulated hexagonal mesoporous silica (HF-1) [9, 11, 13, 16, 17] as hydroformylation catalyst.

The first strategy involves to perform the conventional reaction on a fixed ratio of heptanal and benzaldehyde. In the second strategy the experiment was designed to carry out aldol condensation by stepwise addition of heptanal into the reaction mixture containing benzaldehyde and catalyst under the identical reaction conditions of first strategy, aiming for the maximum consumption of benzaldehyde during the condensation. Stepwise addition of heptanal afforded an appreciable consumption of benzaldehyde of 62% under the present employed experimental designing and reaction conditions. In the third strategy, the hydroformylation of 1-hexene was carryout using HF-1 as heterogeneous catalyst for the formation of heptanal, and thus formed heptanal was separated and used as heptanal source for aldol condensation with benzaldehyde catalysed by CH-1 for the synthesis of jasminaldehyde. This third strategy parallels with the synthesis of jasminaldehyde practiced in industry, where jasminaldehyde is ultimately produced from the castor bean plant and not usually derived from jasmine essential oil. Ricinoleic acid, the principal constituent of castor oil, undergoes cracking to undecylenic acid and heptanal. Thus produced heptanal is separated by distilling and is then reacted with benzaldehyde in the presence of a basic catalyst (*trans*-aldol condensation) to give jasminaldehyde and water. In the fourth strategy the hydroformylation of 1-hexene and aldol condensation were carried out in a single-pot using an autoclave for the jasminaldehyde synthesis. There are many tandem single pot reactions starting from hydroformylation to other valuable products through aldols [6–8, 10, 18, 41–43] hydrogenation [10, 44, 45] and aminomethylations [14, 15, 46]. To the best of our knowledge, this is the first report, if any, for the stepwise addition of heptanal into benzaldehyde over heterogeneous catalyst for the synthesis of jasminaldehyde and also the single pot synthesis of jasminaldehyde via hydroformylation—aldol condensation route. The present work accounts and discusses the effect and significance of the investigated strategies on the selective synthesis of jasminaldehyde.

Materials and methods

Materials

Aminopropyltrimethoxysilane (APTMS), heptanal, tetraethylorthosilicate, chitosan and 1-hexene were procured from Sigma-Aldrich USA. Hexadecyl amine was procured from Sisco Laboratories, India. Syn gas (1:1) was purchased from Alchemie gases and Chemicals Pvt. Ltd., India. Toluene was procured from Spectrochem, India. All chemicals were used without further purification.

Synthesis of catalysts

The catalyst for aldol condensation [24] and hydroformylation [9] were synthesized and characterized according to previous reports.

Aldol catalyst was synthesized by treating chitosan with APTMS. Chitosan (1.5 g) and APTMS (1 g) were dissolved in toluene (25 ml) in a 50 ml RB flask connected with a water condenser. Inert atmosphere was created by nitrogen filled in a balloon. It was refluxed in an oil bath at 110 °C for 24 h. The catalyst was filtered, dried at 100 °C and denoted as CH-1.

Hydroformylation catalyst was synthesized by in situ encapsulation method. Hexadecyl amine (2.7 mmol) was dissolved in an ethanol–water mixture. To this stirring solution $\text{HRh}(\text{CO})(\text{PPh}_3)_3$ (0.07 mmol) was added. To the suspension obtained, TEOS (10.0 mmol) was added drop wise. The precipitate formed was stirred for 1 h and then aged for 18 h. The precipitate was washed with 1:1 ethanol–water mixture and dried in vacuum at room temperature. The catalyst was denoted as HF-1.

Catalyst characterization

CH-1 catalyst was characterized by C, H, N elemental analysis and for accessible amino groups. HF-1 catalyst was characterized by P-XRD, FT-IR, ^{31}P -CPMAS NMR and surface area analysis. FT-IR was recorded using Perkin-Elmer, GX-FTIR by KBr pellet method. Elemental analysis (C, H, N) was carried out with Perkin-Elmer CHNS/O analyzer (Series II, 2400). Powder X-ray diffraction of the catalyst was recorded with Phillips X'Pert MPD system using Ni-filtered $\text{Cu K}\alpha$ radiation ($=1.54050 \text{ \AA}$) over a 2θ range of $1\text{--}8^\circ$ at a step time of $0.05^\circ \text{ s}^{-1}$. ^{31}P -CPMAS NMR was recorded in a Bruker 500 Ultrashield system. The surface area analysis and pore size distribution of the catalyst was determined by N_2 adsorption at 77.4 K using a Sorptometer (ASAP-2010, Micromeritics). Catalyst was degassed at 80 °C for 4 h prior to the measurement.

Methods of reaction: hydroformylation and aldol condensation

For the conventional heterogeneous aldol condensation, heptanal (2.6 g), benzaldehyde (10 g) and CH-1 catalyst (400 mg) were taken in a 100 ml two-necked round bottom flask. One neck was connected to a water condenser and another neck was fixed with a silicon rubber septum. N_2 gas was purged into the reaction mixture for 5 min and then the top of the water condenser was fitted with a balloon filled with N_2 gas to create inert atmosphere. The RB flask was kept in an oil bath at 140 °C kept over a magnetic stirrer. Aliquots of sample were taken at suitable intervals and analyzed using GC (Shimadzu GC-17A).

For the synthesis of jasminaldehyde by slow addition of heptanal to benzaldehyde was conducted as follows. Benzaldehyde (10 g) was taken in round bottom flask and heptanal (2.6 g) was added to keep heptanal to benzaldehyde ratio of 1:4. Chitosan catalyst CH-1 (400 mg) was added and reaction mixture was heated to 140 °C under stirring. After 2 h of reaction, a small quantity of sample was withdrawn and analyzed. Further, 0.35 ml ($\sim 280 \text{ mg}$) heptanal was added to the reaction mixture using a syringe at 2 h. The addition of 0.35 ml was continued at every 30 min up to 8 h. After that, the addition of 0.35 ml was done at every 1 h up to 12 h. The reaction

was continued by adding 0.35 ml at every 1 h interval for 15 h. Aliquots of samples were taken out and analyzed at selected intervals. The product was analyzed by GC.

Hydroformylation was conducted in a 100 ml autoclave reactor (Autoclave Engineers, USA). In an experiment, 15 g of 1-hexene is dissolved in 50 ml of toluene and 200 mg of catalyst (HF-1) was added. The reactor was fitted and flushed twice with N_2 gas and the reactor was heated to 80 °C. The reactor was charged with syn gas ($CO:H_2 = 1:1$) up to 40 bar. Reaction was initiated with stirring at 950 rpm. Aliquots of samples were collected and analyzed in GC.

For the tandem hydroformylation-aldol condensation to jasminaldehyde, the hydroformylation reaction was first conducted for 12 h. The product mixture was separated from the HF-1 catalyst and analyzed with GC. The product mixture was distilled to remove toluene and iso-hexene in a rotavapor, and the final product mixture was weighed. Required amount of the product mixture was weighed out and was mixed with required amount of benzaldehyde (heptanal:benzaldehyde ratio of 1:4) in a RB flask. Catalyst CH-1 (400 mg) was added and the condensation reaction was conducted at 140 °C. The product was analyzed by GC. Conversions were taken with respect to heptanal only and iso-heptanal remained unreacted.

Single pot synthesis of jasminaldehyde was conducted as per the following procedure. 1-Hexene (7.5 g) and benzaldehyde (10 g) were taken in a 100 ml autoclave with 25 mL toluene. 200 mg of HF-1 catalyst and 400 mg of CH-1 catalyst were also added and pressurized with 1:1 syn gas up to 40 bar. Autoclave was heated to 140 °C and stirred for 16 h. Small quantity of sample were withdrawn at specific interval and analyzed. The product mixture was separated by filtration and analyzed by GC. n-Tridecane was used as the internal standard in all of the above reactions.

Results and discussion

Characterization of catalysts

CH-1 catalyst

C, H, N analysis was conducted for chitosan and also APTMS functionalized chitosan (CH-1). The results are: Chitosan (C: 40.02, H: 6.72, N: 8.63) and CH-1 (C: 41.26, H: 6.94, N: 11.02). This confirms the functionalization of chitosan with APTMS. The percentage of accessible amino group was determined by the Schiff base formation of the CH-1 catalyst with salicylaldehyde [24]. The percentage of accessible amino group was found to be 32.6%.

HF-1 catalyst

Catalyst characterization results are given in supplementary material. P-XRD pattern of the catalyst showed a peak at 2θ of 2.08 corresponding to (100) plane of HMS. ^{31}P CPMAS NMR spectrum of the catalyst showed two peaks at 50.0 and 38.60 ppm. N_2 sorption isotherm showed type-IV isotherm. BET surface area and BJH pore diameter of the catalyst were found to be $16.08\text{ m}^2\text{ g}^{-1}$ and 6.6 nm

respectively. These observations match with those reported in our previous studies and confirmed the encapsulation of rhodium complex inside the pores of HMS [9].

Condensation of heptanal with benzaldehyde

The condensation of heptanal with benzaldehyde was carried out using the CH-1 catalyst and the result is given in Table 1 (Entry 1–3). Conversion was 55% at 2 h with 75% selectivity for jasminaldehyde. The reaction gave 99% conversion with respect to heptanal at 6 h. The selectivity to jasminaldehyde was increased to 79% and self-condensed 2-pentyl nonenal was 18%. Benzaldehyde was oxidized to benzoic acid with 3% selectivity in products.

The product distribution [6] for jasminaldehyde formed in mmol was calculated in terms of the conversion of heptanal and the selectivity of aldol products, jasminaldehyde and 2-pentyl 2-nonenal only and the percent of jasminaldehyde was determined (Eq. 1) as follows.

$$\% \text{ of jasminaldehyde} = \frac{\% \text{selectivity of jasminaldehyde} \times 100}{(\% \text{selectivity of jasminaldehyde} + \% \text{selectivity of 2-pentyl 2-nonenal})} \quad (1)$$

The conversion increased almost linearly on increasing the time from 2 to 6 h and the formation of jasminaldehyde increased from 9.4 to 18.4 mmol Table 1 (Entries 1–3). Selectivity remained almost constant but had a slight increase from 75 to 79% with time. This reaction was the conventional aldol reaction. Here benzaldehyde is taken in excess to prevent the self-condensation of heptanal. Hence the atom economy with respect to benzaldehyde was very low. Maximum formation of 18.4 mmol

Table 1 Synthesis of jasminaldehyde by aldol condensation

Entry	Time, h	%Conversion	%Selectivity		
			Jasminaldehyde (mmol of jasminaldehyde) ^c	2-pentyl 2-nonenal	Benzoic acid
1 ^a	2	55	75 (9.4)	25	0
2 ^a	4	86	78 (15.6)	20	2
3 ^a	6	99	79 (18.4)	18	3
4 ^{b1}	2	54	75 (9.3)	24	1
5 ^{b2}	4	64	75 (16.0)	23	2
6 ^{b3}	8	75	72 (29.1)	25	3
7 ^{b4}	12	83	72 (45.2)	23	5
8 ^{b5}	20	97	69 (58.1)	25	6

Reaction conditions: ^a heptanal = 2.6 g (22.8 mmol), benzaldehyde = 10 g (94.2 mmol), catalyst (CH-1) = 400 mg, temperature = 140 °C and agitation speed = 950 rpm

^b benzaldehyde = 10 g (94.2 mmol), catalyst (CH-1) = 400 mg, temperature = 140 °C, heptanal = ¹2.6 g (22.8 mmol), ²3.72 g (32.6 mmol), ³5.96 g (52.2 mmol), ⁴8.2 g (71.8 mmol), ⁵9.32 g (81.6 mmol); Total heptanal by continuous addition = 9.32 g (81.6 mmol)

^c Jasminaldehyde (mmol) = Heptanal (mmol) $\times$ $\frac{\% \text{Conversion of heptanal}}{100}$ $\times$ $\frac{\% \text{ of jasminaldehyde}}{100}$

of jasminaldehyde observed here (Entries 3) from 94.2 mmol of benzaldehyde is indicative of the consumption of ~20% of the benzaldehyde i.e. 80% of benzaldehyde is left unreacted. More insight into the reaction was given by the pictorial representation of conversion and selectivity with respect to time (Fig. 1).

Another reaction more promising in terms of the making more consumption of benzaldehyde, was conducted by slow addition of heptanal by keeping the ratio of heptanal: benzaldehyde ratio near 1:4 in the beginning of the reaction. The rate of addition of heptanal was determined with the help of time dependent analysis of the products. The formation of jasminaldehyde was found to be appreciably increased (Table 1, Entries 4–8) on increasing the addition of heptanal and time. At 2 h, jasminaldehyde formed (Table 1, Entry 4) from 22.7 mmol of heptanal was 9.3 mmol. With slow addition of heptanal and time the formation of jasminaldehyde was continuously increased to 58.1 mmol with 81.6 mmol (Table 1, Entry 8) of the slowly added heptanal. This strategy afforded the formation of 58.1 mmol of jasminaldehyde from 94.2 mmol of benzaldehyde pinpointing that 62% benzaldehyde could be consumed here, which is three times more than conventional process. The rest of 38% percent benzaldehyde is left unreacted. A time dependent study revealed that the conversion increased with increase in time and addition of heptanal. The selectivity of jasminaldehyde was slightly higher at lower time (Table 1, Entry 4, 5). But as the reaction time increased, the addition of heptanal at every half an hour made an increase in heptanal concentration. This made an increase in self-condensation with a slight decrease in selectivity of jasminaldehyde. The former reaction (Entry 3) had 79% selectivity to jasminaldehyde (18.4 mmol), and this reaction (Entry 8) had a lower selectivity of 69% for jasminaldehyde but with a higher yield (58.1 mmol) with respect to benzaldehyde. A clear picture can be obtained with the pictorial

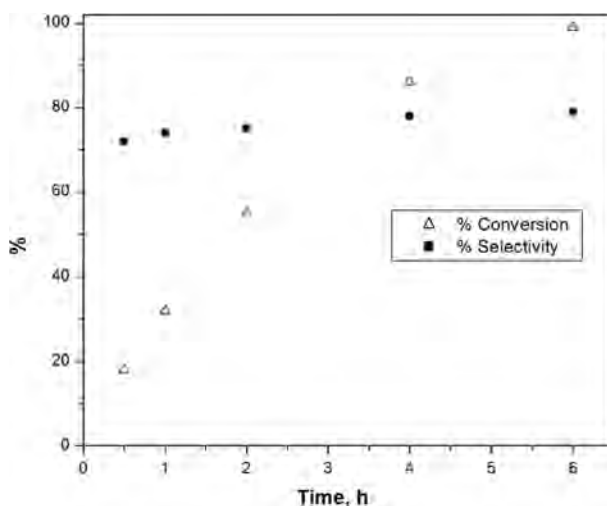


Fig. 1 Time dependent jasminaldehyde synthesis by conventional batch aldol reaction, Reaction conditions: heptanal = 2.6 g (22.8 mmol), benzaldehyde = 10 g (94.2 mmol), catalyst (CH-1) = 400 mg, temperature = 140 °C and agitation speed = 950 rpm

representation of the time dependent conversion and selectivity (Fig. 2). Here the conversion increased largely in the first 2 h, and the slope of increase of conversion got decreased after. This was due to the addition of heptanal to the reaction, which increases the heptanal concentration and requires more time for its conversion. Selectivity was higher at the beginning and started to decrease slightly after the addition of heptanal which is already discussed above. The conversion vs time plot (Fig. 2) indicated that the conversion was increasing and had not attained saturation even after 20 h with addition of 82.5 mmol of heptanal. This strategy showed promising forthcoming to further enhance the consumption of benzaldehyde for which much more specific reactor design and some special automated slow addition of heptanal equipment can have potential to extent this reaction procedure to give a still better activity and selectivity.

Hydroformylation-aldol condensation to jasminaldehyde: a two pot tandem reaction

Another thought was, “from where we can get heptanal?” In the industrial synthesis of jasminaldehyde, distilled heptanal obtained from castor bean plant is used for its cross aldol condensation with benzaldehyde in the presence of a basic catalyst. In view of this hydroformylation is a good tool to obtain heptanal from 1-hexene and accordingly, hydroformylation was conducted with 1-hexene and syngas using a heterogeneous catalyst for that we were expertise by doing a series of experiments [9, 11, 13] The results of hydroformylation are given in Table 2. A 12 h reaction gave 99% conversion of 1-hexene with 39% selectivity to n-heptanal. The n/iso ratio was

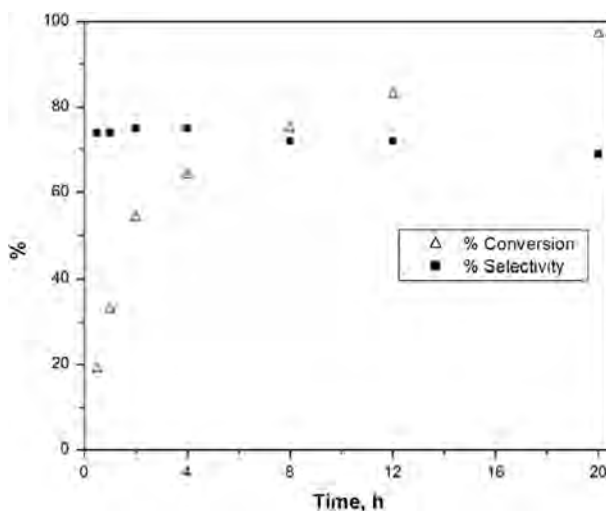


Fig. 2 Time dependent jasminaldehyde synthesis by slow addition of heptanal, Reaction conditions: benzaldehyde = 10 g (94.2 mmol), catalyst (CH-1) = 400 mg, temperature = 140 °C, heptanal added to the reaction in parts according to Table 1 (entries 4 to 8)

Table 2 Hydroformylation of 1-hexene to heptanal

Entry	Time, h	%Conv	%Selectivity				
			iso-hexene	n-heptanal	iso-heptanal	2-ethyl 3-methyl butanal	n/iso
1	2	45	36	44	19	1	2.20
2	4	68	28	43	26	3	1.48
3	8	92	28	42	25	5	1.4
4	12	100	23	39	30	8	1.03

Reaction conditions: 1-hexene = 15 g (178.2 mmol), catalyst (HF-1) = 200 mg, pressure of syn gas = 40 bar, temperature = 80 °C, toluene = 50 mL and agitation speed = 950 rpm

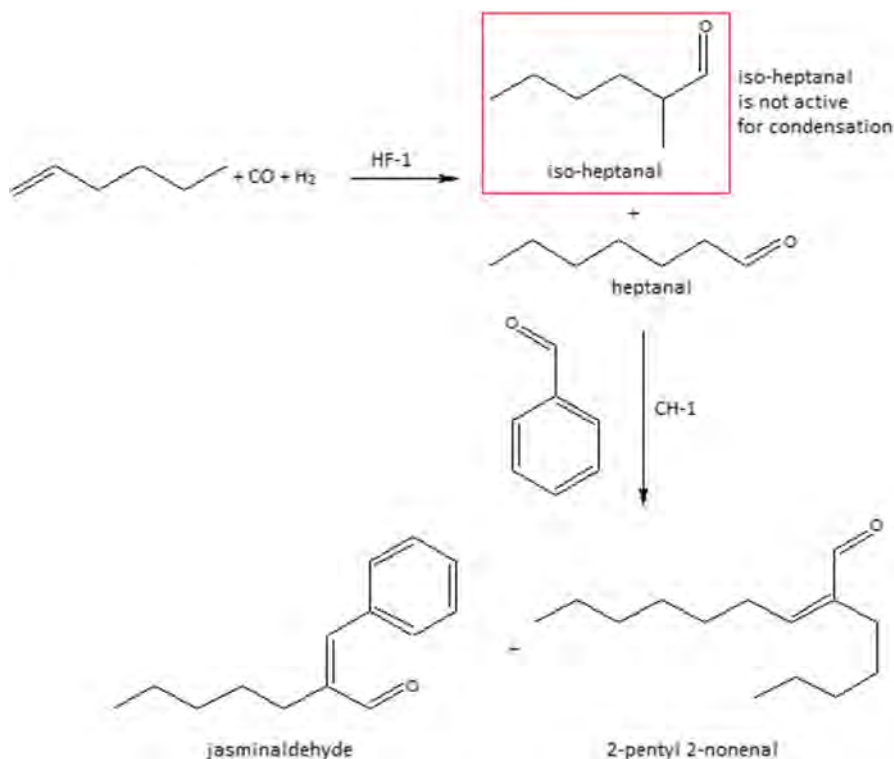
low (1.03) as it is in heterogeneous constrained conditions. It can be seen that the n/iso ratio was better at lower reaction time with low conversion. As the reaction proceeds, more and more 1-hexene converts to iso-hexene over the catalyst leading to formation of iso-heptanal. This makes the decrease in n/iso ratio [9].

The product mixture after 12 h from the reaction given in Table 2 was filtered to remove the catalyst and then the solvent and iso-hexene were distilled off. Now the product mixture contains only heptanal, iso-heptanal and 2-ethyl 3-methyl butanal. 10 g of the product mixture was used as heptanal source for jasminaldehyde synthesis. The jasminaldehyde synthesis was conducted as like a conventional aldol reaction and the results obtained are shown in Table 3. Conversion was calculated with respect to the heptanal and for the selectivity determination; only jasminaldehyde, 2-pentyl nonenal and side product benzoic acid were considered. Other two aldehydes present were not active for aldol condensation in the given conditions and hence is not included in calculating the conversion and selectivity. At lower time of 2 h, the conversion was very low (18%) with jasminaldehyde selectivity 58%. As the reaction time increased, conversion also increased steadily and reached upto 90% at 24 h of reaction. The selectivity to jasminaldehyde was increased to 80%. The

Table 3 Synthesis of jasminaldehyde using the product mixture of reaction from Table 2

Entry	Time, h	%Conversion	%Selectivity		
			Jasmi- nalde- hyde	2-pentyl 2-nonenal	Benzoic acid
1	2	18	58	42	0
2	4	31	66	34	0
3	8	49	75	25	1
4	12	63	78	22	1
5	24	90	80	18	2

Reaction conditions: hydroformylation product mixture = 10 g, benzaldehyde = 20 g, catalyst CH-1 = 400 mg, temperature = 140 °C and agitation speed = 950 rpm



Scheme 2 Single pot synthesis of jasminaldehyde via hydroformylation–aldol condensation

decrease in conversion may be due to the blockage of basic site by the adsorption of iso-heptanal present in the reactant mixture.

Hydroformylation–aldol condensation to jasminaldehyde: a single-pot reaction

A combined reaction of hydroformylation followed by aldol condensation may be a promising reaction sequence for the synthesis of jasminaldehyde. A single pot synthesis of jasminaldehyde via a hydroformylation–aldol condensation tandem reaction in single pot was conducted. This removed the intermediate work up steps between hydroformylation and condensation. The scheme of single pot jasminaldehyde synthesis is shown in Scheme 2.

The synthesis of jasminaldehyde was conducted with calculated amount of 1-hexene and syngas, and benzaldehyde was taken in approximate excess of four times by calculation of the earlier hydroformylation reaction given in Tables 2 and 3. Hydroformylation catalyst and aldol catalysts were added to the reaction mixture to perform single pot synthesis. The result obtained by single pot synthesis is given in Table 4.

Table 4 Single pot synthesis of jasminaldehyde

Entry	Time, h	%Conversion	%Selectivity					
			iso-hexene	n-heptanal	iso-heptanal	Jasmi- nalde- hyde	2-pentyl 2- non- enal	Other products ^a
1	4	36	18	24	35	17	4	2
2	8	58	14	18	28	32	6	3
3	16	70	11	9	30	35	9	6

Reaction conditions: 1-hexene = 7.5 g (89.1 mmol), benzaldehyde = 10 g (94.2 mmol), hydroformylation catalyst (HF-1) = 200 mg, aldol catalyst (CH-1) = 400 mg, syn gas (1:1) = 40 bar, temperature = 140 °C, toluene = 25 ml and agitation speed = 950 rpm

^a2-hydroxyphenylmethyl heptanal & benzoic acid

The conversion was calculated with respect to 1-hexene and all the products from hydroformylation to aldol condensation together were considered for calculation of selectivity. It is observed that, the condensation reaction was slow at lower reaction time. For 4 h, the conversion of 1-hexene was only 36% with selectivity for heptanal (24%), iso-heptanal (35%) and jasminaldehyde (17%). Lower n/iso ratio was due to the condensation of heptanal formed with benzaldehyde to yield jasminaldehyde over CH-1 catalyst. This observation was almost in line with Table 2 (entry 1) showing higher n/iso ratio at lower time. On increasing the reaction time, both conversion and selectivity for jasminaldehyde was increased. Conversion of 70% for 1-hexene was obtained for hydroformylation step and overall selectivity to jasminaldehyde was increased to 35% at 16 h of reaction time. When we calculated for heptanal to jasminaldehyde, 74% of the heptanal formed was converted to jasminaldehyde and self-condensed products with 80% selectivity to jasminaldehyde. A small amount of benzoic acid was formed < 1% as the reaction was done in a closed reactor. Also there had some amount of 2-hydroxyphenylmethyl heptanal, which is the aldol product initially formed. The hydroformylation reaction was slowed down by the presence of base catalyst and benzaldehyde as it is evident from low 1-hexene conversion to aldehydes. Also the possible coordination of carboxylic group of benzoic acid formed with rhodium could be another reason for catalytic poisoning leading to lowering the conversion. Similar carboxylate rhodium complexes and deactivation of catalysts are reported in literature [47]. A promising potential lies in this strategy to increase the conversion of heptanal and selectivity of jasminaldehyde by allowing the reaction for more time.

Mass balance for the best performed strategy

In order to account the mass balance [21] the best performed strategy affording the formation of 58.1 mmol of jasminaldehyde from 94.2 mmol of benzaldehyde with 62% consumption of benzaldehyde, was subjected for quantification of the reactants and products for having an insight in to the reaction system under the employed reaction conditions of entry 8 of Table 1, and corresponding data are listed in Table 5.

Table 5 Mass balance of the best performed strategy of slow addition of heptanal with time for entry 8 of Table 1

Entry	Entrant	mmol
1	Heptanal, M_1	81.6
2	Heptanal reacted (97%), M_2	79.2
3	^a Jasminaldehyde, M_3	58.1
4	^a 2-pentyl 2- nonenal, M_4	21.1
5	Heptanal unreacted, $M_5 (=M_1 - M_2)$	2.4
6	Benzaldehyde, M_6	94.2
7	^b Benzaldehyde reacted for jasminaldehyde, $M_7 (=M_3)$	58.1
8	^b Benzoic acid, M_8	3.6
9	Unreacted benzaldehyde, $M_9 (=M_6 - (M_7 + M_8))$	32.5

Reaction conditions: benzaldehyde = 94.2 mmol, catalyst (CH-1) = 400 mg, time = 20 h, temperature = 140 °C and total heptanal by continuous addition = 81.6 mmol and agitation speed = 950 rpm

^aCalculated in terms of the conversion of heptanal and the selectivity of jasminaldehyde and 2-pentyl 2-nonenal only

^bCalculated in terms of the conversion of benzaldehyde and the selectivity of jasminaldehyde and benzoic acid only

The product distribution [6] for jasminaldehyde was determined by Eqs. 1 given above, and for 2-pentyl 2-nonenal and benzoic acid by Eqs. 2 and 3 respectively.

$$\% \text{ of 2-pentyl 2-nonenal} = \frac{\% \text{ selectivity of 2-pentyl 2-nonenal} \times 100}{(\% \text{ selectivity of jasminaldehyde} + \% \text{ selectivity of 2-pentyl 2-nonenal})} \quad (2)$$

$$\% \text{ of benzoic acid} = \frac{\% \text{ selectivity of benzoic acid} \times 100}{(\% \text{ selectivity of jasminaldehyde} + \% \text{ selectivity of benzoic acid})} \quad (3)$$

The charged amounts of benzaldehyde and heptanal were 94.2 mmol and 81.6 mmol respectively. The reacted heptanal was 79.2 mmol yielding 58.1 mmol of jasminaldehyde and 21.1 mmol of 2-pentyl 2-nonenal. The reacted benzaldehyde (61.7 mmol) was accounted for the major formation of jasminaldehyde (58.1 mmol) and some benzoic acid (3.6 mmol). Neat 32.5 mmol of benzaldehyde remained unconsumed; however it can be further reacted by adding more heptanal and further increasing the time more than 20 h, since Fig. 2 suggested that the conversion was continuing to increase and not attained saturation under the employed reaction conditions.

Comparison of the performance with other heterogeneous catalytic systems

It is of significance to have an insight into the performance of the CH-1 catalyst with other closely related heterogeneous catalyst systems for the synthesis of jasminaldehyde. The CH-1 catalyst compared with other catalysts reported in literature is shown in Table 6.

Table 6 Comparison with other catalytic systems for the jasminaldehyde synthesis

Entry	Catalyst	Heptanal (mmol)	% Conv	Time, h	Temp., °C	Selectivity for Jasmi- naldehyde	References
1	CH-1 (Conventional Batch) ^a	22.6	99	6	140	79	Present work
2	CH-1 (Slow addition of heptanal) ^a	81.6	97	20	140	69	Present work
3	H-Beta Zeolite ^b	10	93	6	125	21	[26]
4	HY-100 Zeolite ^b	10	53	16	125	52	[26]
5	Al MCM-41 ^b	10	99	10	125	56	[26]
6	ALPO ^b	10	96	3	125	86	[26]
7	PTSA-MCM-41 ^b	7.9	62	5	125	86	[27]
8	Chitosan (Dried hydrogel) ^b	7.9	94	8	125	82	[23]
9	Fe-AIP ^b	-	75	4	140	71	[39]
10	Magnesium organo silicate (MOS3) ^b	7.9	99	8	125	82	[48]
11	Natural Talc ^b	7.9	58	8	125	56	[48]
12	Mg-Al(3.5) Hydrotalcite ^b	7.9	98	8	125	86	[49]
13	Ni-Mg-Al ^b	7.9	96	8	125	84	[49]
14	Chitosan-TiO ₂ hybrid ^b	1.0	98	4	80	86	[50]
15	Chitosan-SnO ₂ hybrid ^b	1.0	92	4	80	81	[51]

^aheptanal:benzaldehyde ratio = 1:4^bheptanal:benzaldehyde ratio = 1:5

CH-1 catalyst has comparable conversion and selectivity with some of the reported catalysts, and was better than many of the reported catalysts. ALPO (Entry 6), chitosan (Entry 8), MOS3 (Entry 10), Mg–Al hydrotalcite (Entry 12), Ni–Mg–Al (Entry 13) and chitosan metal oxide hybrid catalysts (Entry 14, 15) were comparable or better performed in terms of conversion and selectivity with that of CH-1 catalyst (Entry 1). Other catalysts like PTSA-MCM-41 (Entry 7) and Fe-AIP (Entry 9) had comparable selectivity for jasminaldehyde but with lesser conversion. The reaction with CH-1 catalyst was performed at higher temperature of 140 °C than other catalysts. The reaction by slow addition of heptanal was also comparable in terms of conversion. Stepwise addition of heptanal could convert 79.1 mmol of heptanal in 20 h (Entry 2). From 94.2 mmol of the benzaldehyde, the reacted benzaldehyde (61.7 mmol) yielded 58.1 mmol of jasminaldehyde. The merit of the present work was on designing the experiments with higher amount of heptanal and benzaldehyde. Also the stepwise slow addition of heptanal could convert much more amount of benzaldehyde.

Conclusions

Jasminaldehyde synthesis over heterogeneous catalysts was performed through different approaches. The studied methods were (1) conventional heterogeneous aldol condensation, (2) an improved aldol condensation by slow addition of heptanal, (3) a tandem reaction of hydroformylation of 1-hexene and aldol condensation with benzaldehyde and (4) a single pot hydroformylation-aldol condensation reaction. Highest selectivity (79%) was observed for the conventional heterogeneous aldol condensation, but about 75% of benzaldehyde was left unreacted. Reaction with slow addition of heptanal to the reaction mixture showed 69% jasminaldehyde selectivity in 20 h with 62% consumption of benzaldehyde, which is further improvable by increasing the time more than 20 h. In the approach of a tandem hydroformylation of 1-hexene to heptanal and aldol condensation with benzaldehyde, a conversion of 90% with respect to heptanal with 80% jasminaldehyde selectivity was obtained. The work was extended to single pot hydroformylation-aldol condensation and gave good conversions and selectivity.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11144-022-02196-9>.

Acknowledgements CSMCRI communication No. IMC 03, CSIR-CSMCRI—128/2021. Authors thank Council of Scientific and Industrial Research (CSIR), New Delhi, India for the financial support through Network Project on the Development of Specialty Inorganic Materials for Diverse Applications. The authors also acknowledge Analytical Division and Central Instrumentation Facility for providing instrumental analysis.

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Article

MMAP/(PH,PH)/1 Queue with Priority Loss through Feedback

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Abstract: In this paper, we consider two single server queueing systems to which customers of two distinct priorities (P_1 and P_2) arrive according to a Marked Markovian arrival process (MMAP). They are served according to two distinct phase type distributions. The probability of a P_1 customer to feedback is θ on completion of his service. The feedback (P_1) customers, as well as P_2 customers, join the low priority queue. Low priority (P_2) customers are taken for service from the head of the line whenever the P_1 queue is found to be empty at the service completion epoch. We assume a finite waiting space for P_1 customers and infinite waiting space for P_2 customers. Two models are discussed in this paper. In model I, we assume that the service of P_2 customers is according to a non-preemptive service discipline and in model II, the P_2 customers service follow a preemptive policy. No feedback is permitted to customers in the P_2 line. In the steady state these two models are compared through numerical experiments which reveal their respective performance characteristics.



Citation: Nair, D.V.; Krishnamoorthy, A.; Melikov, A.; Aliyeva, S.

MMAP/(PH,PH)/1 Queue with Priority Loss through Feedback. *Mathematics* **2021**, *9*, 1797. <https://doi.org/10.3390/math9151797>

Academic Editor: Vladimir V. Rykov

Received: 1 June 2021

Accepted: 12 July 2021

Published: 29 July 2021

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Keywords: queueing system; Marked Markovian arrival process; priority loss; feedback; non-preemptive; preemptive

1. Introduction

In this paper, we discuss two queueing models arising due to feedback of customers from a high priority queue (P_1 queue) joining a lower priority queue (P_2 queue), in a single server queueing system. The P_1 queue is of finite capacity whereas the P_2 queue has unlimited capacity. Immediately after service completion high-priority customers tend to provide feedback on the service again. The probability for feedback at a service completion epoch is θ which is independent of the feedback by other customers and also the number of customers in the system. However, they are to join a low-priority P_2 queue. In addition to this stream of customers in the P_2 queue, customers belonging to such class from outside also join that queue. The two models considered are the preemptive and non-preemptive services of P_2 customers. The flow of customers constitutes a Marked Markovian arrival process. P_1 and P_2 customers have service times following two distinct and mutually independent phase type distributions.

Because some abbreviations appear, mostly in this section, we introduce them at this stage for easy reading.

- MMAP: Marked Markovian Arrival Process;
- MMPP: Markov Modulated Poisson Process;
- FBQ: Feedback Queues;
- IFBQ: Instantaneous Feed Back Queue;
- DFBQ: Delayed Feed Back Queue;
- LIQBD: Level Independent Quasi-Birth-and-Death;
- WV: Working Vacations;
- PU: Primary Users;

- SU: Secondary Users;

In addition to introducing some new notions in feedback and priority queues, this paper extends earlier work to a much more general framework. The salient features of the present paper are:

- It brings down the priority of customers when customers, after first service, feedback to the system. Thus it combines feedback and priority to open a new direction of the investigation.
- Because the customers who feedback join a new queue, our model provides the best way to identify such customers, provided low priority customers are not admitted to the system. In Krishnamoorthy and Manjunath [1] no entry of external customers to the low priority queue, was considered. Thus this paper is a further extension of [1]. Prior to introducing this type of feedback of customers, only two types of feedback were considered in the literature: (i) feedback to the tail end of the queue and (ii) continue service as a feedback customer on completion of the first service. Thus it is hard to identify feedback customers, if at all any, in these cases. In addition, if we allow more than two priorities, then more than one feedback can be allowed to each customer; such feedback customers are directed to the immediately low priority queue. Thus the problems discussed in this paper have several practical utilities.
- This has certain advantages, which include minimizing unnecessarily bothering the server with questions that were clarified in the earlier rounds of service. This feat is achieved through feedback customers being downgraded in their priority.
- The low priority queue accommodates both external customers (of lower priority) and feedback customers from the first priority queue. Though in the literature there is just one paper (see Krishnamoorthy and Manjunath [1]) that considers customer feedback-generated low priority queue, it does not consider external customers of low priority, joining the feedback-driven low priority queue. Besides, in [1] only a very simple case of exponentially distributed service time and Poisson arrival of Priority 1 customer to the system, are considered. In contrast, in this paper MMAP arrival of customers and distinct phase-type distributed service time for the two types of customers, are considered.
- In addition to the above indicated features, a number of extensions of this work is also indicated in the concluding section.

Before going to further details of the paper, we provide literature review on the two topics—feedback and priority queue.

1.1. Literature Review of Feedback Queues

FBQ are extensively discussed in the queueing literature. They are adequate mathematical models of computer communication networks, manufacturing systems, inventory management processes, and many other systems in which a few served customers may require re-servicing for some reason. Indeed, in information transmission systems, erroneously transmitted data (packets, frames, etc.) are re-transmitted; defective items produced during a manufacturing process have to be re-processed, and so on.

Although these systems are widely used in real life, they have not been sufficiently studied for a long time. Such models were first introduced in [2,3]. After these pioneering works, researchers again did not pay attention to them for a long time. Only in the last three decades have such systems been intensively studied by various authors.

In the class of FBQ, it is necessary to distinguish two subclasses: IFBQ, where a return occurs immediately after the service is completed, and DFBQ, in which a request by a customer who was already served out occurs after the elapse of a certain amount of time.

In [4,5], detailed reviews of work conducted on FBQ until 2015 are provided. Therefore, we restrict ourselves to those published from 2016 onwards.

Note that in most of the work reported on FBQ, no separate queue for such customers is considered. In such kinds of systems, it is very hard to control the loss probabilities of

primary and feedback calls. To reduce the loss probabilities of calls, sometimes an orbit(s) for both kind of calls is organized. In recent years, models of feedback queues with reliable and unreliable server subject to various schemes of breakdowns and repairs, have been investigated (see Rajadura et al. [6], Ke et al. [7], Singh et al. [8], Jailaxmi et al. [9], Chang et al. [10], Chang et al. [11], Madheswari et al. [12], Melikov et al. [13], Jain and Kaur [14]). The above-mentioned authors study the IFBQ model with buffers as indicated in the respective papers.

In VijayaLaxmi et al.'s work [15], the model of a feedback M/M/1 queueing system with correlated reneging and WV is examined. To compute the long run system state distribution of the resulting three-dimensional Markov chain, a matrix-geometric method is employed. Using this, several system performance measures are calculated and an optimization problem discussed.

Bouchentouf et al. [16] consider an M/M/1/N feedback queue with server vacation, balking, reneging and holding those reneged customers to the system.

To study a single channel cognitive radio network with a slotted time structure, Zhao and Yue [17] perform analysis and optimization of the system with a finite primary buffer and a probabilistic returning scheme. PU packets can always be transmitted on the channel because the channel is authorized to do so. However, SU packets that access the system, are transmitted opportunistically. To reduce packet loss probability, separate finite buffers are organized for both kinds of users. It is assumed that PU packets have preemptive priority over SU packets, i.e., a PU packet can interrupt an SU packet's transmission. A feedback scheme is accepted for interrupted SU packets, i.e., it is assumed that an interrupted SU packet, in accordance with the Bernoulli scheme, can either be admitted through the SU buffer and wait for the next transmission or be forced to leave the system.

Melikov et al. [13] analyze an IFBQ with heterogeneous servers and MMPP flow. Primary calls are served on a high-speed server. On completion of service, each call either leaves the system or asks for repeated service according to a Bernoulli scheme. Such calls, on completion of service by a slow server, leave the system or return for re-servicing according to the Bernoulli scheme. With the arrival of a primary call, if the queue length of such calls exceeds a certain threshold value and the slow server is free, then the incoming primary call is either directed to the slow server or joins its queue according to the prescribed Bernoulli scheme. Algorithms for calculating the steady-state system probabilities approximately are proposed for both finite and infinite capacity systems. Their high accuracy is demonstrated.

Melikov et al. [18] discuss methods to calculate the system state distribution with instantaneous feedback and a varying arrival rates in a Markov model of a servicing system with one server. At a service completion epoch, the customer either leaves the system or immediately returns to receive the service, which is repeated according to a predefined Bernoulli scheme. Prior to a repeated service, a random server switching time is needed. The rate of arrival of external customers depends on whether the server is in operating or switching mode. The condition for ergodicity of this two-dimensional Markov chain is obtained. Further, three methods for studying it are implemented: the method of generating functions, the method of spectral expansion, and the space merging method.

Melikov et al. [19], analyze a multi-channel queueing system with MMPP flow and delayed feedback. After receiving complete service, customers will decide either to feedback with some state-dependent probability or to leave the system forever with complementary probability. Feedback calls organize an orbit of repeated calls. If all channels of the system are busy when a repeated call arrives, then it either leaves the system with some state-dependent probability or with a complementary probability returns to orbit. Methods to calculate the steady-state probabilities of the appropriate three-dimensional Markov chain, as well as some performance measures of the system under consideration are developed.

1.2. Literature Review of Priority Queues

Priority queues were first considered by White and Christie [20] as a queue with interruption of service of low priority customers to provide service to higher priority

customers. A priority queue with preemptive service can be regarded as a queue with service interruption. For example, a doctor renders their service to a causality patient urgently by interrupting the present consultation in case it is not critical. Jaiswal [21] is in the preemptive priority queue with resumption of service of the low priority customer and Jaiswal [22] discusses time-dependent solutions in priority queues. Cobham [23] considers a non-preemptive priority queue and derived the expected waiting time in equilibrium. A detailed discussion of development in priority queues until 1968 is given in Jaiswal [24]. More recent developments on priority queues could be found in Takagi [25] and Brodal's work [26].

Interruption in service due to server failure is discussed extensively in the literature. In the survey paper by Krishnamoorthy et al. [27], an account of various types of server interruptions is provided. This includes interrupting current service to attend higher priority work.

Customer induced service interruption, as coined by Jacob et al. [28], is in contrast to that of interruption due to server failure. This is carried out for the single server case, where customers who experienced interrupted service are given priority over primary customers. Here self-interrupted customer takes an exponentially distributed time to get out of interruption. This is extended to the multi-server case in Krishnamoorthy and Jacob [29]. All underlying distributions (inter-arrival time, service time, inter-interruption time, interruption fixation time) are assumed to be independent exponential random variables. Dudin et al. [30] extend the above case to a Markovian arrival process and phase type service with c servers and negative customers with a few protected service phases.

Lowering of priority of customers in feedback queues is introduced in Krishnamoorthy and Manjunath [1] (see also the Doctoral thesis by Manjunath [31], Chapter 3). Following these works, we assumed that the P_1 customers who opt for feedback are allotted low priority and so joins the P_2 queue along with those customers who are primary P_2 customers.

We can increase the amount of feedback permitted to both classes of customers up to a finite number. This could be achieved by introducing feedback of P_2 customers after service, placing them in a queue labeled P_3 , in which customers of priority 3 join from outside. This can be repeated up to a maximum of K feedback by P_1 customers, $K - 1$ feedback by P_2 customers, and just 1 feedback by P_K customers. If all queues are allowed to have infinite capacity, then the block matrix entries themselves will be of infinite dimensions. In the simple case of Poisson arrivals of external customers to the respective designated queues and exponentially distributed service time, we will be able to derive explicit expressions for system state probability.

Note that the low priority queue(s) is generated by an internal mechanism as well as an external one in the present paper. However, Krishnamoorthy and Manjunath [1] consider only the internal generation of feedback customers. They were able to employ the matrix geometric method, with all queues having infinite capacity because all underlying distributions were exponential. This helped them achieve a very nice structure for the infinitesimal generator of the resulting continuous-time Markov chain. The block matrix entries in the infinitesimal generator are also of infinite dimensions.

Note that the low priority queue(s) are dependent on the higher priority ones also in its evolution. For example, P_3 depends on P_1 and P_2 when we consider at least three distinct priorities. Various feedback policies in different queuing models are studied in the literature (see, for example, [2,32–37]). An M/G/1 retrial queue with instantaneous customer feedback is considered by Krishnakumar et al. [38]. In this, the feedback customer joins the tail end of the queue. In [39], Krishnakumar et al. analyze a queue with more than one server; here, it is also assumed that the feedback customer joins the tail end of the queue. A queue with collisions and instantaneous feedback of unsatisfied customers is the theme of discussion in Krishnakumar's work [40].

Prior to the introduction of a queue generated by feedback customers, the feedback considered fell into two categories: Either the feedback customer joins the tail end of the queue on completion of service so the service is repeated or he occupies the server

immediately on completion of the service without joining the queue. In these two cases no separate queue for feedback customers is provided. Further, they have the disadvantage of non-identification of feedback customers. Additionally, note that there may be several feedback customers in the system at a time, some of whom have repeated this several times. In the second case, there is at most one feedback customer at any time in the system. If at all there is one, it is the one undergoing service. In the models that we discuss or in their extension to K feedback, we cannot distinguish feedback customers and primary customers in lower priority queues. However, if we do not allow the external flow of customers to queues, except the feedback customers from the immediate higher priorities, then all customers in the remaining queues are feedback customers. This helps in the control of the number of feedback to be allowed in a service system.

The following explanation gives the motivation behind this paper.

The type of feedback which is introduced here is totally different from those discussed in the literature prior to 2018. The feedback customers considered in this paper are given a lower priority. This is because during a service, for example, purchase of equipment, customers are given detailed explanation about the functioning of the system. After this exercise, a few customers may have the tendency to keep on asking questions about the machinery. This is a big nuisance to big organizations who want this process to be brisk. Thus, those customers who are not satisfied by the first service are directed, immediately after service completion, to the lower priority queue. External customers also join this lower priority queue. Thus, here we notice an interplay between feedback and priority. This also could be seen in offices as follows: suppose a customer requests for a service—this may involve search of previous records. So, after providing him/her with the basic service, the system directs the customer to wait in a separate line, which will be attended when the server becomes free from the first queue.

From the application point of view, we can introduce certain control policies: What is the optimal number of feedback which should be allowed to each priority class when there are at least two priority types considered? This involves the computation of the optimal number of waiting lines that should be provided in the system.

The remaining part of the paper is arranged as follows. Description of model I and its mathematical formulation are given in Section 2. Section 3 provides the ergodicity condition and an algorithm to calculate the steady-state probabilities of the LIQBD associated with the problem. Derivation of waiting time of P_1 customers and that of customers in the P_2 queue are discussed in Section 4. Additional performance measures are given in Section 5. Model II is described in Section 6 and further its mathematical formulation is also given in that section. A steady-state analysis of that model can be found in Section 7. The analysis of P_1 customers' waiting time and that of customers in the P_2 queue appear in Section 8. Additional performance measures are recorded in Section 9. Results of numerical experiments are demonstrated in Section 10.

Following notations are used in what follows:

- $\mathbf{e}$: Column vector with all entries 1 of order to be specified.
- $\mathbf{e}'$: Transpose of $\mathbf{e}$.
- CTMC: Continuous time Markov chain.
- I : Identity matrix of appropriate order.
- I_a : Identity matrix of order a .
- $\mathbf{e}_a(b)$: Matrix of order $1 \times b$ having 1 at position a and 0s' elsewhere.
- QBD: Quasi-Birth-and-Death
- $d_{ij}^{(k)}$: entries of D_k , $k = 0, 1$, or 2
- δ_l : l^{th} entry of $D_1\mathbf{e}$
- δ'_l : l^{th} entry of $D_2\mathbf{e}$

2. Mathematical Formulation of Model I

The mathematical formulation of a single server queue to which arrival of customers form an MMAP with parameters D_0 , D_1 , and D_2 is considered. The matrix D_0 governs transitions without an arrival. D_1 and D_2 consist of transition rates with the arrival of class 1 (high priority) and that of class 2 (low priority) respectively. $D = D_0 + D_1 + D_2$ is the infinitesimal generator matrix of the arrival process. The matrices are square matrices of order n . Let ϕ denote the stationary probability vector of D . The stationary arrival rate of class k ($k = 1, 2$) customers is given by $\lambda_k = \phi D_k \mathbf{e}$. Service time of both types of customers is phase-type distributed with representation (α, T) and (β, S) of orders p and q , respectively. The probability of a P_1 customer to feedback is θ on completion of their service. The feedback (P_1) customers, as well as P_2 customers, join the low priority queue. Low priority (P_2) customers are taken for service from the head of the line whenever the P_1 queue is found to be empty at service completion. We assume finite waiting capacity L for P_1 customers and infinite waiting space for P_2 customers. The service of P_2 customers follows a non-preemptive service discipline; that is the arrival of a P_1 customer does not interrupt the ongoing service of a P_2 customer. No feedback is permitted to customers in the P_2 line. It is assumed that the two arrival processes are independent of the service processes.

The QBD Process Associated with Model I

The model described above can be studied as an LIQBD process. First, we introduce the following notations:

$N_1(t)$: the number of P_2 customers in the system at time t ;

$N_2(t)$: the number of P_1 customers in the system at time t .

At time t :

$$S(t) = \begin{cases} 0, & \text{if the server is idle} \\ 1, & \text{if } P_1 \text{ customer in service} \\ 2, & \text{if } P_2 \text{ customer in service} \end{cases}$$

$J(t)$: the phase of the service process when the server is busy;

$M(t)$: the phase of the arrival of the customer.

We can verify that $\{(N_1(t), N_2(t), S(t), J(t), M(t)) : t \geq 0\}$ is an LIQBD with state space

$$\Omega = \cup_{i=0}^{\infty} l(i)$$

where

$l(0) = \{(0, 0, 0, m) / 1 \leq m \leq n\} \cup \{(0, n_2, 1, l, m) / 1 \leq n_2 \leq L; 1 \leq l \leq p; 1 \leq m \leq n\}$ and for $n_1 \geq 1$,

$l(n_1) = \{(n_1, 0, 2, l, m) / 1 \leq l \leq q, 1 \leq m \leq n\} \cup \{(n_1, n_2, 1, l, m) / 1 \leq n_2 \leq L; 1 \leq l \leq p; 1 \leq m \leq n\} \cup \{(n_1, n_2, 2, l, m) / 1 \leq n_2 \leq L; 1 \leq l \leq q; 1 \leq m \leq n\}$

Note that when $N_1(t) = N_2(t) = 0$, the server will be idle and so $J(t)$ need not be considered.

The infinitesimal generator of this CTMC is

$$Q_1 = \begin{bmatrix} B_0 & C_0 & & & \\ B_1 & A_1 & A_0 & & \\ & A_2 & A_1 & A_0 & \\ & & \ddots & \ddots & \ddots \end{bmatrix}.$$

Here, B_0 consists of transitions within the level 0; C_0 contains transitions from level 0 to level 1; B_1 consists of transitions from level 1 to level 0; A_0 contains transitions from level h to level $h + 1$ for $h \geq 1$, A_1 contains transitions within the level h for $h \geq 1$ and A_2 contains transitions from level h to $h - 1$ for $h \geq 2$. The boundary blocks B_0, C_0, B_1 are of orders $n(1 + Lp) \times n(1 + Lp)$, $n(1 + Lp) \times n(q + L(p + q))$, $n(q + L(p + q)) \times n(1 + Lp)$, respectively. A_0, A_1, A_2 are square matrices of order $n(q + L(p + q))$.

Define the entries of $B_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$. Since none or one event alone could take place in a short interval of time with positive probability, in general, a transition such as $(i_1, i_2, j, k, l) \rightarrow (i'_1, i'_2, j', k', l')$ has positive rate only for exactly one of i'_1, i'_2, j', k', l' , different from i_1, i_2, j, k, l .

$$B_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} \alpha \otimes D_1 & i_1 = 0, i_2 = 1; j_2 = 1; 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \\ I_p \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1 + 1; j_1 = j_2 = 1; \\ & 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \\ (1 - \theta)(T^0 \otimes I_n) & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ (1 - \theta)(T^0 \alpha \otimes I_n) & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ D_0 & i_2 = i_1 = 0; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 & 1 \leq i_1 \leq L - 1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus (D_0 + \Delta) & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

where

$$\Delta = \begin{bmatrix} \delta_1 & & & \\ & \delta_2 & & \\ & & \ddots & \\ & & & \delta_n \end{bmatrix}.$$

Define the entries of $C_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (1, i_2, j_2, k_2, l_2)$

$$C_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} \beta \otimes D_2 & i_1 = i_2 = 0; j_1 = 0, j_2 = 2; 1 \leq k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \beta \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq p, 1 \leq k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \\ I_p \otimes D_2 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $B_{1(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(1, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$.

$$B_{1(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} S^0 \otimes I_n & i_1 = i_2 = 0; j_1 = 2, j_2 = 0; 1 \leq k_1 \leq q; 1 \leq l_1, l_2 \leq n \\ S^0 \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq k_1 \leq q, 1 \leq k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $A_{2(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h - 1, i_2, j_2, k_2, l_2)$, where $h > 1$.

$$A_{2(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} S^0 \beta \otimes I_n & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ S^0 \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq k_1 \leq q, 1 \leq k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $A_{1(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$, where $h \geq 1$.

$$A_{1_{(i_1, j_1, k_1, l_1)}}^{(i_2, j_2, k_2, l_2)} = \begin{cases} I_q \otimes D_1 & i_1 = 1, i_2 = 0; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ I_p \otimes D_1 & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 1; \\ & 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \\ I_q \otimes D_1 & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ (1-\theta)(T^0 \beta \otimes I_n) & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq p, 1 \leq k_2 \leq q; \\ & 1 \leq l_1, l_2 \leq n \\ (1-\theta)(T^0 \alpha \otimes I_n) & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ S \oplus D_0 & 0 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq q; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus (D_0 + \Delta) & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; \\ & 1 \leq l_1, l_2 \leq n \\ S \oplus (D_0 + \Delta) & i_1 = i_2 = L; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq q; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

where

$$\Delta = \begin{bmatrix} \delta_1 & & & \\ & \delta_2 & & \\ & & \ddots & \\ & & & \delta_n \end{bmatrix}.$$

Define the entries of $A_{0_{(i_1, j_1, k_1, l_1)}}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which consist of transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h+1, i_2, j_2, k_2, l_2)$, where $h \geq 1$.

$$A_{0_{(i_1, j_1, k_1, l_1)}}^{(i_2, j_2, k_2, l_2)} = \begin{cases} I_q \otimes D_2 & 0 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq q; \\ & 1 \leq l_1, l_2 \leq n \\ I_p \otimes D_2 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \beta \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq p, 1 \leq k_2 \leq q; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq p; 1 \leq l_1, l_2 \leq n \end{cases}$$

Next we proceed for the steady state analysis of the system described.

3. Steady State Analysis

Let $\pi = (\pi_0, \pi_1, \dots, \pi_L)$ denote the steady-state probability vector of the generator

$$A = A_0 + A_1 + A_2 = \begin{bmatrix} F' & G' & & & \\ E' & F & G & & \\ & E & F & G & \\ & & \ddots & \ddots & \ddots \\ & & & E & F & G \\ & & & & E & H \end{bmatrix}$$

where

$$F' = S \oplus D_0 + S^0 \beta \otimes I_n + I_q \otimes D_2, G' = e'_2(2) \otimes (I_q \otimes D_1), E' = e_1(2) \otimes (T^0 \beta \otimes I_n)$$

$$F = \begin{bmatrix} T \oplus D_0 + I_p \otimes D_2 & 0 \\ S^0 \alpha \otimes I_n & S \oplus D_0 + I_q \otimes D_2 \end{bmatrix}.$$

$$G = I_{p+q} \otimes D_1, E = e_1(2)e'_1(2) \otimes (T^0 \alpha \otimes I_n)$$

$$H = \begin{bmatrix} T \oplus (D_0 + \Delta) + I_p \otimes D_2 & 0 \\ S^0 \alpha \otimes I_n & S \oplus (D_0 + \Delta) + I_q \otimes D_2 \end{bmatrix}.$$

i.e.,

$$\pi A = 0, \pi e = 1. \quad (1)$$

The *LIQBD* description of the model indicates that the queueing system is stable (see Neuts [41]) if and only if the left drift exceeds that of the right drift. That is,

$$\pi A_0 e < \pi A_2 e \quad (2)$$

The vector π cannot be obtained directly in terms of the parameters of the model. From (1) we get

$$\pi_i = \pi_{i-1} \mathcal{U}_{i-1}, 1 \leq i \leq L \quad (3)$$

where

$$\mathcal{U}_0 = -G'(F + U_1 E)^{-1}$$

$$\mathcal{U}_i = \begin{cases} -G(F + U_{i+1} E)^{-1} & \text{for } 1 \leq i \leq L-2 \\ -GH^{-1} & \text{for } i = L-1 \end{cases}$$

From the normalizing condition $\pi e = 1$ we have

$$\pi_0 \left(\sum_{j=0}^{L-1} \prod_{i=0}^j \mathcal{U}_i + I \right) e = 1 \quad (4)$$

The inequality (2) gives the stability condition as

$$\begin{aligned} \pi_0 \left[(I_q \otimes D_2) e + (U_0(e_1(2) \otimes (\theta T^0 \beta \otimes I_n)) + I_{p+q} \otimes D_2) e + \sum_{i=1}^{L-1} \prod_{j=0}^i \mathcal{U}_j (e_1(2) e'_1(2) \otimes (\theta T^0 \alpha \otimes I_n)) + I_{(p+q)} \otimes D_2) e \right] \\ < \pi_0 \left[(S^0 \beta \otimes I) e + \sum_{i=0}^{L-1} \prod_{j=0}^i \mathcal{U}_j e_2(2) e'_1(2) (S^0 \alpha \otimes I) e \right] \end{aligned} \quad (5)$$

Let x be the steady state probability vector of Q . We partition this vector as

$$x = (x_0, x_1, x_2, \dots),$$

where x_0 is of dimension $n(1 + Lp)$, $x_1, x_2, \dots$ are of dimension $n(q + L(p + q))$. Under the stability condition, we have

$$x_i = x_1 R^{i-1}, i \geq 2$$

where the matrix R is the minimal nonnegative solution to the matrix quadratic equation

$$R^2 A_2 + R A_1 + A_0 = 0$$

and the vectors x_0 and x_1 are obtained by solving the equations

$$x_0 B_0 + x_1 B_1 = 0 \quad (6)$$

$$x_0 C_0 + x_1 (A_1 + R A_2) = 0 \quad (7)$$

subject to the normalizing condition

$$x_0 e + x_1 (I - R)^{-1} e = 1 \quad (8)$$

Certain probability distributions need to be computed for evaluating the performance of the system being studied. Now we pass on to do that.

4. Waiting Time Analysis

4.1. P_1 Customer without Feedback

Let $W_1(t)$ be the waiting time of a P_1 customer who arrives in the system at time t . In this case, the waiting time is the time until absorption in the Markov process $\{(N(t), S(t), M(t); t \geq 0)\}$, where $N(t)$ is the rank of the customer, $S(t)$ is the status of the server and $M(t)$ is the phase of service at time t . The rank of a customer is r if he is the r th customer in the P_1 queue at time t . The rank of the tagged customer decreases by 1 when the customer ahead of him leaves the system after receiving service. The state-space of the process is given by $\{(h, 1, j) : 1 \leq h \leq r, 1 \leq j \leq p\} \cup \{(r, 2, j) : 1 \leq j \leq q\} \cup \{*\}$, where $*$ denotes the absorbing state denoting that the tagged customer is selected for service. Thus the waiting time can be studied by a phase-type distribution with representation $Ph(\psi_1^{(r)}, W_1)$ where

$$W_1 = \begin{bmatrix} T & & & & \\ T^0 \alpha & T & & & \\ & \ddots & \ddots & & \\ & & T^0 \alpha & T & \\ & & & S^0 \alpha & S \end{bmatrix}, W_1^0 = \begin{bmatrix} T^0 \\ \mathbf{0} \end{bmatrix}$$

Let $w_{r,i,j}$ denote the probability that the tagged customer finds the system in one of the states (h, r, i, j, k) immediately after his arrival. Then,

$$\begin{aligned} w_{1,1,j} &= \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)} \alpha_j}{-d_{k'k'}^{(0)}} x_{0,0,k'}, & 1 \leq j \leq p \\ w_{r,1,j} &= \sum_{h=0}^{\infty} \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)}}{-d_{k'k'}^{(0)} - T_{jj}} x_{h,r-1,1,j,k'}, & r \geq 2, 1 \leq j \leq p \\ w_{r,2,j} &= \sum_{h=1}^{\infty} \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)}}{-d_{k'k'}^{(0)} - S_{jj}} x_{h,r-1,2,j,k'}, & r \geq 1, 1 \leq j \leq q \end{aligned}$$

The initial probability vector $\psi_1^{(r)} = \frac{1}{d_1^{(r)}} \mathbf{w}_1(r)$

where,

$$\mathbf{w}_1(r) = (\mathbf{0}, \dots, \mathbf{0}, w_{r,1,1}, \dots, w_{r,1,p}, w_{r,2,1}, \dots, w_{r,2,q}), d_1^{(r)} = \sum_{k=1}^p w_{r,1,k} + \sum_{k=1}^q w_{r,2,k}$$

Therefore the expected waiting time of the tagged customer according to the state of the system at the time of joining the queue

$$E_{W_1}^{(r)} = (-W_1)^{-1} \mathbf{e}$$

Hence, the expected waiting time of an arbitrary customer in the queue is

$$E(W_1) = \frac{1}{d_1} \sum_{r=1}^{\infty} \mathbf{w}_1(r) E_{W_1}^{(r)}, \text{ where } d_1 = \sum_{r=1}^L d_1^{(r)}$$

4.2. P_1 Customer with Feedback/ P_2 Customer (in P_2 Queue)

Let $W_2(t)$ be the waiting time of a P_1 customer with feedback/ P_2 customer who arrives in the system at time t . In this case, the waiting time is the time until absorption in the Markov process $\{(N_1(t), N_2(t), S(t), K(t), M(t); t \geq 0)\}$, where $N_1(t)$ is the rank of the tagged customer, $N_2(t)$ is the number of P_1 customers in the system, $S(t)$ is the status of the server and $K(t)$ is the phase of service and $M(t)$, the arrival phase at time t . The state space of the process is given by

$\{(h, i, 1, k, l) : 1 \leq h \leq r, 1 \leq i \leq L, 1 \leq k \leq p, 1 \leq l \leq n\} \cup \{(h, i, 2, k, l) : 1 \leq h \leq r, 1 \leq i \leq L, 1 \leq k \leq q, 1 \leq l \leq n\} \cup \{*\}$,

where $*$ denotes the absorbing state denoting that the tagged customer is selected for service. Thus, the waiting time can be studied by a phase-type distribution with representation $Ph(\boldsymbol{\psi}_2^{(r)}, W_2)$ where

$$W_2 = \begin{bmatrix} E & & & \\ F & E & & \\ & F & E & \\ & & \ddots & \ddots \end{bmatrix}, W_2^{00} = \begin{bmatrix} \mathbf{W}_2^{00} \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \end{bmatrix}$$

with

$$\mathbf{W}_2^{00} = \begin{bmatrix} S^0 \otimes \mathbf{e}(n) \\ \mathbf{0} \\ \vdots \\ S^0 \otimes \mathbf{e}(n) \\ \mathbf{0} \\ S^0 \otimes \mathbf{e}(n) \end{bmatrix}$$

and

$$E = \begin{bmatrix} E_{11} & E_{12} & & & & \\ & E_{21} & E_{22} & I_{p+q} \otimes D_1 & & \\ & & E_{31} & E_{22} & I_{p+q} \otimes D_1 & \\ & & & \ddots & \ddots & \\ & & & E_{31} & E_{22} & I_{p+q} \otimes D_1 \\ & & & & E_{31} & E_{22}' \end{bmatrix}$$

where

$$E_{11} = S \oplus (D_0 + \Delta'), E_{12} = \mathbf{e}_2'(2) \otimes (I_q \otimes D_1), E_{21} = \mathbf{e}_1(2) \otimes (T^0 \boldsymbol{\beta} \otimes I_n),$$

$$E_{22} = \begin{bmatrix} T \oplus (D_0 + \Delta') & \\ & S \oplus (D_0 + \Delta') \end{bmatrix}, E_{31} = \mathbf{e}_1(2) \mathbf{e}_1'(2) \otimes (T^0 \boldsymbol{\alpha} \otimes I_n)$$

and

$$E_{22}' = \begin{bmatrix} T \oplus (D_0 + \Delta' + \Delta) & \\ & S \oplus (D_0 + \Delta' + \Delta) \end{bmatrix}.$$

where

$$\Delta' = \begin{bmatrix} \delta_1' & & & \\ & \delta_2' & & \\ & & \ddots & \\ & & & \delta_n' \end{bmatrix}.$$

$$F = \begin{bmatrix} S^0 \boldsymbol{\beta} \otimes I_n & & & \\ & \mathbf{e}_2(2) \mathbf{e}_1'(2) \otimes (S^0 \boldsymbol{\alpha} \otimes I_n) & & \\ & & \ddots & \\ & & & \mathbf{e}_2(2) \mathbf{e}_1'(2) \otimes (S^0 \boldsymbol{\alpha} \otimes I_n) \end{bmatrix}$$

Let $w_{r,i,j,k,l}$ denote the probability that the tagged customer finds the system in one of the states (r, i, j, k, l) immediately after his arrival. Then,

$$\begin{aligned} w_{r,i,1,k,l} &= \sum_{l'=1}^n \frac{d_{l'}^{(2)}}{-d_{l'}^{(0)} - T_{kk}} x_{r-1,i,1,k,l'}, \quad r \geq 1, 1 \leq i \leq L, 1 \leq k \leq p, 1 \leq l \leq n \\ w_{1,i,2,k,l} &= \sum_{l'=1}^n \frac{d_{l'}^{(2)} \beta_k}{-d_{l'}^{(0)}} x_{0,0,l'}, \quad 0 \leq i \leq L, 1 \leq k \leq q, 1 \leq l \leq n \\ w_{r,i,2,k,l} &= \sum_{l'=1}^n \frac{d_{l'}^{(2)}}{-d_{l'}^{(0)} - S_{kk}} x_{r-1,i,2,k,l'}, \quad r \geq 2, 0 \leq i \leq L, 1 \leq k \leq q, 1 \leq l \leq n \end{aligned}$$

The initial probability vector

$$\psi_2^{(r)} = \frac{1}{d_2^{(r)}} (w_2^{(r)})$$

where,

$$w_2^{(r)} = (0, \dots, 0, w_{r,0,2,1,1}, \dots, w_{r,0,2,q,n}, w_{r,1,1,1,1}, \dots, w_{r,1,1,p,n}, w_{r,1,2,1,1}, \dots, w_{r,1,2,q,n}, \dots, w_{r,L,1,1,1}, \dots, w_{r,L,2,q,n})$$

and

$$d_2^{(r)} = \sum_{i=1}^L \sum_{k=1}^p \sum_{l=1}^n w_{r,i,1,k,l} + \sum_{i=0}^L \sum_{k=1}^q \sum_{l=1}^n w_{r,i,2,k,l}$$

Therefore the expected waiting time of the tagged customer according to the state of the system at the time of joining the queue

$$E_{W_2}^{(r)} = (-W_2)^{-1} \mathbf{e}$$

Hence the expected waiting time of an arbitrary customer in the queue is

$$E(W_2) = \frac{1}{d_2} \sum_{r=1}^{\infty} w_2^{(r)} E_{W_2}^{(r)}, \text{ where, } d_2 = \sum_{r=1}^{\infty} d_2^{(r)}$$

5. Other Performance Measures

- The probability that the server is idle:

$$p_{idle} = \sum_{l=1}^n x_{0,0,l}$$

- Mean number of P_1 customers in the system:

$$E_{nsh} = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{k=1}^p \sum_{l=1}^n n_2 x_{n_1,n_2,1,k,l} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{k=1}^q \sum_{l=1}^n n_2 x_{n_1,n_2,2,k,l}$$

- Mean number of P_2 customers in the system:

$$E_{nsl} = \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{k=1}^p \sum_{l=1}^n n_1 x_{n_1,n_2,1,k,l} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{k=1}^q \sum_{l=1}^n n_1 x_{n_1,n_2,2,k,l}$$

- The fraction of time the server is providing service to P_1 customers:

$$T_h = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{k=1}^p \sum_{l=1}^n x_{n_1,n_2,1,k,l}$$

- The fraction of time the server is providing service to P_2 customers:

$$T_l = \sum_{n_1=1}^{\infty} \sum_{n_2=0}^L \sum_{k=1}^q \sum_{l=1}^n x_{n_1, n_2, 2, k, l}$$

6. Mathematical Formulation of Model II

So far, we have discussed the non-preemptive service discipline for priority 2 customers. In this section, we consider the preemptive (repeat identical) case of that. Arrival streams of customers follow MMAP with parameter matrices D_0 , D_1 , and D_2 , as was assumed in the case of model I. This is justified because we are modifying only the pattern of interruption of service of P_2 customers and for comparison of the performance of the two models we should have the same input pattern. The service times of both types of customers are distinct and independent phase-type distributed random variables as in model I. As a P_2 customer is receiving service if a P_1 customer arrives, then the former is preempted by the latter to receive service. The head of the P_2 line is taken for service whenever the P_1 queue is empty.

The QBD Process Associated with Model II

Model II is also an LIQBD process. We employ the matrix geometric method to compute its limiting system state distribution. To start with, we introduce the following notations:

$N_1(t)$: the number of P_2 customers in the system at time t ;

$N_2(t)$: the number of P_1 customers in the system at time t ;

$J(t)$: the phase of the service process when the server is busy;

$M(t)$: the phase of arrival of the customer.

It is easy to verify that $\{(N_1(t), N_2(t), J(t), M(t)) : t \geq 0\}$ is an LIQBD with state space

$$\Omega = \cup_{i=0}^{\infty} l(i)$$

where

$l(0) = \{(0, 0, m) / 1 \leq m \leq n\} \cup \{(0, n_2, l, m) / 1 \leq n_2 \leq L; 1 \leq l \leq p; 1 \leq m \leq n\}$ and for $n_1 \geq 1$,

$l(n_1) = \{(n_1, 0, l, m) / 1 \leq l \leq q; 1 \leq m \leq n\} \cup \{(n_1, n_2, l, m) / 1 \leq n_2 \leq L; 1 \leq l \leq p; 1 \leq m \leq n\}$

Note that when $N_1(t) = N_2(t) = 0$, the server will be idle and so $J(t)$ need not be considered.

The infinitesimal generator of this CTMC is

$$Q_2 = \begin{bmatrix} B_0 & C_0 & & & \\ B_1 & A_1 & A_0 & & \\ & A_2 & A_1 & A_0 & \\ & & \ddots & \ddots & \ddots \end{bmatrix}.$$

where B_0 contains transitions within the level 0; C_0 represents transitions from level 0 to level 1; B_1 represents transitions from level 1 to level 0; A_0 represents transitions from level h to level $h + 1$ for $h \geq 1$, A_1 represents transitions within the level h for $h \geq 1$ and A_2 represents transitions from level h to $h - 1$ for $h \geq 2$. The boundary blocks B_0, C_0, B_1 are of orders $n(1 + Lp) \times n(1 + Lp)$, $n(1 + Lp) \times n(q + Lp)$, $n(q + Lp) \times n(1 + Lp)$ respectively. A_0, A_1, A_2 are square matrices of order $n(q + Lp)$.

Define the entries of $B_{(i_1, j_1, k_1)}^{(i_2, j_2, k_2)}$ as transition submatrices which contains transitions of the form $(0, i_1, j_1, k_1) \rightarrow (0, i_2, j_2, k_2)$. Since none or one event alone could take place in a

short interval of time with positive probability, in general, a transition such as $(i_1, i_2, j, k) \rightarrow (i'_1, i'_2, j', k')$ has positive rate only for exactly one of i'_1, i'_2, j', k' different from i_1, i_2, j, k .

$$B_{0(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \begin{cases} \alpha \otimes D_1 & i_1 = 0, i_2 = 1; 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ I_p \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1 + 1; \\ & 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ (1 - \theta)(T^0 \otimes I_n) & i_1 = 1, i_2 = 0; 1 \leq j_1 \leq p; \\ & 1 \leq k_1, k_2 \leq n \\ (1 - \theta)(T^0 \alpha \otimes I_n) & 2 \leq i_1 \leq L, i_2 = i_1 - 1; 1 \leq j_1 \leq p; \\ & 1 \leq k_1, k_2 \leq n \\ D_0 & i_2 = i_1 = 0; 1 \leq k_1, k_2 \leq n \\ T \oplus D_0 & 1 \leq i_1 \leq L - 1, i_2 = i_1; 1 \leq j_1, j_2 \leq p; \\ & 1 \leq k_1, k_2 \leq n \\ T \oplus (D_0 + \Delta) & i_1 = i_2 = L; 1 \leq j_1, j_2 \leq p; \\ & 1 \leq k_1, k_2 \leq n \end{cases}$$

where

$$\Delta = \begin{bmatrix} \delta_1 & & & \\ & \delta_2 & & \\ & & \ddots & \\ & & & \delta_n \end{bmatrix}.$$

Define the entries of $C_{0(i_1, j_1, k_1)}^{(i_2, j_2, k_2)}$ as transition submatrices which contain transitions of the form $(0, i_1, j_1, k_1) \rightarrow (1, i_2, j_2, k_2)$

$$C_{0(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \begin{cases} \beta \otimes D_2 & i_1 = i_2 = 0; 1 \leq j_2 \leq q; 1 \leq k_1, k_2 \leq n \\ \theta T^0 \beta \otimes I_n & i_1 = 1, i_2 = 0; 1 \leq j_1 \leq p, 1 \leq j_2 \leq q; 1 \leq k_1, k_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ I_p \otimes D_2 & 1 \leq i_1 \leq L, i_2 = i_1; 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \end{cases}$$

Define the entries of $B_{1(i_1, j_1, k_1)}^{(i_2, j_2, k_2)}$ as transition submatrices which contain transitions of the form $(1, i_1, j_1, k_1) \rightarrow (0, i_2, j_2, k_2)$.

$$B_{1(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \{ \mathbf{S}^0 \otimes I_n \quad i_1 = i_2 = 0; 1 \leq j_1 \leq q; 1 \leq k_1, k_2 \leq n$$

Define the entries of $A_{2(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h - 1, i_2, j_2, k_2, l_2)$, where $h > 1$.

$$A_{2(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \{ \mathbf{S}^0 \beta \otimes I_n \quad i_1 = i_2 = 0; 1 \leq j_1, j_2 \leq q; 1 \leq k_1, k_2 \leq n$$

Define the entries of $A_{1(i_1, j_1, k_1)}^{(i_2, j_2, k_2)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1) \rightarrow (h, i_2, j_2, k_2)$, where $h \geq 1$.

$$A_{1(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \begin{cases} \mathbf{e}(q) \otimes (\boldsymbol{\alpha} \otimes D_1) & i_1 = 0, i_2 = 1; \\ & 1 \leq j_1 \leq q, 1 \leq j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ I_p \otimes D_1 & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; \\ & 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ (1-\theta)(\mathbf{T}^0 \boldsymbol{\beta} \otimes I_n) & i_1 = 1, i_2 = 0; 1 \leq j_1 \leq p; 1 \leq j_2 \leq q \\ & 1 \leq k_1, k_2 \leq n \\ (1-\theta)(\mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n) & 2 \leq i_1 \leq L, i_2 = i_1 - 1; 1 \leq j_1, j_2 \leq p; \\ & 1 \leq k_1, k_2 \leq n \\ S \oplus D_0 & i_2 = i_1 = 0; 1 \leq j_1, j_2 \leq q; \\ & 1 \leq k_1, k_2 \leq n \\ T \oplus D_0 & 1 \leq i_1 \leq L-1, i_2 = i_1; 1 \leq j_1, j_2 \leq p; \\ & 1 \leq k_1, k_2 \leq n \\ T \oplus (D_0 + \Delta) & i_1 = i_2 = L; 1 \leq j_1, j_2 \leq p; \\ & 1 \leq k_1, k_2 \leq n \end{cases}$$

where

$$\Delta = \begin{bmatrix} \delta_1 & & & \\ & \delta_2 & & \\ & & \ddots & \\ & & & \delta_n \end{bmatrix}.$$

Define the entries of $A_{0(i_1, j_1, k_1)}^{(i_2, j_2, k_2)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1) \rightarrow (h+1, i_2, j_2, k_2)$, where $h \geq 1$.

$$A_{0(i_1, j_1, k_1)}^{(i_2, j_2, k_2)} = \begin{cases} I_q \otimes D_2 & i_1 = i_2 = 0; 1 \leq j_1, j_2 \leq q; 1 \leq k_1, k_2 \leq n \\ I_p \otimes D_2 & 1 \leq i_1 \leq L, i_2 = i_1; 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \\ \theta \mathbf{T}^0 \boldsymbol{\beta} \otimes I_n & i_1 = 1, i_2 = 0; 1 \leq j_1 \leq p, 1 \leq j_2 \leq q; 1 \leq k_1, k_2 \leq n \\ \theta \mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; 1 \leq j_1, j_2 \leq p; 1 \leq k_1, k_2 \leq n \end{cases}$$

Next we proceed for the steady state analysis of the system described.

7. Steady State Analysis

Let $\boldsymbol{\pi} = (\pi_0, \pi_1, \dots, \pi_L)$ denote the steady state probability vector of the generator

$$A = A_0 + A_1 + A_2 = \begin{bmatrix} F' & G' & & & \\ E' & F & G & & \\ & E & F & G & \\ & & \ddots & \ddots & \ddots \\ & & & E & F & G \\ & & & & E & H \end{bmatrix} \quad \text{where}$$

$$F' = S \oplus D_0 + \mathbf{S}^0 \boldsymbol{\beta} \otimes I_n + I_p \otimes D_2, \quad G' = \mathbf{e}(q) \otimes (\boldsymbol{\alpha} \otimes D_1), \quad E' = \mathbf{T}^0 \boldsymbol{\beta} \otimes I_n$$

$$F = T \oplus D_0 + I_p \otimes D_2$$

$$G = I_p \otimes D_1, \quad E = \mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n$$

$$H = T \oplus (D_0 + \Delta) + I_p \otimes D_2$$

i.e.,

$$\boldsymbol{\pi} A = 0, \boldsymbol{\pi} \mathbf{e} = 1. \quad (9)$$

The LIQBD description of the model indicates that the queueing system is stable (see Neuts [41]) if and only if the left drift exceeds that of right drift. That is,

$$\pi A_0 \mathbf{e} < \pi A_2 \mathbf{e} \quad (10)$$

The vector π cannot be obtained directly in terms of the parameters of the model. From (9), we obtain

$$\pi_i = \pi_{i-1} \mathcal{U}_{i-1}, 1 \leq i \leq L \quad (11)$$

where

$$\begin{aligned} \mathcal{U}_0 &= -G(F + U_1 E)^{-1} \\ \mathcal{U}_i &= \begin{cases} -G(F + U_{i+1} E)^{-1} & \text{for } 1 \leq i \leq L-2 \\ -GH^{-1} & \text{for } i = L-1 \end{cases} \end{aligned}$$

From the normalizing condition $\pi \mathbf{e} = 1$, we have

$$\pi_0 \left(\sum_{j=0}^{L-1} \prod_{i=0}^j \mathcal{U}_i + I \right) \mathbf{e} = 1 \quad (12)$$

The inequality (10) gives the stability condition as

$$\pi_0 \left[(I_q \otimes D_2) \mathbf{e} + (U_0 (\theta T^0 \beta \otimes I_n) + I_p \otimes D_2) \mathbf{e} + \sum_{i=1}^{L-1} \prod_{j=0}^i \mathcal{U}_j (\theta T^0 \alpha \otimes I_n) + I_p \otimes D_2 \right] \mathbf{e} < \pi_0 (S^0 \beta \otimes I) \mathbf{e} \quad (13)$$

Let $\mathbf{x}$ be the steady state probability vector of Q . We partition this vector as

$$\mathbf{x} = (\mathbf{x}_0, \mathbf{x}_1, \mathbf{x}_2, \dots),$$

where $\mathbf{x}_0$ is of dimension $n(1 + Lp)$, $\mathbf{x}_1, \mathbf{x}_2, \dots$ are of dimension $n(q + L(p + q))$. Under the stability condition, we have

$$\mathbf{x}_i = \mathbf{x}_1 R^{i-1}, i \geq 2$$

where the matrix R is the minimal nonnegative solution to the matrix quadratic equation

$$R^2 A_2 + R A_1 + A_0 = 0$$

and the vectors $\mathbf{x}_0$ and $\mathbf{x}_1$ are obtained by solving the equations

$$\mathbf{x}_0 B_0 + \mathbf{x}_1 B_1 = 0 \quad (14)$$

$$\mathbf{x}_0 C_0 + \mathbf{x}_1 (A_1 + R A_2) = 0 \quad (15)$$

subject to the normalizing condition

$$\mathbf{x}_0 \mathbf{e} + \mathbf{x}_1 (I - R)^{-1} \mathbf{e} = 1 \quad (16)$$

Certain probability distributions need to be computed for evaluating the performance of the system being studied.

8. Waiting Time Analysis

8.1. P_1 Customer without Feedback

Let $W_1(t)$ be the waiting time of a P_1 customer who arrives in the system at time t . In this case, the waiting time is the time until absorption in the Markov process $\{(N(t), M(t); t \geq 0)\}$, where $N(t)$ is the rank of the customer and $M(t)$ is the phase of service at time t . The rank of a customer is r if they are the r^{th} customer in the P_1 queue at time t . The rank of the tagged customer decreases by 1 when the customer ahead of him leaves the system after receiving service. The state-space of the process is given by $\{(h, i) : 1 \leq h \leq r, 1 \leq i \leq p\} \cup \{*\}$, where $*$ denote the absorbing state denoting that

the tagged customer is selected for service. Thus the waiting time can be studied by a phase-type distribution with representation $Ph(\boldsymbol{\psi}_1^{(r)}, W_1)$ where

$$W_1 = \begin{bmatrix} T & & & \\ \mathbf{T}^0 \boldsymbol{\alpha} & T & & \\ & \ddots & \ddots & \\ & & \mathbf{T}^0 \boldsymbol{\alpha} & T \end{bmatrix}, \mathbf{W}_1^0 = \begin{bmatrix} \mathbf{T}^0 \\ \mathbf{0} \end{bmatrix}$$

Let $w_{r,j}$ denote the probability that the tagged customer finds the system in one of the states (h, r, j, k) immediately after his arrival.

Then,

$$\begin{aligned} w_{1,j} &= \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)} \boldsymbol{\alpha}_j}{-d_{k'k'}^{(0)}} x_{0,0,k'} + \\ &\quad \sum_{h=1}^\infty \sum_{j'=1}^q \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)} \boldsymbol{\alpha}_j}{-d_{k'k'}^{(0)} - S_{jj}} x_{h,0,j',k'}, \quad 1 \leq j \leq p \\ w_{r,j} &= \sum_{h=0}^\infty \sum_{k=1}^n \sum_{k'=1}^n \frac{d_{k'k}^{(1)} \boldsymbol{\alpha}_j}{-d_{k'k'}^{(0)} - T_{jj}} x_{h,r-1,j,k'}, \quad 2 \leq r \leq L, 1 \leq j \leq p \end{aligned}$$

The initial probability vector

$$\boldsymbol{\psi}_1^{(r)} = \frac{1}{d_1^{(r)}} (\mathbf{w}_1(r))$$

where

$$\mathbf{w}_1(r) = (\mathbf{0}, \dots, \mathbf{0}, w_{r,1}, \dots, w_{r,p}) \text{ and } d_1^{(r)} = \sum_{j=1}^p w_{r,j}$$

Therefore the expected waiting time of the tagged customer according to the state of the system at the time of joining the queue,

$$E_{W_1}^{(r)} = (-W_1)^{-1} \mathbf{e}$$

Hence the expected waiting time of an arbitrary customer in the queue is

$$E(W_1) = \frac{1}{d_1} \sum_{r=1}^\infty \mathbf{w}_1(r) E_{W_1}^{(r)}, \text{ where } d_1 = \sum_{r=1}^L d_1^{(r)}$$

8.2. P_1 Customer with Feedback/ P_2 Customer

Let $W_2(t)$ be the waiting time of a P_1 customer with feedback/ P_2 customer who arrives in the system at time t . In this case, the waiting time is the time until absorption in the Markov process $\{(N_1(t), N_2(t), J(t), K(t); t \geq 0)\}$, where $N_1(t)$ is the rank of the tagged customer, $N_2(t)$ is the number of P_1 customers in the system, $J(t)$ is the phase of service and $K(t)$ is the arrival phase at time t . The state space of the process is given by $\{(h, 0, j, k) : 1 \leq h \leq r, 1 \leq j \leq q, 1 \leq k \leq n\} \cup \{(h, i, j, k) : 1 \leq h \leq r, 1 \leq i \leq L, 1 \leq j \leq p, 1 \leq k \leq n\} \cup \{*\}$, where $*$ denotes the absorbing state denoting that the tagged customer is selected for service. Thus the waiting time can be studied by a phase-type distribution with representation $Ph(\boldsymbol{\psi}_2^{(r)}, W_2)$ where

$$W_2 = \begin{bmatrix} E & & & \\ F & E & & \\ & F & E & \\ & & \ddots & \ddots \end{bmatrix}, \mathbf{W}_2^0 = \begin{bmatrix} \mathbf{S}^0 \otimes \mathbf{e}(n) \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \end{bmatrix}$$

and

$$E = \begin{bmatrix} S \oplus (D_0 + \Delta') & \mathbf{e}(q) \otimes (\boldsymbol{\alpha} \otimes D_1) & & & \\ \mathbf{T}^0 \boldsymbol{\beta} \otimes I_n & T \oplus (D_0 + \Delta') & I_p \otimes D_1 & & \\ & \mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n & T \oplus (D_0 + \Delta') & I_p \otimes D_1 & \\ & & \ddots & \ddots & \\ & & \mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n & T \oplus (D_0 + \Delta') & I_p \otimes D_1 \\ & & & \mathbf{T}^0 \boldsymbol{\alpha} \otimes I_n & T \oplus (D_0 + \Delta + \Delta') \end{bmatrix}$$

where,

$$\Delta' = \begin{bmatrix} \delta'_1 & & & \\ & \delta'_2 & & \\ & & \ddots & \\ & & & \delta'_n \end{bmatrix}$$

$$F = \begin{bmatrix} \mathbf{S}^0 \boldsymbol{\beta} \otimes I_n & 0 \\ 0 & 0 \end{bmatrix}$$

Let $w_{r,i,j,k}$ denote the probability that the tagged customer finds the system in one of the states (r, i, j, k) immediately after his arrival. Then

$$\begin{aligned} w_{1,0,j,k} &= \sum_{k'=1}^n \frac{d_{k'k}^{(2)} \beta_j}{-d_{k'k'}^{(0)}} x_{0,0,k'}, & 1 \leq j \leq q, 1 \leq k \leq n \\ w_{r,0,j,k} &= \sum_{k'=1}^n \frac{d_{k'k}^{(2)}}{-d_{k'k'}^{(0)} - S_{jj}} x_{r-1,0,j,k'}, & r \geq 2, 1 \leq j \leq q, 1 \leq k \leq n \\ w_{r,i,j,k} &= \sum_{k'=1}^n \frac{d_{k'k}^{(2)}}{-d_{k'k'}^{(0)} - T_{jj}} x_{r-1,i,j,k'}, & r \geq 1, 1 \leq i \leq L, 1 \leq j \leq p, 1 \leq k \leq n \end{aligned}$$

The initial probability vector $\boldsymbol{\psi}_2^{(r)} = \frac{1}{d_2^{(r)}} (\mathbf{w}_2(r))$.

where

$$\mathbf{w}_2(r) = (0, \dots, 0, w_{r,0,1,1}, \dots, w_{r,0,q,n}, w_{r,1,1,1}, \dots, w_{r,1,p,n}, \dots, w_{r,L,1,1}, \dots, w_{r,L,p,n})$$

$$d_2^{(r)} = \sum_{j=1}^q \sum_{k=1}^n w_{r,0,j,k} + \sum_{i=1}^L \sum_{j=1}^p \sum_{k=1}^n w_{r,i,j,k}$$

Therefore, the expected waiting time of the tagged customer according to the state of the system at the time of joining the queue,

$$E_{W_2}^{(r)} = (-W_2)^{-1} \mathbf{e}$$

Hence, the expected waiting time of an arbitrary customer in the queue is

$$E(W_2) = \frac{1}{d_2} \sum_{r=1}^{\infty} \mathbf{w}_2(r) E_{W_2}^{(r)}, \text{ where } d_2 = \sum_{r=1}^{\infty} d_2^{(r)}$$

9. Other Performance Measures

- The probability that the server is idle:

$$p_{idle} = \sum_{l=1}^n x_{0,0,l}$$

- Mean number of P_1 customers in the system:

$$E_{nsh} = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{j=1}^p \sum_{k=1}^n n_2 x_{n_1, n_2, j, k}$$

- Mean number of P_2 customers in the system:

$$E_{nsl} = \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{j=1}^p \sum_{k=1}^n n_1 x_{n_1, n_2, j, k} + \sum_{n_1=1}^{\infty} \sum_{j=1}^q \sum_{k=1}^n n_1 x_{n_1, 0, j, k}$$

- The fraction of time the server is providing service to P_1 customers

$$T_h = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{j=1}^p \sum_{k=1}^n x_{n_1, n_2, j, k}$$

- The fraction of time the server is providing service to P_2 customers

$$T_l = \sum_{n_1=1}^{\infty} \sum_{j=1}^q \sum_{k=1}^n x_{n_1, 0, j, k}$$

10. Numerical Results

For the arrival process of customers, we consider the following five sets of matrices for D_0 , D_1 , and D_2 .

1. Exponential (EXP):

$$D_0 = (-1), D_1 = (0.06), D_2 = (0.04)$$

2. Erlang (ERA)

$$D_0 = \begin{bmatrix} -0.3 & 0.3 & 0 \\ 0 & -0.3 & 0.3 \\ 0 & 0 & -0.3 \end{bmatrix}, D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.18 & 0 & 0 \end{bmatrix}, D_2 = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.12 & 0 & 0 \end{bmatrix}$$

3. Hyperexponential (HEXP)

$$D_0 = \begin{bmatrix} -0.34 & 0 \\ 0 & -0.085 \end{bmatrix}, D_1 = \begin{bmatrix} 0.064 & 0.172 \\ 0.01 & 0.039 \end{bmatrix}, D_2 = \begin{bmatrix} 0.004 & 0.1 \\ 0.007 & 0.029 \end{bmatrix}.$$

4. MMAP with negative correlation (MNA)

$$D_0 = \begin{bmatrix} -0.0810 & 0.0810 & 0 \\ 0 & -0.135 & 0 \\ 0 & 0 & -4.0506 \end{bmatrix}, D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0049 & 0 & 0.0702 \\ 2.4474 & 0 & 0.162 \end{bmatrix}, D_2 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0032 & 0 & 0.0567 \\ 1.3602 & 0 & 0.081 \end{bmatrix}$$

5. MMAP with positive correlation (MPA)

$$D_0 = \begin{bmatrix} -0.0810 & 0.0810 & 0 \\ 0 & -0.135 & 0 \\ 0 & 0 & -4.0506 \end{bmatrix}, D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0702 & 0 & 0.0049 \\ 0.162 & 0 & 2.4474 \end{bmatrix}, D_2 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0567 & 0 & 0.0032 \\ 0.081 & 0 & 1.3602 \end{bmatrix}$$

All of these five MMAP processes are normalized so that they have an arrival rate of 0.1. However, these are qualitatively different in the sense that they have different variance and correlation structures. The first three arrival processes, namely EXP, ERA, and HEA, correspond to renewal processes and so the correlation is 0. The arrival processes labeled MNA and MPA have correlated arrivals with the correlation between two successive interarrival times given by -0.4211 and 0.4211 respectively.

For the service time distributions, we consider phase-type distributions,

$$\alpha = [0.8 \quad 0.2], T = \begin{bmatrix} -2 & 2 \\ 0 & -2 \end{bmatrix} \text{ and } \beta = [0.7 \quad 0.3], T' = \begin{bmatrix} -0.5 & 0.5 \\ 0 & -0.5 \end{bmatrix}.$$

10.1. Expected Number of Customers

We fix $\theta = 0.6$.

Tables 1 and 2 show the effect of L on the expected number of P_1 and P_2 customers in the system for model I (non-preemptive case) and model II (preemptive case) respectively. Tables 3 and 4 show the effect of θ on the expected number of P_1 and P_2 customers in the system respectively in both models.

Table 1. Effect of L on expected number of P_1 customers in the system (when $\theta = 0.6$).

L	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
2	0.1003	0.0748	0.1080	0.1324	0.0901	0.0561	0.0542	0.0569	0.0661	0.0576
3	0.1024	0.0749	0.1114	0.1363	0.1054	0.0565	0.0542	0.0575	0.0675	0.0748
4	0.1027	0.0750	0.1121	0.1369	0.1231	0.0565	0.0542	0.0576	0.0676	0.0915
5	0.1027	0.0750	0.1122	0.1370	0.1413	0.0565	0.0542	0.0576	0.0676	0.1072

Table 2. Effect of L on expected number of P_2 customers in the system (when $\theta = 0.6$).

L	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
2	0.3280	0.2275	0.3445	0.4024	3.5013	0.4020	0.3107	0.4340	0.4848	3.5894
3	0.3328	0.2776	0.3522	0.4014	3.4494	0.4035	0.3107	0.4364	0.4841	3.5394
4	0.3335	0.2776	0.3537	0.4011	3.4134	0.4036	0.3107	0.4366	0.4840	3.5098
5	0.3337	0.2776	0.3540	0.4011	3.3864	0.4036	0.3107	0.4366	0.4840	3.4860

Table 3. Effect of θ on expected number of P_1 customers in the system (when $L = 3$).

θ	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
0	0.0806	0.0630	0.0863	0.1211	0.0983	0.0565	0.0542	0.0575	0.0675	0.0748
0.2	0.0879	0.0669	0.0948	0.1261	0.1007	0.0565	0.0542	0.0575	0.0675	0.0748
0.4	0.0951	0.0709	0.1032	0.1311	0.1031	0.0565	0.0542	0.0575	0.0675	0.0748
0.6	0.1024	0.0749	0.1114	0.1363	0.1054	0.0565	0.0542	0.0575	0.0675	0.0748
0.8	0.1096	0.0792	0.1195	0.1415	0.1078	0.0565	0.0542	0.0575	0.0675	0.0748
1	0.1169	0.0836	0.1273	0.1469	0.1103	0.0565	0.0542	0.0575	0.0675	0.0748

Table 4. Effect of θ on expected number of P_2 customers in the system (when $L = 3$).

θ	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
0	0.1581	0.1425	0.1615	0.1831	1.6508	0.1728	0.1488	0.1789	0.2039	1.5060
0.2	0.2115	0.1867	0.2182	0.2465	2.1553	0.2400	0.1994	0.2519	0.2843	2.0596
0.4	0.2693	0.2317	0.2812	0.3188	2.7507	0.3163	0.2532	0.3370	0.3771	2.7312
0.6	0.3328	0.2776	0.3522	0.4014	3.4494	0.4035	0.3107	0.4364	0.4841	3.5391
0.8	0.4034	0.3247	0.4336	0.4959	4.2660	0.5037	0.3724	0.5532	0.6075	4.5053
1	0.4832	0.3734	0.5284	0.6047	5.2175	0.6196	0.4392	0.6912	0.7504	5.6559

From Table 1, we can see that the expected number of P_1 customers increases as L increases for all arrival processes in both the models as expected. Additionally, the expected number of P_1 customers in the case of preemptive service discipline is much less than that in the non-preemptive case. In both models, this rate of increase decreases, as L increases due to the diminished effect of L .

From Table 2, we can see that the expected number of P_2 customers increases as L increases in the case of EXP, HEXP, ERA (the rate of increase decreases as L increases), decreases in the case of MNA and MPA for both the models. This is due to the feedback effect and correlation effect in the case of MNA and MPA (also the arrival rate for all processes is fixed to a small value (0.1)). Furthermore, the expected number of P_2 customers in the case of preemptive service discipline is much more than that in the non-pre-emptive case (see Figure 1; the blue curve is invisible in the graph as there is not much difference in the expected number of P_1 customers in case of MPA and MNA). In the figures, E_n denotes the expected number of customers in the system and E_{wt} denote the expected waiting time of customers in the system.

From Table 3, we can see that as θ increases, the expected number of P_1 customers increases for all arrival processes in the case of model I and remains constant for different arrival processes in the case of model II. This is due to the feedback effect in the case of model I, but feedback does not affect P_1 customers in the case of model II.

From Table 4, we can see that as θ increases, the expected number of P_2 customers increases for all arrival processes in the case of model I and model II. This is due to the feedback effect (see Figure 2. The blue curve is invisible in the graph as there is not much difference in the expected number of P_1 customers in the case of MPA and MNA).

From Tables 2 and 4, we can see that the expected number of P_2 customers is much bigger for MPA. This is due to the correlation effect. From Tables 1 and 3, we can see that the expected number of P_1 customers is lesser for model II compared to model I. Also, from Tables 2 and 4, we can see that the expected number of P_2 customers is much bigger for model II compared to model I (in the case of MPA, the expected number becomes bigger when the feedback rate is high due to correlation effect).

10.2. Expected Waiting Time

Tables 5 and 6 show the effect of L on the expected waiting time of P_1 and P_2 customers in the system for model I(non-preemptive case) and model II (preemptive case) respectively. Tables 7 and 8 show the effect of θ on the expected waiting time of P_1 and P_2 customers in the system respectively in both models.

Table 5. Effect of L on expected waiting time of P_1 customers in the system (when $\theta = 0.6$).

L	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
2	1.0712	1.0414	1.0493	1.0796	1.0907	0.9018	0.8989	0.9028	0.9114	0.9121
3	1.0766	1.0419	1.0543	1.0852	1.1001	0.9021	0.8989	0.9033	0.9124	0.9274
4	1.0775	1.0419	1.0553	1.0861	1.1150	0.9021	0.8989	0.9033	0.9125	0.9428
5	1.0776	1.0419	1.0555	1.0863	1.1317	0.9021	0.8989	0.9033	0.9125	0.9573

Table 6. Effect of L on expected waiting time of P_2 customers in the system (when $\theta = 0.6$).

L	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
2	3.6377	3.5569	3.6488	3.6766	9.2171	4.0102	3.7665	4.1070	4.7945	9.4702
3	3.6422	3.5567	3.6550	3.6740	8.7061	4.0118	3.7665	4.1097	4.7937	9.3680
4	3.6429	3.5567	3.6563	3.6733	8.4624	4.0119	3.7665	4.1099	4.7936	9.2446
5	3.6430	3.5567	3.6565	3.6732	8.3021	4.0119	3.7665	4.1099	4.7936	9.1352

Table 7. Effect of θ on expected waiting time of P_1 customers in the system (when $L = 3$).

θ	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
0	0.9828	0.9579	0.9815	1.0069	1.0300	0.9021	0.8997	0.9031	0.9117	0.9252
0.2	1.0114	0.9842	1.0059	1.0304	1.0519	0.9021	0.8994	0.9031	0.9119	0.9259
0.4	1.0426	1.0121	1.0303	1.0564	1.0752	0.9021	0.8992	0.9032	0.9121	0.9266
0.6	1.0766	1.0419	1.0543	1.0852	1.1001	0.9021	0.8989	0.9033	0.9124	0.9274
0.8	1.1140	1.0738	1.0779	1.1172	1.1268	0.9021	0.8986	0.9034	0.9127	0.9283
1	1.1551	1.1082	1.1009	1.1529	1.1556	0.9021	0.8983	0.9035	0.9131	0.9293

Table 8. Effect of θ on expected waiting time of P_2 customers in the system (when $L = 3$).

θ	Model I					Model II				
	EXP	ERA	HEXP	MNA	MPA	EXP	ERA	HEXP	MNA	MPA
0	3.5018	3.4623	3.5047	3.5223	6.3444	3.8240	3.9046	3.6427	4.6121	6.7614
0.2	3.5413	3.4912	3.5461	3.5626	7.0025	3.8738	3.9579	3.6784	4.6594	7.4534
0.4	3.5874	3.5225	3.5954	3.6124	7.7768	3.9354	4.0250	3.7193	4.7190	8.3122
0.6	3.6422	3.5567	3.6550	3.6740	8.7061	4.0118	4.1097	3.7665	4.7937	9.3680
0.8	3.7081	3.5946	3.7283	3.7505	9.8063	4.1073	4.2172	3.8212	4.8878	10.6518
1	3.7885	3.6368	3.8196	3.8458	11.1251	4.2273	4.3543	3.8850	5.0066	12.2415

From Table 5, we can see that the expected waiting time increases as L increases for all arrival processes in both models. This happens since when L increases, the expected number of P_1 customers increases, and hence the expected number of feedback customers also increases. As expected, the expected waiting time of P_1 customers in the case of pre-emptive service discipline is less than that in the non-pre-emptive case.

From Table 6, we can see that the expected waiting time decreases as L increases for all arrival processes except in the cases of EXP and HEXP in model I. This is because when L increases, the expected number of P_1 customers increases in the cases of EXP, ERA, and HEXP and decreases in the case of MNA and MPA due to the correlation effect. However, the rate of increase in the case of ERA is very small. This all happens as we fixed our arrival rate to a small quantity (0.1). However, both, the rate of increase and the rate of decrease, decrease as L increases. This happens as when L increases, the expected number of P_1 customers increases, and when it hits L , the effect of L decreases. As a result, the expected number of feedback customers increases in the case of EXP and HEXP and decreases in all other cases. Hence, the expected waiting time decreases for all arrival processes except in the cases of EXP and HEXP as L increases.

Again, from Table 6, in the case of model II, the expected waiting time of P_2 customers is more than that in model I, as we expected. In this case, the expected waiting time increases in the cases of EXP and HEXP, decreases and then stays constant in the case of ERA and decreases in the cases of MNA and MPA as L increases. This is because when L increases, the expected number of P_1 customers increases in the cases of EXP and HEXP, stays constant in the case of ERA and decreases in the case of MNA and MPA (due to the correlation effect). This all happens as we fixed our arrival rate to a small quantity (0.1). As expected, the expected waiting time of P_2 customers in the case of preemptive service discipline is more than that in the non-preemptive case. (see Figure 3. The blue curve is invisible in the graph as there is not much difference in the expected waiting time of P_1 customers in the case of MPA and MNA).

Next, we fixed $L = 3$. From Table 7, we can see that the expected waiting time of P_1 customers increases as θ increases for all arrival processes in model I. This happens since when θ increases, the expected number of feedback customers increases, and the expected number of customers in the P_2 queue increase. As the service discipline is non-

pre-emptive, this will also affect P_1 customers. Hence, the expected waiting time of P_1 customers increases as θ increases for all arrival processes.

Again, from Table 7, we can see that the expected waiting time decreases as θ increases for all arrival processes in model II. This happens since when θ increases, the expected number of feedback customers increases, and the expected number of customers in the P_2 queue increases. However, as the service discipline is preemptive, this will not have much effect on P_1 customers.

From Table 8, we can see that the expected waiting time of P_2 customers increases as θ increases for all arrival processes in both model I and model II. This happens since when θ increases, the expected number of feedback customers increases, and the expected number of customers in the P_2 queue increases. (see Figure 4. The blue curve is invisible in the graph as there is not much difference in the expected waiting time of P_1 customers in the case of MPA and MNA).

We can see that, in both the models, among all arrival processes, the expected waiting time is longer in the case of MPA. This is due to the correlation effect.

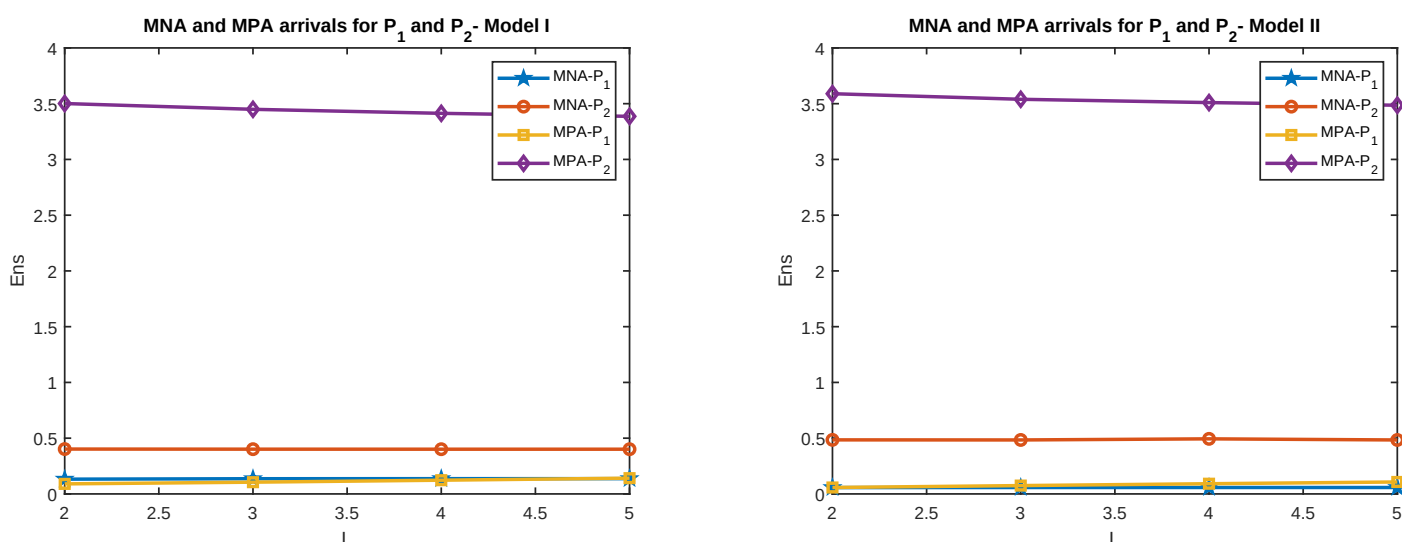


Figure 1. Effect of L on expected number of P_1 and P_2 customers in the system for MNA and MPA arrival processes with correlation of -0.4211 and 0.4211 , respectively (when $\theta = 0.6$).

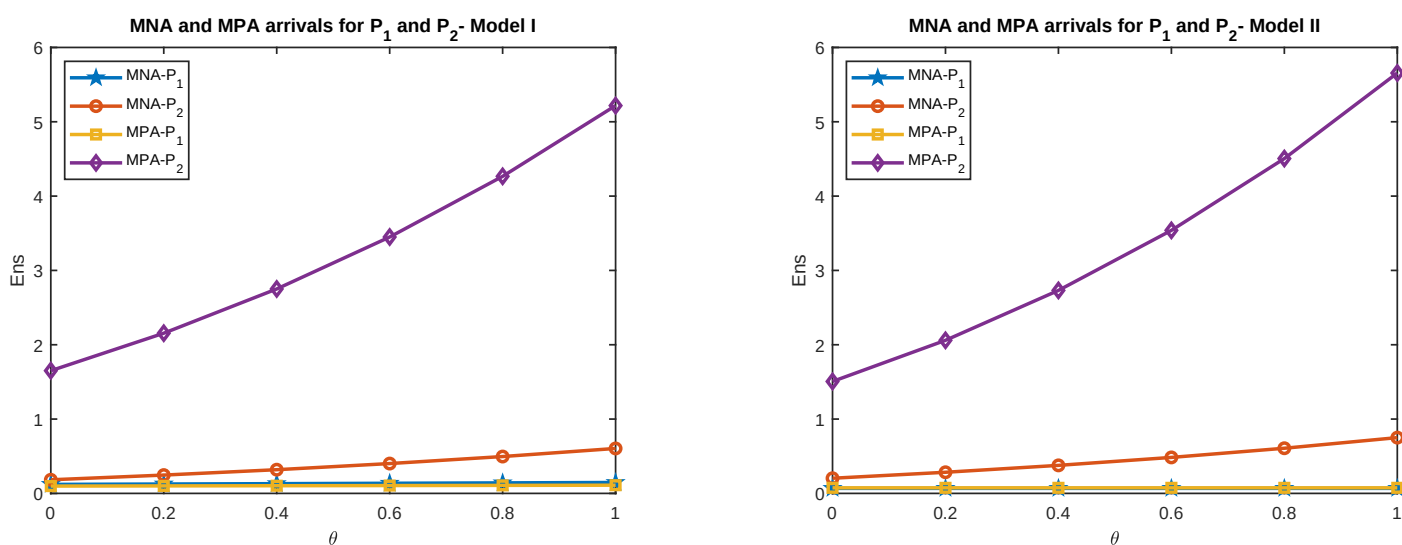


Figure 2. Effect of θ on expected number of P_1 and P_2 customers in the system for MNA and MPA arrival processes with correlation of -0.4211 and 0.4211 , respectively (when $L = 3$).

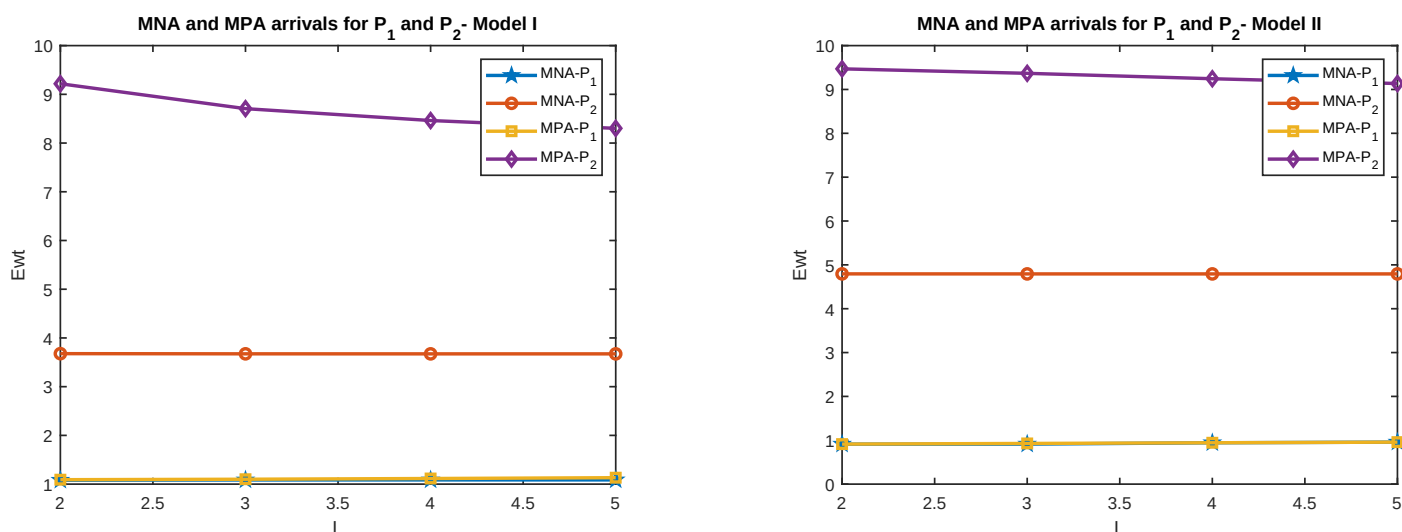


Figure 3. Effect of L on expected waiting time of P_1 and P_2 customers in the system for MNA and MPA arrival processes with correlation of -0.4211 and 0.4211 , respectively (when $\theta = 0.6$).

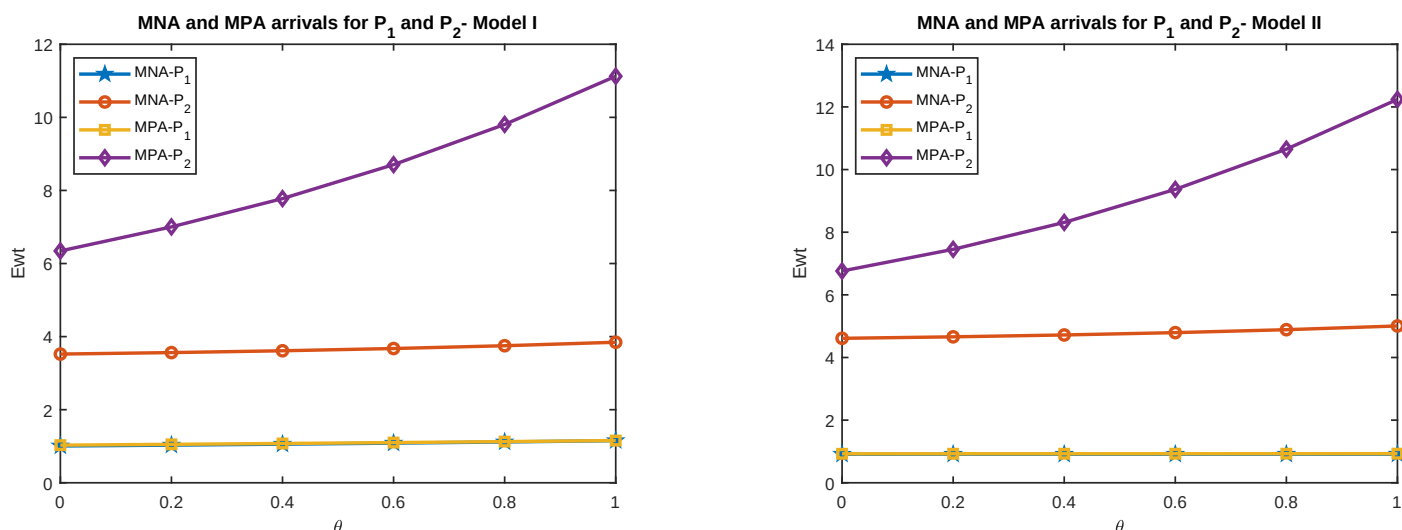


Figure 4. Effect of θ on expected waiting time of P_1 and P_2 customers in the system for MNA and MPA arrival processes with correlation of -0.4211 and 0.4211 , respectively (when $L = 3$).

11. Conclusions

In this paper, we analyzed two single server queueing models with priority customers. High priority customers were allowed exactly one feedback if they wished so. However, feedback results in the loss of their priority status. The inflow of customers forms an MMAP and service of the distinct class of customers are according to two independent, non-identical phase-type distributions. The stationary distributions of both systems are derived and expressions for several performance characteristics are obtained. The numerical illustrations provided throw light on the relative performance of the two models.

One possible extension of this work is to examine the case of infinite capacity waiting stations for all classes of priority. This can be approached through the method of roots as discussed in papers by Chaudhry et al. [42]. It is also interesting to compare two different situations: in one, the external customers join only in the highest priority line, and in the second, low priority customers join in the respective queues.

Author Contributions: Conceptualization, A.K.; Data curation, D.V.N.; Formal analysis, D.V.N.; Funding acquisition, S.A.; Investigation, A.K. and A.M.; Methodology, A.K.; Supervision, A.K.; Validation, D.V.N.; Visualization, D.V.N.; Writing—original draft, A.K.; Writing—review and editing, A.K., D.V.N., A.M. and S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors are grateful to the Referees for the valuable suggestions and constructive criticisms which lead to considerable improvement in the presentation of this paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Secondary Metabolites and their Antioxidant Activities: An Overview

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Received: July 14, 2020; **Published:** September 19, 2020

Abstract

Free radicals/reactive oxygen species (ROSs)/reactive nitrogen species (RNSs) are produced by cells through endogenous reactions, various physiochemical events or pathological issues. Antioxidants (AOX) are compounds scavenge or regulate these radicals. The balance between these molecules and AOXs is a pre-requisite for healthy physiological functions of the body. If free radicals/ROS/RNS over expresses in the system results into oxidative stress and other life style disorders i.e., oxidize lipids, proteins, nucleic acids and trigger a number of human diseases. Therefore, inclusion of antioxidants in food can assist in balancing this sort of oxidative bursts. Chemical antioxidants like butylated hydroxy anisole, butylated hydroxy toluene, propyl gallate and tert-butyl hydroquinone were employed in food resulted in to dangerous ill effects. Thus, the search for plant based biomolecules with antioxidative activity has been explored in recent times. The present overview provides an account of plants employed by the local people as antioxidants or functional foods or stress reliever in alleviating human diseases. The secondary metabolites analyzed in the present study as antioxidants include polyphenolics, carotenoids, alkaloids, terpenoids and flavonoids. These molecules function either as free radical scavengers or function by retarding chain initiation or involved in repair of damaged biomolecules. The output of the study will assist in providing knowledge regarding appropriate applications of the natural bioactive components in the regular diet as nutraceuticals.

Keywords: Antioxidant; Free Radicals; Oxidative Stress; Secondary Metabolites; Ethnic People

Introduction

Reactive radicals such as reactive oxygen species (ROSs) and reactive nitrogen species (RNS), are produced inside the living systems through multiple metabolic pathways. The cell system is in built with antioxidants that safe guard the cells from oxidative burst caused by these free radicals. Antioxidants (AOX) like glutathione, thioredoxin and AOX enzymes (superoxide dismutase, glutathione peroxidase, catalase regulates the oxidative stress and protect lipids, proteins, and nucleic acids. Similarly, tocopherols, ascorbate, carotenoids, flavonoids, amino acids are also natural AOXs present in the vegetables, fruits and leaves. There is a high demand for such foods balanced with AOXs and probiotics that may vitalize the human health. AOXs had a growing demand in pharmaceutical field owing to their protective roles in scavenging ROS/RNS against oxidative stress induced pathological event. Exploration of AOX features of herbals requires proper protocols, which address the mode of AOX activity and its kinetics of the reactions. Many reports were correlated about the AOX potentialities of plant based secondary metabolites using wide protocols with human health. Protocols like inhibited autoxidation connected

with termination-enhancing AOXs, for chain-breaking AOXs, and preventive AOXs are unique. The easy protocol is *in vitro* determination of AOX power of food items. Some of the methods are 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate), 2,2-diphenyl-1-picrylhydrazyl radical scavenging, Fe^{3+} - Fe^{2+} transformation method, ferric reducing antioxidant power protocol, cupric ions reducing power, Folin-Ciocalteu reducing power, peroxy radical ($\text{ROO}\cdot$), superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen ($^1\text{O}_2$), nitric oxide radical ($\text{NO}\cdot$) scavenging and chemiluminescence assay. Many phytochemicals from indigenous plants have been found to possess antioxidant activity under *in vivo* and *in vitro* assays. In fact, only a few species have been validated therapeutically useful under *in vivo* conditions due to their interference with physiological processes like absorption, distribution, metabolism, storage and excretion. However, the phytochemicals are being screened only in terms of *in vitro* antioxidant activity and the data are then directly employed to their therapeutic usefulness. In this juncture, the overview of the present study refers selected ethnic plants used by the native people and their applications.

Materials and Methods

The plants were selected based on the traditional usage by the native people or tribes of the locality. The algal species includes *Gracilaria dura*, *Kappaphycus alvarezii* and *Hypnea musciformis*; bryophytes: *Plagiochila beddomei*, *Leucobryum bowringii*, *Octoblepharum albidum*, *Marchantia polymorpha*, *Marchantia linearis*, *Pallavicinia lyellii*, *Thuidium tamariscellum*; angiosperms: *Solanum aculeatissimum*, *Solanum mauritianum*, *Artemisia japonica*, *A. nilagirica*, *Premna serratifolia*, *Orthosiphon aristatus*, *Pouteria campechiana*, *Pogostemon benghalensis*, *P. cablin*, *Thottea siliquosa*, *Tylophora Subramanii*, *Begonia cultivars*, *Osbeckia* species *Clerodendron infortunatum*, *Tectona grandis* and Wild Balsam Species. Protocols used were 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate), 2,2-diphenyl-1-picrylhydrazyl radical scavenging, Fe^{3+} - Fe^{2+} transformation method, ferric reducing antioxidant power protocol, cupric ions reducing power, Folin-Ciocalteu reducing power, peroxy radical ($\text{ROO}\cdot$), superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen ($^1\text{O}_2$) scavenging assay.

Results and Discussion

Sumayya, *et al.* [1] explored the local usage of seaweeds as sources of ailments for curing many human disorders along the coastal belts of Kerala and established that they are proven sources of biologically active metabolites. Many of the active principles produced by marine seaweeds were used in traditional and complementary medicine. Hence, a preliminary phytochemical survey of the seaweeds will be needed to reveal its secondary metabolite constituents and their by channeling towards its therapeutic values. In this study, the methanol extract of *Gracilaria dura* was subjected to phytochemical analysis to know the secondary metabolites present in the extract. The crude extract was purified by column chromatography. The resultant fraction was subject to GC-MS analysis revealed a pool of terpenoids. Four major terpenoids such as dodecanal, hexadecane, tetradecanal, heptadecane and 6, 4, 10-trimethyl-2-pentadecanone (sesquiterpenoid) were noticed. The purified terpenoid extract showed remarkable antioxidant activity in terms of scavenging hydroxyl radicals in a concentration dependent manner. The extract exhibited remarkable profile on the FRAP assay and strong DPPH scavenging potential. The IC_{50} values were significant and comparable with the synthetic antioxidants like ascorbate and quercetin. The obtained results suggest the possible use of red algae, *G. dura* as a good candidate in terms of functional food supplement and also in combating carcinogenesis and inflammatory disorders. Thus, *G. dura* may be considered for future application in medicine, food and cosmetic industries [2].

Similarly, the edible marine algae *Kappaphycus alvarezii* and *Hypnea musciformis*, were evaluated for their phytochemicals followed by its quantification, purification of terpenoids and evaluation of their *in vitro* antioxidant activity in terms of ABTS, FRAP reducing power, superoxide, hydroxyl radical scavenging and hydrogen peroxide radical assay, the metal chelating activities. Qualitative analysis revealed the phytochemicals such as carbohydrates, proteins, alkaloids, glycosides, flavonoids, steroids, saponins, phlobatannins and polyphenolics. The quantified data of *K. alvarezii* were carbohydrate - 38.28 mg/g, protein - 1.06 mg/g and fat - 1.02 mg /g of dry wt., whereas *H. musciformis* revealed 18.02, 1.2 and 0.934 mg/g of dry wt. respectively. Polyphenol forms the major secondary metabolites especially

the terpenoids. The column chromatography purified terpenoids composition from *H. musciformis* and *K. alvarezii* were fractionated by GC-MS analysis. They showed remarkable antioxidant activities in terms of scavenging hydroxyl radicals in a concentration dependent manner. Further, the extract exhibited strong inhibition on the H_2O_2 and DPPH scavenging assay. The IC_{50} values were significant and comparable with ascorbate and quercetin. Thus, the selected seaweeds may be included in daily dietaries to combat energy malnutrition and micronutrient deficiencies [3].

Remesh and Manju [4] documented the ethnobryological usage of bryophytes by the ethnic tribes from Western Ghats, India. In this scenario following bryophytes were evaluated scientifically for their importance.

Methanol extract of *Plagiochila beddomei* the liverwort was evaluated in terms of antioxidant and antimicrobial activities. Total phenolics was fractionated by high performance liquid chromatography showed the phenolic acids such as coumaric, ferulic, gallic, caffeic, protocatechol, cinnamic, sinapate, chlorogenate and hydroxyl benzoate. Methanolic extract displayed broad spectrum of antimicrobial activity against various bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and fungi like *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Aspergillus niger*, *A. flavus*, *A. terreus* and *Mucor indicus*. Poor survivorship curve of bacteria and inhibition of fungal spore germination substantiated the antimicrobial power of the extract. *P. beddomei* displayed significant antioxidant activity in terms of 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging ability, ferric-reducing antioxidant power, ferric thiocyanate assay and hydroxyl radical-scavenging activities. Phenols and flavonoids were noticed in high levels. Total phenol content was showed the highest correlation with FRAP assay (R^2 0.966). MTT assay also revealed the non-toxic nature of the extract [5].

In addition, Manoj, *et al.* [6] screened *Leucobryum bowringii* and *Octoblepharum albidum*, the bryophytes of Western Ghats in terms of total flavonoids, phenolic content, its fractionation by RP-HPLC and HPLC-PAD. Antioxidant potentialities were examined using DPPH, Ferric Reducing Antioxidant Power, Hydroxyl radical assay, FTC assay and DNA nick assay. Phytochemical study revealed the presence of phenols, saponins, flavonoids, glycosides and tannins. Significant antioxidant potentialities in terms of FRAP, DPPH, hydroxyl radical scavenging and FT assay. Phenols and flavonoids showed many phenolic acids and flavonoids such as quercetin, kaempferol and rutin analogues. Significant linear correlation was observed between total phenolic content and FRAP assay. Reduction in the formation of nicked DNA and simultaneously with increased native form of DNA. Thus, it can be summarized that the selected bryophyte extracts could be an important source of phenolic compounds with high antioxidant capacity comparable with red wine or beverages like tea.

Currently, different therapeutic strategies have been designed for the prevention and treatment of reactive oxygen species mediated diseases, with special emphasis on antioxidant therapy, that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. Among the different phytochemicals, phenolic compounds are established free radical scavengers and antioxidants common among plants; they possess many biological effects, mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals. Present investigation on *Marchantia polymorpha* a bryophyte has been an attempt to screen the phytochemicals and their potential roles in antioxidant potentialities. To complete the objectives, several parameters such as solvent extracts using nonpolar to polar, qualitative analysis of the secondary metabolites, estimation of total phenolic content and its fractionation by RP-HPLC. Antioxidant potentialities were examined with aqueous and ethanolic extracts using DPPH*, FRAP, Hydroxyl radical assay, FTC assay and DNA nick assay. The phytochemical study revealed the presence of phenols, saponins, flavonoids, glycosides and tannins. Significant antioxidant potentialities in terms of FRAP, DPPH, hydroxyl radical scavenging and FT assay. Phenols showed the peaks of many phenolic acids such as gallate, vanilate, chlorogenate, cinnamate, protocatechol, coumarate, ferulate, sinapic, caffeate and hydroxyl benzoate. Significant linear correlation was observed between total phenolic content and FRAP assay. Reduction in the formation of nicked DNA and increased native form of DNA. Aqueous and ethanolic extracts of *Marchantia polymorpha* could be a source of polyphenolic compounds with high antioxidant capacity comparable with standard ascorbate and butylated hydroxytoluene [7].

Remya Krishnan and Murugan [8] screened the phytochemicals and evaluated the antioxidant potentials of ethanolic and water extracts of *Marchantia linearis* (Bryophyta). The goals are achieved in terms of evaluating total flavonoids, phenolic content and its fractionation by RP-HPLC and HPLC-PAD. Antioxidant potentialities was validated by accessing free-radical (DPPH*, ABTS*, FRAP, phosphomolybdenum, H_2O_2 and $\cdot OH$) scavenging, iron chelating activity and scavenging of superoxide radicals were examined. RP-HPLC analysis revealed phenolic acids such as coumarate, sinapate, vanillic and gallic acids. Flavonoids noticed were quercetin, luteolin and apigenin. *M. linearis* extracts presented a remarkable capacity to scavenge all the tested reactive species with IC_{50} values being found at the $\mu g/mL$ level. Ethanolic extract was shown to have the highest phenolics while lowest IC_{50} values for the DPPH*, ABTS* radical scavenging capacities, iron chelating scavenging efficiency, significant activities in scavenging of superoxide radicals, hydrogen peroxide and hydroxyl radicals. The obtained results suggest the potential of *M. linearis* as a potent candidate against free-radical-associated oxidative damage.

Lubaina., *et al.* [9] studied the phytochemical constituents and antioxidant potentialities of petroleum ether, ethyl acetate and water extracts of *Pallavicinia lyellii* a thalloid liverwort. Qualitative aqueous extract analysis of phytochemicals showed the presence of glycosides, tannins, coumarins, alkaloids, saponins, flavonoids, phenols, steroids, reducing sugar and terpenoids. Total flavonoids and phenols were revealed significant levels. Total phenols was fractionated by reverse phase high performance liquid chromatography exhibited phenolic acids such as gallate, chlorogenate, cinnamate, protocatechol, hydroxybenzoate, coumarate, caffeate and ferulate. The *in vitro* antioxidant activity determined through 2,2-diphenyl-1-picrylhydrazyl, ferric reducing antioxidant potentiality, hydroxyl radical scavenging activity, superoxide anion radicals quenching effect and metal chelating assay. Among them aqueous extract showed the remarkable free radical quenching activity in terms of FRAP ($478 \pm 1.76 \mu M/g$), DPPH ($89 \pm 0.23\%$) and hydroxyl radical scavenging activity ($83.1 \pm 0.23\%$) whereas ethyl acetate fraction exhibited the highest superoxide anion scavenging and iron reduction ability with inhibition rate of $90 \pm 0.23\%$ and $25.3 \pm 0.96 \mu g/ml$ respectively at $1000 \mu g/ml$. The significant antioxidant potentialities may be contributed by the phenolic constituents. The results suggest that *P. lyelli* has promising antioxidant activity and could serve as potential source of natural antioxidants.

Greeshma., *et al.* [10] field surveyed the ignored medicinal bryophytes of the biological world at Neyyar wildlife sanctuary, Trivandrum, Kerala and subsequently scientifically validated the AOX potentialities of *Thuidium tamariscellum*. The analysis includes the phytochemicals screening and its antioxidant potentialities in terms of DPPH, ABTS, H_2O_2 , FRAP and metal chelating ability. Total terpenoids level was remarkable. Interestingly, a concentration dependent free radical scavenging potential was noticed and was comparable with the synthetic antioxidant ascorbate. Further, the FTIR analysis of petroleum ether, chloroform, ethyl acetate, and methanol extract confirmed the presence of alcohols/phenols, primary, secondary amines, amides, alkanes, aldehydes, saturated aliphatic alkenes aromatics and aromatic amines, nitro compounds, carboxylic acids, esters, ethers, aliphatic amines, alkyl halides and carbonyls in the moss indicating the presence of medicinally important compounds like flavonoids, terpenoids and alkaloids in the various solvent extracts of the moss. Thus, the overall result of the present study showed that the moss is rich in important pharmaceutical compounds and was reflected as its antioxidant potential. Further studies are warranted to isolate, identify and purify the lead terpenoid present in the moss [11].

Ayyanar and Ignacimuthu [12] documented ethno botanical medicinal plants commonly used by Kani tribals in Tirunelveli hills of Western Ghats. Based on the document, Meenu Krishnan., *et al.* [13] analyzed antihaemolytic, anti-lipid peroxidative potential by purified protease inhibitors from the fruits of *Solanum aculeatissimum*. In human erythrocytes against hydrogen peroxide toxicity. Protease inhibitor was isolated and purified from the fruits of *S. aculeatissimum* (SAPI) via four sequential step procedures i.e., salt precipitation to sepharose affinity chromatography. The purity was confirmed by reverse phase HPLC chromatography. The molecular mass was detected using size elution chromatography (22.2 kDa). It has deep roots in history, being one of the major botanicals used in traditional medicine to treat conditions ranging from diabetes, malaria, to snakebites. Subsequently, antihaemolytic and anti-lipid peroxidative potential was carried. Prior to the addition of H_2O_2 to induce haemolysis, different concentrations (50 - 500 $\mu g/ml$) of SAPI was added to 2 ml of 4% erythrocyte suspension and allowed to incubate for 5 minutes at room temperature. The mixture was centrifuged and the colour density

of the supernatant was measured spectro photometrically. Quercetin was used as internal standard. The % haemolysis and IC_{50} values were calculated. The PI was potent against haemolysis of the erythrocyte in concentration dependent manner with $IC_{50} = 368.54 \mu\text{g/ml}$. IC_{50} value of quercetin was $224 \mu\text{g/ml}$. The lower the IC_{50} the more protection offered against haemolysis by SAPI. These results suggest that the SAPI is ideal anti-haemolytic agent and offered significant biological action compared with standard drug employed.

Murugan, *et al.* [14] investigated the *in vivo* genotoxicity of *Artemisia japonica* and *A. nilagirica* and its potential antigenotoxicity against cyclophosphamide (CP) induced DNA damage. Initially, the essential oil (EO) was analyzed by GC-MS. *A. japonica* and *A. nilagirica* (250 and 500 mg/kg b.w.) was administered to Swiss white mice of both sexes for 15 days, and the animals received an injection of saline or CP (0.9% NaCl) 24 h before they were euthanized. The GC-MS analysis revealed the presence of camphor, 8-cineole, borneol, artemisia ketone and β -thujone the major compounds in *A. nilagirica*. Meanwhile *A. japonica* contains linalool, caryophyllene oxide, *trans*-linalool oxide, p-cymene and 1, 8 -cineole. Significant inhibitions were observed in the evaluated parameters, demonstrating the absence of cytotoxic and genotoxic effects of EOs at all tested doses. In liver, kidney, cardiac and bone marrow cells, EO reduced the DNA damage induced by CP. Results showed a dose–response action of EO. Furthermore, the various tissues showed a difference in the potential antigenotoxic effects. In conclusion, EO of *Artemisia* was not genotoxic and inhibited the genotoxicity induced by CP. Further investigations are needed to purify the potential compound from EO and also to analyze the effects over human health.

Jayakumar, *et al.* [15] evaluated the mitigating effect of purified solasodine from *Solanum mauritianum* against hydrogen peroxide induced oxidative damage in human erythrocytes. *Solanum mauritianum* (Solanaceae) is an exotic species from South America. *Solanum* species are known for alkaloid content which marks them medicinally important. Herbal products have received considerable attention in recent years due to their diverse pharmacological properties, including antioxidant and antitumor potential. Solasodine, the nitrogen analogue of diosgenin, has been reported as potential steroidal precursor for the supplementary source of the commercial synthesis of diverse steroidal drugs. Free radicals are formed during the course of normal metabolic process in the biological system. They are highly reactive molecules due to the presence of unpaired electron. In this juncture, present study was aimed to isolate and purify the alkaloid solasodine from fruits and leaf and to evaluate its antioxidant activity via the assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide, hydrogen peroxide, superoxide anion scavenging activities etc. The alkaloid solasodine was isolated from leaves and fruits of the species using non polar to polar solvents and subsequently purified using column chromatography. Remarkable antioxidant activities are shown by the alkaloid solasodine. IC_{50} values were comparable with synthetic antioxidants such as ascorbate, rutin. Moreover, the solasodine exhibited concentration dependent inhibitory activity against the free radicals. Significant scavenging property was noticed especially with DPPH radicals. Antihaemolytic activity of the solasodine was also confirmed from this study. Thus, it can be suggested that solasodine show more protective effects through antioxidant potential against H_2O_2 induced oxidative damage on erythrocytes. So, solasodine can effectively be used as natural antioxidant for the treatment and prevention of lipid peroxidation related disorders.

Similarly, Jayakumar, *et al.* [16] proved the antioxidant and antihemolytic activities of purified caulophyllumine-A from *Solanum mauritianum*. Ripened fruits of *Solanum mauritianum* are used by the local people as vegetable during famine periods and also the fruits and leaves are used to cure various ailments. In this scenario, the present investigation was undertaken to isolate the lead alkaloid molecule from *S. mauritianum* and to evaluate its antioxidant potentialities. In the first phase, the crude alkaloid was isolated, purified by column chromatography yielded a bluish coloured fraction caulophyllumine- A and was further confirmed by NMR. Further, the antioxidant activity was assayed using the DPPH radical scavenging and other assays. The IC_{50} values ranged from 66.5 to $121 \mu\text{g/ml}$. Protective effects of caulophyllumine-A, against H_2O_2 induced oxidative damage in plasmid pBR322 DNA was remarkable at the tested doses ($\mu\text{g/ml}$). Finally, the antihemolytic potential of caulophyllumine- A was analyzed against human blood erythrocytes, whereby the % lysis of RBCs was found to be in the minimal range of 4.5 to 12.4% comparable with the control ascorbate.

Lubaina and Murugan [17] documented the reactive oxygen species and ascorbate–glutathione interplay in signaling and stress responses in *Sesamum orientale* against *Alternaria sesami*. *Sesamum orientale* the wild and cultivar Thilarani exposed to *Alternaria sesami*

infection triggered the signal cascade H_2O_2 content that was positively correlated with lipid peroxidation. The data were also supported by H_2O_2 localization as observed by scanning electron microscopy. Parallely, infection altered chloroplasts marginally and mitochondria effectively in susceptible cultivar than wild sesame. Deformities in the structure of these organelles were accompanied by changes in antioxidant machinery. H_2O_2 can be effectively detoxified via the ascorbate–glutathione cycle. Increases in ascorbate peroxidase, and glutathione reductase activities concomitant with ascorbate (AsA) and glutathione interplay, as well as AsA regeneration ability, function to keep the balance of cellular H_2O_2 under pathogenicity. Dehydroascorbate reductase and monodehydroascorbate reductase are responsible for AsA regeneration. Oxidative damage in Thilarani cultivar compared to wild sesame is attributed by a lower induction of the ascorbate–glutathione cycle as an antioxidant defense system and was not sufficient to protect mitochondria but prevent ultra-structural damage of chloroplasts. Overall, the availability of antioxidants and the induction of antioxidant enzyme activities for detoxifying reactive oxygen species (ROS) are regulated effectively in wild sesame against *A. sesami* induced oxidative stress. The experiments using ROS scavengers demonstrate that the antioxidant defense system is modulated by O_2^{--} or H_2O_2 signals.

Documenting and validating traditional and indigenous system of medicine in India has been continuously increasing from the last few decades. The current study was made to evaluate the phytochemical constituents and antioxidant potentiality of *Premna serratifolia* an aromatic medicinal plant known as ‘Agnimantha’ in Ayurvedic system of medicine. It has immense ethno pharmacological importance in folk medicine for curing various human ailments. The qualitative phytochemical screening of the aqueous and ethanolic leaf extracts of *P. serratifolia* revealed the presence of glycosides, flavonoids, alkaloids, tannins, terpenoids and steroids. Subsequently, the quantification of major secondary metabolites revealed remarkable level of phenols followed by terpenoids and flavonoids. The *in vitro* antioxidant activity was determined by DPPH, FRAP, hydroxyl radical scavenging activity, hydrogen peroxide scavenging, superoxide anion radicals quenching effect and metal chelating assay. The aqueous extracts showed positive correlation between concentrations of the extracts with DPPH radical scavenging capacity. Similarly, the other antioxidant potentials also displayed remarkable activities when comparable with synthetic antioxidants such as ascorbate and BHA. The results suggest that the aqueous extract of *P. serratifolia* are potential source for natural antioxidants due to the presence of polyphenols and that could have a protective role as well as prevention from life style diseases [18].

Oxidative stress plays significant role in pathophysiologic events of acute and chronic diseases. Intracellular biomolecules such as lipids, proteins and nucleic acids are damaged via oxidation by excessive ROS. Protease inhibitor was isolated and purified from the fruits of *Solanum aculeatissimum* (SAPI) via four sequential step procedure i.e. salt precipitation to Sepharose affinity chromatography. Subsequently, the antioxidant power is analysed using DPPH, H_2O_2 , O_2^{--} , ABTS, $OH\cdot$ radicals scavenging activity, reducing power potential, metal chelating ability and FRAP (Ferric reducing antioxidant power) method. SAPI exhibited significant IC_{50} values for most of the AOX assays. DPPH radical scavenging, reducing power, metal chelating ability, ABTS and $OH\cdot$ radical scavenging activities were comparable with the synthetic antioxidants like ascorbate and BHT. Further studies are warranted to trace the molecular mechanism of AOX activity by SAPI using *in vivo* animal models [19].

The use of traditional medicine is expanding to newer horizons and plants still remain as the novel source of structurally important compounds that lead to the development of innovative drugs. It is anticipated that plants can provide potential bioactive compounds for the development of new ‘leads’ to combat various diseases. The systematically performed *in vitro* assays revealed that the tested plant extract may find in therapy as agent with high pharmaceutical value. In this juncture, the present study was undertaken to analyze the antioxidant, antimicrobial and anticancer potentiality of the medicinal herb *Orthosiphon aristatus* Benth. The results reveal marginal antibacterial, anti-inflammatory and anticancer activity than the respective standards. Considering anticancer activity, the extract showed high IC_{50} values for all the cell lines tested. The data on anti-inflammatory activity indicate an inhibition percentage of 19.38, when treated with 10 μ g/ml of extract while indomethacin produced 54.4% inhibition. For antibacterial activity, inhibitory values at higher concentrations were quite good. In general, more activity was obtained when using more concentrated decoctions of the plant extract studied. The best results (60.5% inhibition) were obtained with antioxidant activity. This study provides evidence that the plant extract have an-

tioxidant properties, as tested through the DPPH method. Therefore, the plant may have great relevance in the prevention and therapies of diseases in which oxidant or free radicals are implicated. The ability of the extracts to scavenge the free radical is an indication of the broad spectrum antioxidant potential of *O. aristatus*, which make the plant a candidate for bio prospecting for antioxidant drugs. In addition, the plant is a good candidate for further phytochemical and chromatographic studies to isolate and fully characterize the compound related to this *in vitro* biological activity [20].

Sunila and Murugan [21] aimed to study the changes in the level of phenols and flavonoids during fruit development of *Pouteria campechiana* and its antioxidant potential. The number of experiments conducted was twenty for each case. Polyphenols at different ontological stages may help producers and food technologists to identify which cultivar and/or maturity stage are most adequate for their need. Egg fruits were harvested and classified into six developmental stages based on week after pollination (WAP): stage I (4WAP); stage II (8 WAP); stage III (12WAP); stage IV (16WAP); stage V (20 WAP) and stage VI (24 WAP). The total phenolics and flavonoids of egg fruits at different developmental stages were investigated. The antioxidant capacities of ethanolic and aqueous extracts were determined by different assays such as FRAP, DPPH, ABTS, superoxide anion, hydroxyl radical and H₂O₂ scavenging assays. The total phenolic contents varied from 30.35 to 2.26 mg chlorogenic acid equivalents/g dry weight (DW), and the total flavonoid contents ranged from 0.683 to 3.37 mg rutin equivalents/g DW. Total phenolics showed an initial increase and subsequently decreased during development. In contrast, flavonoids increased from stage I to VI. Antioxidant assays showed varied patterns of inhibition. Significant correlations were observed between antioxidant capacities and total phenolic and flavonoid contents.

Essential oils (Eos) are complex mixtures of volatile lipophilic components and are obtained from leaf, twigs, fruits, flowers of higher plants. These oils are isolated commonly through hydrodistillation. *Pogostemon* species are well known for their essential oils and therapeutic values. Chemical composition and antioxidant activity of Eos from two species viz. *Pogostemon benghalensis* and *P. cablin* were evaluated. Essential oil from both species was extracted by hydrodistillation using Clevenger type apparatus. The essential oil obtained was subjected to GC-Fid analysis followed by GC-MS. The antioxidant activity was studied using DPPH, ABTS, FRAP, metal chelating, hydrogen peroxide, super oxide radical, reducing power, hydroxyl radical scavenging assays using ascorbic acid and butylated hydroxytoluene as standards. 36 to 41 compounds were identified from the essential oil of *Pogostemon* species. α -Cadinol (35.78%) and patchouli alcohol (34.85%) were the major components in these oils. In addition to these compounds 1,8 cineole, aromadendrene, β -patchoulene, α -caryophyllene, β -caryophyllene, α -patchoulene, germacrene A were identified as the other predominant compounds. The antioxidant activity of Eos was significant when compared with standards. 75.3 to 91.5% inhibition activity was noticed against various free radical scavenging assays. The Eos showed remarkable IC₅₀ value which suggests their potency to use as a natural antioxidant in various industries, foods and therapeutics [22].

Ethnic usage reveals that most of the plants possess a wide array of biological and pharmacological potentialities that may protect tissues from oxidative damages. In the present part, various solvent extracts of root and leaves of *Thottea siliquosa* was screened to evaluate their potent *in vitro* antioxidant activity, total phenol, alkaloid, saponins and flavonoid contents in order to find possible sources for future novel antioxidants for pharmaceutical formulations. Thorough study was performed with the petroleum ether and ethyl acetate extracts of root and leaf of the plant by *in vitro* chemical analysis and antioxidant potentialities. Preliminary qualitative analysis revealed the presence of alkaloids, tannins, glycosides, coumarins, flavonoids and saponins. Phenols (2.5 - 4.8 mg/g), alkaloids (43 - 287 μ g/g) and saponins (66-303 μ g/g) represented the highest level both in the petroleum ether and ethyl acetate extracts of root and leaves. The antioxidant potentialities in terms of DPPH radical, hydroxyl radical, superoxide anion and nitric oxide quenching capacity revealed varying responses. The IC₅₀ values are comparable with the standards such as ascorbate and butylhydroxy toluene. Lipid peroxidation level was comparatively inhibited by the extracts. Results from present study suggest that the phytochemicals in the extracts of *T. siliquosa* act as antioxidant agents as it was substantiated by its free radical scavenging activities [23].

Artemisia species are used in traditional medicine for the treatment of various ailments such as indigestion, infection, irregular menstruation, cramp, cold, epilepsy, typhoid, tuberculosis, urinary calculi, colic, fever, asthma, bronchitis, sciatica, flatulence, anaemia, insom-

nia, gout, depression, nervousness, hysteria, measles, bruise, chilblain, leprosy, malaria and cancer. Similarly, most species has been used as antiseptic, antioxidant, anthelmintic, expectorant, diuretic and insect repellent. The biological activity shown by the plants was due to the essential oil present in them. The present study describes the fractionation of essential oil and its antioxidant potentialities in two allied species - *Artemisia japonica* Thunb. and *A. nilagirica* (Clarke) Pamp. The essential oil from *Artemisia japonica* and *A. nilagirica* was isolated by hydrodistillation and analyzed by GC-MS. 30 and 19 compounds were noticed in the oil respectively. The predominant phytochemicals in the essential oil of *A. nilagirica* were camphor (41%), 8-Cineole (17.0%), Borneol (8.6%) Artemisia ketone (6.4%) and β -Thujone (6.0%). Meanwhile *A. japonica* contain Linalool (70.4%), Caryophyllene oxide (6.7%), *trans*-Linalool oxide (4.5%), p-cymene (3.4%) and 1, 8-Cineole (2.3%). Significant antioxidant potentialities was displayed in terms of DPPH scavenging, beta carotene bleaching, total antioxidant activity, soybean oil rancidity and sun flower oil free fatty acids and iodine values. Interestingly, *A. nilagirica* showed remarkable antioxidant potentialities and was comparable with synthetic antioxidants such as ascorbate, butylated hydroxytoluene and BHA. The present study suggests that the tested *Artemisia* species revealed a pool of potential biomolecules in the essential oil and also exerted remarkable antioxidant values. Further studies are warranted to isolate and purify the principle component from the essential oil [24].

Tylophora subramanii, a climbing endemic species of Southern Western Ghats belongs to Asclepiadaceae. The local people use this plant for curing many rheumatic ailments. The objective of this study is to explore the phytochemistry and the antioxidant potential of ethanolic leaf extract of *T. subramanii* which is considered traditionally as an important medicinal plant. The methods adapted includes (a) phytochemical analysis to find out the presence of various bioactive compounds (b) *in vitro* antioxidant analysis of ethanolic leaf extract by 2,2-diphenyl-1-picrylhydrazyl assay, nitric oxide scavenging assay, superoxide scavenging potential, *ex vivo* superoxide dismutase assay, catalase assay. The major outcomes are the following: ethanolic leaf extract showed remarkable levels of the phytochemicals such as flavonoids, tannins, terpenoids, saponins, alkaloids, proteins and carbohydrates. Similarly, it also showed potential antioxidant activities. High catalase and superoxide dismutase activity with low lipid peroxide level, which were comparable with the synthetic antioxidant L-ascorbic acid. Thus, the work concludes that *T. subramanii* display a wide range of pharmacologically useful phytochemicals which exhibited significant antioxidant potentials. Future works are planned to elucidate the lead molecule and its biological potentialities [25].

Aswathy and Murugan [26] documented the ethnobotanical knowledge about herbals used by tribes of Kerala and Tamil Nadu with special reference to *Begonia malabarica*. Anthocyanins from vegetables and flowers have fascinated pharmaceutical industries are proven nutraceutical health. *Begonia* species forms diverse hyper group and are distinguished on the basis of morphological parameters. *Begonia rex-cultorum* (Baby rainbow) and *Begonia malabarica* exhibited the highest antioxidant activities. The anthocyanin concentration positively correlates with the antioxidant potentialities among the cultivars. The IC_{50} value related with DPPH and metal chelating activities of the *B. rex-cultorum* (Baby rainbow) extract are 32.3 μ g/mL and 18.7 μ g/mL respectively. Remarkable scavenging potentialities are displayed against metal chelating, carotene bleaching, ABTS radical, FRAP assays and the results are comparable with synthetic antioxidant like BHT. The diversity in radical scavenging in these assays may be due to factors like stereo selectivity of the radicals or due to the differential solubility of anthocyanin molecules in the crude extract. Further studies are warranted to isolate and fractionate the major anthocyanins in the cultivars [27].

Bosco Lawrence and Murugan [28] reported the folklore use of *Osbeckia* species from Munnar hills, Kerala and subsequently validated the information. Health-benefit properties of natural pigments have been intensely studied, especially the anthocyanins. In the last few decades, research on anthocyanins has attracted biologists by the increasing evidence of their health beneficial effects. *Osbeckia*, belongs to Melastomataceae and is well-known for colouring pigments and other bioactive compounds. In the present study, total anthocyanin and antioxidant capacity indicators were evaluated from 8 *Osbeckia* spp. and anthocyanin was extracted from *in vitro* cultures of *O. aspera* and *O. reticulata*. The antioxidant effect was studied using ABTS (2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay, the FRAP, the scavenging ability of hydroxyl radicals and the superoxide anion scavenging activity. Anthocyanins extracted from *in vitro* cultures were purified and fractionated using column chromatography and LC-MS MS analysis. *In vitro* cultures

of *O. aspera* was obtained in MS medium fortified with various combinations of Benzyl Adenine (BA), Naphthalene acetic acid (NAA) and 2, 4-D. The chromatograms of *O. aspera* revealed the presence of malvidin-3 -diglucoside, peonidin, delphinidin and cyanindin whereas *O. reticulata* cultures accumulated large amounts of malvidin, cyanindin and cyanidin aglycone. Thus, the results tempt to suggest that the *Osbeckia* species are rich in anthocyanin and therefore displayed potential AOX power. *O. aspera* and *O. reticulata* callus was induced for the *in vitro* production of anthocyanins. The pool of anthocyanins was purified and fractionated by LC-MS/MS and AOX assays were performed with the purified anthocyanin which showed higher level activities [29].

Greeshma Murukan and Murugan [30] reported the natural dye yielding plants used by the tribes of Wayanad, Kerala, India. They validated the hepatoprotective and antioxidant activity of purified anthocyanin extracted from the cell suspension culture of *Clerodendron infortunatum*. A protocol has been developed for the induction of callus proliferation from leaf and nodal explants of *C. infortunatum*. The explants were inoculated on murashige and skoog (MS) medium supplemented with diverse combinations of 2, 4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) for triggering callus formation. Subsequently, the green compact callus has been sub-cultured in the medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin for anthocyanin synthesis. Cell suspension culture was also established and the elicitor, salicylic acid was used for triggering anthocyanin synthesis. Three different chromatographic columns (solid phase extraction by Sepharose C18 column, Oasis-MCX and Amberlite XAD 7+Sephadex LH 120 sorbents) were employed to purify the *in vitro* synthesized anthocyanin from cell suspension cultures. For purity evaluation, high-performance liquid chromatography (HPLC) and molar absorptivity assay was used. Further, hepatoprotective and antioxidant activity was evaluated comparing with silymarine, as standard in rats. *In vitro* antioxidant scavenging activity was analysed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay. After 1 month, the leaf explants yielded remarkable green compact callus on murashige and skoog (MS) medium containing 2.0 mg/l benzylaminopurine (BAP) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Salicylic acid enhanced anthocyanin synthesis. The mean purity values obtained by high-performance liquid chromatography (HPLC) were $90.9\% \pm 1.9$ and $80.60\% \pm 2.3$ for Oasis MCX, Amberlite XAD-7+Sephadex LH-20 column respectively. However, the purity calculated by molar absorptivity was found to be less. The highest purity achieved using molar absorptivity analysis was with MCX cartridges i.e., $85.9 \pm 3.8\%$. High-performance liquid chromatography (HPLC) yielded 12 anthocyanin fractions. Remarkable antioxidant scavenging activity was noticed as revealed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay. The hepatoprotective activity (25, 50, 100 mg/100g b. w) was compared with silymarine (25 mg/kg b. w) against carbon tetrachloride (CCl_4) induced toxicity. Anthocyanin extract improved the aspartate aminotransferase (AST), alanine aminotransferase (ALT) and recovered the activity of kidney function by decreasing the urea and creatinine content. In addition, the administration of anthocyanin significantly inhibited the oxidative stress via its scavenging of the reactive oxygen species formed by carbon tetrachloride (CCl_4) stress. Further, a decrease in the malondialdehyde (MDA), hydrogen peroxide (H_2O_2), nitric oxide (NO) accumulation and an increase of glutathione (GSH) content were noticed. Similarly, improved lipid profiles, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were also observed suggesting that anthocyanin significantly suppress the toxicity via its activation of antioxidant enzymes [glutathione transferase (GST), catalase (CAT) and superoxide dismutase (SOD)]. The overall results showed that the purified anthocyanin of *C. infortunatum* functions as an antioxidant and thereby hepatoprotective protection against carbon tetrachloride (CCl_4) induced toxicity in animal models [31].

Greeshma Murukan and Murugan [32] purified and characterized anthocyanin from *in vitro* culture of teak and its antioxidant potential. Anthocyanin was extracted from *in vitro* culture, purified by using amberlite lite XAD column and fractionated by Liquid chromatography mass spectrometry (LC-MS/MS). Various antioxidant assays were carried such as 2,2-diphenyl 1-picryl-hydrazyl-hydrate (DPPH), 2,2'-azino-bis-3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), Oxygen radical absorbance capacity (ORAC), Nitric oxide (NO) and Hydrogen peroxide (H_2O_2). Liquid chromatography mass spectrometry (LC-MS/MS) revealed the major fraction as cyanidin 3-(2-xylosyl-rutinoside) with unknown peaks. The amount of anthocyanin was 15.23 mg/g monomeric anthocyanin. Further, the potential antioxidant capacity of the teak anthocyanin was comparable to common vegetables and fruits. Similarly, high correlations of anthocyanin with

antioxidant activity, such as oxygen radical absorbance capacity (ORAC), 2,2'-azino-bis-3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) ($r = 0.95, 0.93$, and 0.80) were found. The high anthocyanins content and potential antioxidant activity suggests that teak anthocyanin may be applied in the food industry as a good source of natural pigments.

Arathy, *et al.* [33] evaluated the antioxidant activities of purified anthocyanin from wild balsam species. Initially, anthocyanin was extracted from floral leaves of wild balsam species and purified by chromatographic techniques. The major fractions identified were hesperidin, dimethoxy antirrhinin and trimethoxy antirrhinin. Further, the anthocyanin extracts were subjected to *in vitro* protocols like 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, DPPH scavenging assay, ferric reducing antioxidant power (FRAP), beta carotene bleaching assay, metal chelating and H_2O_2 scavenging power. Interestingly, ABTS, FRAP analyzes yielded significant results as compared to others. The data were comparable with that of synthetic antioxidants like ascorbate and catechin. Meanwhile, beta carotene and H_2O_2 scavenging assay showed moderate results. DPPH and metal chelating protocols displayed the values 71% and 64% respectively at 25 $\mu\text{g/ml}$ concentration. The antioxidant results were supported by NMR and LC MS analysis. This study provides model systems for the evaluation of natural antioxidants like anthocyanin. Future *in vivo* clinical studies are warranted to confirm the obtained data.

Conclusion

The overview of the present report infers that these plant extracts possess optimal antioxidant properties. Thus, the extracts can be beneficial in treating stress caused due to multiple of factors. Over the past few years, significant scientific knowledge has been documented regarding plant redox characters and its AOX defense. Plants synthesize and accumulate many non-enzymatic antioxidants like ascorbate, glutathione and polyphenolics. Some of these AOXs occur constitutively, while others are synthesized in response to abiotic and biotic stress events. Almost all plant based phytochemicals exhibit certain level of antioxidant activity under *in vitro* assays. However, for *in vivo* studies, plant antioxidants have to channel through multiple process. However, many pharmaceutical industries tapping such native knowledge of people in to economically important nutraceutical products. In this juncture, the above said species could be further screened for isolating unique antioxidants that can be supplemented to human diet.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgements

The authors thank the study group for their collaboration.

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Volume 8 Issue 10 October 2020

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Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Evaluation of *in vitro* and *in vivo* potential of *Bacillus subtilis* MBTDCMFRI Ba37 as a candidate probiont in fish health management

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ARTICLE INFO

Keywords:

Bacillus subtilis Ba37
Antibacterial activity
Growth performance
Immune response
V. anguillarum
E. suratensis

ABSTRACT

Bacillus subtilis MBTDCMFRI Ba37 (*B. subtilis* Ba37), an antibacterial strain isolated from the tropical estuarine habitats of Cochin, was evaluated for *in vitro* and *in vivo* potential, and its application as a candidate probiont in fish health management. *B. subtilis* Ba37 was characterized using their morphological and biochemical properties. It exhibited exoenzymatic activities, tolerance to various physiological conditions and a wide spectrum of antibacterial activity against aquaculture pathogens such as *Vibrio* and *Aeromonas*. In co-culture assay, *B. subtilis* Ba37 inhibited *Vibrio anguillarum* O1 (*V. anguillarum* O1) even with the initial cell count of 10^4 CFU mL⁻¹. Cytotoxicity assay performed using the cell free supernatant (CFS) of *B. subtilis* Ba37 revealed its non toxic nature. A twenty one days of feeding trial was conducted in juveniles of *Etroplus suratensis* (*E. suratensis*) by administering *B. subtilis* Ba37 to evaluate its efficacy on growth, immune parameters and antioxidant enzyme activities. Overall the supplementation of *B. subtilis* Ba37 enhanced significantly ($P < 0.05$) the survival rate, weight gain, specific growth (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and feed efficiency (FE) of the fed animals as compared with the control. The immune parameters and antioxidant activities such as total protein, alkaline phosphatase (ALP), superoxide dismutase (SOD) and catalase were also improved significantly ($P < 0.05$) while serum alanine aminotransferase (SGOT) and serum aspartate aminotransferase (SGPT) activities were decreased slightly than the control. After fifteen days of challenge test, the fish fed with *B. subtilis* Ba37 showed higher relative percentage survival (RPS) than the control. Thus the study indicated the advantages of *B. subtilis* Ba37 to be used as a candidate probiont, which could be effectively utilized in managing diseases in aquaculture systems and to improve the health of the host.

1. Introduction

Aquaculture is considered as a rapidly expanding food production sector in the world due to the increasing demand for high quality diets and safer animal proteins with rich nutritional value. The industry offers higher income and support the socio-economic development of the nation and improves employment opportunity for both skilled and unskilled individuals [1,2]. In recent years, an intensification in the aquaculture practices is necessary owing to the increased fish consumption due to the overexploitation of the natural fish stocks. Since the expansion of the industry may probably invite increased disease outbreaks to the associated animals, as it offers only a confined and poor

environment with high stocking density [3,4]. *Etroplus suratensis* (pearl spot), the State fish of Kerala popularly considered as a food source with high demand. The drastic reduction happened in the total fish landings of *E. suratensis* is considered mainly due to the overfishing and various infectious diseases [5–7].

Bacterial diseases are measured to be the foremost limiting factor leading to economic loss in the aquaculture industry all over the world [8,9]. Among the different pathogenic microorganisms, one of the most common microorganism is *Vibrio anguillarum* which was widely reviewed and evaluated its pathogenic impact in aquaculture [10,11]. *Vibrio anguillarum* is a major aquatic pathogen causing vibriosis, a haemorrhagic septicaemic disease which leads to mortalities to fishes,

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<https://doi.org/10.1016/j.micpath.2020.104610>

Received 5 February 2019; Received in revised form 20 July 2020; Accepted 9 November 2020

Available online 16 November 2020

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crustaceans and bivalves [12]. These bacterial infections can be partially controlled through the applications of suitable antibiotics and chemotherapeutics. However, these are often hazardous as the residues which are left behind adversely affect the environment and also due to the disturbances of the gastrointestinal bacterial populations of the host animal, development and transfer of antimicrobial resistance among pathogens, immunosuppression and reduced disease resistance [13,14].

In order to overcome the adverse effects of using antibiotics and chemotherapeutics, search for a naturally derived and environmental friendly alternative has been necessitated worldwide to control the disease outbreaks. In this regard, the use of probiotic bacteria is receiving wide acceptance in aquaculture [8,14]. The use of probiotics was reported to benefit the host health by controlling the bacterial infections, improving the microbial balance, water quality, enzymatic functions and providing essential nutrients [15–17]. Besides, they also enhance the important immune parameters of the host which play an important role in boosting the immunity to fight against infectious diseases. Further, the probiotics will protect their cells from oxidative damage by improving the production of antioxidant enzyme like SOD, catalase etc which correlate directly with the immune and health benefits of fish [2,18–20]. Pronounced use of probiotic bacteria from the division Gammaproteobacteria (*Vibrio* spp., and *Pseudomonas* spp.) and Firmicutes (*Lactobacillus* spp., and *Bacillus* spp.) are the common practice aided in aquaculture [21–23]. The beneficial characteristics of *Bacillus* sp. have previously been reported by several researchers, which led to their approval to use as potential probiont in aquaculture [9,24,25]. *B. subtilis* is considered as one among the universal candidate to be used as probiotic bacteria and a good biocontrol agent in aquaculture [26–29].

It is ideal for exploring the mangrove ecosystem in search of probiotics as they are one of the productive habitats for a good number of various living organisms [30]. *B. subtilis* Ba37 previously isolated from a mangrove environment with a broad spectrum of antimicrobial activity especially against aquaculture pathogens was selected for this study [31]. The properties of *B. subtilis* Ba37 encouraged us for the further exploration and thus the present study aims to evaluate the *in vitro* and *in vivo* potential of *B. subtilis* Ba37 as a candidate probiont in fish health management. Moreover, the effect of probiotic supplementation of *B. subtilis* Ba37 on the growth, immune response and disease resistance against *V. anguillarum* O1 infection in *E. suratensis* also will be examined. The results of the study may provide the basic information to develop a putative probiont as well as a novel antimicrobial agent against aquaculture pathogens especially belonging to *Vibrio* species.

2. Materials and methods

2.1. Bacterial strains

A bacterial strain isolated from the sediment sample collected from Mangalavanam (mangrove ecosystem) of tropical estuarine habitats of Cochin, Kerala with a broad spectrum of antibacterial activity against aquaculture pathogens prompted us to explore its potential to be used as probiont/biocontrol agent. The bacterial strain was identified as *Bacillus subtilis* MBTDCMFRI Ba37 (*B. subtilis* Ba37) [31]. A reference strain *B. subtilis* MTCC441 (Microbial Type Culture Collection, Chandigarh, India) was used for the characterization studies.

For the antagonistic assay, the test organisms used were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101 (courtesy Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (courtesy National Centre for Aquatic Animal Health, Cochin University of Science and Technology), *A. veronii* (courtesy CMFRI culture collection), *V. vulnificus* MTCC1145, *V. parahaemolyticus* MTCC451 (courtesy Microbial Type Culture Collection, Chandigarh, India). All bacterial isolates were preserved at -80°C as glycerol stocks and were inoculated into freshly prepared broth when necessary.

2.2. Characterization of *B. subtilis* Ba37

B. subtilis MTCC441 was used for the comparison of morphological and phenotypic analysis of *B. subtilis* Ba37. Phenotypic characterization was conducted viz., Gram's and spore staining, catalase test, oxidase activity, reduction of nitrate, indole production, methyl red test, Voges-Proskauer reactions, H_2S production, citrate utilization, oxidative-fermentative test and carbohydrate fermentation [32].

2.3. Exoenzymatic assay

To assess hydrolytic extracellular enzymatic activities, overnight grown *B. subtilis* Ba37 was inoculated in nutrient agar with 1% sodium chloride as experimental medium (Himedia) supplemented with the specific substrate such as starch for amylolytic activity, skimmed milk for proteolytic activity, tributyrin for lipolytic activity, gelatin for gelatinase activity, carboxy methyl cellulose for cellulase activity, phenolphthalein phosphate agar (Himedia) for phosphatase activity and urea agar (Himedia) for urease activity, respectively using agar diffusion method [33]. The plates were incubated at $30 \pm 2^{\circ}\text{C}$ for 24 h. The amylolytic activity was detected by flooding with 1% iodine solution on plates and measuring the transparent zone around the colony [34]. To detect the presence of gelatinase activity, the appearance of the transparent zone around the colony after flooding the plates with 15% mercuric chloride (HgCl_2) was measured [35]. The proteolytic activity was determined by the zone of clearance of skim milk degradation around the colony [33]. The cellulolytic activity was visualized by clearance zone around the isolates when plates were overlaid with Congo-red solution (1%) and then washed with 1 M NaOH solution [36]. Lipase production was determined by the halo formation of precipitated fatty acids around the colony [37]. Phosphatase and urease activity was determined by the pink colour formation on corresponding media [31]. Evaluation of the qualitative exo-enzymatic potential was carried out based on the zone of clearance around the colony [38].

2.4. Physicochemical tolerance assay

The tolerance of *B. subtilis* Ba37 towards various physicochemical parameters viz., temperature, pH, and salinity was carried out by already mentioned methods [31]. The temperature resistance of the isolates was determined by inoculating them in nutrient agar medium and incubating in a wide range of temperatures (20°C to 55°C). The salinity tolerance was tested in a medium containing peptone, yeast extract supplemented with respective concentrations of salt (NaCl) (0%–30% (w/v)). The pH tolerance was examined by growing isolates in nutrient agar medium with different pH gradients 4–10 (adjusted using 0.1 N HCl and 0.1 N NaOH). The plates were incubated at $30 \pm 2^{\circ}\text{C}$ for 24 h. For each assay, the plates were incubated at $30 \pm 2^{\circ}\text{C}$ for 24 h, and the tolerance of the isolates to various stress conditions was detected by measuring the intensity of growth of the strains.

2.5. Antibiotic susceptibility test

The antibiotic susceptibility pattern of the *B. subtilis* Ba37 was evaluated by the standard disc diffusion method using commercially available antibiotics impregnated discs (Himedia, Mumbai, India) including Penicillin (P-10U), Bacitracin (B-10U), Erythromycin (E-15mcg), Tetracycline (T-30mcg), Streptomycin (S-10mcg), Ciprofloxacin (Cf-5mcg), Chloramphenicol (C-30mcg), Gentamicin (G-10mcg), Ampicillin (A-10mcg), Kanamycin (K-10mcg), Cefoperazone (CPZ-75mcg), Cephalothin (CEP-30mcg), Colistin (CL-10mcg), Cefuroxime (CXM-30mcg), Levofloxacin (LE-5mcg), Lomefloxacin (LOM-10mcg), Nitrofurantoin (NIT-300mcg), Norfloxacin (NX-10mcg) and Cefoxitin (CX-30mcg). Briefly, 100 μl of overnight grown *B. subtilis* Ba37 was swabbed on Muller-Hinton agar (Himedia, Mumbai, India) plates and antibiotic discs were placed above it and incubated for 24 h at

$30 \pm 2^\circ\text{C}$ [39]. After incubation, the zone of inhibition was measured (mm) using the HiAntibiotic Zone scale and interpreted using the zone size interpretation chart (Himedia, Mumbai, India) [40].

2.6. Antibacterial potential

The initial screening of antibacterial activity of *B. subtilis* Ba37 was carried out by spot diffusion assay against the test pathogens [31]. In brief, isolated bacteria were spotted over pre-swabbed plates with aquaculture pathogens which are mentioned as test organisms above. The antibacterial assay of cell-free supernatant was done by well diffusion method and disc diffusion method. The isolate was inoculated in nutrient broth supplemented with 1% NaCl and incubated at $30 \pm 2^\circ\text{C}$ for 72 h in a shaker incubator (100 rpm). The culture was centrifuged at $8000 \times g$ for 15 min and filtered through a $0.2 \mu\text{m}$ syringe filter (Pall Life science, USA). For the disc diffusion assay, 40 μL of this cell free preparation was impregnated on 6 mm diameter sterile discs (HiMedia, India) and air-dried [41]. These discs were placed on Muller Hinton agar plates with 1.5% sodium chloride concentration (HiMedia, India) pre-swabbed with the test pathogens. For the well diffusion assay, 40 μL of cell-free supernatant was introduced into the wells on the medium which were previously swabbed with the test organisms [42]. Then the plates were incubated at $30 \pm 2^\circ\text{C}$ for 24 h, and the zone of inhibition around the discs and wells were measured using HiAntibiotic Zone scale (Himedia, Mumbai, India) and recorded against target organisms. The antibacterial activity of the selected isolate was compared with commercialized antibiotics against tested pathogens.

2.7. Generation time and growth curve

Five mL of nutrient broth with 1.5% NaCl was inoculated with a single colony from the plates and incubated overnight at $30 \pm 2^\circ\text{C}$ on a rotary shaker (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20–24 h. One percentage of overnight grown culture was inoculated to 100 mL nutrient broth with 1.5% sodium chloride. Bacterial cultures were kept in a shaking incubator at $30 \pm 2^\circ\text{C}$ for 24–48 h. One mL of samples (bacterial cultures) were taken at every half an hour interval from 0 to 8 h for the determination of generation time. For the growth curve (change in cell number against time) studies, samples were taken at different time intervals from 0 h to 48 h. Growth was determined by measuring the OD at 600 nm using MULTISCAN GO, Thermo, Finland and viable cell count by serial dilution followed by plating on nutrient agar with 1% NaCl and the bacterial growth curve is plotted [43]. The determination of the generation time of the cultures was carried out as per the methodology described earlier [44].

2.8. Co-culture inhibitory assay

Co-culture inhibitory assay was performed to assess the ability of antagonistic *B. subtilis* Ba37 in inhibition of pathogenic *V. anguillarum* O1 on co-existence. Cultures were grown separately in nutrient broth supplemented with 1% NaCl and incubated at $30 \pm 2^\circ\text{C}$ in a shaker at 120 rpm overnight. The above cultures were inoculated in nutrient broth (with 1% NaCl) to obtain three different concentrations of *V. anguillarum* O1 (cell count of approximately 10^2 , 10^3 , 10^4 CFU mL⁻¹) each of which is being separately added with six different concentrations of *B. subtilis* Ba37 (0 , 10^4 , 10^5 , 10^6 , 10^7 and 10^8 CFU mL⁻¹ respectively) [45]. The control cultures were kept without mixing each other. The cultures were incubated at $30 \pm 2^\circ\text{C}$ in a shaker (120 rpm), and all samples (1 mL) were withdrawn at 24 h intervals for 4 days to determine the cell count. Counts of the pathogen were monitored by withdrawing daily 1 mL samples that were serially diluted 10-fold and 100 μL aliquots of each were spread plated on TCBS agar and nutrient agar (with 1% NaCl) supplemented with 5 mM potassium chromate (K_2CrO_4) plates. The plates were incubated at $30 \pm 2^\circ\text{C}$ for 24 h and the colonies obtained on the plates were counted and expressed as log₁₀ CFU mL⁻¹.

2.9. Haemolytic assay

Haemolytic assay to detect the pathogenic nature of *B. subtilis* Ba37 and *V. anguillarum* O1 to lyse the RBC cells were carried out using the already established method [2] with suitable modifications. Briefly, the isolates were spot inoculated on nutrient agar supplemented with 5% (v/v) of defibrinated sheep blood and incubated at $30 \pm 2^\circ\text{C}$ for 24 h. Haemolytic activity was detected by clear zone of haemolysis (zone of clearance) around the colonies.

2.10. Cytotoxicity assay

In vitro cytotoxicity was used to determine the toxic effects of the metabolite of *B. subtilis* Ba37 and *V. anguillarum* O1 on fibroblast cell lines of *Cyprinus carpio* (Koi carp). The confluent fish cell monolayer was grown in Leibovitz's Medium (L-15) supplemented with 5% fetal calf serum (FCS) in a 24 well flat bottomed plates (Nunc, New York). For cytotoxicity assays, cells were seeded from monolayer at a density of 10^5 cells/well, with 1 mL of the medium is added into 24 well plates and incubated at $30 \pm 2^\circ\text{C}$ in a humid atmosphere containing 5% CO_2 for 5 days [46]. Cell-free supernatant was obtained by centrifugation of 10 mL of 18 h culture broth of *B. subtilis* Ba37 and *V. anguillarum* O1 and filtered through a $0.2 \mu\text{m}$ syringe filter (Pall life science, USA). Cells in the plates were infected with 100 μL of cell-free supernatant and sterile Luria Bertani (LB) medium as a control. L-15 media were added into the wells and incubated at $30 \pm 2^\circ\text{C}$ for a week and examined daily. To assess the cytotoxic character of cell free supernatants of isolates to cell lines, cells were subjected to microscopic examination to observe the morphological changes occurred to cell monolayers. Any detected alterations in the morphology of monolayer were scored as a positive cytotoxic response [47].

2.11. Experimental fish and feeding trail

To evaluate the *in vivo* potential of *B. subtilis* Ba37, healthy juveniles of *E. suratensis* (average fish weight: 0.35 ± 0.08 g and length: 2.5 ± 0.4 cm) was collected from Matsyafed Hatchery, Azhikode, Kerala. Prior to the experiment, all fishes were acclimatized to the laboratory conditions for 15 days and fed with commercial feed. Basic physicochemical parameters of the water (temperature, dissolved oxygen, ammonia, and pH) were at $26 \pm 2.0^\circ\text{C}$, 6.09 ± 0.02 mg L⁻¹, 0.04 ± 0.001 mg L⁻¹, 7.01 ± 0.03 , respectively) were monitored. A suspension of *B. subtilis* Ba37 was prepared by sub-culturing the 20 h old bacterial inoculum in nutrient broth (1% NaCl) at $30 \pm 2^\circ\text{C}$ for 24 h. The culture was pelleted, washed twice with sterile $1 \times$ phosphate buffer saline (PBS) with pH of 7.4. The cells were resuspended in sterile PBS to OD₆₀₀ of 1.0, corresponding to 4.2×10^9 CFU mL⁻¹. The cell count was determined by plating 10-fold serial dilution in nutrient agar with 1% NaCl by spread plate method. Before experiment, the fishes were starved for 24 h. During the experimental period, the water was not renewed to maintain the stable water environment and bacterial concentration. A basal diet were prepared with 45% crude protein and 9% lipid and this diet was used in the experiment (Table 1). The experiments were carried out in triplicates. A total of 60 juveniles of *E. suratensis* were divided randomly into two experimental groups. At the beginning of the experiment, fishes with similar sizes were weighed and distributed into 6 glass tanks of 15 L capacity with 10 fishes in each tank. Throughout the experiment, the water salinity was maintained at 10–12 ppt, water temperature at $30 \pm 2^\circ\text{C}$, under natural light and dark cycles with continuous aeration for 21 days.

The following treatments groups were maintained: (i) Group which fed with basal diet (T_1), (ii) Group fed with basal diet and *B. subtilis* Ba37 at a final concentration of 4.2×10^6 CFU mL⁻¹ (T_2). During the feeding trial, fishes were manually fed with basal diet twice daily till apparent satiation and it was ensured that no feed was leftover. All group of fishes were monitored for any signs of disease or mortality up to 21 days. After

Table 1

Ingredient formulation and proximate composition of the experimental diet (% dry matter).

Ingredients	(g/kg)
Marine protein mixture	382
Soy Flour	330
Wheat Flour	200
Sardine Oil	42
Lecithin	1
Vitamin C	3
Vitamin Mixture	20
Mineral Mixture	20
Antifungal	1
Antioxidant	1
Proximate composition (% Dry matter)	
Moisture	5.03
Crude protein	45.02
Crude lipid	9.05
Crude ash	9.84
Gross energy (MJ kg ⁻¹)	19.46

completion of the experiment, one fish from each tank of the experimental group (T₂) was randomly selected and sacrificed to evaluate the presence of *B. subtilis* Ba37.

2.12. Growth performance

Fishes were weighed both at the start and end of the feeding trial to calculate the growth performances as follows.

Weight gain % (WG) = 100(final weight – initial weight)/initial weight.

Specific growth rate (SGR) = 100 (ln final weight - ln initial weight)/no. of days feeding trial.

Survival (%) = (final number of fish/initial number of fish) × 100.

Feed conversion ratio (FCR) = Feed intake (g dry weight)/weight gain (g).

Protein efficiency ratio (PER) = Total wet weight gain (g)/crude protein fed (g).

Feed efficiency (FE) = 100 × (weight gain, g)/(dry diet intake, g).

2.13. Sample preparation

At the termination of the feeding trial, four fishes from each tank were anaesthetized with clove oil and sacrificed for further analysis. The fishes were weighed and homogenized with 0.25 M chilled sucrose on a wet basis (pH 7). The homogenate was centrifuged at 6000 rpm for 10 min at 4 °C (HERMLE Labortechnik GmbH, Germany). After centrifugation, the supernatant solution was aliquoted in 1.5 mL micro-centrifuge tubes. The samples were stored at -80 °C for further analysis.

2.14. Immune and antioxidant parameters

The SOD activity was assayed using a commercial test kit (Sigma-Aldrich) following the manufacturer's instruction. The change in the absorbance was measured at 450 nm. SOD activity (inhibition rate %) was calculated by subtracting the slope of all linearity of absorbance's and multiply with 100. SOD activity was expressed as U mg protein⁻¹.

Catalase assay kit (Sigma-Aldrich) was used to evaluate the activity. A colorimetric assay was performed (at 520 nm) following the procedures as mentioned in the kit. CAT activity was expressed as U mg protein⁻¹. The difference in absorbance through time (ΔA520) is inferred as the measure of catalase activity.

The total protein activity was measured at 550 nm using the assay kit (Biuret method- Coral clinical systems). Total protein was expressed as U mg protein⁻¹. Total protein was calculated by multiplying the mean absorbance change per minute (ΔA/minute) with the standard at 37 °C.

Alkaline phosphatase activity was determined by the use of kit (pNPP

kinetic method- Coral clinical systems) and the optical density was measured at 405 nm. Samples and reagents were prepared and their alkaline phosphatase activities were evaluated by the procedures mentioned by the manufacturer. The Alkaline phosphatase activity (U/L) was calculated by multiplying the mean absorbance change per minute (ΔA/minute) with 2754.

SGOT activity (U/L) was determined using the kit (SGOT/ASAT kit; Mod IFCC method- Coral clinical systems) and absorption was measured at 340 nm following the procedures as mentioned in the kit. The SGOT activity (U/L) was calculated by multiplying the mean absorbance change per minute (ΔA/minute) with 952 at 30 °C.

SGPT (ALAT) kit was used to find the activity which used the modified IFCC method (Coral clinical systems). The procedures were followed as mentioned in the kit and the absorbance was recorded at 340 nm. The SGOT activity (U/L) was calculated by multiplying the mean absorbance change per minute (ΔA/minute) with 952 at 30 °C.

2.15. Challenge study

V. anguillarum O1 was inoculated into fresh Luria Bertani broth (LB) broth with 1% NaCl and incubated at 28 °C with shaking for 18 h, followed by centrifugation (10,000 rpm for 20 min at 4 °C) and re-suspension in sterilized PBS. During the challenge experiments, the fishes were maintained in glass tanks containing 5 L water of 10 ppt salinity at 30 ± 2 °C. The experimental fishes after 21 days feeding trial were randomly divided into two infected groups – (i) group fed with basal diet infected with *V. anguillarum* O1 (T₁) and (ii) group fed with basal diet and *B. subtilis* Ba37 infected with *V. anguillarum* O1 (T₂). Five fishes from each tank of feeding trial were bath challenged with PBS containing *V. anguillarum* O1 at a final concentration of 7.5 × 10⁴ CFU mL⁻¹. All experiments were performed in triplicates. The challenged fishes were monitored daily for 15 days to record the mortality. During challenge tests, no feed was provided and water was not exchanged. Dead fishes were subjected to bacteriological examination immediately after death. Percentage of survival was calculated with respect to control. The relative percentage survival (RPS) was calculated using the following equation, RPS (%) = [1 - {mortality in treated group (%) / mortality in control group (%)}] × 100.

2.16. Statistical analysis

One-way analysis of variance (ANOVA) was carried out with SPSS20.0 (SPSS, USA, ver. 20.0) to assess for any significant differences between the means. The differences in means values were identified by Scheffe's multiple range tests to determine whether significant difference (*P* < 0.05) existed among different treatments.

3. Results

3.1. Characterization

B. subtilis Ba37 was found to be aerobic, Gram-positive, rod shaped (size ~0.4–0.7 × 1.5–3.0 μm), motile, with ellipsoidal endospores and observed at sub-terminal or central positions as swollen sporangia. Colonies were smooth, slightly undulate, irregular, slightly raised, creamish white, opaque and large after 72 h of growth at 30 ± 2 °C on nutrient agar with 1% NaCl. Morphological and biochemical characteristics of *B. subtilis* Ba37 were found to be similar with the reference strain *B. subtilis* MTCC441 (Table 2).

3.2. Exoenzymatic assay

On examination of the zone of hydrolysis around the colonies, *B. subtilis* Ba37 was found to produce lipolytic, amylolytic, cellulolytic proteolytic, and gelatinolytic enzymes. Hemolytic and phospholytic activities were not observed (Fig. 1).

Table 2Morphological and biochemical characterization of *B. subtilis* Ba37.

Morphological Characteristics	<i>B. subtilis</i> MBTDCMFRI Ba37	<i>B. subtilis</i> MTCC 441
Colony colour on nutrient agar (1%NaCl)	Creamish white	Creamish white
Size	Large	Large
Form	Irregular	Irregular
Margin	Undulate	Undulate
Elevation	Flat	Flat
Cell Shape	Rod	Rod
Gram stain	G+	G+
3% KOH	–	–
Endospore formation	+	+
Pigmentation	–	–
Biochemical Characteristics		
Catalase reaction	+	+
Oxidase reaction	+	+
Nitrate reduction	+	+
Indole production	–	–
Methyl red test	–	–
Voges -Proskauer test	+	+
H ₂ S production	–	–
Citrate utilization	+	+
Arginine dihydrolase	–	–
Lysine decarboxylase	–	–
Ornithine decarboxylase	–	–
Carbohydrate fermentation		
Arabinose	–	–
Galactose	–	–
Glucose	+	+
Inositol	–	–
Mannitol	+	+
Mannose	–	–
Melibiose	–	–
Raffinose	–	–
Salicin	+	+
Trehalose	+	+
Xylose	–	–

“+” = Positive reaction; “–” =Negative reaction

3.3. Physicochemical tolerance assay

The survivability of *B. subtilis* Ba37 in varied physiological conditions showed that it can adapt to the wide range of fluctuating environmental conditions (Table 3). The results showed that they can grow well up to 55 °C. It was also observed that the species grow well in regular as well as hyper-saline conditions and the species can survive up to 10% of salt concentration. pH tolerance study confirmed that they can resist the variations in pH (4–10) and can survive in acidic, basic and neutral conditions.

3.4. Antibiotic susceptibility test

The isolate *B. subtilis* Ba37 was tested for their antibiotic resistance using nineteen commercially available antibiotics (Fig. 2). *B. subtilis* Ba37 was susceptible to sixteen tested antibiotics except for bacitracin (B-10U), ampicillin (A-10mcg) and colistin (CL-10mcg).

3.5. Antibacterial potential

Antibacterial activity of *B. subtilis* Ba37 was tested against seven major aquaculture pathogens using spot diffusion, well diffusion, and disc diffusion methods (Table 4, Fig. 3). It was found that the culture, as well as the cell-free supernatant of *B. subtilis* Ba37, demonstrated an inhibitory potential towards all the tested aquaculture pathogens. The inhibitory activity was greater against *V. vulnificus* MTCC1145 and *V. harveyi* 101 whereas the least against *A. hydrophila* and *A. veronii*. To have a comparison of results, the same pathogens were also tested against ten commercially available antibiotics (Table 5).

3.6. Growth curve and generation time

V. anguillarum was selected for further experiments viz., growth curve and generation time, co-culture inhibitory assay, cytotoxicity and haemolytic assay etc as it is one of the major disease causing bacterial pathogens in aquaculture rearing systems inflicting economic loss in larviculture [12]. The growth curve of the *V. anguillarum* O1 against the isolate *B. subtilis* Ba37 was illustrated in Fig. 4. The growth rate demonstrated by the curves showed a small lag phase of 3 h, after which the microorganisms entered into a rapid logarithmic phase for *V. anguillarum* O1. For *B. subtilis* Ba37 there was a typical lag phase of 3 h which then slowly extending into a logarithmic phase. The generation time was calculated from the growth data at the 3 h (8.1×10^4 CFU mL⁻¹) and 0 h (2.4×10^3 CFU mL⁻¹). The generation time for *B. subtilis* MBTDCMFRI Ba37 and *V. anguillarum* O1 was found to be 59 & 49 min generation⁻¹ respectively.

Table 3Physiochemical characteristics of *B. subtilis* Ba37.

Physiological properties	Growth of <i>B. subtilis</i> Ba37
Temperature	20–55 °C
pH	4–10
NaCl (%)	0–10

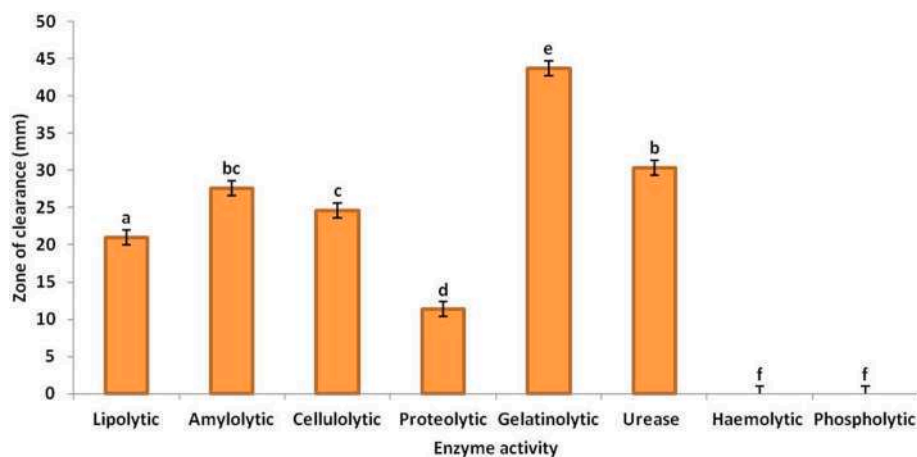


Fig. 1. Enzymatic activity of *B. subtilis* Ba37. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation. values (a-f) with different letters are significantly different ($P < 0.05$).

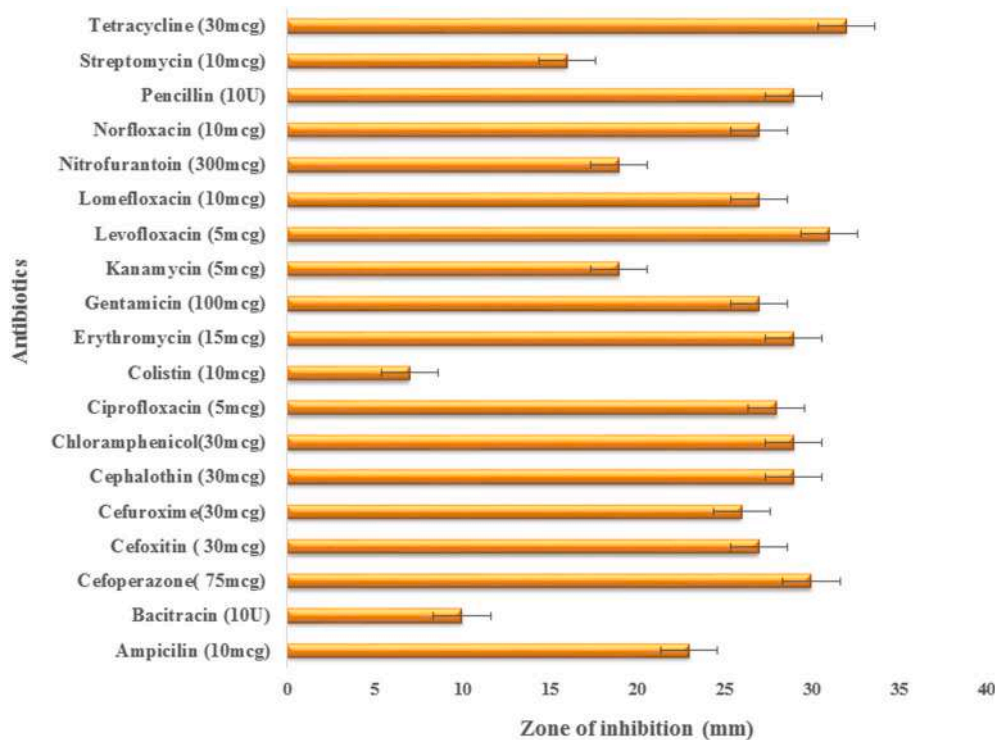


Fig. 2. Antibiotics susceptibility pattern of *B. subtilis* Ba37 against commercially available antibiotics.

Table 4

Antibacterial activity of *B. subtilis* Ba37 against pathogens.

Assay Method	Zone of Inhibition (mm)						
	Vvul	Vpar	Ahyd	Valg	Vhar	Vang	Aver
Spot diffusion	19.67 ^{ap} ±1.62	10.37 ^{bp} ±1.53	10.28 ^{bp} ±0.58	18.06 ^{ap} ±2.00	19.67 ^{ap} ±2.52	17.27 ^{ap} ±0.82	12.18 ^{bcp} ±1.85
Well diffusion	16.08 ^{apq} ±1.87	12.23 ^{abcp} ±1.02	10.04 ^{bcp} ±2.00	14.17 ^{abcq} ±1.00	15.29 ^{abp} ±1.72	16.12 ^{ap} ±1.84	9.67 ^{cp} ±1.29
Disc diffusion	15.31 ^{aq} ±1.15	11.33 ^{abp} ±1.58	9.00 ^{bp} ±1.45	12.30 ^{abq} ±1.50	15.11 ^{ap} ±0.97	15.33 ^{ap} ±2.06	11.05 ^{abp} ±1.52

Data are the mean values of triplicate and expressed as mean ± standard deviation. Row (a-c) and column (p-q); values with different letters are significantly different ($P < 0.05$). Vvul- *Vibrio vulnificus* MTCC1145; Vpar- *V. parahaemolyticus* MTCC 451; Ahyd- *Aeromonas hydrophila*; Valg - *V. alginolyticus* 101; Vhar - *V. harveyi* 101 , Vang- *V. anguillarum* O1 and Aver – *Aeromonas veronii*. The measurement indicates the inhibition zone (mm) formed around the isolates. Values not sharing common alphabets are significantly different ($P < 0.05$).

3.7. Co-culture inhibitory assay

It was observed that *B. subtilis* Ba37 can specifically grow on nutrient agar supplemented with 5 mM K_2CrO_4 while *V. anguillarum* O1 cannot grow in the same. This property was used for estimating *B. subtilis* counts in co-culture assay. Co-culture assay was performed further to confirm the antagonistic perspective of *B. subtilis* Ba37 against the major aquaculture pathogen *V. anguillarum* O1. It was observed that the growth of *V. anguillarum* O1 at different concentrations (10^2 - 10^4 CFU mL⁻¹) was inhibited by *B. subtilis* Ba37 (10^4 - 10^8 CFU mL⁻¹) (Fig. 5). *B. subtilis* Ba37 inhibited *V. anguillarum* O1 in co-culture when the initial cell count of the probiont was even at 10^4 CFU mL⁻¹. Even though the lower concentrations of *B. subtilis* Ba37 (10^4 CFU mL⁻¹) allowed initial growth of *V. anguillarum* O1; the continued incubation never allowed the cell densities of the pathogen to reach to a level beyond control. At higher concentrations of *B. subtilis* Ba37 (10^6 - 10^8 CFU mL⁻¹), the growth exhibited by *V. anguillarum* O1 was completely hindered after 96 h (Fig. 5).

3.8. Cytotoxicity and haemolytic assay

Cytotoxicity assay showed the viability of more than 95% in koi carp cell lines after treating with filtered cell free supernatant of *B. subtilis*

Ba37, while the cell line treated with the supernatant of *V. anguillarum* O1 showed only less than 10% viability. Microscopic observation revealed that the cells treated with *B. subtilis* Ba37 remained intact without any morphological changes confirming its non-toxic nature to koi carp cell line. A complete ruptured pattern by shrinking, vacuolization of cells, and finally, monolayer destruction was observed for those cell line treated with *V. anguillarum* O1 showing its elevated toxicity potential towards cell line (Fig. 6). Absence of clearance zone around *B. subtilis* Ba37 on blood agar plates depicted its non haemolytic activity while the presence of a clear zone around *V. anguillarum* O1 exhibited its haemolytic potential.

3.9. Growth performance

B. subtilis Ba37 was confirmed to be harmless by administrating it in juveniles of *E. suratensis*. After feeding trial, juveniles of *E. suratensis* did not show any signs of disease or mortality in Group T₂ and *B. subtilis* Ba37 was reisolated from the fishes. Hence, it can be considered as safe for using diverse treatment conditions. Growth performances of the group fed with basal diet (T₁) and the group fed with basal diet and *B. subtilis* Ba37 (T₂) are shown in Table 6. In 21 days period, percentage weight gain of fish fed with basal diet and *B. subtilis* Ba37 (217.53 ± 3.25) were significantly ($P < 0.05$) higher than those fed with the basal

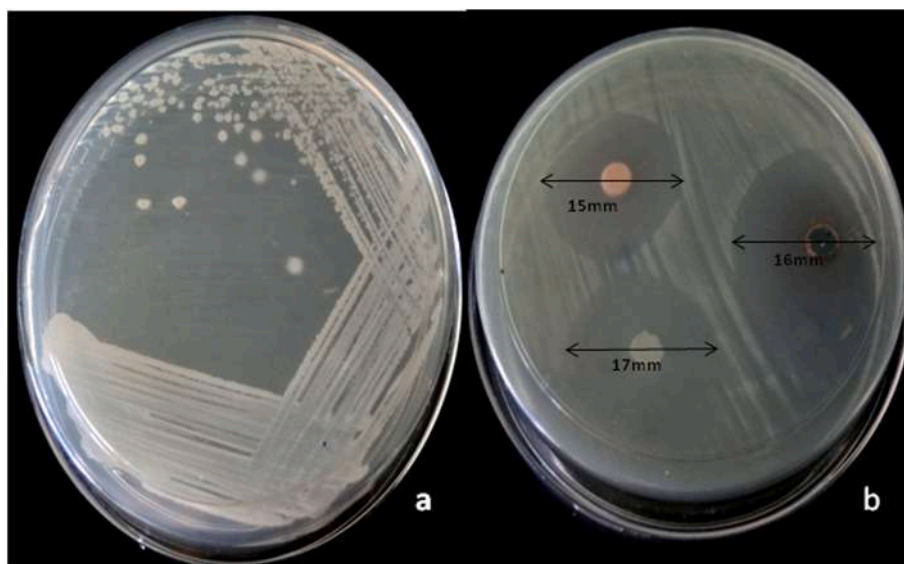


Fig. 3. a. *B. subtilis* Ba37 on nutrient agar supplemented with 1% sodium chloride, b. Antagonistic activity of cell free supernatants of *B. subtilis* Ba37 against *V. anguillarum* O1 by spot diffusion, disc diffusion, and well diffusion methods.

Table 5

Antibiotic profile of standard commercial antibiotics against test pathogens.

Zone of inhibition of antibiotics (mm)										
Test organisms	P	B	E	T	S	Cf	C	G	A	K
<i>Vibrio vulnificus</i> MTCC1145	33	6*	32	33	16	40	34	27	30	6
<i>Vibrio parahaemolyticus</i> MTCC 451	6	6	15	20	16	20	25	19	6	20
<i>Aeromonas veronii</i>	6	6	15	25	16	23	21	24	6	20
<i>Aeromonas hydrophila</i>	6	6	11	20	9	30	30	27	6	7
<i>Vibrio harveyi</i> 101	6	6	13	21	11	18	23	15	6	13
<i>Vibrio anguillarum</i> O1	6	6	15	28	14	14	33	24	6	20
<i>Vibrio alginolyticus</i> 101	6	6	11	15	12	17	19	16	6	13

P - Penicillin (10U), B - Bacitracin (10U), E – Erythromycin (15mcg), T- Tetracyclin (30mcg), S - Streptomycin (10mcg), Cf - Ciproflaxacin (5mcg), C - Chloramphenicol (30mcg), G- Gentamycin (10mcg), A - Ampicillin (10mcg), K - Kanamycin (30mcg). *6' indicate no zone of inhibition as the disc size is 6mm

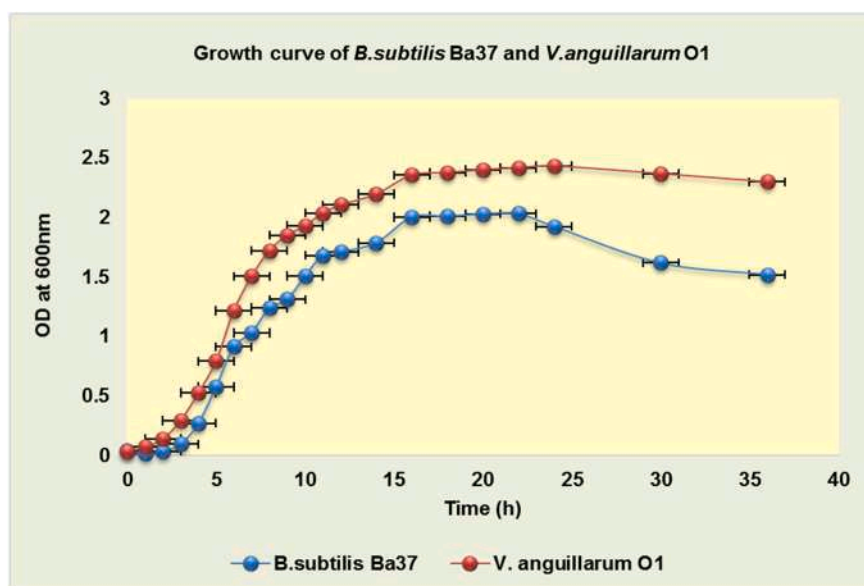


Fig. 4. Growth curve of *B. subtilis* Ba37 and *V. anguillarum* O1. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation.

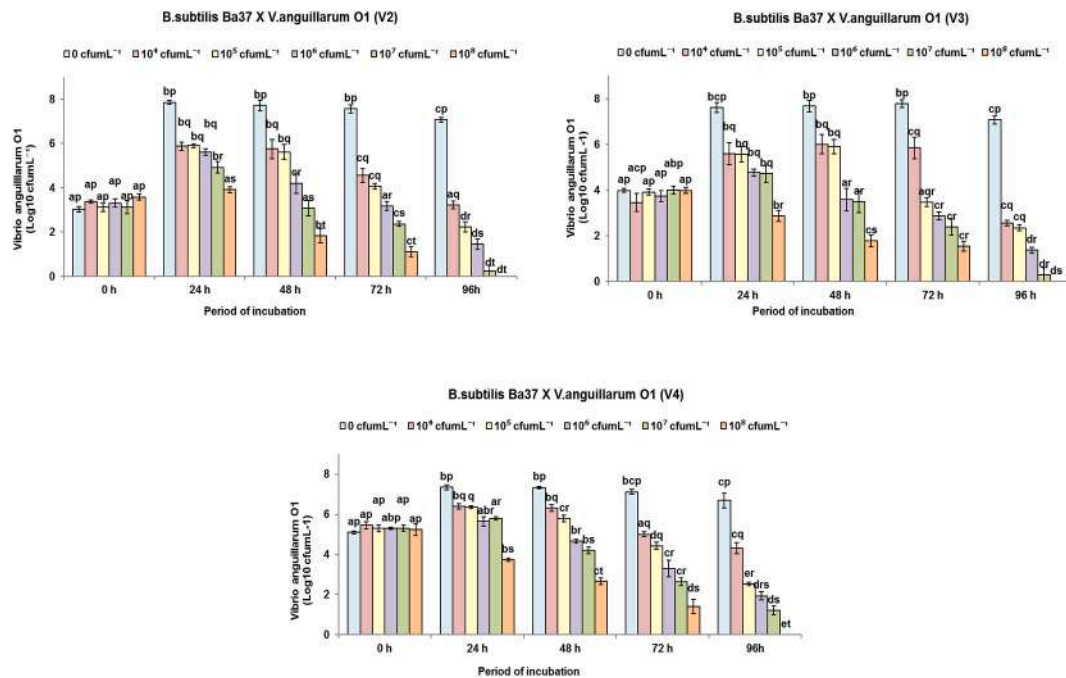


Fig. 5. Inhibitory activity of *B. subtilis* Ba37 against *V. anguillarum* O1 in coculture at increasing cell densities. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation. values with different letters [for period of incubation (a-d) and for concentrations (p-t)]; are significantly different ($P < 0.05$).

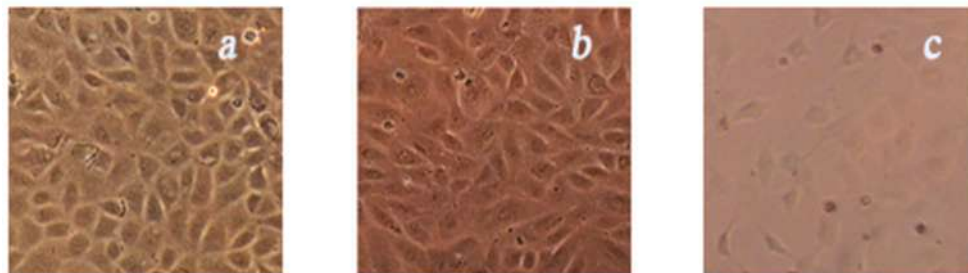


Fig. 6. Cytotoxicity assay of cell free supernatant of *B. subtilis* Ba37 and *V. anguillarum* O1. a. Control cell line. b - Cell line treated with CFS of *B. subtilis* Ba37. c - Cell line treated with CFS of *V. anguillarum* O1.

Table 6

Growth performance of *E. suratensis* after feeding with basal diet (T_1) and basal diet supplemented with *B. subtilis* Ba37 (T_2).

Parameters	Experimental diets	
	T_1	T_2
Percentage weight gain(%WG)	143.11 $\pm$ 5.09	217.53 $\pm$ 3.25
Specific growth rate (SGR)	4.23 $\pm$ 0.8	5.50 $\pm$ 0.08
Percentage survival	80.0 $\pm$ 5.77	100 $\pm$ 0.00
Feed conversion ratio (FCR)	1.47 $\pm$ 0.03	1.13 $\pm$ 0.03
Protein efficiency ratio (PER)	0.88 $\pm$ 0.12	1.03 $\pm$ 0.15
Feed efficiency (FE)	0.44 $\pm$ 0.06	0.63 $\pm$ 0.08

diet (143.11 $\pm$ 5.09). The survival rate of the group fed with basal diet and *B. subtilis* Ba37 (T_2) and the group which fed with basal diet (T_1) showed 100% and 80% respectively. The specific growth rate of the group fed with basal diet and *B. subtilis* Ba37 (T_2) (5.50 $\pm$ 0.08) was also significantly higher as compared with the group fed with the basal diet (T_1) (4.23 $\pm$ 0.8) ($P < 0.05$). The PER and FE were higher for the fishes fed with basal diet and *B. subtilis* Ba37 (T_2) (1.03 $\pm$ 0.15 and 0.63 $\pm$ 0.08 respectively) whereas a lower value of FCR (1.13 $\pm$ 0.03) was observed for the same group.

3.10. Effects of *B. subtilis* Ba37 on immune and antioxidant activities

Effects of dietary administration of *B. subtilis* Ba37 on immune parameters and antioxidant activities were shown in Figs. 7 and 8. Upon evaluation of the sample obtained from the juveniles of *E. suratensis*, it was found that the diet incorporated with *B. subtilis* Ba 37 significantly increased the SOD as well as catalase activities. SOD and catalase activities of the group fed with the diet containing *B. subtilis* Ba37 were 45.14 $\pm$ 0.82 and 42.27 $\pm$ 1.41 U/mg of protein, respectively ($P < 0.05$) whereas the value was much lesser for the animals fed with the basal diet (30.71 $\pm$ 1.67 and 36.11 $\pm$ 1.29 U/mg of protein respectively). The total proteins levels of experimental groups of fishes fed with diet containing *B. subtilis* Ba37 was significantly higher ($P < 0.05$) (6.67 $\pm$ 0.62 U/mg of protein) that of the group fed with the basal diet (4.01 $\pm$ 0.19 U/mg of protein). On the other hand, the alkaline phosphatase (ALP) levels did not increase significantly in the group fed with diet containing *B. subtilis* Ba37 than basal diet. But the alkaline phosphatase activity was found to be enhanced (6.85 $\pm$ 0.17 U/L) as compared to control diet (5.25 $\pm$ 0.09 U/L). SGOT and SGPT showed a decreasing trend in activity than control groups. SGOT and SGPT activities of the group fed with the diet containing *B. subtilis* Ba37 was lower (29.63 $\pm$ 0.34 and 19.57 $\pm$ 0.56 U/L respectively) than that of animals fed with basal diet (32.53 $\pm$ 0.45 and 20.49 $\pm$ 0.48 U/L respectively).

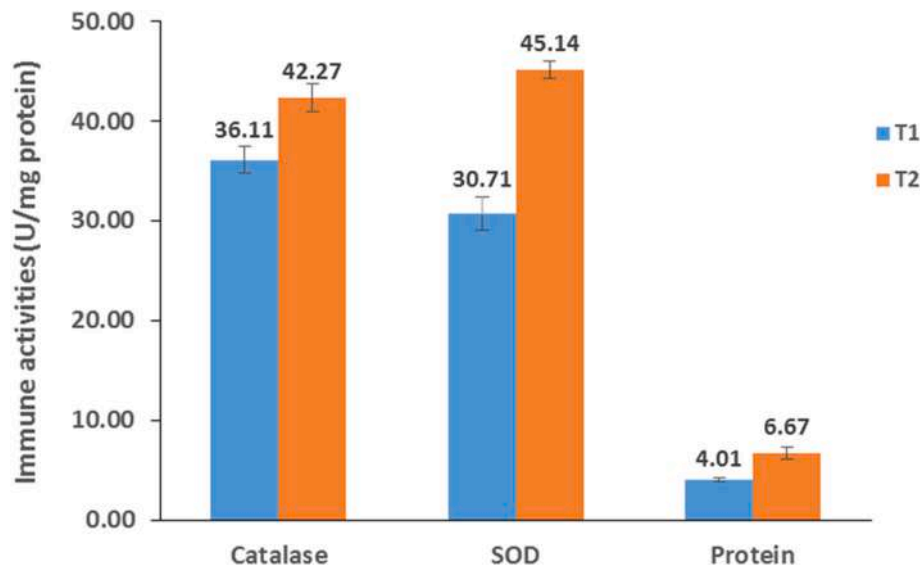


Fig. 7. Effect of supplementation of *B. subtilis* Ba37 on protein level, superoxide dismutase (SOD) and catalase activities in *E. suratensis*. T₁ - Group which fed with basal diet, T₂ - Group fed with basal diet with *B. subtilis* Ba37. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation.

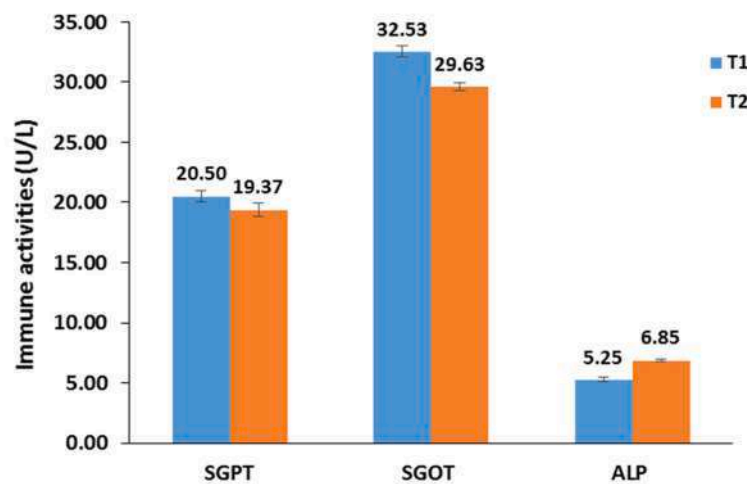


Fig. 8. Effect of supplementation of *B. subtilis* Ba37 on serum aspartate aminotransferase (SGOT), serum alanine aminotransferase (SGPT) and alkaline phosphatase (ALP) activities in *E. suratensis*. T₁ - Group which fed with basal diet, T₂ - Group fed with basal diet with *B. subtilis* Ba37. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation.

3.11. Resistance to *V. anguillarum* O1 challenge

The challenge study revealed that group fed with basal diet and *B. subtilis* Ba37 infected with *V. anguillarum* O1 (T₂) (RPS; $83.33 \pm 3.33\%$), possess significantly higher ($P < 0.05$) tolerance to *V. anguillarum* O1 infection. It showed significantly lower mortality rate than that of the group fed with basal diet infected with *V. anguillarum* O1 (T₁) (RPS; $53.33 \pm 6.67\%$) (Fig. 9). *V. anguillarum* O1 was reisolated from the group fed with basal diet infected with *V. anguillarum* O1 (T₁) and not from the group fed with basal diet and *B. subtilis* Ba37 infected with *V. anguillarum* O1 (T₂).

4. Discussion

The present study illustrated a potential probiotic bacterial strain, *B. subtilis* MBTDCMFRI Ba37, isolated from the sediments of the mangrove ecosystem (Mangalavanam), a tropical estuarine habitat, Cochin. *B. subtilis* is a ubiquitous bacterium and is a relatively rich

source of antimicrobial activity since many species of this genus synthesize a multitude of antimicrobial peptides [48] inhibiting a wide range of aquaculture, plant and animal pathogens [45,49]. They have been used as a promising antagonistic bacteria as well as a good biocontrol agent in aquaculture [26,29]. The production of antimicrobial compounds, low antibiotic resistance, production of a multitude of enzymes, tolerance to abiotic stresses, non-pathogenicity revealed by *in vitro* and *in vivo* studies and other added health benefits, etc. are the prime factors to be considered on selecting a putative probiotic strain. The ability of the isolate *B. subtilis* Ba37 to inhibit the growth of major aquaculture pathogens belonging to the genus *Vibrio* and *Aeromonas* suggests the fact that they could be effectively used in aquaculture directly or as a feed additive to control fish diseases. Other studies also proved that *Bacillus* spp. could be the best candidates for controlling the major bacterial infections in aquaculture sector as it has a broad spectrum of antimicrobial activity [2,8,19]. Our results further illustrated that the antibacterial activity exhibited by *B. subtilis* Ba37 is as better as commercial antibiotics like ampicillin (10 mcg), penicillin (10 U),

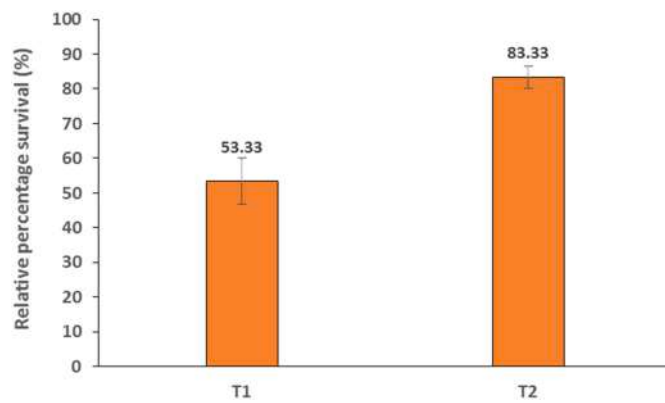


Fig. 9. Effect of supplementation of *B. subtilis* Ba37 on the survival of fish after challenge with *V. anguillarum* O1. T₁ - Group fed with basal diet infected with *V. anguillarum* O1, T₂ - Group fed with basal diet and *B. subtilis* Ba37 infected with *V. anguillarum* O1 Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation.

bacitracin (10 U), erythromycin (15 mcg), kanamycin (30 mcg) and streptomycin (10 mcg) (Table 5).

The suitability and robustness of biological agents depend on their response to environmental conditions in which they need to perform [48]. Physiological conditions often affect the functionality of biological agents, and they must remain functional and effective over wide-ranging physiological conditions [50]. *B. subtilis* Ba37 grown well under a wide range of temperature, salinity, and pH would help its better adaptation towards the host, and the aquatic environment promises its potential to act as a candidate biocontrol agent in aquaculture systems [27,49]. Studies showed that *Bacillus* can grow within the intestinal tract and rather may exert a unique symbiotic relationship with their host and possibly be considered as temporary residents and hence there is an intense interest in using *Bacillus* sp in aquaculture [51]. Spore forming capability of *Bacillus* spp. has also provided excellent ecological advantages that allowed for long-term storage, resistance to toxic compounds, extreme temperature, desiccation and radiation; thus allowing for the relatively easy formulation and commercialization of *Bacillus*-based stable microbial products 'MPs' [16]. Other than the natural antibacterial potential, a good probiotic is also judged with its additional functional properties which could improve the health aspects of the host by catalyzing natural processes such as metabolism, feed digestion, and utilization, prevention of intestinal disorders, feed efficiency, predigestion of anti-nutritional factors present in the ingredients etc., [17,29,49]. Fish growth in aquaculture is greatly affected by the bioavailability of nutrients, which is influenced by its digestive enzyme production [52]. The ability of *B. subtilis* Ba37 to produce several hydrolytic enzymes may support/improve digestion and enhance nutrient uptake by the host by degrading a variety of natural substrates leading to nutrient cycling and also enhances water quality [14,53]. It was reported that prospective probiotics should not carry the genes for antibiotic resistance and gram-positive probiotics minimize the chance of transfer of antibiotic resistant genes [13,54]. Both *B. subtilis* Ba37 showed resistance pattern towards sixteen antibiotics confirms the fact that it bears a minimum number of antibiotic resistance genes and thus only a trifling possibility of respective gene transmission when used as a probiotic strain. Therefore the use of a gram positive bacteria with non transferable antibiotic resistance genes suggests their safety for using in aquaculture applications.

This is the first report of using 5 mM potassium chromate in nutrient agar as differential/specific medium for the growth of *B. subtilis* Ba37 while the same medium will inhibit the proliferation of *V. anguillarum* 101. We have observed that *V. anguillarum* 101 cannot grow in nutrient agar supplemented with 5 mM potassium chromate whereas *Bacillus* can flourish in the same medium. This property was utilized to differentiate

the count between two organisms used in co-culture. Results of the co-culture experiment showed that the growth of *V. anguillarum* O1 was controlled when the concentration of *B. subtilis* Ba37 increased under *in vitro* conditions. The growth of the pathogens was successfully inhibited by antagonistic bacteria in co-culture, where the inhibitory activity enhanced with an increase in biomass of *B. subtilis* Ba37 (Fig. 5). This may be mainly due to the increase in the production of bacteriocins, organic acids or any other metabolites produced by the probiotic or by preventing the pathogens from adhering and establishing in the host by competitive exclusion [55]. Even if with a little long lag period and doubling time, *B. subtilis* Ba37 exhibited a proven ability to control the growth of pathogens. This revealed the higher inhibitory potential of the selected strain against the pathogen. This result further leads to the efficiency of antibacterial metabolites produced by the strain even in lower concentrations will be sufficient enough to inhibit the pathogen [15,29].

The primary requisite of an isolate to be used as a probiotic strain in aquaculture should be its non pathogenic character towards the host [17]. Cell free supernatant obtained from *B. subtilis* Ba 37 exhibited >95% viability on koi carp cell line discloses the non-toxic nature of antagonistic metabolite of the isolates. Our study further endorses that *B. subtilis* MBTDCMFRI Ba37 has no haemolytic activity as it lacks the zone formation on blood agar plates thereby reducing the chance of host infection affirms its non-pathogenic nature [2] This further helps to prove the plausible character of *B. subtilis* Ba37 as a good probiotic which can be used in aquatic systems. *In vitro* analysis is only considered as a preliminary screening tool to establish the probiotic character of selected strains as it may vary according to the experimental conditions when administered in a host. It is thus imperative to assess the *in vivo* aspects of the strain to confirm its safety to host in using as a candidate probiotic in aquaculture system [22,49]. The administration of *B. subtilis* Ba37 did not cause any harmful effects, disease signs or mortality in juveniles of *E. suratensis* even at a dose of 10^6 CFU/mL⁻¹ affirming the safety of the selected probiotic isolates. The present study revealed that the growth, immunity and survival rate of the host were enhanced by the administration of *B. subtilis* Ba37 along with the basal diet. The significantly higher value observed for percentage weight gain, SGR, PER, FE and survival percentage to the fishes fed with the basal diet and *B. subtilis* Ba37 indicated its efficiency to improve the growth rate of the host.

Our results showed that the administration of diet incorporated with *B. subtilis* Ba 37 significantly increased the SOD as well as catalase activities in the juveniles of *E. suratensis*. SOD and CAT are antioxidant enzymes present in fish cells, which protect them from oxidative damages [18,19]. This implies the ability of *B. subtilis* Ba37 to reduce the oxidative damage by improving the antioxidant enzyme levels in the host. Our results indicated that the fish supplemented with *B. subtilis* Ba37 diet recorded a significantly higher level of total protein in the sample. The protein level is related to the defensive mechanism level of fish against infectious diseases [19]. Thus it is evident that the supplementation of *B. subtilis* Ba37 in the diet will enhance the immunity of fishes against pathogens. Other researchers also identified that fish *Catla catla* fed with *B. subtilis* containing diet had improved their protein levels [56,57]. Alkaline phosphatase is considered as an antibacterial enzyme with hydrolytic activity [58]. The enhanced alkaline phosphatase activity in the group fed with diet containing *B. subtilis* Ba37 indicated the increased expression of this enzyme by macrophages leading to improved antibacterial potential [18,59]. It was reported by other researchers that the ALP activity of fishes was increased by feeding diets supplemented with *Bacillus licheniformis* Dabhl and *Lactococcus rhamnosus* [19,60]. The higher level of SGOT and SGPT in normal conditions is the indication of liver damage in fish due to oxidative damage or by pathogenic infections [20]. In our studies, the SGOT and SGPT activities of the group fed with the diet containing *B. subtilis* Ba37 was lower than that of the group fed with basal diet indicated the absence of any adverse effect on host animals. It is clear from the above observation that

B. subtilis Ba37 is a good growth promoter and immune enhancer. Many other researchers also reported the similar findings [15,29,61]. The successful reisolation of the probiotic candidate *B. subtilis* Ba37 from the gut of experimental animals after experimentation indicated its ability to colonize on its gastrointestinal tract. This reveals its probiotic character due to which it may benefit the host by reducing the growth of harmful or unwanted bacteria [62].

The challenge experiments are useful in evaluating the effectiveness of the used probiotic bacteria to the host animal against pathogens infection. It was observed that the supplementation of *B. subtilis* Ba37 significantly increased the survival of *E. suratensis* challenged against *V. anguillarum* O1 (Fig. 9). The higher survival rate recorded with the fish fed with basal diet and *B. subtilis* Ba37 indicated its enhanced resistance against aquaculture pathogens. Other researchers also reported similar observations that the use of *B. subtilis* and other *Bacillus* species improved the immunity of *Oreochromis niloticus*, *Oreochromis mossambicus* etc when challenged against *S. agalactiae* and *A. hydrophila* [19,61]. The elevated growth, immune responses and antioxidant status observed with the experimental animals may be due to the intake of probiotic supplemented diet which in turn enhanced their resistance against *V. anguillarum* O1.

5. Conclusion

In general, the present study revealed that the *B. subtilis* Ba37 possess unique properties such as extensive antibacterial property towards aquatic pathogens, tolerance to a wide range of physico-chemical conditions and suppress the *Vibrio* population on co-culture assay. Moreover, the administration of *B. subtilis* Ba37 showed better growth performances, enhanced immune responses and antioxidant activities resulted in higher relative percentage survival in juveniles of *E. suratensis* after challenging with *V. anguillarum* O1. It is concluded that *B. subtilis* Ba37 is an ideal probiotic strain to be used in aquatic rearing systems to improve the resistance against pathogenic infections, growth as well as the immune parameters of the host.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Acknowledgements

The authors are thankful to the Indian Council of Agricultural Research (ICAR) for funding the study through the Application of Microorganisms in Agriculture and Allied Sectors (AMAAS project). We also express our sincere gratitude to the Director, ICAR- Central Marine Fisheries Research Institute for providing necessary facilities to carry out the research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2020.104610>.

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Antibacterial assay guided isolation of a novel hydroxy-substituted pentacyclo ketonic compound from *Pseudomonas aeruginosa* MBTDCMFRI Ps04

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Received: 3 May 2020 / Accepted: 12 November 2020

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Abstract

Antimicrobial compounds from the natural source have gained greater relevance because of their wide spectrum of possible applications, especially in the aquaculture industry where pathogenic threat and antibacterial resistance are serious concerns. In this regard, *Pseudomonas aeruginosa* MBTDCMFRI Ps04 (*P. aeruginosa* Ps04) strain isolated from the tropical estuarine habitats of Cochin was evaluated for its antibacterial potential against major aquaculture pathogens. The physiological conditions for the maximum production of the active metabolite were also optimized. An activity-guided approach was employed further to isolate and characterize the secondary metabolite responsible for the inhibitory potential. It was found that the cell free supernatant (CFS) of *P. aeruginosa* Ps04 exhibited strong antibacterial activity against major aquaculture pathogens belonging to the genus *Vibrio* and *Aeromonas* and retained its potential even at 30% (v/v) dilution. The highest antibacterial activity was detected from 3rd day culture, grown in glycerol alanine media (1% each) as carbon and nitrogen source, respectively, at 30 °C, pH 7.0 and at a salinity of 20 parts per thousand (ppt). The activity of the antagonistic principle was found to be stable against variations in pH (pH 2–pH 12), temperature (up to 120 °C) and enzymatic treatments. Bioassay-guided purification followed by spectroscopic characterization of active fractions of *P. aeruginosa* Ps04 revealed that the compound 4-Hydroxy-11-methylpentacyclo [11.8.0.0^{2,3}.0^{11, 12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one is responsible for its major antibacterial activity. The results of this study indicated that *P. aeruginosa* Ps04 has beneficial antibacterial properties which could be used in developing novel antimicrobial therapeutics against a variety of aquaculture pathogens.

Keywords *P. aeruginosa* Ps04 · Antibacterial compound · FT-IR, LC-MS, NMR

Introduction

Bacterial disease outbreaks are considered a major threat which adversely affects the aquaculture industry by infecting almost all animals irrespective of their size and environment. Antibiotics and other chemicals are generally used to control

or prevent such infectious diseases. However, the use of antibiotics leads to generate antimicrobial resistance in host animals and other environmental related issues [1, 2]. The use of probiotic organisms was considered an effective, safe and promising alternative in disease control against aquaculture pathogens [3, 4]. Prophylactic administration of such biocontrol agents was used to enhance the health, immune response and metabolism of the host animals. Supplementation of such biocontrol agents was considered an effective measure to suppress the pathogenic proliferation as well as to enhance the host resistance by synthesizing a wide range of bioactive compounds with varied properties [5, 6].

Several studies specified the use of *Pseudomonas* spp. as an effective biocontrol agent against many of the infectious diseases in both plants and animals [7–10]. *Pseudomonas* strains were well-known resource of various bioactive compounds which inhibited the growth of pathogens belonging to genus *Vibrio*, *Aeromonas*, *Photobacterium*, *Staphylococcus*,

Responsible Editor: Fernando R. Pavan

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etc. [11–14]. The compounds isolated from *Pseudomonas* were structurally diverse like pseudopeptides, pyroles, phloroglucinol, quinolone, quinoline, moiramides and benzaldehyde which further include antimicrobial compounds like phenazine and phthalate derivatives [12, 13, 15]. These antimicrobial compounds efficiently act on pathogens causing cell lysis, nitrous oxide or acetyl-CoA carboxylase inhibition [14]. Among various *Pseudomonas* sp., *P. aeruginosa* is the most studied strain due to their high abundance and metabolic diversity [4, 16–18]. Studies showed that *P. aeruginosa* produced a variety of compounds such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide, (2E, 5E)-phenyltetradeca-2,5-dienoate and pyorubrin with biological potential [13, 15, 19, 20]. It was also known to produce a wide range of secondary metabolites with antimicrobial potential like pyocyanin, phenazines and 2-n-heptyl-4-hydroxyquinolinol [12, 21–23].

P. aeruginosa isolated from the marine habitats were not studied much as compared with the terrestrial isolates which harbour a variety of bioactive compounds. Moreover the ecological diversity offered by the marine environment may enrich the *Pseudomonas* spp. with the untapped resource of bioactive metabolites which need to be explored further [14, 18]. Antimicrobial activity of *P. aeruginosa* derived from the marine habitats was as effective as synthetic antibiotics like tetracycline and bacitracin [24]. In this regard, our study aimed to isolate and characterize the potent antibacterial compound from a marine isolate of *P. aeruginosa* Ps04 with a broad spectrum of antibacterial activity. The study also focused to optimize the fermentation medium and physiological conditions for mass productions of antibacterial metabolites. The findings of this study could help in identifying novel therapeutic agents that may find its application in aquaculture to control bacterial infections.

Materials and methods

Microbial strains

A marine bacterium *Pseudomonas aeruginosa* MBTDCMFRI Ps04 (*P. aeruginosa* Ps04) with demonstrated antimicrobial activity against aquaculture pathogens was selected for this study [3]. The isolate was characterized using morphological and biochemical characteristics and 16S ribosomal RNA (rRNA) gene sequencing method [3, 25]. Spot diffusion, well diffusion and disc diffusion assays were used to establish the antibacterial potential of *P. aeruginosa* Ps04 against aquatic pathogens [3, 10, 26]. The culture was spotted on pre-swabbed Mueller Hinton agar (MHA) plates with test organisms (pathogens) for spot diffusion assays.

Filter-sterilized CFS of the culture was used for well diffusion and disc diffusion assays. The test organisms used for antibacterial assay were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101 (courtesy Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (courtesy National Centre for Aquatic Animal Health, Cochin University of Science and Technology) *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (courtesy Microbial Type Culture Collection, Chandigarh, India). All the bacterial isolates were maintained with the Microbial Culture Collection of Marine Biotechnology Division (MCC-MBTD), Central Marine Fisheries Research Institute (CMFRI). All the bacterial isolates were preserved at -80°C as glycerol stocks.

Optimization of medium and physiochemical conditions

P. aeruginosa Ps04 streaked on nutrient agar supplemented with 50% seawater (SW) and incubated at $30 \pm 2^{\circ}\text{C}$ for 24 h to obtain a pure colony. The pure colony thus obtained was inoculated into 5 mL of nutrient broth (with 50% SW) and kept for incubation (at 30°C , 200 rpm, for 20–24 h) on a rotary shaker (New Brunswick Scientific Co. Inc., NJ, USA). To optimize the medium, one percentage (1%) of this overnight grown culture (starter culture) was inoculated to 100 mL of different mediums (in 50% SW) like nutrient broth, Zobells marine broth, tryptone soya broth and glycerol alanine medium. The composition of glycerol alanine medium was used (1 L containing 10.0 g DL-alanine; 20 mL glycerol; 0.139 g dipotassium hydrogen phosphate (K_2HPO_4); 4.06 g magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$); 14.2 g sodium sulphate (Na_2SO_4); 0.01 g ferric citrate at pH 7 ± 0.2) as described in the previous study [23]. All the flasks were incubated for a period of 7 days (at 30°C at 200 rpm) in duplicates. One millilitre of the culture was drawn at every 24-h interval to check the cell count and antibacterial assay (by disc diffusion method). The cell counts were determined by serial dilution.

The optimal physiochemical conditions for growth and production of the antibacterial compound by *P. aeruginosa* Ps04 were assessed by growing the culture in the optimized glycerol alanine medium at various temperatures (25, 30, 35 and 40°C), pH (6.0, 7.0, 8.0 and 9.0), salinity (5, 10, 15, 20, 25, 30 and 35 ppt), carbon sources (glycerol and glucose at varying concentrations from 0.5–1%) and nitrogen sources (alanine and glutamic acid at varying concentrations from 0.5–1%) for a period of 7 days (at 30°C at 200 rpm) in duplicates. Samples for cell count and antibacterial activity were drawn at every 24 h.

Preliminary characterization of antibacterial metabolites

Stability of the CFS of *P. aeruginosa* Ps04 towards heat, pH and enzyme treatments was carried out. To analyse the thermal sensitivity, the CFS of *P. aeruginosa* Ps04 was kept in a water bath for 30 min at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C and autoclaved for 15 min at 120 °C. CFS of *P. aeruginosa* Ps04 kept at 37 °C was used as control. Each of the heat treated and control samples was tested for antibacterial activity by the disc diffusion method. The stability of CFS obtained from *P. aeruginosa* Ps04 on lysozyme and proteolytic enzymes like proteinase K and trypsin treatments was tested using 1, 2 and 5 mg/mL concentration of enzymes. After 2 h incubation at 37 °C, enzymatic action was inactivated by keeping in a boiling water bath for 5 min [27, 28]. Antibacterial activities of the treated samples were tested against pathogens using the disc diffusion method with CFS of *P. aeruginosa* Ps04 as control. The pH stability was carried out by adjusting the pH of the CFS of *P. aeruginosa* Ps04 from 2 to 12 using HCl (1 molL⁻¹) and NaOH (1 molL⁻¹), with the help of a pH meter, and incubating for 2 h. CFS of *P. aeruginosa* Ps04 was used as control and the antibacterial activity was determined by disc diffusion method [27]. Effect of dilution was detected by diluting the CFS of *P. aeruginosa* Ps04 up to 10% (v/v) using sterile phosphate buffered saline, pH 7.4 [10]. The inhibitory activity against pathogens was tested by the disc diffusion method.

Extraction of crude bioactive metabolite from *P. aeruginosa* Ps04

P. aeruginosa Ps04 was inoculated into 200 mL of glycerol alanine medium (pH 7; 20 ppt salinity) and incubated on a rotary shaker overnight (New Brunswick Scientific Co. Inc., NJ, USA) (30 °C at 200 rpm) for 3 days to prepare the starter culture. One percentage of starter culture was inoculated to 10 L of glycerol alanine medium (20 ppt salinity, pH 7) and kept for incubation on a rotary shaker (at 30 °C in 150 rpm). After 3 days of incubation, the culture was centrifuged (8000 rpm for 15 min at 4 °C), and the supernatant was filter-sterilized (0.2 µm). The supernatant thus obtained was lyophilized to obtain 10 g of powder which was further extracted using ethylacetate (EtOAc)/methanol (MeOH) (1:1, v/v) mixture and refluxed for 2.5 h. The extract was then filtered through anhydrous sodium sulphate (Na₂SO₄). The filtrate thus obtained was evaporated (40 °C) using a rotary evaporator (Buchi, Germany) under vacuum to dryness at a temperature below 42 ± 2 °C to obtain a crude dark pinkish oily residue (4.5 g).

Purification of antibacterial compound

The crude residue obtained was initially separated and evaluated using thin layer chromatography (TLC). The presence of compounds was analysed on precoated silica gel TLC plates (TLC Silica gel 60, Merck Millipore) using various solvent systems and identified dichloromethane (DCM) methanol (MeOH) gradient at 95:5 ratio (v/v) as solvent system. The compounds separated on TLC plate were observed in UV chamber by using 254 nm UV light. Thus it is identified that DCM - MeOH gradient is effective for column elution. Thus the sample was eluted through the silica column initially with DCM then with a gradient of DCM/MeOH followed by 100% MeOH to get 14 fractions. The antibacterial activity was evaluated using disc diffusion method and pooled according to their TLC profile to get four fractions. The third and fourth fractions (PA₃₋₄, 2.834 g) with similar TLC profiles and higher antimicrobial potential were pooled together and labelled as PA₃. PA₃ was re-purified using column chromatography (silica) with solvents n-hexane, then the gradient of hexane with increasing concentration of EtOAc followed by a gradient of EtOAc with increasing concentration of MeOH and finally with 100% MeOH to get six fractions. The antibacterial activity potential was assayed and found that the third fraction (PA_{3.3}, 879 mg) exhibited higher inhibitory potential and hence purified further using preparative TLC plate (silica) using 0.5% MeOH in DCM to get another six fractions according to TLC profile. The sixth fraction (PA_{3.3.6}, 52 mg) exhibited the highest antibacterial potential, and good purity (according to TLC profile) was selected for spectroscopic characterization. The purified fraction (PA_{3.3.6}) with antibacterial activity was concentrated by a rotary evaporator and purged with nitrogen for final drying. All solvents used were of analytical or chromatographic reagent grade and were obtained from E-Merck (Darmstadt, Germany).

Structural characterization of the antibacterial compound

Fourier transform infrared (FT-IR) spectrophotometry

The purified fraction (PA_{3.3.6}) was subjected to FT-IR (Fourier transform infrared) spectrometric analysis to trace the spectra of the compounds. The sample was mixed thoroughly with KBr powder, and KBr pellets were formed. The surface of the pellets was exposed to infrared radiation to get the spectra and was recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted into mid IR range, i.e. between 4000 and 400 cm⁻¹.

Liquid chromatography - mass spectroscopy (LC-MS)

The LC-MS analysis of purified fraction (PA_{3.3.6}) was carried out on Quattro Premier XE (Micromass), an electrospray ionization (ESI) - triple quadrupole mass spectrometer, which is attached to Acquity UPLC (Waters). For each run, 5 µL of samples was injected onto a C₁₈ column (Acquity UPLC BEH, 1.7 µm, 2.1 mm × 50 mm; Waters). A linear gradient elution was done utilizing water (H₂O; solvent A) and methanol (MeOH; solvent B) (0–20 min.: 50–90% B; 20–23 min.: 90% B; 23–25 min.: 90–50% B), and the flow rate was maintained at 0.2 mL min⁻¹. Eluents from the chromatographic column pass through photodiode array (PDA) detector and then enter into the mass spectrometer. Ultraviolet (UV) absorbance data in the range of 210–400 nm were recorded by the PDA detector. Mass spectrometric data were acquired in positive ion polarity of ESI, and the *m/z* range of detection was set to 50–1500. The ESI source parameters were capillary 3 kV, cone 30 V, extractor 5 V, source temperature 100 °C, desolvation temperature 300 °C, desolvation gas flow 800 l/h (L/h) and cone gas flow 50 L/h.

Nuclear magnetic resonance spectroscopy (NMR)

For nuclear magnetic resonance (NMR) spectrum analysis of the purified compound (PA_{3.3.6}), all data acquisitions and processing were facilitated by MassLynx (Waters) followed by nuclear magnetic resonance (¹H and ¹³C NMR) spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in deuterated chloroform (CDCl₃) as aprotic solvent at ambient temperature with tetramethylsilane (TMS) as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ¹³C NMR signals was determined from DEPT 135 experiments. Standard pulse sequences were used for DEPT 135, ¹H–¹H COSY, two-dimensional NOESY, HSQC and HMBC experiments.

Statistical analysis

One-way analysis of variance (ANOVA) was carried out with SPSS 20.0 (SPSS, USA, ver. 20.0) to assess for any significant differences between the means. The differences in means values were identified by Scheffe's multiple range tests to determine whether significant difference (*P* < 0.05) existed among different treatments.

Results

Antibacterial activity

P. aeruginosa Ps04 was gram negative, small rod-shaped (size ~0.5–0.8 µm X 1.5–3.0 µm) bacterium. The morphological characteristics of the colony were opaque, smooth, slightly undulate and medium in size. It produced bluish green pigmentation on nutrient agar (50% SW) after 48 h of incubation at 30 °C. Through biochemical and 16S ribosomal RNA (rRNA) gene sequencing methods, the isolate was identified as *P. aeruginosa*. The sequence was deposited in the GenBank database with accession number JF719801. *P. aeruginosa* Ps04 exhibited a significantly higher inhibitory potential (*P* < 0.05) against both *V. vulnificus* MTCC 1145, *V. anguillarum* O1 and *A. hydrophila* (zone of inhibition 19–22 mm). A lower inhibitory potential was observed against *V. alginolyticus* 101 and *V. parahaemolyticus* MTCC451 (zone of inhibition 14–16 mm) (Table 1).

Optimization of physiochemical conditions

The glycerol alanine medium was found to be the most suitable media for the growth and maximum production of the antibacterial metabolite from *P. aeruginosa* Ps04 (from 3rd day onwards) (Fig. 1a). The antibacterial compound production of *P. aeruginosa* Ps04 was observed in the late phase of culture (about 40 h) in the glycerol alanine medium. The activity reached the maximum in the stationary phase and remained stable for two more days with small flux in the activity. Glycerol (1%) and alanine (1%) were found to be the best carbon and nitrogen source, respectively, for optimum production of antimicrobial metabolite. The optimum temperature for the growth and production of the antibacterial component was recorded as 30 °C with a maximum inhibitory zone of 12–21 mm (from the 3rd day) against tested pathogens (Fig. 1b). There was variation in the growth of *P. aeruginosa* Ps04 at different temperatures (20–40 °C). The increased antibacterial compound production was achieved at 20 ppt salinity, and maximum antagonistic potential was observed (from the 3rd day) with a clearing zone of 13–21 mm against tested pathogens (Fig. 2a). It was observed that the growth of *P. aeruginosa* Ps04 was not much affected by variation in the salinity (0–30 ppt). The antibacterial metabolite production was highest at pH 7.0 followed by pH 8.0 (after 3 days of culture) with a clearing zone of 12–20 mm (Fig. 2b). The growth and inhibitory compound production by *P. aeruginosa* Ps04 were found to be not much affected by varying the pH.

Table 1 Antibacterial activity of *P. aeruginosa* MBTDCMFRI Ps04 against pathogens

Assay method	Zone of inhibition (mm)					
	Vvul	Vpar	A hyd	Valg	Vhar	Vang
Spot diffusion	21.67 ^{ap} ± 1.53	16.00 ^{aq} ± 1.00	21.33 ^{ap} ± 1.53	14.00 ^{aq} ± 1.00	20.33 ^{aq} ± 1.53	22.00 ^{ap} ± 1.00
Well diffusion	22.00 ^{ap} ± 1.00	16.00 ^{aq} ± 1.00	21.00 ^{ap} ± 1.00	14.33 ^{aq} ± 0.58	17.00 ^{bcq} ± 1.00	21.0 ^{ap} ± 1.00
Disc diffusion	20.67 ^{apw} ± 1.15	14.67 ^{aq} ± 0.58	19.00 ^{aprw} ± 1.00	16.00 ^{aqrs} ± 1.00	18.00 ^{acts} ± 1.00	22.00 ^{au} ± 1.00

Vvul - *Vibrio vulnificus* MTCC1145; Vpar - *V. parahaemolyticus* MTCC 451; A hyd - *Aeromonas hydrophila*; Valg - *V. alginolyticus* 101; Vhar - *V. harveyi* 101; Vang - *V. anguillarum* O1. The measurement indicates the inhibition zone (mm) formed around the isolates. Data are the mean values of triplicate and expressed as mean ± standard deviation. Values with different letters [for different diffusion assays (a–c) and for pathogens (p–u)] are significantly different ($P < 0.05$)

Preliminary characterization of antibacterial metabolites

The inhibitory potential of the CFS of *P. aeruginosa* Ps04 retained its activity by diluting to 30% against all the tested aquaculture pathogens except *V. alginolyticus* 101 (Table 2). Diluting the CFS even up to 10% was also noted to be effective against the growth of *V. anguillarum* O1 and *V. vulnificus* MTCC1145. In all the tested pH range, it was observed that the CFS of *P. aeruginosa* Ps04 was effective ($P < 0.05$) against *V. harveyi* 101, *V. anguillarum* O1 and *V. vulnificus* MTCC1145. The results also revealed that the effectiveness of the CFS to reduce the growth of the tested pathogens was better in neutral pH range (pH 6–8). The inhibitory effect was lower ($P > 0.05$) against *V. alginolyticus* 101 and *V. parahaemolyticus* MTCC 451 (Table 2). Temperature variation studies followed the same trend as observed in pH that the CFS of *P. aeruginosa* Ps04 retained its potential from room temperature to 120 °C without any significant reduction in its activity (Table 2). The antimicrobial compound was found to be stable (15–22 mm) when treated with lysozyme, proteinase K and trypsin (Table 2).

Purification of antibacterial compound

The crude residue was chromatographed over silica column (60–120 mesh) with a stepwise gradient of solvents from DCM, DCM:MeOH and finally MeOH (100%, 50%, 30% and 100%). The fractions were collected and concentrated under vacuum. TLC was evaluated, and fractions with the same TLC profile were pooled together to furnish four fractions (PA_{1–4}). The subfraction PA₃ and PA₄ which exhibited high antibacterial activity (zone size of 16–28 mm against various pathogens) was collected together and purified using column chromatography with *n*-hexane, *n*-hexane:EtOAc, EtOAc, EtOAc:MeOH and finally with MeOH (100:0, 50:50, 75:25) to give another six subfractions. After concentrating, all fractions were checked for antagonistic activity and TLC evaluation. The subfraction PA_{3,3} exhibited more

antibacterial activity (zone size of 14–23 mm) against various pathogens. The subfraction PA_{3,3} was re-purified using preparative thin layer chromatography over Si gel GF₂₅₄ (particle size 15 µm) (0.5% MeOH:DCM) to obtain six subfractions of which the subfraction PA_{3,3,6} exhibited higher antibacterial potential and was found to be pure. Its R_f value was 0.37. This compound was selected for structural analysis and characterization using spectroscopic methods.

Structural characterization of antibacterial compound

The FTIR spectra of the compound showed major bands at 3396.01 cm⁻¹, 2928.88 cm⁻¹, 1726.24 cm⁻¹, 1510.88 cm⁻¹, 1726 cm⁻¹ and 1046.43 cm⁻¹ (Fig. 3). The LC-MS analysis of the compound exhibited a base peak at $m/z = 359.46$ (100%). The molecular ion peak was observed at 321.76 ([M + H]) (9.28%). Other major fragment peaks at $m/z = 321.76$, 313.89 (11.4%), 280.07 (12%), 137.48 (25.29%), 124.53 (24.29%) and 105.25 (18.75%). Proton NMR analysis of the compound revealed three sets of peaks between δ 7–8, a singlet at δ 6.2, and different sets of multiplets between δ 0.8–4.8 (Supplementary Fig. 1). Evaluation of the ¹³C spectra of the compound contained 22 carbon signals ranging from δ 14 to 171 (Supplementary Fig. 2). The seven CH₂ signals between δ 20 and 70 were confirmed by evaluating the DEPT 135 spectrum (Supplementary Fig. 3). The ¹H-¹H and ¹H-¹³C correlations were evaluated using ¹H-¹H COSY (Supplementary Fig. 4), HSQC and HMBC spectrums.

Amorphous brownish solid; UV (MeOH) $\lambda_{\max}$ (log ϵ): 273nm; TLC (Si gel GF₂₅₄ 15 mm; Chloroform (CHCl₃)/MeOH 10:90, v/v) R_f : 0.37; LC (Column: Acquity UPLC BEH C18, 1.7 µm, 2.1 mm × 50 mm (Waters); gradient elution using H₂O (solvent A) and MeOH (solvent B) for 25 min: 50% B - 90% B in 20 min, hold at 90% B for 3 min, and 90% B - 50% B in 2 min; flow rate: 0.2 mL/min; volume of sample injection: 5 µL; elemental analysis found: C, 82.46; H, 7.55; O, 9.99; IR (KBr, cm⁻¹). $\nu_{\max}$ 762.18 cm⁻¹ γ_r (C-H alkanes), 1510.88 cm⁻¹ and 1596.11 cm⁻¹ ν (C-C aromatic ring), 1726.24 cm⁻¹ ν (C=O), 2928.88 cm⁻¹ ν (C-H alkanes)

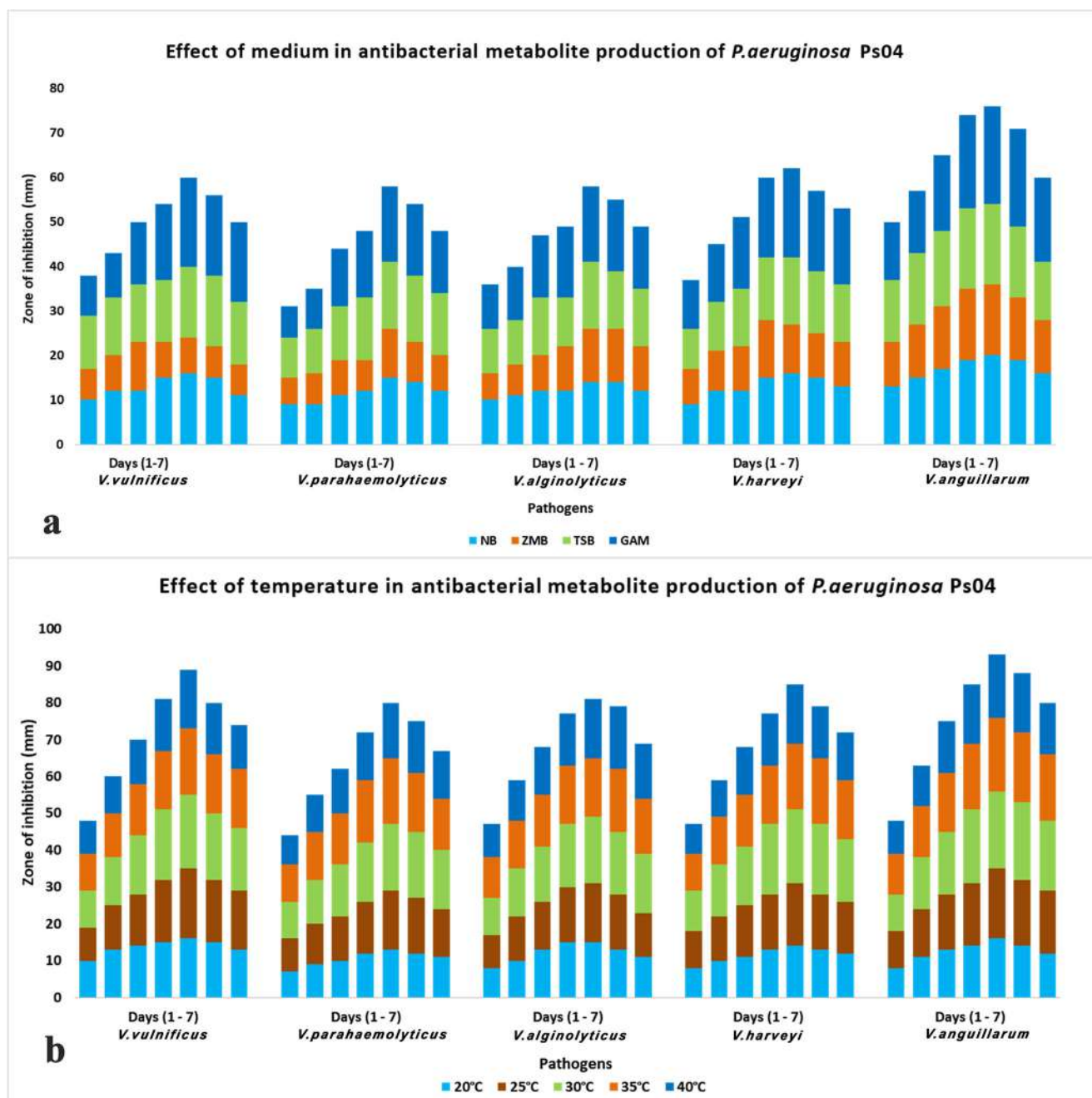


Fig. 1 Effect of medium (a) and temperature (b) for the production of antibacterial metabolite in *P. aeruginosa* MBTDCMFRI Ps04. NB- nutrient broth, ZMB- Zobells Marine agar, TSB- tryptone soya broth, GAM- glycerol alanine medium

and 3396.01 cm^{-1} ν (C-H alkanes). ^1H NMR (CDCl_3 , 500 MHz, δ ppm) and ^{13}C NMR (CDCl_3 , 125 MHz, δ ppm) data (Table 3); ESI MS m/z (rel. Int. %): 350.58 (M+Na) (100%), 321.76 ([M+H]) (9.28%), 313.89 (11.4%), 280.07 (12%), 137.48 (25.29%), 124.53 (24.29%) and 105.25 (18.75%). From the above data, the structure of the antibacterial compound was proposed to be 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosal, 1,3,5,8(9), 17-penten-14-one, molecular formula was determined to be $\text{C}_{22}\text{H}_{24}\text{O}_2$ (Fig. 4).

Discussion

Bacterial infections are one of the major challenges faced by the aquaculture sector which will devastate its contribution towards the global food production sector. The remedial measures practiced against these infections, such as the usage of chemicals or antibiotics, are inappropriate concerning their adverse health impacts on the host and to the environment. Antimicrobial metabolites derived from natural resources can be considered a green and safe alternative against synthetic

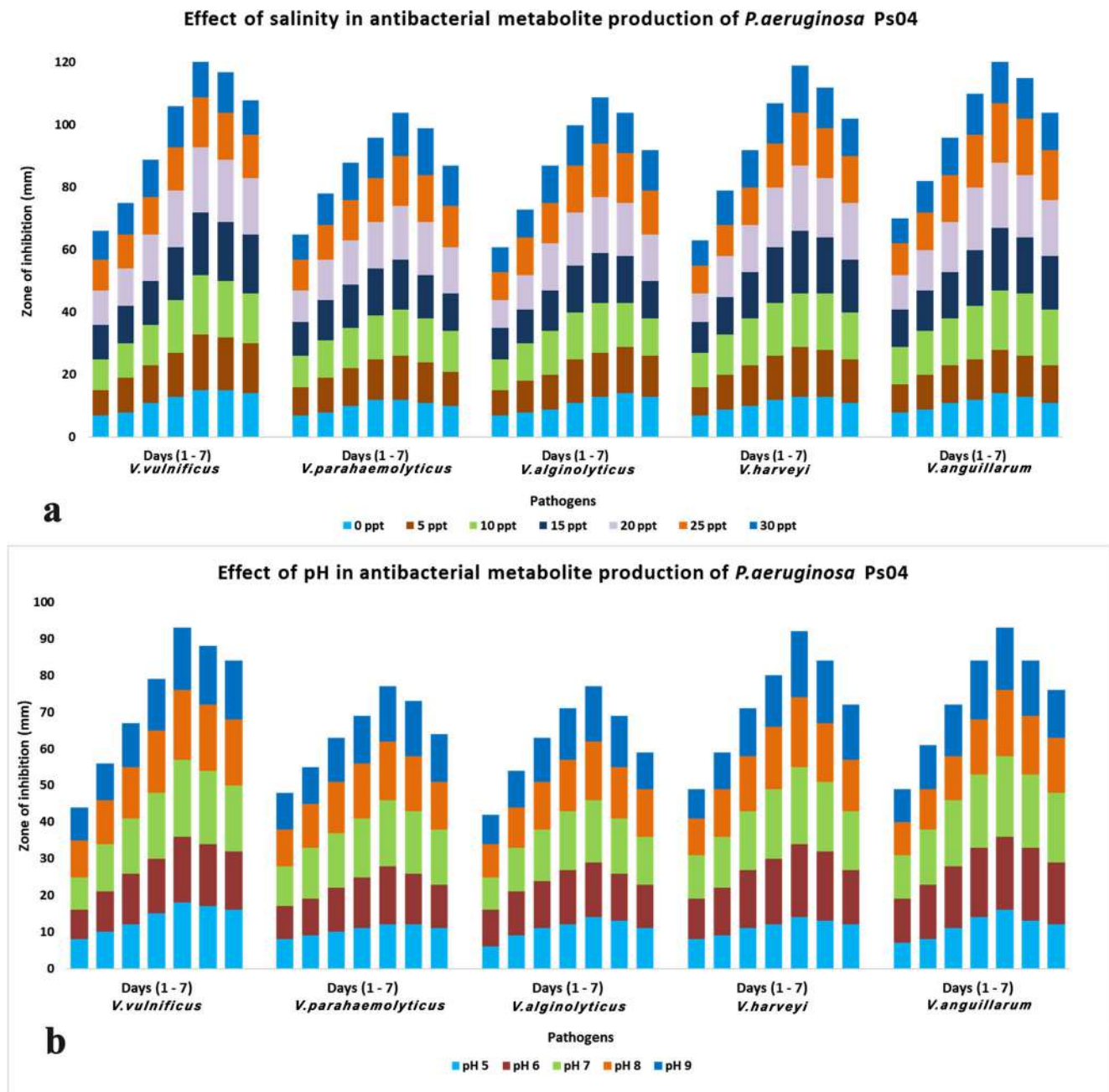


Fig. 2 Effect of salinity (a) and pH (b) for the production of antibacterial metabolite in *P. aeruginosa* MBTDCMFRI Ps04

compounds to suppress these bacterial outbreaks [2, 3, 15]. On this account, *P. aeruginosa* could be considered an important species with high metabolic diversity having ecological and clinical significance [17, 18, 29]. Their structurally diverse secondary metabolites are already known to have inhibitory potential against aquaculture pathogens especially belonging to *Vibrio* and *Aeromonas* [3, 30, 31]. Thus *P. aeruginosa* Ps04 can be well explored to be used as an antibacterial agent in aquaculture. Our study revealed that the strain *P. aeruginosa* Ps04 isolated from the tropical estuarine habitats displayed antibacterial activity against the tested aquaculture pathogens. Thus, we have employed activity-

guided isolation and characterization of the antibacterial secondary metabolite from *P. aeruginosa* Ps04 with high inhibitory potential against aquaculture pathogens.

The productions of secondary metabolites by the organisms are greatly influenced by the physical and chemical environment and their growth conditions [7, 32–35]. Thus, we have addressed the effects of fermentation medium, incubation time and fermentation conditions such as pH, salinity and temperature to ensure the enhanced production of the antibacterial metabolite from *P. aeruginosa* Ps04. Glycerol alanine medium maintained at 30 °C, pH 7.0 and a salinity of 20 ppt was found to be most suitable for the maximal antibacterial

Table 2 Preliminary characterization of the cell-free supernatant of *P. aeruginosa* MBTDCMFRI Ps04

Dilution	Zone of inhibition (mm)				
	A	B	C	D	E
10%	0 ^a ± 0	0 ^a ± 0	1.33 ^{ab} ± 0.58	2.00 ^b ± 0.58	0 ^a ± 0
20%	0 ^a ± 0	0 ^a ± 0	3.00 ^b ± 0.58	3.33 ^b ± 1.53	0 ^a ± 0
30%	6.67 ^a ± 0.58	0 ^b ± 0	6.00 ^{ab} ± 1.53	6.33 ^a ± 1.15	2.33 ^c ± 0.58
40%	7.67 ^a ± 0.58	3.33 ^b ± 1.00	7.67 ^a ± 1.00	7.67 ^a ± 1.53	6.67 ^a ± 1.00
50%	8.33 ^{ab} ± 0.58	6.00 ^a ± 1.15	9 ^b ± 1.53	10.67 ^b ± 2.08	6.00 ^a ± 1.53
60%	7.33 ^a ± 1.15	7.67 ^b ± 1.00	10.67 ^b ± 1.00	12.00 ^b ± 1.53	6.67 ^a ± 1.00
70%	8.00 ^a ± 1.53	8.00 ^a ± 0.58	11 ^b ± 1.15	11.33 ^b ± 1.53	7.00 ^a ± 1.73
80%	11.00 ^a ± 1.00	7.67 ^b ± 1.53	15 ^b ± 1.53	15.67 ^b ± 1.15	8.67 ^a ± 1.53
90%	12.33 ^a ± 1.15	8.00 ^b ± 1.15	16.33 ^c ± 1.53	16.00 ^c ± 1.53	9.00 ^{ab} ± 1.53
Control (100%)	14.67 ^a ± 0.58	10.00 ^b ± 1.53	17.33 ^c ± 2.00	16.00 ^a ± 1.53	10.33 ^b ± 1.15
Temperature	Zone of inhibition (mm)				
	A	B	C	D	E
40 °C	16.00 ^{ap} ± 1.00	10.00 ^{bp} ± 1.00	14.67 ^{ap} ± 1.15	14.00 ^{apqr} ± 1.00	13.33 ^{abp} ± 1.53
50 °C	15.67 ^{apq} ± 1.15	9.33 ^{bp} ± 0.58	14.00 ^{apq} ± 1.00	14.33 ^{apq} ± 1.15	13.00 ^{apqr} ± 1.00
60 °C	14.33 ^{apq} ± 0.58	9.00 ^{bp} ± 1.00	13.67 ^{apq} ± 0.58	14.00 ^{apqr} ± 1.00	12.33 ^{apqr} ± 0.58
70 °C	14.00 ^{apq} ± 1.00	9.00 ^{bp} ± 1.00	13.33 ^{acpq} ± 0.58	13.00 ^{acpqr} ± 1.00	11.33 ^{bcpqr} ± 0.58
80 °C	13.00 ^{apq} ± 1.00	8.67 ^{bp} ± 0.58	12.00 ^{apq} ± 1.00	12.33 ^{apqr} ± 0.58	11.00 ^{abpqr} ± 1.00
90 °C	12.67 ^{apq} ± 1.53	8.33 ^{bp} ± 0.58	12.67 ^{apq} ± 1.53	11.33 ^{abpqr} ± 0.58	10.67 ^{abpqr} ± 0.58
100 °C	12.00 ^{aq} ± 1.00	8.00 ^{bp} ± 1.00	12.00 ^{apq} ± 1.00	11.00 ^{abqrs} ± 1.00	10.00 ^{abpq} ± 1.00
120 °C	12.00 ^{aq} ± 1.00	7.67 ^{bp} ± 0.58	10.67 ^{aq} ± 1.53	10.67 ^{ar} ± 0.58	9.67 ^{abqr} ± 0.58
Control (RT)	15.67 ^{apq} ± 0.58	10.33 ^{bp} ± 1.15	15.00 ^{ap} ± 1.00	14.67 ^{ap} ± 1.53	14.00 ^{ar} ± 1.00
pH	Zone of inhibition (mm)				
	A	B	C	D	E
pH 2	12.67 ^{ap} ± 1.53	5.00 ^{bp} ± 1	13.67 ^{ap} ± 1.53	11.00 ^{ap} ± 1.00	5.67 ^{bp} ± 0.58
pH 3	14.33 ^{ap} ± 1.15	5.67 ^{bp} ± 1.15	14.00 ^{ap} ± 1.00	12.00 ^{apq} ± 1.00	6.33 ^{bp} ± 0.58
pH 4	15.00 ^{ap} ± 1.00	7.33 ^{bpq} ± 0.58	15.33 ^{ap} ± 0.58	14.33 ^{apqrs} ± 1.53	6.67 ^{bpq} ± 0.58
pH 5	13.33 ^{ap} ± 1.15	7.33 ^{bpq} ± 1.53	15.67 ^{ap} ± 1.53	14.67 ^{apqrs} ± 0.58	6.67 ^{bpq} ± 0.58
pH 6	15.00 ^{ap} ± 1.00	8.33 ^{bpq} ± 1.15	16.67 ^{ap} ± 1.15	15.33 ^{aqrs} ± 0.58	7.67 ^{bpq} ± 1.15
pH 7	15.67 ^{ap} ± 0.58	8.33 ^{bpq} ± 1.53	16.33 ^{ap} ± 1.15	16.67 ^{aqrs} ± 0.58	8.00 ^{bpq} ± 1.00
pH 8	16.33 ^{ap} ± 1.15	11.33 ^{bq} ± 1.53	17.33 ^{ap} ± 1.15	18.00 ^{aqrs} ± 1.00	10.00 ^{bq} ± 1.00
pH 9	14.67 ^{ap} ± 1.53	9.67 ^{bpq} ± 2.08	17.33 ^{ap} ± 0.58	17.00 ^{aqrs} ± 1.00	8.00 ^{bpq} ± 1.00
pH 10	14.33 ^{ap} ± 0.58	9.67 ^{bpq} ± 1.53	16.33 ^{ap} ± 0.58	16.00 ^{aqrs} ± 1.00	7.33 ^{bpq} ± 0.58
pH 11	14.67 ^{ap} ± 1.53	9.33 ^{bpq} ± 0.58	15.67 ^{ap} ± 0.58	15.00 ^{aqrs} ± 1.00	6.00 ^{cp} ± 1.00
pH 12	13.67 ^{ap} ± 0.58	6.67 ^{bpq} ± 1.15	14.00 ^{ap} ± 1.00	13.33 ^{ap} ± 1.15	5.67 ^{bp} ± 0.58
Control	16.00 ^{ap} ± 0.58	9.00 ^{bpq} ± 1.00	17.67 ^{ap} ± 1.53	17.00 ^{aqrs} ± 1.00	9.00 ^{bpq} ± 1.00
Enzyme	Zone of inhibition (mm)				
	A	B	C	D	E
Lysozyme (1 mg/ml)	15.33 ^a ± 0.58	9.33 ^b ± 0.58	15.33 ^a ± 0.58	14.33 ^a ± 0.58	10.64 ^b ± 1.53
Lysozyme (2 mg/ml)	15.05 ^a ± 1.04	8.67 ^b ± 0.64	15.04 ^a ± 1.01	14.09 ^a ± 1.05	10.33 ^b ± 1.15
Lysozyme (5 mg/ml)	13.47 ^a ± 1.01	8.33 ^b ± 0.47	13.46 ^a ± 1.06	12.03 ^a ± 0.94	9.67 ^b ± 0.58
Trypsin (1 mg/ml)	15.67 ^a ± 1.15	9.09 ^b ± 1.03	15.67 ^a ± 0.58	13.06 ^{ac} ± 1.03	11.04 ^{bc} ± 1.05
Trypsin (2 mg/ml)	15.24 ^a ± 0.85	8.33 ^b ± 0.58	15.18 ^a ± 0.69	11.67 ^c ± 0.58	9.75 ^b ± 0.45
Trypsin (5 mg/ml)	15.03 ^{ac} ± 0.58	8.05 ^{ab} ± 1.02	14.23 ^c ± 0.33	10.45 ^{ac} ± 1.53	8.67 ^b ± 0.58
Proteinase K (1 mg/ml)	15.14 ^a ± 1.05	9.05 ^b ± 1.04	13.53 ^a ± 0.94	12.33 ^a ± 0.58	8.33 ^b ± 0.91
Proteinase K (2 mg/ml)	14.23 ^a ± 0.85	8.33 ^b ± 0.85	12.37 ^a ± 1.15	10.78 ^a ± 1.06	7.05 ^b ± 1.03
Proteinase K (5 mg/ml)	14.67 ^a ± 0.76	7.67 ^b ± 0.58	13.05 ^a ± 1.03	9.77 ^{ab} ± 1.06	6.03 ^b ± 1.08
Control	15.67 ^a ± 0.58	9.67 ^b ± 0.42	15.71 ^a ± 1.05	14.52 ^a ± 1.04	10.84 ^b ± 1.04

A - *Vibrio harveyi* 101; B - *V. alginolyticus* 101; C - *V. anguillarum* 01; D - *V. vulnificus* MTCC1145; E - *V. parahaemolyticus* MTCC 451. Data are the mean values of triplicate and expressed as mean ± standard deviation. In each section, values with different letters [(a-c) row wise and (p-s) column wise] are significantly different ($P < 0.05$)

compound production. An optimal production rate of the antimicrobial metabolite was observed between third to fifth days (Fig. 1a). Other studies supported our result that the antibacterial compound production from *P. aeruginosa* strain using similar glycerol alanine medium was initially less and reached optimum after 66 h [23]. Dilution of CFS obtained from *P. aeruginosa* Ps04 even up to 30% of its initial concentration was found to be effective against tested pathogens. A similar observation was reported where the culture supernatant from *Pseudomonas* sp. was effective against *V. harveyi* 101 at a concentration of 30% [10]. The antibacterial activity of CFS of *P. aeruginosa* Ps04 was not much affected significantly with pH, enzymes and temperature. This indicated that the antibacterial compound produced by the *P. aeruginosa*

Ps04 is stable against temperature, enzymes and pH variations. The inertness of the CFS to proteolytic enzymes like proteinase K and trypsin suggested that the compound responsible for the inhibitory potential is not proteinaceous in nature [10]. The optimized medium and conditions for the mass production of the bioactive secondary metabolite from *P. aeruginosa* Ps04 could be applied for various industrial applications.

Bacteriocins produced by the microorganisms with structural diversity are responsible for their antimicrobial potential in aquatic ecosystems [11, 36]. Phenazine alkaloids, diketopiperazines, pyocyanin, phenazine 1 carboxylic acid, phenazine carboxymide, phloroglucinol, pseudo peptide pyrrolidinedione, pyrroles, moiramides, pyorubrin, etc. are

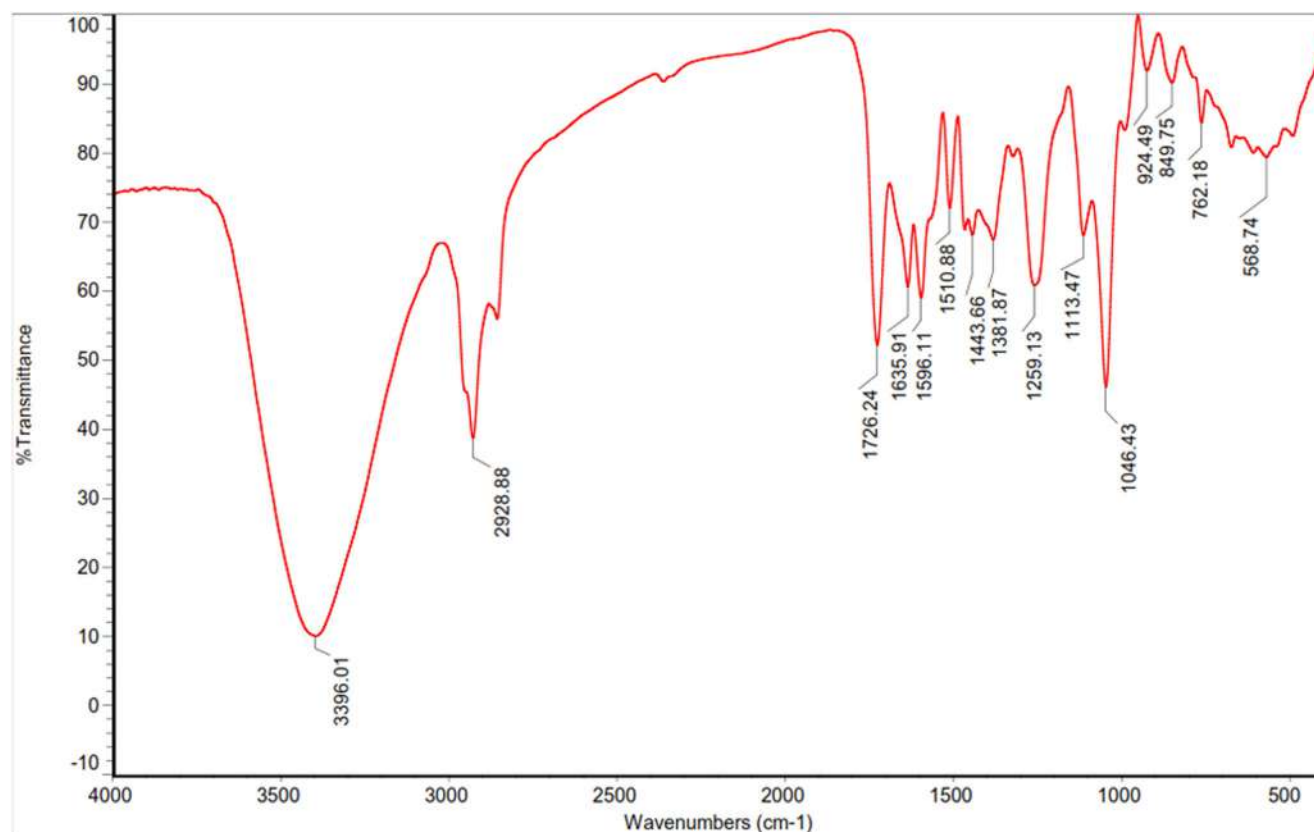


Fig. 3 FT-IR spectrum of 4-Hydroxy-11-methylpentacyclo [11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one

some of the secondary metabolites identified from the *Pseudomonas* sp. [12, 14, 15, 23]. These compounds may act against pathogenic infections in various ways [14, 37, 38]. Thus the isolation and characterization of the bioactive principle from *P. aeruginosa* Ps04 responsible for its antibacterial property is important in terms of its application in various sectors. The bioassay-guided isolation and characterization of the CFS of *P. aeruginosa* Ps04 furnished a novel ketonic compound with high antibacterial activity.

The molecular formula of the bioactive compound was assigned to be C₂₂H₂₄O₂, based on their FT-IR, LC-MS, 1D (one dimensional) and 2D (two dimensional) NMR spectral data analysis. Upon detailed evaluation of the FT-IR spectra, it was found that the IR band at 1046 cm⁻¹ is due to δ OH deformation mode [39]. The peak at 1510.88 cm⁻¹ is due to C–C stretches in an aromatic ring. The strong peak at 1726.88 cm⁻¹ indicates the presence of C=O functional group. A broad band around 3396 cm⁻¹ confirms the presence of –OH functional group in the compound [40]. The aromatic C–H stretching vibrations were observed around 2928 cm⁻¹ [40]. The CH₂ bending modes were observed around 1500 cm⁻¹. The FT-IR peak appeared around 1259 cm⁻¹ is due to C–C–O absorption which is highly specific to O–H absorption of phenolic compounds [41]. By analysing the IR spectra, it could be assessed that our compound contained an aromatic phenolic

centre, a ketonic group and many CH₂ functional groups. The base peak was M + K peak (359.583, 100%). M + H peak was observed at 321.763 (9.28%). M + Na peak was also observed at 343.063 (11.89%). Other major peaks were obtained at 313 (11.4%), 280 (12%), 124 (24%), 137 (25%) and 105 (18.75%). From the mass spectral data, it was clear that the molecular mass of the compound was 320, and FT-IR data confirmed the presence of OH and C=O functional groups. Proton NMR signals obtained between δ 7 and 8 revealed the presence of an aromatic ring within the compound (Supplementary Fig. 1) [42]. After evaluating the 2D spectra, the corresponding carbon atoms were assigned as C-1 to C-6 and as the part of aromatic six membered ring [43] (Supplementary Fig. 2). The ¹³C spectra revealed that the compound contained 22 carbon atoms. DEPT 135 showed signals to prove the presence of seven CH₂ groups (C-10, C-13, C-15, C-19, C-20 and C-21). Evaluation of the ¹³C spectra in combination with DEPT 135 indicated that the compound contained 22 carbon atoms of which 8 were quaternary, 7 were CH₂ and the rest as CH or CH₃. The peak at quaternary carbon signal obtained at δ 178 confirmed the presence of C=O (C14) functional group which causes maximum deshielding to carbon atom [44]. Similar is the result in the case of carbon atom that attached with an electronegative OH functional group in an aromatic system that appeared at δ 171

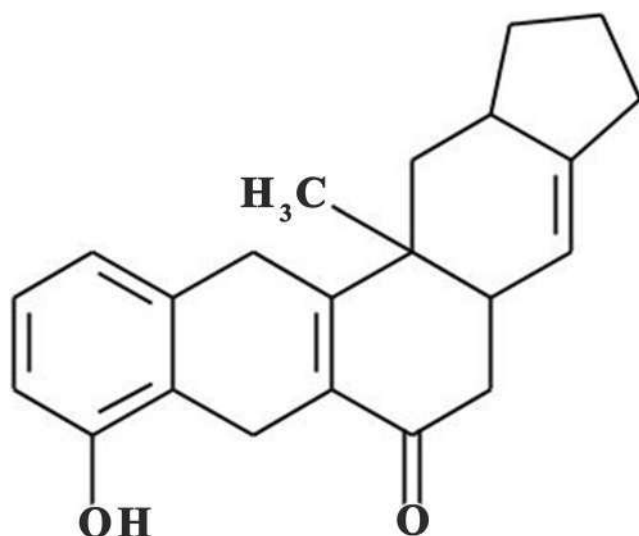


Fig. 4 Structure of 4-Hydroxy-11-methylpentacyclo [11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one

(C-4). Moreover, a singlet peak obtained at δ 6.2 in proton NMR was assigned due to hydrogen atom of OH group which

is deshielded due to the presence of oxygen atom and appeared in the downfield region. It was reported that the phenolic type of proton will appear as a sharp singlet between δ 4 and δ 7, whereas the hydroxylic peak in alcohols were found between δ 0.5 and δ 0 4.0 [41]. This confirmed the presence of a OH group attached to a six membered aromatic ring. From these points, it can be concluded that the molecule contains 22 carbon and two oxygen and 24 hydrogen atoms. Out of these two oxygen atoms, one is a hydroxyl functional group and another one is a ketonic moiety. The carbon signals revealed that there were 10 unsaturated carbon atoms of which 6 were quaternary and 4 were CH groups. The absence of carbon signals after δ 100 in DEPT 135 revealed that the CH₂ groups present in the compound were not part of any unsaturated framework. All the DEPT 135 signals were observed between δ 21 and 66 which indicated that they are not shielded or deshielded (attached to any electron withdrawing or donating groups) but are the part of an aliphatic system. Out of the eight quaternary carbon atoms, seven of them exhibited carbon signals between δ 108 and 171 (C-2, C-3, C-4, C-8, C-9, C-14 and C-17) which confirmed the fact that all of them are the

Table 3 NMR spectroscopic data of 4-Hydroxy-11-methylpentacyclo [11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one in CDCl₃

Carbon no.	¹³ C NMR (DEPT)	H	$\delta^1\text{H}$ NMR (int., mult., <i>J</i> in Hz) ^b	¹ H- ¹ H COSY	HMBC (¹ H- ¹³ C)
1	123.77 (CH)	H1	7.53 (m, 1H)	H-6	C-2, C-6
2	140.01 (C)				
3	117.84 (C)				
4	171.12 (C)				
5	108.33 (CH)	H5	7.32(m, 1H)	H-6	C-4, C-6
6	131.93 (CH)	H6	8.2 (t, <i>J</i> = 7.5 Hz, 1H)		
7	29.17 (CH ₂)	H7	3.60 (s, 2H)		C-2, C-8
8	171.46 (C)				
9	124.69 (C)				
10	28.96 (CH ₂)	H10	3.73 (m, 2H)		C-3, C-9
11	28.79 (C)				
12	68.1 (CH)	H12	2.51 (m, 1H)	H-18	C-18, C-11
13	65.24 (CH ₂)	H13	2.55 (d, <i>J</i> = 17.5 Hz, 2H)	H-12	C-12, C-14
14	178.94 (C)				
15	65.38 (CH ₂)	H15	1.26 (s, 2H)	H-16	C-11, C-16
16	20.8 (CH)	H16	2.08 (m, 1H)	H-19	C-17
17	154.51 (C)				
18	125.51 (CH)	H18	4.81 (m, 1H)		C-17
19	31.77 (CH ₂)	H19	0.86 (m, 2H)	H-20	C-16, C-20
20	22.63 (CH ₂)	H20	1.23 (m, 2H)		C-21
21	31.64 (CH ₂)	H21	2.01(m, 2H)	H-20	C-17
22	14.04 (CH ₃)	H22	1.17 (s, 3H)		C-11
23		OH	6.2 (s, 1H)		

^a NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometer

^b Values in ppm, multiplicity and coupling constants (*J*/4 Hz) are indicated in parentheses. Assignments were made with the aid of the ¹H-¹H COSY, HSQC and HMBC experiments

part of an unsaturated framework. The quaternary carbon signal obtained at δ 28.7 (C-11) is identified as a normal saturated carbon atom [45]. The position CH_3 , $\text{C}=\text{O}$ and OH functional groups were assigned with the help of both 1D and 2D spectral data. Carefully evaluating the spectral data the structure of compound was assigned as 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one (Fig. 4). Most of the studies conducted by other researchers identified phenazine-like compounds responsible for the activity of *P. aeruginosa* [19, 20, 23]. A hydroxy-substituted quinoline compound with a capacity to enhance the antibacterial ability of various antibacterial agents was also isolated from *P. aeruginosa* [21]. Similar to this, our study also identified a novel hydroxy-substituted cyclo ketonic compound from *P. aeruginosa* Ps04 responsible for its broad spectrum of antibacterial potential.

Conclusion

The present work signified that *P. aeruginosa* Ps04 possesses potential antibacterial properties against aquaculture pathogens especially belonging to genus *Vibrio* and *Aeromonas*. This work described the bioassay-guided isolation and characterization of a novel compound with antibacterial activity as 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one from *P. aeruginosa* Ps04 grown in optimized medium and physiochemical conditions. The secondary metabolite produced by *P. aeruginosa* Ps04 was found to be stable in different pH and temperature. The inertness of the metabolite on treatment with proteolytic enzymes suggested its non-proteinaceous nature. The antimicrobial effects of the newly derived compound could be utilized as a green alternative to control bacterial infections. The detailed study of the new compound and their functional activities can serve as a breakthrough in developing new antimicrobial therapeutics ideal for aquaculture applications.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42770-020-00404-z>.

Acknowledgements The authors are thankful to the Indian Council of Agricultural Research (ICAR) for funding the study through the Application of Microorganisms in Agriculture and Allied Sectors (AMAAS project). We also express our sincere gratitude to the Director, Central Marine Fisheries Research Institute (CMFRI), for providing necessary facilities to carry out the research. The authors would like to thank the Sophisticated Analytical Instruments Facility (SAIF) - IIT, Chennai; Centre for Bio-Separation Technology (CBST) - Vellore Institute of Technology University, Vellore; and Sophisticated Test and Instrumentation Centre (STIC) - CUSAT, Kochi, for providing assistance in NMR and FT-IR analysis, respectively. The authors are thankful to Dr. V. Sabareesh, Vellore Institute of Technology, for his support in LC-MS analysis of the sample.

Funding The work was supported by the Indian Council of Agricultural Research (ICAR) through the Application of Microorganisms in Agriculture and Allied Sectors (AMAAS project, Institute Project Code Number 2020600004).

Data availability Nucleotide sequences have been submitted to the GenBank database with accession number JF719801.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflicts of interest.

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Isolation and characterization of a novel antimicrobial oxatetracycloketone from *Bacillus stercoris* MBTDCMFRI Ba37 isolated from the tropical estuarine habitats of Cochin

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Received: 11 February 2020 / Accepted: 11 January 2021

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Abstract

Antimicrobial compounds from the safest source have gained greater relevance because of their wide spectrum of possible applications, especially in aquaculture industry, where pathogenic threat and antibacterial resistance are serious concerns. *Bacillus stercoris* MBTDCMFRI Ba37 isolated from mangrove environment of tropical estuarine habitats of Cochin exhibited a wide spectrum of antibacterial activity against major aquaculture pathogens belonging to genus *Vibrio* and *Aeromonas*. The structural characterization of the antibacterial compound from this strain may help in identifying their role as a bio-control agent in aquaculture and allied sectors. The highest antibacterial activity was detected in 3rd day culture, grown in a modified *Bacillus* medium containing 1% of glycerol and 0.5% of glutamic acid at 30 °C, pH 8.0 and 15 ppt saline conditions. The inhibitory activity of the cell free supernatant was evident even at 20% v/v dilution. Preliminary studies on the nature of antibacterial action indicated that the bioactive principle is stable at temperatures up to 70 °C, between pH 6–9 and instable to lysozyme and proteolytic enzymes. Bioassay guided purification followed by spectroscopic characterization of active fractions of *B. stercoris* MBTDCMFRI Ba37 revealed that the compound 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one, is responsible for its major antibacterial activity. This is the first report on isolation and characterization of an antibacterial compound from the species *B. stercoris*. The results of this study indicated that *B. stercoris* MBTDCMFRI Ba37 has beneficial antibacterial properties which could be useful in developing novel antimicrobial therapeutics against a variety of aquaculture and other pathogens.

Keywords *Bacillus stercoris* · Antibacterial compound · FT-IR · LC–MS · NMR

Introduction

Large scale outbreaks of pathogenic infections remain a basic restricting component for the aquaculture industry, where worldwide fish production peaked at around 171 million tons, with aquaculture representing 47 percent of the aggregate [1]. Majority of species belonging to *Vibrio* and *Aeromonas* have been mostly associated with larval mortalities in aquaculture [2]. Uses of antibiotics were employed as an effective way to control these pathogenic microbes worldwide. Many studies indicated the adverse effect of using antibiotics as a remedial measure, since it cause many issues including immune suppression and reduced disease resistance [3, 4]. In order to overcome the hostile effects of using antibiotics and chemotherapeutics, naturally derived secondary metabolites with antibacterial potential can be an effective alternative.

Supplementary Information The online version of this article (<https://doi.org/10.1007/s11033-021-06146-x>) contains supplementary material, which is available to authorized users.

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Members of the genus *Bacillus* are considered as microbial factories, capable of producing a vast array of active metabolites with different structure and functions [5]. This genus includes a great variety of species that produce bacteriocins or bacteriocin like substances (BLS), lipopeptides, non-peptide based antibiotics etc. which are having inhibitory potential [6, 7]. Among the various species of *Bacillus*, *B. subtilis* is a common candidate to be used as probiotic bacteria in aquaculture [8, 9]. It is referred as an ideal biocontrol agent used as an alternative against a wide range of pathogens [10–12]. It was reported that 4–5% of its genome are dedicated to antibiotic synthesis with a capacity to produce more than two dozens of structurally diverse antimicrobial compounds [13, 14]. This versatile bacterium is considered as one of the important producers of metabolites including bacteriocin, subtilisin A, subtilin, bacilysin etc. having a wide spectrum of activities against pathogenic microorganisms [11, 15, 16]. Characterization of these metabolites would help in understanding the detailed mechanisms of antibacterial activity exhibited by this species [17]. Based on several genomic and chemotaxonomic studies *Bacillus subtilis* was further characterized into four subspecies *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *spizizenii* [18], *Bacillus subtilis* subsp. *inaquosorum* [19] and *Bacillus subtilis* subsp. *stercoris* [20]. Based on their genomic identity and the presence of secondary metabolite they are now promoted to the species status as *Bacillus spizizenii*, *Bacillus inaquosorum* and *Bacillus stercoris* [21].

From our previous research which aimed to understand the diversity of culturable bacteria with antimicrobial properties in the sediment and sub-surface water of Cochin estuary, we have screened about 4,870 isolates to evaluate their inhibitory potential [22]. The study identified *Bacillus subtilis* MBTDCMFRI Ba37 as a promising isolate which could be explored further for their application in aquaculture. Recently the strain MBTDCMFRI Ba37 was further reconfirmed as *Bacillus stercoris* based on genomic studies. In view of the antibacterial potentials exhibited by *Bacillus stercoris* MBTDCMFRI Ba37, the present study aimed to optimize the medium and physiological conditions for the mass productions of the antimicrobial metabolite followed by their preliminary characterization. The bioactive principle responsible was further chromatographically purified based on their activity and characterized using spectroscopic methods. To the best of our knowledge, this is the first report describing the characterization of antibacterial compound from *Bacillus stercoris*. The findings of this study envisage as a footprint towards the production and usage of novel therapeutic agents to use against bacterial infections especially in the aquaculture sector.

Materials and methods

Bacterial strains

The bacterial strains isolated from the sediment sample of Mangalavanam (Mangrove ecosystem) of tropical estuarine habitats, Cochin were assayed for their antibacterial properties against aquatic pathogens [22]. Among the isolates obtained from the preliminary study, the isolate *B. stercoris* MBTDCMFRI Ba37 was found to be a most promising isolate based on the consistency and broad spectrum of antibacterial activity and selected as the candidate strain for isolation and characterization of bioactive compound.

The aquatic bacterial pathogens used in the study were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101 (courtesy Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (courtesy National Centre for Aquatic Animal Health, Cochin University of Science and Technology), *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (courtesy Microbial Type Culture Collection, Chandigarh, India).

Bacterial identification

The isolate was identified by employing morphological characteristics, biochemical methods, and 16S ribosomal RNA (rRNA) gene sequencing using the primers AGAGTTTGA TCCTGGCTCAG (forward) and ACGGCTACCTTGTTA CGACTT (reverse) [23, 24] and further confirmed using housekeeping genes like *gyrA* (*GyrA*-f (5' CAG TCA GGA AAT GCG TAC GTC CTT 3') and *GyrA*-r (5' CAA GGT AAT GCT CCA GGC ATT GCT 3') [25] and *rpoB* (*Rpo B*-F (5' GTT GGC TTC ATG ACT TGG GA 3') and *Rpo B*-R (5' ACG TTC CAT ACC TAA ACT TTG 3') [26]. The 16S rRNA gene sequence similarity was calculated using EzBio- Cloud (<http://www.ezbiocloud.net/identify>) [27]. 16S rRNA, *gyrA* and *rpoB* gene sequences of all closely related species of the genus *Bacillus* were extracted from the NCBI database (www.ncbi.nlm.nih.gov). Concatenated tree was constructed using partial sequences of the 16S, *gyrA* and *rpoB* genes from 14 type strains of closely related *Bacillus* species and *B. cereus* ATCC 14579T was used as an outgroup taxon. Sequences of each gene from all strains were aligned using ClustalX2.0.12. Genes sequence lengths were: 16S rRNA-1179 bp, *gyrA*-860 bp, and *rpoB*-758 bp. These sequences were concatenated into a single sequence of 2797 bp long in a series of 16S-*gyrA*-*rpoB*. The phylogenetic tree was constructed in MEGA 7 software [28]. The evolutionary history was calculated through the neighbour joining method [29].

Antimicrobial activity of cell free supernatant

The antibacterial activity of the strain *B. stercoris* MBTDCMFRI Ba37 was carried out by spot diffusion assay. The culture was spotted on pre-swabbed Mueller Hinton agar (MHA) plates with aquatic pathogens [22]. The antimicrobial activity of cell free supernatant (CFS) of *B. stercoris* MBTDCMFRI Ba37 was determined by agar well diffusion and disc diffusion assay [2, 30]. For agar well diffusion technique, wells were created aseptically on MHA plate already swabbed with aquatic pathogens. CFS of *B. stercoris* MBTDCMFRI Ba37 (30 µl) were added to the wells and the plates were incubated for 24 h at 30 °C. Similarly for disc diffusion assay, CFS of *B. stercoris* MBTDCMFRI Ba37 (30 µl) was added to a sterile disc of 6 mm diameter. After drying, discs were placed on MHA plate already swabbed with aquatic pathogens and kept for incubation at 30 °C for 24 h. Disks impregnated with tetracycline (30 mcg) and sterile distilled water (40 µl) were used as positive and negative controls [31].

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of CFS of *B. stercoris* MBTDCMFRI Ba37 was evaluated using the broth macrodilution method [32]. The bacterial pathogens used for this study were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101. For the broth macrodilution method, 1 ml of aquatic bacterial pathogenic suspension of 5×10^5 CFU/ml was added to the wells of a sterile 12-well titer plate already containing 1 ml of serially double diluted *B. stercoris* MBTDCMFRI Ba37 CFS in Mueller Hinton broth (MHB), which was then incubated for 24 h at 30 °C. Control wells were prepared with 1 ml MHB, 1 ml of MHB with 1 ml bacterial pathogenic suspension and 1 ml CFS of *B. stercoris* MBTDCMFRI Ba37. The contents of each well were mixed. MIC was defined as the lowest concentration of CFS of *B. stercoris* MBTDCMFRI Ba37 showing no visible turbidity of pathogenic strains after 24 h of incubation on the basis of metabolic activity [31]. All the experiments were done in triplicates.

Optimization of production medium

B. stercoris MBTDCMFRI Ba37 was taken from glycerol stock at -80 °C and streaked on nutrient agar (with 1% NaCl) and incubated at 30 °C for 24 h and a single colony obtained was inoculated into 5 ml of nutrient broth supplemented with 1% NaCl and incubated at 30 °C on a rotary shaker overnight (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20–24 h. 1% of overnight grown culture (starter culture) was inoculated to 100 ml of various media like

nutrient broth (with 1% NaCl), Zobell marine broth, nutrient broth (with 50% seawater) and modified *Bacillus* media [33] (g/l) containing 5.0 g glutamic acid; 10 ml glycerol; 0.5 g KH_2PO_4 ; 0.5 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$; 0.015 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at pH 7. All the flasks were incubated at 30 °C for a period of 7 days in triplicate. Media with tetracycline (10 µg/ml) and sterile distilled water were used as positive and negative controls. Samples for antibacterial activity were drawn every 24 h interval and 1 ml of the culture was processed for evaluating the inhibitory potential against aquaculture pathogens by the disc diffusion method.

Optimization of the physiochemical conditions

The optimal conditions for growth and production of the antimicrobial compound by *B. stercoris* MBTDCMFRI Ba37 was assessed by growing the culture in modified *Bacillus* media at various temperature (25, 30, 35 and 40 °C), pH (5.0, 6.0, 7.0, 8.0 and 9.0), salinity (5, 10, 15, 20, 25, 30 and 35 ppt), carbon (glycerol and glucose at varying concentrations from 0.5 to 1%) and nitrogen sources (alanine and glutamic acid at varying concentrations from 0.5 to 1%) for a period of 7 days in triplicate. Tetracycline (10 µg/ml) and sterile distilled water under the experimental conditions were used as positive and negative controls. Samples for cell count and antibacterial activity were drawn every 24 h. One milliliter of the culture was processed for evaluating the inhibitory potential against pathogens by the disc diffusion method as explained earlier.

Preliminary characterization of antibacterial compound

Sensitivity of antibacterial compound to enzymes, pH and heat treatment was carried out using cell free culture supernatant of *B. stercoris* MBTDCMFRI Ba37. Heat sensitivity of the inhibitory substance was tested by heating the cell free supernatant of *B. stercoris* MBTDCMFRI Ba37 in a water bath for 30 min at 40 to 100 °C, and autoclaving for 15 min at 120 °C. Cell free supernatant at 37 °C was used as control. Each of the treated and untreated supernatants was tested for inhibitory activity as described above. The sensitivity of the antibacterial principle to lysozyme and proteolytic enzymes like proteinase K and trypsin was tested using 1, 2 and 5 mg/ml concentration. After 2 h incubation at 37 °C, enzymatic action was inactivated by keeping at boiling water bath for 5 min. Antibacterial activity was tested against pathogens using the disc diffusion method with cell free supernatant as control. The pH stability was tested by adjusting the pH of the cell free supernatant to 2 to 12 using 1 mol l^{-1} HCl and 1 mol l^{-1} NaOH, with the help of a pH meter,

and incubating for 2 h and the activity was tested against pathogens as mentioned above. Cell free supernatant with its pH adjusted to 7.0 is used as control. Effect of dilution of the cell free supernatant was detected by diluting the filtrate containing the potential antibacterial compound up to 10% (v/v) using sterile phosphate buffered saline (pH 7.4). The antimicrobial activity against pathogens was tested by the method as stated earlier.

Bioassay guided isolation and purification of antibacterial compounds from *B. stercoris* MBTDCMFRI Ba37

Six litres of 3 days old *B. stercoris* MBTDCMFRI Ba37 culture in modified *Bacillus* medium incubated at 30 °C at 150 rpm was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant was filter sterilized (0.2 µm) and lyophilized to obtained 15 g of supernatant powder which was partitioned between ethyl acetate (EtOAc)/methanol (MeOH) (1:1 v/v) and reflux for 2.5 h and filtered through anhydrous sodium sulphate (Na₂SO₄). The filtrate thus obtained was evaporated using rotary evaporator under vacuum to dryness at a temperature below 45 ± 2 °C to get pale yellow extract (5 g). The crude fraction thus obtained was evaluated using TLC using various solvent systems and identified that dichloromethane (DCM) methanol (MeOH) gradient is effective for column elution. Thus the sample was eluted through silica column initially with DCM then with a gradient of DCM / MeOH followed by 100% MeOH to get 23 fractions. They were evaluated and pooled according to their TLC profile to get seven fractions. The second fraction (BS₂, 1.2 g) with highest antimicrobial potential was re-purified using column chromatography (silica) with solvents n-hexane, then the gradient of hexane with increasing concentration of EtOAc followed by a gradient of EtOAc with increasing concentration of MeOH and finally with 100% MeOH to get thirteen fractions which were pooled according to TLC profile to get seven fractions finally. The antibacterial activity potential was assayed and found that the second fraction (BS_{2.2}, 220 mg) exhibited higher inhibitory potential and hence purified further using preparative TLC plate (silica) using 15% EtOAc in n-hexane to get five fractions according to TLC profile. The fourth and fifth fractions with almost similar TLC profile and antibacterial potential were pooled together (20 mg) and chromatographed over preparatory TLC plates (silica) using 15% EtOAc in n-hexane to get further three fractions. The second fraction exhibited higher antibacterial potential and good purity (according to TLC profile) was selected for spectroscopic characterisation (Fig. S1).

General experimental procedures

The purified fraction (BS_{2.2.4-5.2}) was subjected to FT-IR (Fourier Transform Infra Red) spectrometric analysis to trace the spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to the mid IR range, i.e., between 4000 and 400 cm⁻¹. The LC-MS experiments were carried out on Quattro Premier XE (Micromass), an electrospray ionization (ESI)-triple quadrupole mass spectrometer, which is attached to Acquity UPLC (Waters). For each run, 5 µl of samples were injected onto a C₁₈ column (Acquity UPLC BEH, 1.7 µm, 2.1 mm × 50 mm; Waters). A linear gradient elution was done utilizing water (H₂O; solvent A) and methanol (MeOH; solvent B) (0–20 min.: 50–90% B; 20–23 min.: 90% B; 23–25 min.: 90–50% B) and the flow rate was maintained at 0.2 ml min⁻¹. Eluents from the chromatographic column pass through photo diode array (PDA) detector and then enter into the mass spectrometer. Ultraviolet (UV) absorbance data in the range 210–400 nm were recorded by PDA detector. Mass spectrometric data were acquired in positive ion polarity of ESI and the *m/z* range of detection was set to 50–1500. The ESI source parameters were: capillary 3 kV; Cone 30 V; Extractor 5 V; Source Temperature 100 °C; Desolvation Temperature 300 °C; Desolvation Gas Flow 800 l/h (l/h); Cone Gas Flow 50 l/h. All data acquisitions and processing were facilitated by MassLynx (Waters) followed by nuclear magnetic resonance (¹H and ¹³CNMR) spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in CDCl₃ as aprotic solvent at ambient temperature with tetramethylsilane (TMS) as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ¹³C NMR signals was determined from DEPT experiments. Standard pulse sequences were used for DEPT, ¹H–¹H COSY, two-dimensional NOESY, HSQC, and HMBC experiments. All solvents were of analytical, spectroscopic or chromatographic reagent grade, and were obtained from E-Merck (Darmstadt, Germany).

Statistical analysis

One-way analysis of variance (ANOVA) was carried out with SPSS20.0 (SPSS, USA, ver. 20.0) to assess for any significant differences between the means. The differences in means values were identified by Scheffe's multiple range tests to determine whether significant difference (*P* < 0.05) existed among different treatments.

Results

Identification of candidate strain and its antibacterial activity

Morphological characteristics of the isolate were Gram-positive, rods shaped (size $\approx 0.4\text{--}0.7 \times 1.5\text{--}3.0 \mu\text{m}$), motile, endospores were ellipsoidal in shape and observed at sub-terminal or central positions in swollen sporangia. The isolate was identified as *B. stercoris* by constructing phylogenetic tree based on 16S rRNA, *gyrA* and *rpoB* gene sequences concatenated from the strain along with 14 closely related type strains under *Bacillus subtilis* group (Fig. 1). The 16S rRNA gene sequence was deposited in GenBank data base with accession number JF719798. The strain *B. stercoris* MBTDCMFRI Ba37 was authenticated and deposited under general deposit in a national culture collection, National Centre for Microbial Resource (NCMR), Pune, with accession number MCC 4444. The

antibacterial activity of the filtered supernatant of *B. stercoris* MBTDCMFRI Ba37 exhibited greater inhibitory activity against *V. vulnificus* MTCC1145 followed by *V. harveyi* 101 and least against *A. hydrophila*. The zone of inhibition against the tested pathogen by spot diffusion, well diffusion and disc diffusion method were around 9–22 mm were given in Fig. 2. The CFS of *B. stercoris* MBTDCMFRI Ba37 was used for MIC assay. The count of *B. stercoris* MBTDCMFRI Ba37 was found to be 2×10^8 cfu/ml. It showed that MIC was 1 ml CFS of *B. stercoris* MBTDCMFRI Ba37 against *V. harveyi* 101 and *V. alginolyticus* 101 and 500 μl of CFS against *V. anguillarum* O1.

Optimization of medium and physiochemical conditions for the production of antibacterial compound

Among different media used, modified *Bacillus* medium was found to be more effective in maximum production of antibacterial principle from *B. stercoris* MBTDCMFRI Ba37 followed by nutrient broth with 1% NaCl (Fig. S2a).

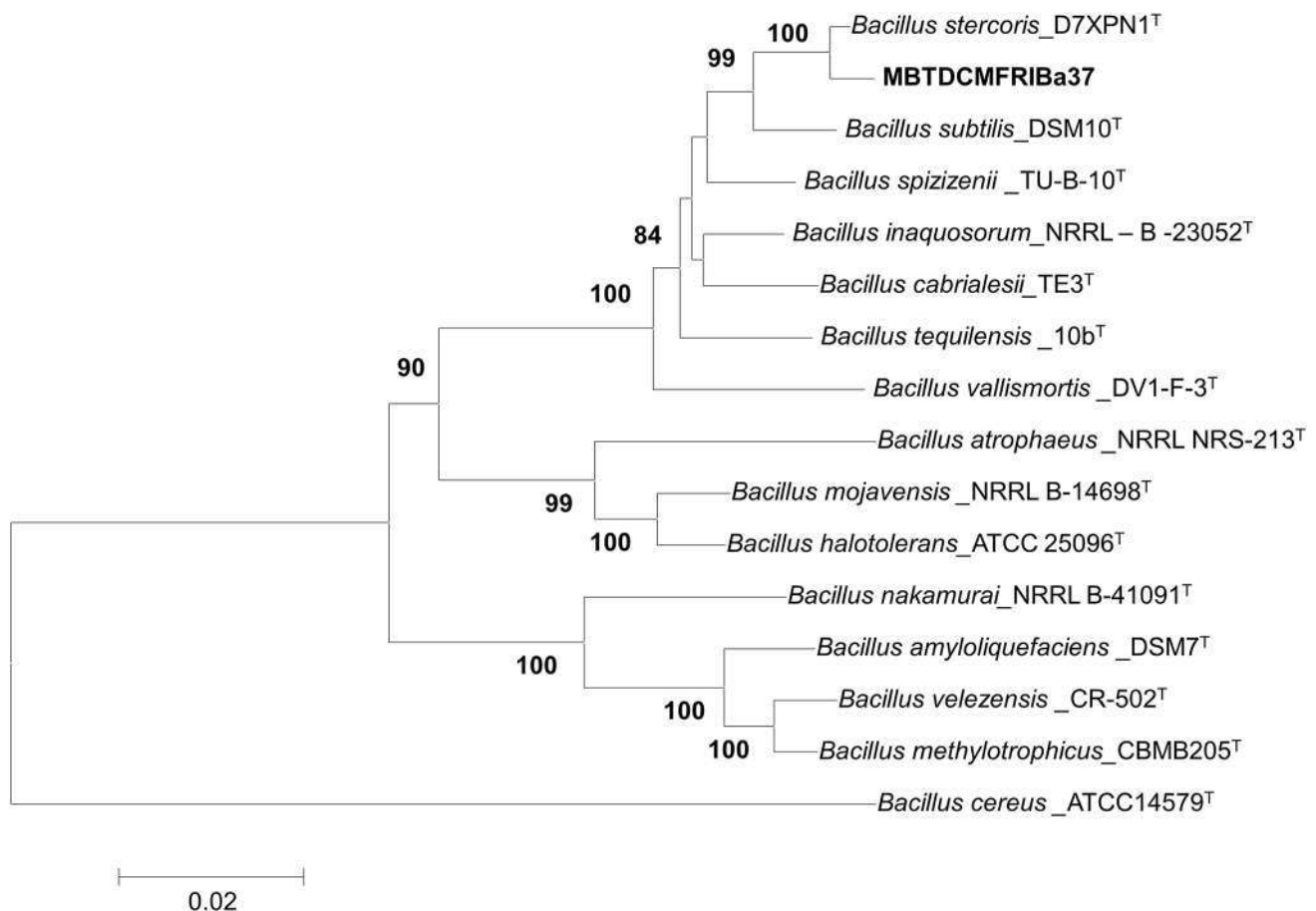
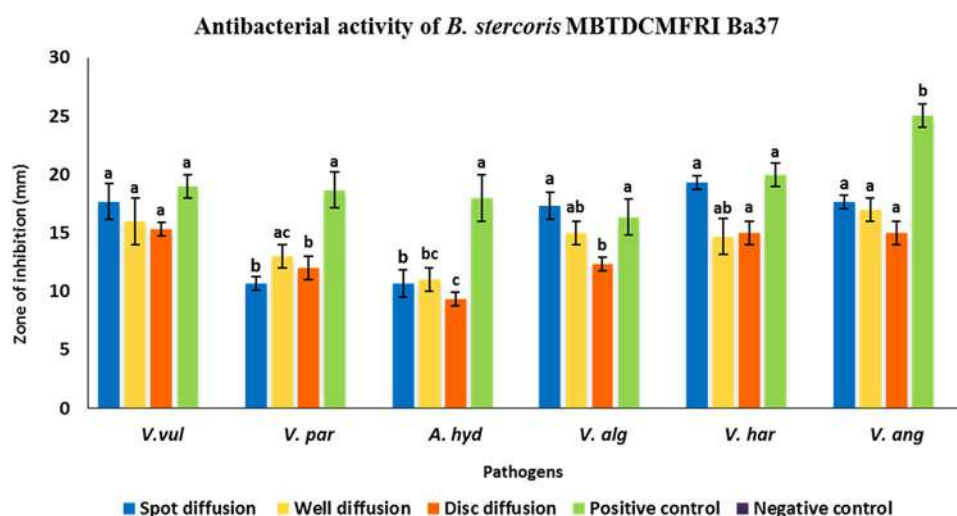


Fig. 1 Phylogenetic tree based on 16S rRNA, *gyrA* and *rpoB* gene sequences concatenated from strain *B. stercoris* MBTDCMFRI Ba37 along with 14 closely related type strains under *Bacillus subtilis* group. The tree was constructed using the neighbor-joining method

with MEGA 6.0. Bootstrap values over 50% (1000 replications) were shown at each node. The bar represents 0.02 substitutions per site. *Bacillus cereus* ATCC 14579^T was used as the out group

Fig. 2 Antibacterial activity of *B. stercoris* MBTDCMFRI Ba37 against aquatic pathogens. Vvul—*Vibrio vulnificus* MTCC1145; Vpar—*V. parahaemolyticus* MTCC 451; Ahyd—*Aeromonas hydrophila*; Valg—*V. alginolyticus* 101; Vhar—*V. harveyi* 101 and Vang—*V. anguillarum* O1. The measurement indicates the inhibition zone (mm) formed around the isolates. Data are the mean values of triplicate and expressed as mean $\pm$ standard deviation. Values with different letters (a–c) are significantly different ($P < 0.05$)



The best carbon and nitrogen source for optimum production was found to be glycerol (1%) and glutamic acid (0.5%) respectively. *B. stercoris* MBTDCMFRI Ba37 can grow from 25 to 40 °C, the optimum temperature for growth and the production of the antibacterial compound was recorded at 30 °C, with a maximum inhibitory zone of 12–21 mm on the 3rd day against tested pathogens (Fig. S2b). Enhanced production was achieved at 15 ppt salinity and maximum antibacterial compound was produced on the 3rd day with a clearing zone of 13–21 mm. The inhibitory activity of the culture supernatants was less for higher and lower salinity of 35 ppt and 5 ppt respectively (Fig. S3a). The pH does not influence much with inhibitory compound production. There was no significant variation in the growth of *B. stercoris* MBTDCMFRI Ba37 or antibacterial activity with the tested pH range. However, the inhibitory activity was highest at pH 8.0 followed by pH 7.0 with 3 days of culture with a clearing zone of 12–20 mm (Fig. S3b). The negative controls showed no activity, while positive control exhibited greater antibacterial activity (with a clearing zone ranging from 12 to 30 mm for different experiments) against various pathogens.

Preliminary characterization of antibacterial principle

The inhibitory compound production of *B. stercoris* MBTDCMFRI Ba37 was observed in the late phase of culture (about 40 h) in modified *Bacillus* medium and activity reached a maximum in the stationary phase and remained stable for two more days with small flux in the activity. From the dilution studies, it was observed that diluting even up to 20% of the cell free supernatant of *B. stercoris* MBTDCMFRI Ba37 retained its activity against all the tested aquaculture pathogens (Fig. S4a). Temperature variation studies revealed that the cell free supernatant of *B. stercoris*

MBTDCMFRI Ba37 is highly effective at room temperature and retained its potential even up to 70 °C without any significant alteration ($P > 0.05$) (Fig. S4b). Its activity against pathogens reduced at higher temperatures (above 80 °C). Activity was totally lost after incubation at 121 °C. While in case of stability at different pH, the antibacterial component was found to be stable at pH 6–9, but the activity was lesser at the extremes (pH > 9 and pH < 6) (Fig. S5). However antibacterial compound was found to be unstable to lysozyme and proteolytic enzyme treatments.

Purification and structural characterisation of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

The EtOAc/MeOH extract was chromatographed over silica column (60–120 mesh) with a stepwise gradient of solvents from DCM, DCM: MeOH and finally MeOH (100:0, 96:4, 90:10, 85:15, 80:20, 60:40, 20:80, 0:100). The fractions were collected and concentrated under vacuum and TLC was evaluated and fractions with the same TLC profile were pooled together to furnish seven fractions (Bs₁₋₇). The column fractions obtained were evaluated for its inhibitory activity against aquaculture pathogens. The sub fraction Bs₂ (7.8% yield) which exhibited high antibacterial activity (zone size of 12–23 mm against various pathogens) was further purified using column chromatography with *n*-hexane, *n*-hexane:EtOAc (100:0, 97:3, 94:6, 91:9, 85:15, 75:25, 60:40, 50:50 and, 0:100) and then with EtOAc:MeOH (90:10, 80:20, 0:100) to give another seven sub fractions. After concentrating and TLC evaluation, fractions with same TLC profile were pooled together to furnish seven sub fractions (Bs_{2, 1-7}) and all fractions were checked for antibacterial activity. The sub fraction Bs_{2, 2} (220 mg) which exhibited higher antibacterial potential (inhibition zone of

15–22 mm against various pathogens) was re-purified using preparative thin layer chromatography over Si gel GF₂₅₄ (particle size 15 μ m) (15% EtOAc: *n*-hexane) to obtain five sub fractions of which the two sub fraction Bs_{2.2.4} and Bs_{2.2.5} exhibited higher antibacterial potential (inhibition zone of 16–22 mm against various pathogens) were pooled (Bs_{2.2.4-5}, 20 mg yield) and further purified by preparative thin layer chromatography over Si gel GF₂₅₄ (particle size 15 μ m) (15% EtOAc: *n*-hexane) and were found to be single compound (Bs_{2.2.4-5.0.2}, 8 mg,) which exhibited the antibacterial potential (inhibition zone of 18–22 mm against various pathogens). Its R_f value is 0.68. The schematic representation of the purification pattern is given by (Fig. S1).

Analysis of the IR spectra revealed a split peak around 2900 cm⁻¹, a broad peak around 3449 cm⁻¹ and peaks at 1728 cm⁻¹ and 1265 cm⁻¹ (Fig. 3). The LC–MS analysis of the compound exhibited a molecular ion peak (M + H peak) at *m/z* = 391.45 and fragment peaks at *m/z* = 279.33, 181.18, and 167.16 (Fig. S6). Peaks were also observed at *m/z* = 413, 429 and 803.68. Analysis of the ¹³C NMR spectrum (Table 1) with the DEPT-135 together showed one ketonic carbon (C-5, δ 211.36), three oxygenated CH groups (C-15, 17 and 24 with δ 71.31, 71.30 and 81.33 respectively), four CH₂ groups (C-4, C-6, C-11 and C-14 with δ 51.59, 51.57, 29.70, 29.36 respectively) and other 17 CH and CH₃ carbons together (δ values between 14 and 42) (Fig. S7 & S8). The ¹H spectrum showed a quartet with a *J* value of 6.4 Hz appeared at a downfield shift of δ 3.96 ppm of two hydrogen atoms (Fig. S9). Similarly another quartet

with a *J* value of 6.4 Hz appeared at a downfield shift of δ 3.80 ppm of one hydrogen atom. Consequently the CH₃ groups attached to CH groups were appeared as doublets around δ 1.2 ppm (C-18 and C-19) and δ 1.01 ppm (C-27). The structural correlation between hydrogen atoms bound to a particular carbon was further identified by ¹H-¹H COSY. The presence of CH groups attached to electronegative centers were further confirmed by the presence of ¹³C signals appeared around δ 71.31, 71.30 and 81.33 ppm (Fig. S7). The ¹³C signals appeared around δ 211.36 ppm confirms the presence of a C=O group attached to the ring system (C-5). HSQC correlations observed between H-15 & 17 (δ 3.9 ppm) and C-15 and 17 (δ 71.2 ppm) further confirms the proposed structure. Similarly HSQC correlation was also prominent between H-24 and C-24. Proton at H-2 exhibited HMBC correlation with C-23 and C-10. H-27 exhibited HMBC correlations with C-25 and C-6, whereas H-6 showed a correlation with C-24. The doublets formed at δ 1.01 and 1.2 with a *J* value of 6.4 Hz also support the present proposed structure that these CH₃ groups are only associated with a neighbouring CH protons.

1-(1-Hydroxyethyl)-1,7,10,12,13,15,17-heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

Amorphous yellow solid; UV (MeOH) λ_{max} (log ϵ): 247 nm (3.91); TLC (Si gel GF254 15 mm; CHCl₃/MeOH 10:90, v/v) R_f: 0.55; LC (Column: Acquity UPLC

Fig. 3 IR spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17-heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

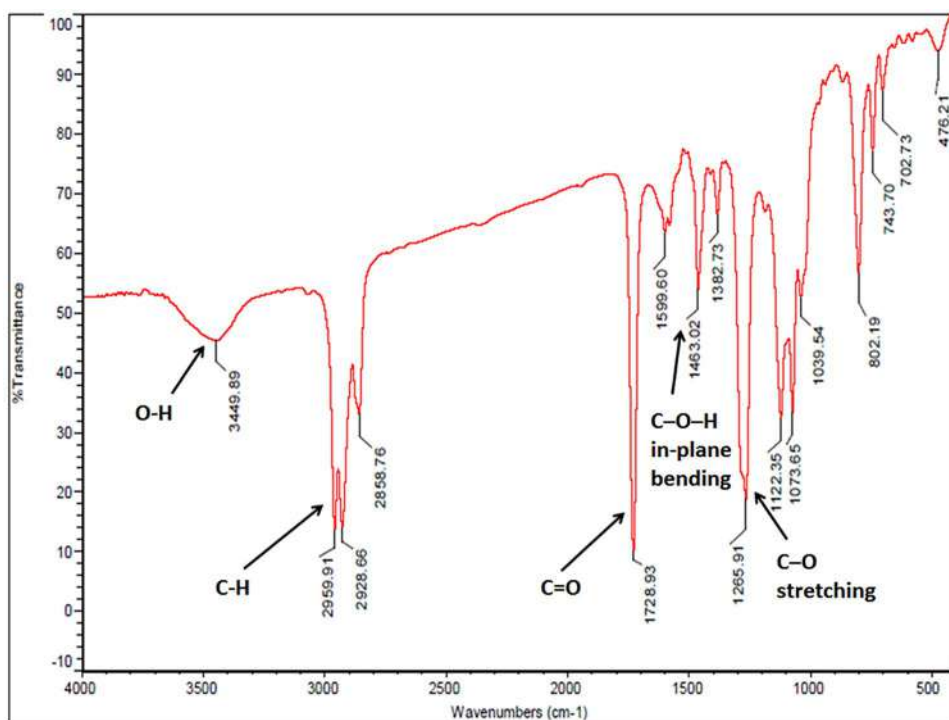


Table 1 NMR spectroscopic data of 1-(1-Hydroxy-ethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one in CDCl₃

Carbon no	¹³ C NMR (DEPT)	H	¹ H NMR (int., mult., <i>J</i> in Hz) ^a	¹ H- ¹ H COSY	HMBC (¹ H- ¹³ C)
1	34.24 (C)		–		
2	42.81 (CH)	H-2	1.00 (m, 1H)	H-7, H-3	C-23, C-10
3	41.38 (CH)	H-3	1.77 (m, 1H)	H-4, H-10	
4	51.59 (CH ₂)	H-4	3.6 (m, 2H)		
5	211.36 (C)		–		
6	51.67 (CH ₂)	H-6	3.59 (s, 2H)		C-24
7	22.69 (CH)	H-7	1.54 (m, 1H)	H-8	
8	41.29 (CH)	H-8	3.57 (m, 1H)	H-11, H-9	
9	40.91 (CH)	H-9	3.52 (m, 1H)	H-14	
10	22.93 (CH)	H-10	1.18 (m, 1H)	H-9	
11	29.70 (CH ₂)	H-11	1.20 d (m, 2H)		
12	23.71 (C)		–		
13	23.73 (C)		–		
14	29.36 (CH ₂)	H-14	1.20 (m, 2H)		
15	71.31 (CH)	H-15	3.99 (q, <i>J</i> = 6.4 Hz, 1H)	H-18	
16	O		–		
17	71.30 (CH)	H-17	3.99 (q, <i>J</i> = 6.4 Hz, 1H)	H-19	
18	22.69 (CH ₃)	H-18	1.21 (d, <i>J</i> = 6.4 Hz, 3H)		
19	22.67 (CH ₃)	H-19	1.21 (d, <i>J</i> = 6.4 Hz, 3H)		C-21, C-14
20	21.81 (CH ₃)	H-20	2.22 (m, 3H)	H-12	C-11, C-18
21	21.79 (CH ₃)	H-21	2.22 (m, 3H)	H-13	
22	21.59 (CH ₃)	H-22	1.19 (m, 3H)	H-10	C-3, C-9
23	14.09 (CH ₃)	H-23	1.19 (m, 3H)	H-7	C-25, C-8, C-11
24	81.33 (CH)	H-24	3.79 (q, <i>J</i> = 6.4 Hz, 1H)	H-27	
25	18.82 (CH ₃)	H-25	2.17 (s, 3H)		
26	O (OH)		–		
27	14.09 (CH ₃)	H-27	1.01 (d, <i>J</i> = 6.4 Hz, 3H)		C-25, C-6
28	O		–		

NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometer

^aValues in ppm, multiplicity and coupling constants (*J*/Hz) are indicated in parentheses. Assignments were made with the aid of the ¹H-¹H COSY, HSQC, HMBC and NOESY experiments

BEH C18, 1.7 μ m, 2.1 mm $\times$ 50 mm (Waters); gradient elution using H₂O (solvent A) and MeOH (solvent B) for 25 min.: 50% B–90% B in 20 min., hold at 90% B for 3 min and 90% B–50% B in 2 min.; flow-rate: 0.2 ml/min.; volume of sample injection: 5 μ l) Rt: 20.52 min. (Photo Diode Array (PDA) detection in the range, 200–400 nm); Elemental analysis found: C, 72.42; H, 7.13; N, 3.67; O, 16.78; IR (KBr, cm⁻¹) $\nu_{\max}$ 721.40 cm⁻¹ ν (C–H alkanes), 1034.84 cm⁻¹ ν (C–N), 1168.90 1313.57 1377.22 cm⁻¹ ν (C–H), 1464.02 cm⁻¹ δ (C–H of alkanes), 1600.97 1665.59 δ (N–H), 1741.78 cm⁻¹ ν (C=O), 2728.40, 2852.81, 2924.18, 2956.01 cm⁻¹ ν (C–H alkanes). ¹H NMR (CDCl₃, 500 MHz, δ ppm) and ¹³C NMR (CDCl₃, 125 MHz, δ ppm) data, (Table 1); ESI MS *m/z* (rel. int. %): 803.68 ([2M+Na]⁺) (48), 413.44 (4.5), 391.45 ([M+H]⁺) (100), 279.33 (14), 181.18 (22), 167.16 (17).

Discussion

The present study illustrated a potential antibacterial strain *B. stercoris* CMFRIMBTD Ba37, isolated from the sediments of mangrove ecosystem (Mangalavanam), a tropical estuarine habitat, Cochin. Further, the bioactive molecule behind the antibacterial activity was also characterized, which exhibited a broad spectrum of inhibitory activity against aquaculture pathogens. Many species of *Bacillus* were reported to synthesize a multitude of antimicrobial metabolites which inhibit a wide range of aquaculture pathogens [17, 34]. They have been used as a promising probiotic bacteria as well as a good biocontrol agent in aquaculture [8, 12]. Other than the production of antimicrobial compounds, the inhibitory effects of *Bacillus* may also due to their ability to outcompete pathogens for nutrients or mucosal space or to the alteration of pH in the growth medium [22, 35, 36].

The production of antimicrobial peptides by *Bacillus* spp. was thought to be under complex genetic regulation and several antimicrobial peptides are produced by a single strain simultaneously or at least at different time intervals or growth conditions [37]. Modifications in culture conditions and nutrients may induce the production of different peptide antibiotics [38]. Modified *Bacillus* medium optimized in this study has shown an increased production of antibacterial compound, probably because this medium satisfies all the necessary requirements for bioactive compound production than commercially available heterotrophic rich medium [33]. The production pattern of the antibacterial compound in synthetic media showed that production starts at exponential phase and remained constant during the stationary phase indicating that this substance is a secondary metabolite [33, 39].

In order to use *B. stercoris* MBTDCMFRI Ba37 in aquaculture system or for its further application, the data on minimum inhibitory concentration of cell free supernatants for inhibiting the growth of pathogen is vital [2]. The cell free supernatant can be diluted up to 20% of its initial concentration to retain its inhibitory potential and activity was maintained up to 70 °C and pH 6–9, indicating the bioactive compound was relatively heat stable and possibility of their application in industrial level production of antimicrobial compound. In addition, the cell free supernatant was sensitive to proteases tested, suggesting that a peptide moiety is associated with its activity [40]. Our results indicated that *B. stercoris* MBTDCMFRI Ba37 is a potential microorganism for the production of antibacterial compounds, and the optimal conditions standardized for the mass production of bioactive metabolites can be applied in various industrial activities.

Antibacterial substances produced by different bacteria seem to play an important role in aquatic ecosystems [41]. Bacteriocins may perform a defensive role to hinder the invasion of other strains or species into an occupied niche [42]. One single *Bacillus* strain is often able to produce several types of molecules stable over a wide range of pH and temperature and partially resistant to enzyme treatments. Therefore, research for new metabolites with antimicrobial activity is very important in this field. The identification and chemical characterization of antibacterial compounds produced by the *Bacillus* spp. and exploration of their activity against pathogens addresses this subject [39]. The species *Bacillus stercoris* contain characteristic genes which help to produce the antibiotic metabolites like subtilosin and bacilysin, the lipopeptides fengycin and surfactin [21]. The bioactive compound obtained from *B. stercoris* MBTDCMFRI Ba37 was isolated, purified and identified. The molecular formula of the bioactive compound was assigned to be $C_{25}H_{42}O_3$, based on their IR, MS, ID and 2D NMR spectral data analysis. In the IR spectra the split peaks around

2900 cm^{-1} which are due to C–H stretching confirms the presence of CH_3 groups [43]. The peak at 2858 is due to C–H asymmetric stretching of C_2 Hgroups [43]. The peak around 3449 cm^{-1} is due to O–H stretching [44]. The strong C=O stretching frequency appeared around 1728 cm^{-1} confirmed the fact that the ketonic group is attached to a six membered ring system [45]. For five membered or lower ring system, due to higher ring strain the C=O stretching frequency will be 1745 cm^{-1} or higher [45]. The molecular ion peak (M+H peak) at $m/z = 391.45(C_{25}H_{42}O_3 + H)$ and fragments peaks at $m/z = 279.33$, 181.18, and 167.16 are due to fragments $C_{18}H_{31}O_3$, $C_{12}H_{21}O$ and $C_{11}H_{19}O$. The peak obtained at $m/z = 803.68$ is due to $2\text{ M} + \text{Na}$. The $\text{M} + \text{Na}$ and $\text{M} + \text{K}$ (Fig. S6) peaks at 413 and 429 also confirmed the molecular weight of the compound as 390.610.

A quartet with a J value of 6.4 Hz appeared at a downfield shift of δ 3.96 ppm of two hydrogen atoms indicated the presence of two CH groups (C-15 and C-17) attached to an electronegative centre (oxygen atom) and to a CH_3 group [46]. Similarly another quartet with a J value of 6.4 Hz appeared at a downfield shift of δ 3.80 ppm of one hydrogen atom indicated the presence of a CH group (C-24) attached to an electronegative centre (oxygen atom) and to a CH_3 group. The appearance of quartets further confirmed the fact that both these CH groups are further attached to quaternary C atoms (C-1, C-12 and C-13) without any further H atoms to split the quartets formed. Consequently, the CH_3 groups attached to CH groups have appeared as doublets around δ 1.2 ppm (C-18 and C-19) and δ 1.01 ppm (C-27). The presence of CH groups attached to electronegative centers were further confirmed by the presence of ^{13}C signals appeared around δ 71.31, 71.30 and 81.33 ppm. The ^{13}C signals appeared around δ 211.36 ppm confirmed the presence of a C=O group attached to the ring system (C-5) [47]. HSQC correlations observed between H-15 and 17 (δ 3.9 ppm) and C-15 and 17 (δ 71.2 ppm) further confirmed the proposed structure. Similarly HSQC correlation was also prominent between H-24 and C-24. The doublets formed at δ 1.01 and 1.2 with a J value of 6.4 Hz also supported the present proposed structure that these CH_3 groups are only associated with a neighbouring CH protons. H-24 (3.61 ppm) showed HSQC correlation with C-24 (81.33 ppm) indicating that they are directly connected and moreover a downfield appearance of the proton and carbon signals indicated the presence of an electronegative group (OH) attached directly with C-24. Proton at H-4 (3.6 ppm) position exhibited HSQC correlation with C-4 (51.59). Similarly correlation between H-6 and C-6 were confirmed by their HSQC correlation signals appeared at 3.58 ppm and 51.59 ppm. Proton at H-2 exhibited HMBC correlation with C-23 and C-10. H-27 exhibited HMBC correlations with C-25 and C-6, whereas H-6 showed a correlation with C-24. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the

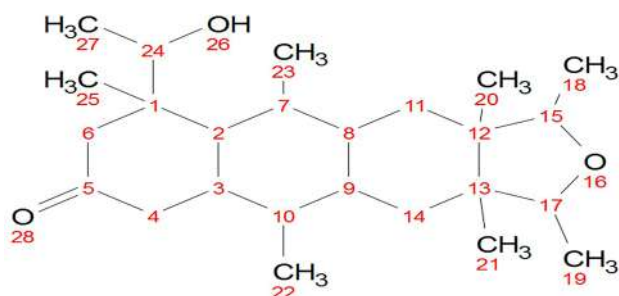


Fig. 4 Structure of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}] heptadecan-5-one

occurrence of 25 carbon atoms in the molecule. The relative positions of the different carbon atoms and corresponding H atoms were further assigned from the ¹H–¹H COSY, HSQC, HMBC, and NOESY spectra to confirm the established skeleton of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}] heptadecan-5-one (Fig. 4). This study for the first time, reports a novel antibacterial compound from *B. stercoris* which may be exploited its application in aquaculture sectors.

Conclusions

Our findings suggested that *B. stercoris* MTDCMFRI Ba37 possess an extensive antibacterial property against a spectrum of aquaculture pathogens. To the authors' knowledge, this is the first report describing the structural characterization of antibacterial compound from *B. stercoris*. From this study, it is concluded that *B. stercoris* MTDCMFRI Ba37 is a potential microorganism for the production of bioactive compounds. Bioassay-guided chromatographic purification techniques lead to the isolation and characterization of a novel antibacterial compound 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one, from this strain. This compound can be further considered in drug development program wherein they may be chemically modified further to increase their activity. A further detailed study of the new compound and their can serve as a breakthrough in the development of new antimicrobial therapeutics ideal for aquaculture in the near future.

Acknowledgements The authors are thankful to the Indian Council of Agricultural Research (ICAR) for funding the study through the Application of Microorganisms in Agriculture and Allied Sectors (AMAAS project). We also express our sincere gratitude to the Director, Central Marine Fisheries Research Institute (CMFRI) for providing necessary facilities to carry out the research. The authors would like to thank the Sophisticated Analytical Instruments Facility (SAIF)–IIT, Chennai, Centre for Bio-Separation Technology (CBST)–Vellore Institute

of Technology University, Vellore and Sophisticated Test and Instrumentation Centre (STIC)–CUSAT, Kochi, for providing assistance in NMR, and FT-IR analysis respectively. The authors are thankful to Dr. V. Sabareesh, Vellore Institute of Technology for his support in LC MS analysis of sample.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent is not applicable to this research as it does not include any research involving Human Participants.

Research involving human and/or animals participants The authors confirm that there has been no research involving Human Participants and/or Animals in this study.

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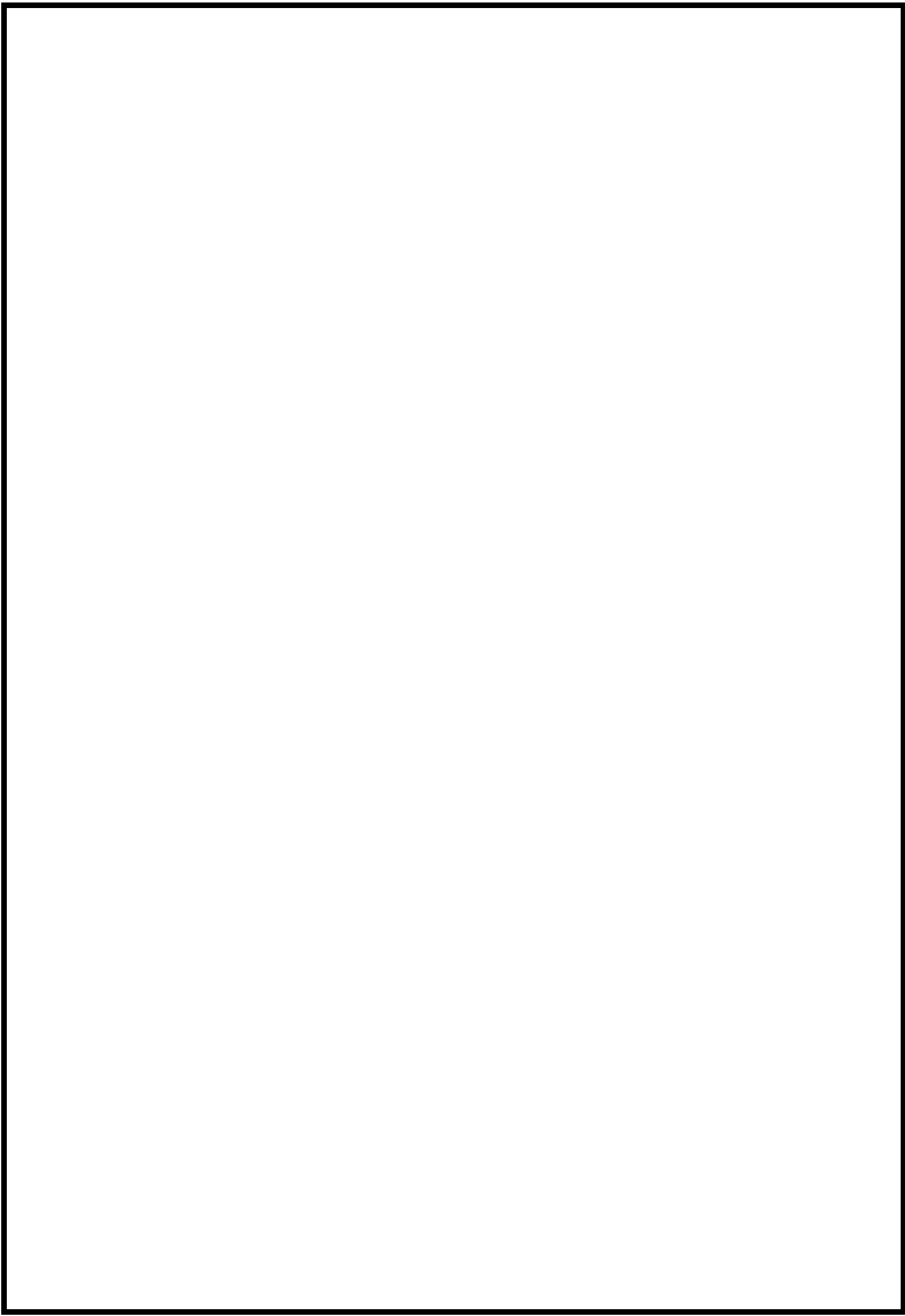
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On a Queueing System with Processing of Service Items under Vacation and N-policy with Impatient Customers

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(Received March 2019 ; accepted April 2020)

Abstract: We assume that customers arrive at a single server queueing system according to Markovian Arrival process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level permitted is L . The inventory processing time follows phase type distribution. The server returns from vacation when there are N customers in the system. Service time follows two distinct phase type distributions depending on whether the processed item is available or not at service commencement epoch. Customers join the queue with probability p and balk with probability $1-p$. Also customers waiting for service become impatient and renege after a random time period which is exponentially distributed. We find the distribution of time until the number of customers hit N . Several system performance characteristics are computed. Also we computed Laplace Stieltjes Transform of the waiting time distribution for the case of no reneging. For the special case of no reneging, some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy are also discussed.

Keywords: Balking and reneging of customers, individual and social optimal strategy, L - and N - policies, Laplace-Stieltjes transform, Markovian arrival process, phase type service and inventory processing, server vacation to produce inventory, waiting time distribution.

1. Introduction

In vacation queueing systems, the server may not be available for a period of time due to working on some supplementary jobs, doing some maintenance work or server's failure that interrupt customer service or simply taking a break. Levy and Yechiali [11] introduced the concept of server vacation. Doshi [5] gives an excellent review of work done in vacation queueing models until 1985. Further developments could be accessed from Takagi [13] and Tian and Zhang [14].

In most of the work reported in queueing theory it is implicitly assumed that if the

server is ready to serve and the customers are available to receive service then the service process proceeds. Either availability of "additional" items required to provide service is not taken into consideration/ignored or its abundance is taken for granted. In the latter case the holding cost incurred is completely ignored. Sometimes the item(s) required for service may not be available. In such cases service cannot be provided even when the server is readily available and customer(s) are waiting. Typical example in medical case is operation theatre. In the absence of 'stent' for a heart patient in need of it, surgery cannot be performed. In a vehicle repair shop a vehicle requiring a specific part replacement, cannot be serviced if spares are not available.

(Expansions of various abbreviations are given in 'Notations and abbreviations' below the introduction.)

Thus in several service systems, availability of both customers and servers cannot guarantee service. This will be explained in detail later. This naturally leads to the investigation of availability of additional item(s) required to provide service. As a consequence some optimization problems arise— how much of additional item(s) to be processed at a stretch (optimal value of L) and what should be the optimal value of N ? This leads to the consideration of holding cost, shortage cost and associated revenue loss. Kazimirisky [10] seems to be the first to introduce 'additional items needed for service'. He considered a single server queue with Batch Markovian Arrival and General Service time distribution denoted as BMAP/G/1 queue, with the server engaged in producing additional items whenever the customers are not waiting. Exactly one processed item is required for each customer. The customer service time distribution depends on whether processed item is available or not. Thus there are two distinct service time distributions.

Baek *et al.* [2] considered MMAP (Marked Markovian Arrival Process) of customers of two types— type I (high priority) and type II (low priority). Both type of customers require a certain minimum number of additional items to start their service. Type I customers do not have a waiting space. If a type I customer is in service while another type I customer arrives, the latter leaves the system. On the other hand if a type II customer is in service, the former is pushed out of the system by the type I arrival provided the number of additional items available is at least equal to the minimum number required to start its service. Else, it leaves the system without changing the status. Type II customers have an infinite capacity waiting space. Additional items arrive to the system according to MAP. Dhanya *et al.* [3] extend the above to retrial queueing set up.

Hanukov *et al.* [8] analyze a simple queueing system where again additional items are needed for service of a customer (one item for each customer). Arrival process is Poisson and service time is exponentially distributed. Accumulation of N customers (N -policy) in queue to commence service, once server becomes idle, was introduced by Yadin and Naor [18]. This has the advantage that the length of a busy period becomes larger when server is

activated on accumulation of N or more customers, thereby bringing down the expected cost incurred per unit time. Divya *et al.* [4] considered single server queue in which the customers arrive according to Markovian Arrival Process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level permitted is L . The inventory processing time follows phase type distribution [12]. The server returns from vacation when there are N customers in the system. The service time follows two distinct phase type distributions depending on whether the processed item is available or not at service commencement epoch. They analysed the distribution of time until the number of customers hit N or the inventory level reaches L , distribution of idle time, the distribution of time until the number of customers hit N and also the distribution of number of inventory processed before the arrival of first customer. They also provided Laplace Stieltjes Transform of busy cycles in which no item is left in the inventory and atleast one item is left in the inventory. They performed some numerical computations to evaluate the expected idle time, standard deviation and coefficient of variation of idle time of the server.

In real life, people become impatient while waiting for service. Hence to model reality, we should take into consideration customers' impatience. To characterize customers' impatience, some terminologies like balking, reneging and retrials are employed in queueing system. Balking customers decide not to join the queue if it is too long and reneging customers leave the queue if they have waited too long for service. Retrial queues study systems where the customers do not wait in a line when server is found to be busy; instead they keep repeating their attempts to access the server at random time points (see Falin and Templeton [6], Artalejo and Gomaz-Corral [1]). Wang *et al.* [16] has presented a review on queueing systems with impatient customers.

Yechiali [19] considers an M/M/c queue, where $c = 1$, or $1 < c < \infty$, or $c = \infty$. At random epochs, the system as a whole gets shock and as a consequence it breaks down. This results in the loss of all customers who are either on the wait for service or undergoing service. When the system is down and undergoing repair process, newly arriving customers become impatient. Their impatience is reflected through a timer of random duration—each customer has his own timer. If a timer expires before the system is repaired, that customer abandons the queue. He analyzed the above described model and derived various quality of service measures such as mean sojourn time of a served customer; proportion of customers served; rate of lost customers due to disasters; and rate of abandonments due to impatience.

Wang and Zhang [17] consider a single-server service-inventory system where customers arrive according to a Poisson process and service times are independent and exponentially distributed. A customer takes exactly one item from the inventory upon service completion. A continuous review policy is adopted to replenish the inventory. With two different information levels, i.e. the fully unobservable case and the partially observable

case, arriving customers decide whether to join or to balk the system. They investigated customers individually optimal and socially optimal strategies, and further consider the optimal pricing issue that maximises the servers revenue. Some numerical experiments were carried out to show that the individually optimal joining probability (or threshold) is not always greater than that of socially optimal one. It was observed that, to maximise the servers revenue, concealing some system information from customers may be more profitable. Conversely, to maximise the social welfare, customers need more system information. Finally, numerical results in the fully unobservable case illustrate a reasonable phenomenon that the revenue maximum is equal to social optimum in most cases.

The present paper extends the work of Hanukov *et al.* [8] to a single server, Markovian Arrival, Phase Type distributed processing time and Phase Type distributed service time (Two distinct types—one when the processed item is available and the other when processed is not available) which we denote as MAP/(PH,PH)/1 queue under N -policy with requirement of one additional item to each customer. At service completion epoch, if the server becomes idle for want of customers, he starts processing additional items required to serve customers. A maximum of L items are processed at a stretch. However during this process, if number of customers in the system hits N , then the server immediately commences service. Processing time of items have Phase type distribution. Service time also follows Phase type distribution; however two distinct distributions are considered—one when an additional item is available to start service and the other when no additional item is available. This is already discussed in Divya *et al.* [4]. In this paper we extend that to the case where the customers are impatient. In addition we investigate the individual, social and system optimal strategies by introducing appropriate costs associated with certain system parameters.

Next we turn to further details of the present work. We consider a single server queueing system in which the customers arrive according to Markovian Arrival process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level is L . The inventory processing time follows phase type distribution. The server returns from vacation when there are N customers in the system. Service time follows two distinct phase type distributions according as there is no processed item or there are processed items at the beginning of service. Customers join the queue with probability p and balk with probability $1 - p$. Also the customers waiting for service may become impatient and renege after a random time period which is exponentially distributed. Whereas Wang and Zhang [17] follow replenishment policy through external sources in the context of queueing-inventory, we investigate the system in which the item is processed by the server himself. Further, in Wang and Zhang model, the server has to stay idle when inventory level drops to zero; in the present model the server processes the item and serves customer if at a service

commencement epoch the item is not available.

In all papers cited above that deal with additional items for service/inventory /processed item, it is assumed that service time duration for a customer in the presence of additional items are shorter than that starts in the absence of such an item. Thus the availability of such an item reduces the waiting time of customers in the system. This additional items are in some cases reusable. In this paper we consider the case of non-reusable items.

The rest of the paper is arranged as follows. The model description and mathematical formulation are given in section 2. Section 3 provides steady state analysis of the model, the distribution of time until the number of customers hit N and some other performance measures. For the special case of no reneging some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy are discussed in section 4. Section 5 contains a special case in which the system is working in normal mode.

Notations and abbreviations used in the sequel:

- $e(a)$ = Column vector of 1s' of order a .
- e'_a : Transpose of e_a .
- e = Column vector of 1s' of appropriate order.
- $CTMC$: Continuous time Markov chain.
- I_a = identity matrix of order a .
- $e_a(b)$ = column vector of order b with 1 in the a th position and the remaining entries zero.
- PH : Phase Type
- MAP : Markovian Arrival Process
- LST : Laplace-Steiltjes Transform
- $LDQBD$: Level Dependent Quasi-Birth and-Death
- $d_{ij}^{(k)}$: entries of D_k , $k = 0$ or 1
- δ_l : l^{th} entry of $D_1 e$.

Highlights of this paper are:

- Extends Hanukov *et al.* [8] to the case of Markovian arrival process. In addition, we set an upper bound (L) on the maximum number items processed by the server at a stretch. Further we introduce the N -policy for the number of customers to accumulate, in order to start a fresh service cycle.

- The inventory processing time and service time distributions follow more general distributions (phase type).
- Extends Divya *et.al.* [4] by introducing the two types of customer impatience: balking and reneging. Thus the system turns out to be a level dependent QBD.
- For the special case of no reneging, some numerical experiments to compute individual optimal strategy, maximum revenue to the server and social optimal strategy are also discussed.

2. Model Description and Mathematical formulation

We assume that the customers arrive at a single server queueing system according to MAP with representation (D_0, D_1) of order n . At the end of a service if the system is left with no customer, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level is restricted to L . Processing time for each item in the inventory follows phase type distribution $PH(\alpha, T)$ of order m_1 . Server returns from vacation when there are N customers in the system. Service time follows $PH(\beta, S)$ of order m_2 when there is no processed item and it follows $PH(\gamma, U)$ of order m_3 when there are processed items. Customers join the queue with probability p and balk with probability $1 - p$. Also the customers waiting for service may become impatient and renege after a random time period which is exponentially distributed with parameter $(n-1)\phi$, $n \geq 1$, where n is the number of customers in the system.

Let $Q^* = D_0 + D_1$ be the generator matrix of arrival process and π^* be its stationary probability vector. Hence π^* is the unique (positive) probability vector satisfying

$$\pi^* Q^* = 0, \pi^* e = 1.$$

The constant $\beta^* = \pi^* D_1 e$, referred to as *fundamental rate*, gives the expected number of arrivals per unit of time in the stationary version of the MAP. It is assumed that arrival process is independent of the inventory processing and service process.

The model described in above can be studied as a level dependent quasi-birth-and-death (LDQBD) process. First we introduce the following notations:

At time t :

$N(t)$: the number of customers in the system at time t ,

$I(t)$: the number of processed inventory,

$$J(t) = \begin{cases} 0, & \text{when the server is on vacation,} \\ 1, & \text{when the server is busy serving a customer.} \end{cases}$$

$K(t)$: the phase of the inventory processing/service process,

$M(t)$: the phase of arrival of customer.

It is easy to verify that $\{(N(t), I(t), J(t), K(t), M(t)) : t \geq 0\}$ is a LDQBD with state space

$$l(0) = \{(0, i, 0, k_1, l) : 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \quad \{(0, L, 0, l) : 1 \leq l \leq n\}.$$

For $1 \leq h \leq N-1$,

$$l(h) = \{(h, i, 0, k_1, l) : 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \quad \{(h, L, 0, l) : 1 \leq l \leq n\} \\ \{(h, 0, 1, k_2, l) : 1 \leq k_2 \leq m_2, 1 \leq l \leq n\} \quad \{(h, i, 1, k_3, l) : 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n\}$$

and for $h \geq N$,

$$l(h) = \{(h, 0, 1, k_2, l) : 1 \leq k_2 \leq m_2, 1 \leq l \leq n\} \quad \{(h, i, 1, k_3, l) : 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n\}.$$

Note that when $0 \leq N(t) \leq N-1$ and $I(t) = L$, the server will be idle as the inventory level reaches its maximum level 'L' and the number of customers doesnot hit 'N'. So $K(t)$ neednot be considered.

The infinitesimal generator of this CTMC is

$$_1 = \begin{bmatrix} B_0 & C_0 & & & & & & & \\ B_1 & E_1 & I \otimes pD_1 & & & & & & \\ & B_2 & E_2 & I \otimes pD_1 & & & & & \\ & & & & B_{N-2} & E_{N-2} & I \otimes pD_1 & & \\ & & & & & B_{N-1} & E_{N-1} & F & \\ & & & & & & B'_N & A_1^{(N)} & A_0^{(N)} \\ & & & & & & & A_2^{(N+1)} & A_1^{(N+1)} & A_0^{(N+1)} \end{bmatrix}.$$

The boundary blocks B_0, C_0, B_1 are of orders $(Lm_1 + 1)n \times (Lm_1 + 1)n$, $(Lm_1 + 1)n \times ((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n)$, $((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n) \times (Lm_1 + 1)n$, respectively. For $2 \leq h \leq N-1$, B_h and for $1 \leq h \leq N-1$, E_h are square matrices of order $(m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n$. F and B'_N are of orders $((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n) \times (m_2 + Lm_3)n$ and $(m_2 + Lm_3)n \times ((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n)$, respectively. For $h \geq N$, $A_0^{(h)}$, $A_1^{(h)}$ and for $h \geq N+1$, $A_2^{(h)}$ are square matrices of order $(m_2 + Lm_3)n$. Define the entries $B_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, $C_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ and $B_{1(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$, $(0, i_1, j_1, k_1, l_1) \rightarrow (1, i_2, j_2, k_2, l_2)$ and $(1, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$, respectively. Define $E_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, $B_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, F and B'_N as transition

submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$, where $1 \leq h \leq N-1$, $(h, i_1, j_1, k_1, l_1) \rightarrow (h-1, i_2, j_2, k_2, l_2)$, where $2 \leq h \leq N-1$, $(N-1, i_1, j_1, k_1, l_1) \rightarrow (N, i_2, j_2, k_2, l_2)$ and $(N, i_1, j_1, k_1, l_1) \rightarrow (N-1, i_2, j_2, k_2, l_2)$, respectively. Define the entries $A_2^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$, $A_1^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$ and $A_0^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h-1, i_2, j_2, k_2, l_2)$, where $h \geq N+1$, $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$ and $(h, i_1, j_1, k_1, l_1) \rightarrow (h+1, i_2, j_2, k_2, l_2)$, where $h \geq N$, respectively. Since none or one event alone could take place in a short interval of time with positive probability, in general, a transition such as $(h_1, i_1, j_1, k_1, l_1) \rightarrow (h_2, i_2, j_2, k_2, l_2)$ has positive rate only for exactly one of h_1, i_1, j_1, k_1, l_1 different from h_2, i_2, j_2, k_2, l_2

$$B_0^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} T^0 \alpha \otimes I_n & i_2 = i_1 + 1, 0 \leq i_1 \leq L-2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n & i_1 = L-1, i_2 = L; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T \oplus \Delta & i_1 = i_2, 0 \leq i_1 \leq L-1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \Delta & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \end{cases}$$

where

$$\Delta = D_0 + (1-p) \begin{bmatrix} \delta_1 & & \\ & \delta_2 & \\ & & \ddots \\ & & & \delta_n \end{bmatrix},$$

$$C_0^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} I_{m_1} \otimes pD_1, & 0 \leq i_1 \leq L-1; i_1 = i_2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ pD_1, & i_2 = i_1 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$B_1^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S^0 \alpha \otimes I_n, & i_1 = i_2 = 0; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq m_2, 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ U^0 \alpha \otimes I_n, & 1 \leq i_1 \leq L; i_2 = i_1 - 1; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $1 \leq h \leq N-1$,

$$E_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} T^0 \alpha \otimes I_n, & 0 \leq i_1 \leq L-2, i_2 = i_1 + 1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n, & i_1 = L-1, i_2 = L; j_1 = j_2 = 0; 1 \leq k_1 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2, 0 \leq i_1 \leq L-1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2 = 0, j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ U \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_3, 1 \leq l_1, l_2 \leq n \\ \Delta - (h-1)\phi I_n, & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $2 \leq h \leq N-1$,

$$B_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} (h-1)\phi I_{m_1 n}, & 0 \leq i_1 \leq L-1, i_1 = i_2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_n, & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S^0 \beta \otimes I_n + (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_{m_3 n}, & 1 \leq i_1 \leq L, i_1 = i_2; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$F_{(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} e(m_1) \otimes (\beta \otimes pD_1), & i_1 = i_2 = 0; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_2} \otimes pD_1, & i_2 = i_1 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ I_{m_3} \otimes pD_1, & i_2 = i_1, 1 \leq i_1 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ e(m_1) \otimes (\gamma \otimes pD_1), & 1 \leq i_1 \leq L-1; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ \gamma \otimes pD_1, & i_1 = i_2 = L; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$B'_{N(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} S^0 \beta \otimes I_n + (N-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ (N-1)\phi I_{m_3 n}, & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $h \geq N+1$,

$$A_2^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S^0 \beta \otimes I_n + (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & i_2 = i_1 - 1, 2 \leq i_2 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_{m_3 n}, & i_2 = i_1, 1 \leq i_2 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $h \geq N$,

$$A_1^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S \oplus \Delta - (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0, j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ U \oplus \Delta - (h-1)\phi I_{m_3 n}, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_3, 1 \leq l_1, l_2 \leq n. \end{cases}$$

$$A_0^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} I_{m_2} \otimes pD_1, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_3} \otimes pD_1, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

Remarks: When $L = 0$ (that is, no item processed during vacation) the problem discussed reduces to classical N -policy.

3. Steady State Analysis

3.1. Stability condition

Lemma 3.1. *The system under consideration is stable.*

Proof. We use the following result to prove this(see Tweedie [15]).

Result (Tweedie [15]) Let $\{X(t)\}$ be a Markov process with discrete state space and rates of transition q_{sr} , $s, r \in S$, $\sum_r q_{sr} = 0$. Assume that there exist

1. a function $\psi(s)$, $s \in S$, which is bounded from below (this function is said to be a Lyapunov or test function);
2. a positive number ϵ such that:

- variables $y_s = \sum_{r \neq s} q_{sr} (\psi(r) - \psi(s)) < \infty$ for all $s \in S$;
- $y_s \leq -\epsilon$ for all $s \in S$ except perhaps a finite number of states.

Then the process $\{X(t)\}$ is regular and ergodic.

For the model under discussion, we consider the following test function:

$$\psi(s) = \psi(h, i, j, k, l) = h.$$

The mean drifts

$$\begin{aligned} y_s &= \sum_{r \neq s} q_{sr} (\psi(r) - \psi(s)) \\ &= q_{s,s+1} - q_{s,s-1}. \end{aligned} \tag{1}$$

We have $q_{s,s+1} = r_1$, say (a constant) and $q_{s,s-1} = r_2 + (s-1)\phi$, where r_2 is a constant. Hence from (1), $y_s = r_1 - r_2 - (s-1)\phi$, which depends only on the level s . Now,

$$\lim_{s \rightarrow \infty} y_s = -\infty.$$

Thus the assumptions of Tweedie's result hold and hence the Markov process under consideration is regular and ergodic. Hence the system is stable.

3.2. Steady state vector

By finite truncation method we get steady state vectors of the LDQBD approximately. In this method, we truncate the infinitesimal generator at a finite level K . The level K is chosen in such a way that probability of customer loss due to truncation is small. To get an appropriate level, say K_f , we start with an initial value for K and increasing it in unit steps until a properly chosen cut-off criterion is satisfied. Here, we use the algorithm by Artalejo *et al.* [1], the steps of which are explained below.

With K as cut-off level, the modified generator is

$$\bar{Q}(K) = \begin{bmatrix} B_0 & C_0 & & & & & & & & \\ B_1 & E_1 & I \otimes pD_1 & & & & & & & \\ & B_2 & E_2 & I \otimes pD_1 & & & & & & \\ & & & & & & & & & \\ & & & B_{N-2} & E_{N-2} & I \otimes pD_1 & & & & \\ & & & B_{N-1} & E_{N-1} & F & & & & \\ & & & & B'_N & A_1^{(N)} & A_0^{(N)} & & & \\ & & & & & A_2^{(N+1)} & A_1^{(N+1)} & A_0^{(N+1)} & & \\ & & & & & & & & & \\ & & & & & & A_2^{(K-1)} & A_1^{(K-1)} & A_0^{(K-1)} & \\ & & & & & & & A_2^{(K)} & \theta^{(K)} & \end{bmatrix},$$

where $\theta^{(K)} = A_1^{(K)} + A_0^{(K)}$. Let $\bar{\pi}$ be the stationary distribution of $\bar{Q}(K)$ which satisfies

$$\begin{aligned} \bar{\pi} \bar{Q}(K) &= 0, \\ \bar{\pi} e &= 1, \end{aligned} \quad (2)$$

where $\bar{\pi} = [\bar{\pi}(0), \bar{\pi}(1), \dots, \bar{\pi}(K)]$. Define $\mathbf{y} = [\mathbf{y}_0(K), \mathbf{y}_1(K)]$ with

$$\begin{aligned} \mathbf{y}_0(K) &= [\bar{\pi}(0), \bar{\pi}(1), \dots, \bar{\pi}(K-1)], \\ \mathbf{y}_1(K) &= \bar{\pi}(K). \end{aligned}$$

Now $\mathbf{y}(K, i) = \bar{\pi}(i)$, $0 \leq i \leq K$. Here $\mathbf{y}_0(K)$ is a row vector of dimension $m = (Lm_1 + 1)n + (N-1)[m_2n + L(m_1 + m_3)n + n] + (K-N)(m_2 + Lm_3)n$ and $\mathbf{y}_1(K)$ is a row vector of dimension $(m_2 + Lm_3)n$. Now from (2), we have

$$[\mathbf{y}_0(K), \mathbf{y}_1(K)] \begin{bmatrix} H_{00}(K) & H_{01}(K) \\ H_{10}(K) & H_{11}(K) \end{bmatrix} = [\mathbf{0}_m, \mathbf{0}_{(m_2 + Lm_3)n}], \quad (3)$$

where $H_{00}(K)$ is obtained from $\bar{Q}(K)$ by deleting the last column matrices and last row matrices. $H_{01}(K) = [0, 0, \dots, 0, A_0^{(K-1)}]^T$, $H_{10}(K) = [0, 0, \dots, 0, A_2^{(K)}]$ and $H_{11}(K) = \theta^{(K)}$. These are block structured matrices with $K \times K$, $K \times 1$, $1 \times K$ and 1×1 blocks respectively. $\mathbf{0}_m$ and $\mathbf{0}_{(m_2 + Lm_3)n}$ are row vectors of dimensions m and $(m_2 + Lm_3)n$, respectively, with all entries equal to zero. From (3), we get

$$\mathbf{y}_1(K) H_{10}(K) H_{00}^{-1}(K) = -\mathbf{y}_0(K), \quad (4)$$

$$\mathbf{y}_1(K)[H_{11}(K) - H_{10}(K)H_{00}^{-1}(K)H_{01}(K)] = \mathbf{0}_{(m_2+Lm_3)n}. \quad (5)$$

Also we have

$$H_{00}(K) = \begin{bmatrix} H_{00}(K-1) & H_{01}(K-1) \\ J_0(K-1) & J_1(K-1) \end{bmatrix},$$

where

$$\begin{aligned} J_0(K-1) &= [0, \quad, 0, A_2^{(K-1)}], \\ J_1(K-1) &= A_1^{(K-1)}. \end{aligned}$$

The inverse of matrix $H_{00}(K)$ can be determined using theorem 4.2.4 in Hunter [9] as

$$H_{00}^{-1}(K) = \begin{bmatrix} M_{00}(K) & M_{01}(K) \\ M_{10}(K) & M_{11}(K) \end{bmatrix},$$

where

$$\begin{aligned} M_{00}(K) &= [H_{00}(K-1) - H_{01}(K-1)J_1^{-1}(K-1)J_0(K-1)]^{-1}, \\ M_{01}(K) &= -J_1^{-1}(K-1)J_0(K-1)M_{00}(K), \\ M_{11}(K) &= [J_1(K-1) - J_0(K-1)H_{00}^{-1}(K-1)H_{01}(K-1)]^{-1}, \\ M_{10}(K) &= -H_{00}^{-1}(K-1)H_{01}(K-1)M_{11}(K). \end{aligned}$$

Now we can see that the structure of the block matrices $H_{01}(K-1)$ and $J_0(K-1)$ simplify the above set of equations. We have

$$H_{00}^{-1}(K-1)H_{01}(K-1) = \begin{bmatrix} M_{01}(K-1) \\ M_{11}(K-1) \end{bmatrix} A_0^{(K-2)}.$$

$$\text{Also } J_0(K-1)H_{00}^{-1}(K-1)H_{01}(K-1) = A_2^{(K-1)}M_{11}(K-1)A_0^{(K-2)}.$$

By example 4.2.2 (Hunter [9]), we have $(X + AYB)^{-1} = X^{-1} - X^{-1}A(Y^{-1} + BX^{-1}A)^{-1}BX^{-1}$.

Then we have

$$(X + AYB)^{-1} = [I - X^{-1}A(Y^{-1} + BX^{-1}A)^{-1}B]X^{-1}.$$

Here, we have $X = H_{00}(K-1), A = -H_{01}(K-1), Y = J_1^{-1}(K-1)$ and $B = J_0(K-1)$.

Finally, we get

$$\begin{aligned} M_{00}(K) &= [I - M_{01}(K)J_0(K-1)]H_{00}^{-1}(K-1), \\ M_{11}(K) &= [J_1(K-1) - A_2^{(K-1)}M_{11}(K-1)A_0^{(K-2)}]^{-1}, \\ M_{01}(K) &= -\begin{bmatrix} M_{01}(K-1) \\ M_{11}(K-1) \end{bmatrix} A_0^{(K-2)}M_{11}(K), \\ M_{10}(K) &= -J_1^{-1}(K-1)J_0(K-1)M_{00}(K). \end{aligned}$$

Thus the computation of the vector $\mathbf{y}_1(K)$ reduces to solving the system of equations (5) subject to the normalizing condition

$$\bar{\pi}(K)[\mathbf{e} - H_{10}(K)H_{00}^{-1}(K)\mathbf{e}] = 1.$$

The vector $\mathbf{y}_0(K)$ can be solved by substituting $\mathbf{y}_1(K)$ in (4). To get the cut-off value, successive increments of K are made, starting from $N+2$ and we stop at the point $K = K_c$ when

$$\max_{0 \leq i \leq K_c} \|\mathbf{y}(K_c, i) - \mathbf{y}(K_c - 1, i)\|_\infty < \epsilon,$$

where $\epsilon > 0$ is infinitesimal quantity and $\|\cdot\|_\infty$ is the infinity norm (see Goswami and Selavaraju [7]).

Now we suppose that $x_{h,i,j,k,l}$ represent the steady state probability that system is in state (h, i, j, k, l) .

3.3. Distribution of time until the number of customers hit N

We show that this is a phase type distribution where the underlying Markov process has state space

$$\{(h, i, j, k) : 0 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq j \leq m_1, 1 \leq k \leq n\}$$

$$\cup \{(h, L, k) : 0 \leq h \leq N-1, 1 \leq k \leq n\} \cup \{*\},$$

where $*$ denotes the absorbing state indicating the number of customers reaching N . The infinitesimal generator is

$$\mathbf{V}_1 = \begin{bmatrix} V_1 & V_1^{(0)} \\ \mathbf{0} & \mathbf{0} \end{bmatrix}, \text{ where}$$

$$\mathbf{V}_1 = \begin{bmatrix} H_0 & I_{Lm_1+1} \otimes pD_1 & & & \\ & H_0 & I_{Lm_1+1} \otimes pD_1 & & \\ & G_2 & H_2 & I_{Lm_1+1} \otimes pD_1 & \\ & \ddots & \ddots & \ddots & \\ & & G_{N-2} & H_{N-2} & I_{Lm_1+1} \otimes pD_1 \\ & & & G_{N-1} & H_{N-1} \end{bmatrix},$$

$$V_1^{(0)} = \begin{bmatrix} \mathbf{0} \\ \mathbf{0} \\ \mathbf{e}(Lm_1+1) \otimes p\delta \end{bmatrix}, \text{ with } H_0 = \begin{bmatrix} T \oplus \Delta & T^0 \alpha \otimes I_n & 0 & 0 \\ 0 & T \oplus \Delta & T^0 \alpha \otimes I_n & 0 \\ 0 & 0 & T \oplus \Delta & T^0 \otimes I_n \\ 0 & 0 & 0 & \Delta \end{bmatrix}.$$

For $2 \leq h \leq N-1$,

$$G_h = (h-1)\phi I_{(Lm_1+1)n}$$

and

$$H_h = \begin{bmatrix} T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \alpha \otimes I_n & 0 & 0 \\ 0 & T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \alpha \otimes I_n & 0 \\ 0 & 0 & T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \otimes I_n \\ 0 & 0 & 0 & \Delta - (h-1)\phi I_{m_1n} \end{bmatrix}.$$

The initial probability vector is

$$\psi_1 = \left(\frac{1}{d_1}\right)(w_{0,0,1,1}, \dots, w_{0,0,1,n}, \dots, w_{0,0,m_1,1}, \dots, w_{0,0,m_1,n}, \dots, w_{0,L-1,m_1,1}, \dots, w_{0,L-1,m_1,n}, 0),$$

where

$$w_{0,0,k,l} = \sum_{k'=1}^{m_2} \frac{\sigma_{k'} \alpha_k}{-d_{ll}^{(0)} - S_{k'k'}} x_{1,0,1,k',l} + \sum_{k'=1}^{m_3} \frac{\tau_{k'} \alpha_k}{-d_{ll}^{(0)} - U_{k'k'}} x_{1,1,1,k',l},$$

$$w_{0,i,k,l} = \sum_{k'=1}^{m_3} \frac{\tau_{k'} \alpha_k}{-d_{ll}^{(0)} - U_{k'k'}} x_{1,i+1,1,k',l}, \quad \text{with } 1 \leq i \leq L-1,$$

and

$$d_1 = \sum_{l=1}^n \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} w_{0,i,k,l}, \text{ where } \mathbf{0} \text{ is a zero matrix of order } 1 \times ((N-1)Lm_1n + n).$$

Here, $\sigma_{k'}$ represents the absorption rate to phase k' from $PH(\beta, S)$, $\tau_{k'}$ represents the absorption rate to phase k' from $PH(\gamma, U)$, $S_{k'k'}$ represent the $k'k'$ th entry of S , $U_{k'k'}$ represent the $k'k'$ th entry of U , α_k denote the k th component of the initial distribution of inventory processing and $d_{ll}^{(0)}$ represent the diagonal entry in l th row of D_0 .

3.4. Some other Performance Measures

- Probability that the server is idle,

$$P_{idle} = \sum_{h=0}^{N-1} \sum_{l=1}^n x_{h,L,0,l}.$$

- The fraction of time the server is idle waiting for N th customer,

$$P_{id} = \sum_{l=1}^n x_{N-1,L,0,l}.$$

- The fraction of time the server is busy in inventory production,

$$P_{pro} = \sum_{h=0}^{N-1} \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n x_{h,i,0,k,l}.$$

- The fraction of time the server is busy serving,

$$P_{ser} = \sum_{h=1}^K \sum_{k=1}^{m_2} \sum_{l=1}^n x_{h,0,1,k,l} + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n x_{h,i,1,k,l}.$$

- Expected number of customers in the system,

$$\begin{aligned} E(S) = & \sum_{h=1}^{N-1} \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n h x_{h,i,0,k,l} + \sum_{h=1}^{N-1} \sum_{l=1}^n h x_{h,L,0,l} + \sum_{h=1}^K \sum_{k=1}^{m_2} \sum_{l=1}^n h x_{h,0,1,k,l} \\ & + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n h x_{h,i,1,k,l}. \end{aligned} \quad (6)$$

- Expected number of items in the inventory,

$$E(it) = \sum_{h=0}^{N-1} \sum_{i=1}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n i x_{h,i,0,k,l} + \sum_{h=0}^{N-1} \sum_{l=1}^n L x_{h,L,0,l} + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n i x_{h,i,1,k,l}. \quad (7)$$

- Expected rate at which the inventory processing is switched on,

$$E(ipo) = \sum_{k=1}^{m_2} \sum_{l=1}^n \sigma_k x_{1,0,1,k,l} + \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n \tau_k x_{1,i,1,k,l}.$$

4. Special Cases

1. $p = 1, \phi = 0$

In this case, the present model reduces to Divya *et al.* [4]. We see that the model can be studied as a LIQBD process.

2. $\phi = 0$

In this case also, the model can be studied as a LIQBD process with obvious modifications in Divya *et al.* [4].

From now on we concentrate in the case $\phi = 0$.

First, we find the LST of the waiting time distribution.

4.1. Waiting time analysis

To find the waiting time of a customer who joins for service at time t , we have to consider different possibilities depending on the status of server at that time. The server may be on vacation or in normal mode. Let $W(t)$ be the waiting time of a customer in the system who arrives at time t and $W^*(s)$ be the corresponding LST.

Case I. (Vacation mode)

Let Ev_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(h'+1, i', 0, k', l')$ or in the state $(h'+1, L, 0, l')$, where $0 \leq h' \leq N-2$, $0 \leq i' \leq L-1$, $1 \leq k' \leq m_1$, $1 \leq l' \leq n$.

In this case, the waiting time is the time until absorption in a Markov process whose state space is given by $\{(h, i, k_1, l) : 1 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \cup \{(h, L, l) : 1 \leq h \leq N-1, 1 \leq l \leq n\} \cup \{(h^*, 0, k_2) : 1 \leq h^* \leq N-1, 1 \leq k_2 \leq m_2\} \cup \{(h^*, i, k_3) : 1 \leq h^* \leq N-1, 1 \leq i \leq L, 1 \leq k_3 \leq m_3\} \cup \{*\}$ where (h, i, k_1, l) denote the states that correspond to the server being on vacation with h customers in the system, i , items in inventory, k_1 , the processing phase and l , the arrival phase, (h, L, l) denote the state that correspond to the server being on vacation mode with h customers in the system, L items in inventory and l , the arrival phase. $(h^*, 0, k_2)$ denote the states that correspond to the tagged customer being in the position h^* when the server is on normal mode, k_2 , the service phase when there is no processed item, (h^*, i, k_3) denote the states that correspond to the tagged customer being in position h^* when the server is in normal mode with i processed items in the inventory and k_3 denote the service phase and $*$ denote the absorbing state indicating the service completion of the tagged customer. Thus the conditional waiting time can be studied by a phase type distribution with representation $PH(\psi_1, W_1)$ where

$$W_1 = \begin{bmatrix} M_{11} & M_{12} \\ 0 & M_{22} \end{bmatrix}, \quad W_1^0 = \begin{bmatrix} \mathbf{0} \\ M^0 \end{bmatrix},$$

where

$$M^0 = \begin{bmatrix} M^{00} \\ \mathbf{0} \end{bmatrix}, \quad \text{with} \quad M^{00} = \begin{bmatrix} S^0 \\ e(L) \otimes U^0 \end{bmatrix},$$

$$M_{11} = \begin{bmatrix} E & I_{Lm_1+1} \otimes D_1 \\ & E & I_{Lm_1+1} \otimes D_1 \\ & & E \end{bmatrix}, \quad \text{where}$$

$$E = \begin{bmatrix} T \oplus \Delta & T^0 \alpha \otimes I_n & & \\ & T \oplus \Delta & T^0 \alpha \otimes I_n & \\ & & T \oplus \Delta & T^0 \otimes I_n \\ & & & \Delta \end{bmatrix},$$

$M_{12} = e_{N-1}(N-1)e_{h_1}'(N-1) \otimes F$, where

$$F = \begin{bmatrix} e(m_1) \otimes (p\delta \otimes \beta) & & \\ & e(m_1) \otimes (p\delta \otimes \gamma) & \\ & & p\delta \otimes \gamma \end{bmatrix},$$

$$M_{22} = \begin{bmatrix} G & & \\ H & G & \\ & & H & G \end{bmatrix}, \quad \text{where } G = \begin{bmatrix} S & \\ & I_L \otimes U \end{bmatrix}, \quad H = \begin{bmatrix} S^0 \beta & 0 & 0 \\ U^0 \beta & 0 & 0 \\ 0 & I_{L-1} \otimes (U^0 \gamma) & 0 \end{bmatrix}.$$

Thus the conditional LST,

$$W^*(s | Ev_1) = \psi_1(sI - W_1)^{-1}W_1^0,$$

where ψ_1 is the initial probability vector which ensures that the Markov chain always starts from the level h .

Case II. (Normal mode)

Let Ev_2 denote the event that the tagged customer immediately after his joining finds the system in the state $(h'+1, 0, 1, k'', l')$, where $h' \geq 1, 1 \leq k'' \leq m_2, 1 \leq l' \leq n$ or in the state $(h'+1, i', 1, k''', l')$, where $1 \leq h' \leq N-1, 1 \leq i' \leq L-N+h', 1 \leq k''' \leq m_3, 1 \leq l' \leq n$ or in the state $(h'+1, i', 1, k''', l')$, where $h' \geq N, 1 \leq i' \leq L, 1 \leq k''' \leq m_3, 1 \leq l' \leq n$.

In this case, the waiting time is the time until absorption in a Markov process whose state space is given by $\{(h, 0, k) : 2 \leq h \leq K, 1 \leq k \leq m_2\} \cup \{(h, i, k) : 2 \leq h \leq N-1, 1 \leq i \leq L-N+h, 1 \leq k \leq m_3\} \cup \{(h, i, k) : N \leq h \leq K, 1 \leq i \leq L, 1 \leq k \leq m_3\} \cup \{*\}$ where $(h, 0, k)$ denote the states that correspond to the server being in normal mode with h customers in the system, service phase k when there is no processed item, (h, i, k) denote the states that correspond to the server being in normal mode with h customers in the system, service phase k when there are i processed items and $*$ denote the absorbing state indicating the service completion of the tagged customer and K is chosen in such a way that

$P\left(\sum_{h=0}^K x_h e^h > 1 - \epsilon\right) \rightarrow 0$ for every $\epsilon > 0$. Thus the conditional waiting time can be studied by a truncated phase type distribution with representation $Ph(\psi_2, W_2)$ where

$$W_2 = \begin{bmatrix} G_1 & & & & & \\ H_1 & G_2 & & & & \\ & & H_{N-2} & G_{N-1} & & \\ & & & H_{N-1} & G & \\ & & & & H & G \\ & & & & & H & G \end{bmatrix},$$

$$W_2^0 = \begin{bmatrix} E^0 \\ \mathbf{0} \\ \mathbf{0} \end{bmatrix}, \text{ where } E^0 = \begin{bmatrix} S^0 \\ e(L-N+1) \otimes U^0 \end{bmatrix}.$$

For $1 \leq h \leq N-1$,

$$G_h = \begin{bmatrix} S \\ I_{L-N+h} \otimes U \end{bmatrix}, \quad H_h = \begin{bmatrix} S^0 \beta & 0 \\ U^0 \beta & 0 \\ 0 & I_{L-N+h} \otimes U^0 \gamma \end{bmatrix},$$

$$E = \begin{bmatrix} S \\ I_L \otimes U \end{bmatrix}, \quad F = \begin{bmatrix} S^0 \beta & 0 & 0 \\ U^0 \beta & 0 & 0 \\ 0 & I_{L-1} \otimes U^0 \gamma & 0 \end{bmatrix}.$$

Thus the conditional LST,

$$W^*(s | Ev_2) = \psi_2 (sI - W_2)^{-1} W_2^0,$$

where ψ_2 is the initial probability vector which ensures that the Markov chain always starts from the level h .

Let $w_{h,i,j,k,l}$ and $w_{h,L,0,l}$ denote the probability that the tagged customer finds the system in the state (h,i,j,k,l) and $(h,L,0,l)$ respectively immediately after his arrival. Then

$$w_{h,i,0,k_1,l} = \sum_{l'=1}^n \frac{p d_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1 k_1}} x_{h-1,i,0,k_1,l'},$$

$$1 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n$$

$$w_{h,L,0,l} = \sum_{l'=1}^n \frac{p d_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'}} x_{h-1,L,0,l'}, \quad 1 \leq h \leq N-1, 1 \leq l \leq n$$

$$w_{h,0,1,k_2,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2 k_2}} x_{h-1,0,1,k_2,l'}, \quad 2 \leq h \leq N-1, \text{ or } h \geq N+1, \\ 1 \leq k_2 \leq m_2, 1 \leq l \leq n$$

$$w_{N,0,1,k_2,l} = \sum_{l'=1}^n \sum_{k_1=1}^{m_1} \frac{pd_{l'l}^{(1)} \beta_{k_2}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1 k_1}} x_{N-1,0,0,k_1,l'} \\ + \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2 k_2}} x_{N-1,0,1,k_2,l'}, \quad 1 \leq k_2 \leq m_2, 1 \leq l \leq n$$

$$w_{h,i,1,k_3,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{k_3 k_3}} x_{h-1,i,1,k_3,l'}, \\ 2 \leq h \leq N-1, 1 \leq L-N+h-1, 1 \leq k_3 \leq m_3, 1 \leq l \leq n$$

$$w_{h,i,1,k_3,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{k_3 k_3}} x_{h-1,i,1,k_3,l'}, \quad h \geq N+1, 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n$$

$$w_{N,i,1,k_3,l} = \sum_{l'=1}^n \sum_{k_1=1}^{m_1} \frac{pd_{l'l}^{(1)} \gamma_{k_3}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1 k_1}} x_{N-1,i,0,k_1,l'} \\ + \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2 k_2}} x_{N-1,i,1,k_2,l'}, \quad 1 \leq i \leq L, 1 \leq k_2 \leq m_2, 1 \leq l \leq n.$$

Thus we have the following theorem.

Theorem 4.1. *The LST of the waiting time is given by*

$$W^*(s) = \frac{1}{d_2} \left[\sum_{h'=1}^{N-1} \sum_{i'=0}^{L-1} \sum_{k'=1}^{m_1} \sum_{l'=1}^n \psi_1(sI - W_1)^{-1} W_1^0 w_{h',i',0,k',l'} + \sum_{h'=1}^{N-1} \sum_{l'=1}^n \psi_1(sI - W_1)^{-1} W_1^0 w_{h',L,0,l'} \right. \\ + \sum_{h'=1}^{\infty} \sum_{k''=1}^{m_2} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',0,1,k'',l'} + \sum_{h'=1}^N \sum_{i'=1}^{L-N+h'-1} \sum_{k'''=1}^{m_3} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',i',1,k''',l'} \\ \left. + \sum_{h'=N+1}^{\infty} \sum_{i'=1}^L \sum_{k'''=1}^{m_3} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',i',1,k''',l'} \right], \quad (8)$$

where

$$d_2 = \sum_{h'=1}^{N-1} \sum_{i'=0}^{L-1} \sum_{k'=1}^{m_1} \sum_{l'=1}^n w_{h',i',0,k',l'} + \sum_{h'=1}^{N-1} \sum_{l'=1}^n w_{h',L,0,l'} + \sum_{h'=1}^{\infty} \sum_{k''=1}^{m_2} \sum_{l'=1}^n w_{h',0,1,k'',l'} \\ + \sum_{h'=1}^N \sum_{i'=1}^{L-N+h'-1} \sum_{k'''=1}^{m_3} \sum_{l'=1}^n w_{h',i',1,k''',l'} + \sum_{h'=N+1}^{\infty} \sum_{i'=1}^L \sum_{k'''=1}^{m_3} \sum_{l'=1}^n w_{h',i',1,k''',l'}. \quad (9)$$

Now, we assume that each customer receives a reward of R units after service completion and he has to pay a price q ($0 \leq q < R$) for an item. Let h_w denote the waiting

cost per unit time of a customer in the system.

4.2. Individual equilibrium strategy

Define

$$F_1(p) = R - q - h_w E(W).$$

We have to find an equilibrium strategy according to which the customers join the system.

4.3. Revenue maximization

We have to find an optimal price q to maximize the revenue of the server given by

$$F_2(q) = p_e q \pi^* D_1 e - h_1 E(S) - h_2 E(it) - cE(ipo),$$

where

h_1 : holding cost/unit customer/unit time,

h_2 : holding cost/unit item/unit time,

c : switching on cost of inventory processing/unit time,

p_e : Individual equilibrium strategy corresponding to q .

4.4. Social optimal strategy

Next we consider social optimal strategy. For a given price q and a joining probability p , the surplus of all customers S_1 and the server revenue S_2 are given by

$$S_1 = \pi^* p D_1 e (R - q - h_w E(W)) \text{ and } S_2 = p q \pi^* D_1 e - h_1 E(S) - h_2 E(it) - cE(ipo).$$

Therefore, the expected social welfare per unit time is,

$$F_3(p) = S_1 + S_2 = \pi^* p D_1 e (R - h_w E(W)) - h_1 E(S) - h_2 E(it) - cE(ipo).$$

4.5. Numerical results

$$\text{We fix } N = 3, L = 2, \alpha = \beta = [1 \ 0], \gamma = [0.8 \ 0.2], T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix},$$

$$S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}, U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

We find the individual optimum and social optimum corresponding to the above parameters.

Table 1. Effect of p on various performance measures, when $D_0 = (-20)$, $D_1 = (20)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.5045	1.0248	1.8794	0.6445	-10.2243	76.2876
0.2	0.2571	1.0514	1.7601	1.2429	2.1430	238.8498
0.3	0.1762	1.0806	1.6421	1.7921	6.1878	339.5609
0.4	0.1368	1.1129	1.5254	2.2882	8.1599	472.8811
0.5	0.1138	1.1486	1.4102	2.7271	9.3077	607.5573
0.6	0.0991	1.1881	1.2965	3.1041	10.0449	743.7425
0.7	0.0891	1.2316	1.1848	3.4149	10.5461	881.5515
0.8	0.0821	1.2794	1.0752	3.6551	10.8954	1021.0406
0.9	0.0773	1.3325	0.9680	3.8207	11.1356	1162.1853
1.0	0.0743	1.3925	0.8635	3.9081	11.2870	1304.8471

Table 2. Effect of p on various performance measures, when $D_0 = (-25)$, $D_1 = (25)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.4052	1.0312	1.8494	0.7985	-5.2584	108.9862
0.2	0.2084	1.0657	1.7009	1.5239	4.5807	273.3550
0.3	0.1446	1.1045	1.5545	2.1694	7.7701	439.4306
0.4	0.1138	1.1486	1.4102	2.7271	9.3077	607.5573
0.5	0.0962	1.1986	1.2684	3.1882	10.1881	778.0391
0.6	0.0853	1.2549	1.1297	3.5441	10.7364	951.0852
0.7	0.0783	1.3187	0.9946	3.7865	11.0843	1126.7485
0.8	0.0743	1.3925	0.8635	3.9081	11.2869	1304.8471
0.9	0.0728	1.4827	0.7371	3.9027	11.3611	1484.8406
1.0	0.0741	1.6035	0.6156	3.7651	11.2950	1665.6008

Table 3. Effect of p on various performance measures, when $D_0 = (-30)$, $D_1 = (30)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.3392	1.0378	1.8196	0.9496	-1.9586	141.7398
0.2	0.1762	1.0806	1.6421	1.7921	6.1878	339.5609
0.3	0.1240	1.1303	1.4676	2.5151	8.8023	540.0395
0.4	0.0991	1.1881	1.2965	3.1041	10.0449	743.7425
0.5	0.0853	1.2549	1.1297	3.5441	10.7365	951.0857
0.6	0.0773	1.3324	0.9680	3.8207	11.1356	1162.1853
0.7	0.0734	1.4260	0.8124	3.9215	11.3322	1376.6660
0.8	0.0732	1.5501	0.6636	3.8363	11.3401	1593.3114
0.9	0.0777	1.7424	0.5222	3.5570	11.1132	1809.3387
1.0	0.0898	2.1045	0.3886	3.0780	10.5080	2018.3028

In Tables 1,2 and 3, $E(W)$ denotes the expected waiting time of an arbitrary customer. We can see that the $E(W)$ decreases as p increases upto some p_1 (shown in bold letters) and after that it increases. This is due to the effect of N -policy. As p increases (upto p_1), the number of customers in the system hit N more fast so that the server stops processing of service items and start serving customers and hence $E(W)$ decreases. When p becomes p_1 , $E(W)$ starts increasing due to the diminished effect of N . Hence F_1 increases as p increases upto p_1 and after that it decreases. As we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers are served in a cycle. $E(ipo)$ increases upto p_1 , as p increases. This is due to the effect of N -policy. As p increases, the number of customers in the system hit N more rapidly and hence customers leave the system quickly sothat the server can switch on to processing at a faster rate. When p increases beyond p_1 , $E(ipo)$ decreases as p increases due to the diminished effect of N .

From Tables 1, 2 and 3, We can see that F_1 is strictly increasing on $[0, p_1]$ and strictly decreasing on $[p_1, 1]$. Thus,

1. If $F_1(p_1) \leq 0$, then $F_1(p) \leq 0$ for all $p \in [0, 1]$. In this case, the maximum benefit is negative which implies that customers do not join the system even if there is no customer in the system.
2. If $F_1(0) > 0$ and $F_1(1) > 0$, then $F_1(p) > 0$ for all $p \in [0, 1]$. In this case, customers prefer to join the system, because the minimal benefit is positive.
3. If $F_1(p_1) \geq 0$ and $F_1(0) < 0$, $\exists p_e \in [0, p_1]$ such that $F_1(p_e) = 0$.
4. If $F_1(p_1) \geq 0$ and $F_1(1) < 0$, $\exists p_e \in [p_1, 1]$ such that $F_1(p_e) = 0$.
5. If $F_1(p_1) \geq 0$, $F_1(0) < 0$ and $F_1(1) < 0$ then $\exists p_e \in [0, p_1]$ such that $F_1(p_e) = 0$ and $p'_e \in [p_1, 1]$ such that $F_1(p'_e) = 0$.

Hence, if, either of the cases 3,4 and 5 happen, then customers are indifferent between joining and balking the system. Suppose that, case 3 holds. Then the above discussions imply that when the joining probability p adopted by other customers is greater than p_e , the expected net benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p < p_e$ because then the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individual's best response is an increasing function of the strategy selected by other customers. Therefore, we expect a crowd situation in this case due to the effect of N -policy.

Next, suppose that, case 4 holds. Then the above discussions imply that when the joining probability p adopted by other customers is smaller than p_e , the expected net

benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p > p_e$ because then the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individual's best response is a decreasing function of the strategy selected by other customers. Therefore, we can avoid a crowd situation. This is due to the diminished effect of N -policy.

Next, suppose that case 5 holds, then the above discussions imply that when the joining probability p adopted by other customers is greater than p_e and less than p'_e , the expected net benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p < p_e$ or $p > p'_e$ because then the expected net benefit is negative. If $p = p_e$ or $p = p'_e$, every strategy is the best response since the expected net benefit is always 0.

Table 4. Individual optimum when $D_0 = (-20)$, $D_1 = (20)$.

$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-50.11	-60.11	-70.11	-80.11	-90.11	-100.11	-110.11	-120.11
0.1	39.78	29.78	19.78	9.78	-0.22	-10.22	-20.22	-30.22
0.2	52.14	42.14	32.14	22.14	12.14	2.14	-7.86	-17.86
0.3	56.19	46.19	36.19	26.19	16.19	6.19	-3.81	-13.81
0.4	58.16	48.16	38.16	28.16	18.16	8.16	-1.84	-11.84
0.5	59.31	49.31	39.31	29.31	19.31	9.31	-0.69	-10.69
0.6	60.04	50.04	40.04	30.04	20.04	10.04	0.04	-9.96
0.7	60.55	50.55	40.55	30.55	20.55	10.55	0.55	-9.45
0.8	60.90	50.90	40.90	30.90	20.90	10.90	0.90	-9.1
0.9	61.14	51.14	41.14	31.14	21.14	11.14	1.14	-8.86
1.0	61.29	51.29	41.29	31.29	21.29	11.29	1.29	-8.71

Table 5. Individual optimum when $D_0 = (-25)$, $D_1 = (25)$.

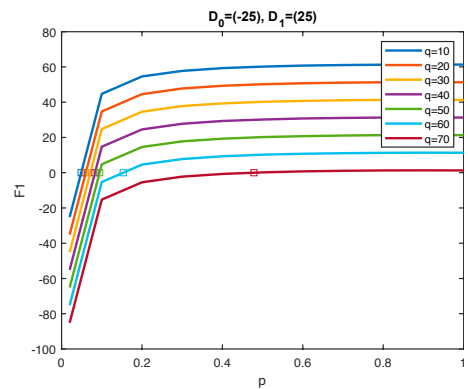
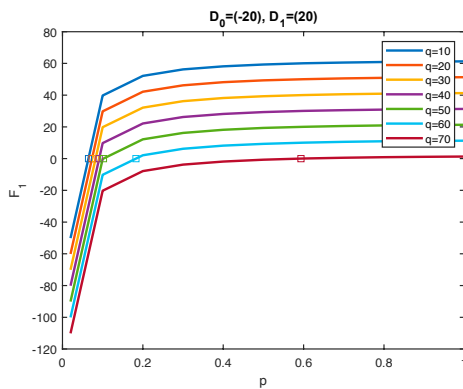
$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-25.12	-35.12	-45.12	-55.12	-65.12	-75.12	-85.12	-95.12
0.1	44.74	34.74	24.74	14.74	4.74	-5.26	-15.26	-25.26
0.2	54.58	44.58	34.58	24.58	14.58	4.58	-5.42	-15.42
0.3	57.77	47.77	37.77	27.77	17.77	7.77	-2.23	-12.23
0.4	59.31	49.31	39.31	29.31	19.31	9.31	-0.69	-10.69
0.5	60.19	50.19	40.19	30.19	20.19	10.19	0.19	-9.81
0.6	60.74	50.74	40.74	30.74	20.74	10.74	0.74	-9.26
0.7	61.08	51.08	41.08	31.08	21.08	11.08	1.08	-8.92
0.8	61.29	51.29	41.29	31.29	21.29	11.29	1.29	-8.71
0.9	61.36	51.36	41.36	31.36	21.36	11.36	1.36	-8.64
1.0	61.30	51.30	41.30	31.30	21.30	11.30	1.30	-8.70

Table 6. Individual optimum when $D_0 = (-30)$, $D_1 = (30)$.

$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-8.46	-18.46	-28.46	-38.46	-48.46	-58.46	-68.46	-78.46
0.1	48.04	38.04	28.04	18.04	8.04	-1.96	-11.96	-21.96
0.2	56.19	46.19	36.19	26.19	16.19	6.19	-3.81	-13.81
0.3	58.80	48.80	38.80	28.80	18.80	8.80	-1.20	-11.20
0.4	60.05	50.05	40.05	30.05	20.05	10.05	0.05	-9.95
0.5	60.74	50.74	40.74	30.74	20.74	10.74	0.74	-8.26
0.6	61.14	51.14	41.14	31.14	21.14	11.14	1.14	-8.86
0.7	61.33	51.33	41.33	31.33	21.33	11.33	1.33	-8.67
0.8	61.34	51.34	41.34	31.34	21.34	11.34	1.34	-8.86
0.9	61.12	51.12	41.12	31.12	21.12	11.12	1.12	-8.88
1.0	60.51	50.51	40.51	30.51	20.51	10.51	0.51	-9.49

From Tables 4,5 and 6, we get the values of F_1 corresponding to different values of p and q when the arrival rates are 20, 25 and 30 respectively.

In our experiment, $\exists$ a q_1 such that $F_1(p_1) \geq 0$, $F_1(0) < 0$, $F_1(1) \geq 0$ and $\exists$ exactly one equilibrium p_e in $(0, p_1]$ for all $q \in [0, q_1)$ where $0 < q_1 < R$ (in Table 6, $q_1 = 70.51$). Also p_e is strictly increasing for all q in $[0, q_1)$ (in Figure 1, $(p_e, 0)$ corresponding to different q 's are plotted using squares). This is due to the effect of N -policy. Also, $\exists$ a q_2 , where $q_1 \leq q_2 < R$ such that when $q_1 \leq q \leq q_2$, $\exists p_e \in [0, p_1]$ and $p'_e \in [p_1, 1]$ such that p_e is strictly increasing and p'_e is strictly decreasing in $[q_1, q_2]$ (in Table 6, $q_2 = 71.34$). This case is shown in Figure 2. When $q \in (q_2, R]$, $F_1(p) < 0$ for $p \in [0, 1]$ and there is no equilibrium probability. Hence, if q increases (up to q_1), more customers are supposed to join the queue, since the server can start service only if the number of customers in the system hit N . When q increases from q_2 to R , customers do not join the system since the maximum benefit is negative.



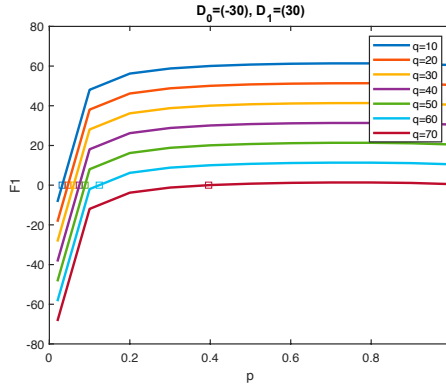


Figure 1. Effect of $q (< q_1)$ on individual equilibrium strategy.

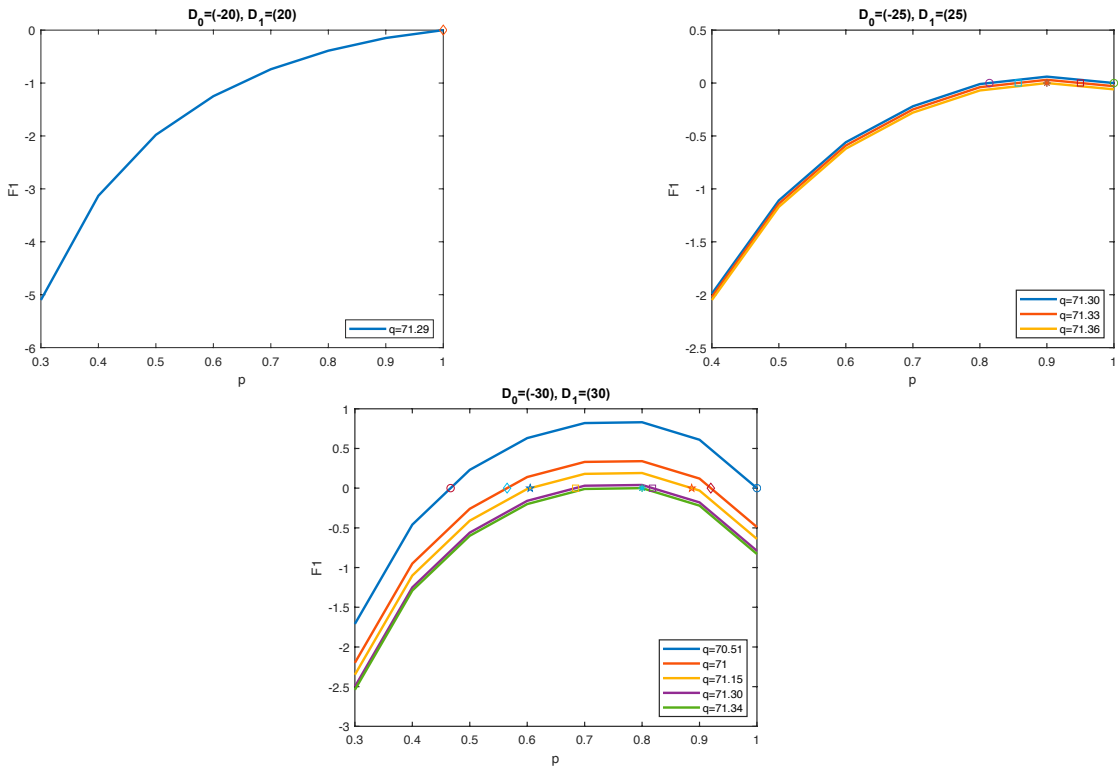


Figure 2. Effect of $q (q_1 \leq q \leq q_2)$ on individual equilibrium strategy.

Figure 1 shows individual equilibrium probabilities p_e as q varies ($0 < q < q_1$), corresponding to different arrival rates. We can see that p_e increases as q increases for the three different arrival rates. But p_e decreases as arrival rate increases. Figure 2 shows individual equilibrium probabilities p_e, p'_e as q varies ($q_1 \leq q \leq q_2$) corresponding to different arrival rates. We see that p_e increases and p'_e decreases as q increases and coincides when $q = q_2$ for three different arrival rates.

Tables 7 and 8 show the effect of q on revenue of the server. Here, we see that F_2

decreases as q increases upto q_1 . This happens because when q increases upto q_1 , p_e increases and hence the rate of hitting N becomes faster so that $E(ip_o)$ increases. But we see that when q increases in $[q_1, q_2]$, after a certain q -value, revenue function increases if p_e is the joining probability. This is due to the diminished effect of N -policy. Here, in all the cases, maximum revenue occur corresponding to $q = 10$ and the revenue decreases if a higher q is levied upto q_1 . But when q increases beyond q_1 , after a certain q -value, revenue increases if a higher q is levied.

Table 7. Revenue Maximization ($0 < q < q_1$).

q	$D_0 = (-20), D_1 = (20)$		$D_0 = (-25), D_1 = (25)$		$D_0 = (-30), D_1 = (30)$	
	p_e	F_2	p_e	F_2	p_e	F_2
10	0.0646	-15.31	0.0327	-11.22	0.0320	-12.45
20	0.0735	-16.82	0.0477	-14.45	0.0461	-16.08
30	0.0824	-18.32	0.0628	-17.67	0.0603	-19.67
40	0.0913	-19.82	0.0778	-20.81	0.0745	-23.20
50	0.1018	-21.56	0.0929	-23.92	0.0886	-26.65
60	0.1827	-34.50	0.1535	-35.91	0.1240	-35.01
70	0.5932	-84.23	0.4784	-84.63	0.3960	-84.29

Table 8. Revenue Maximization ($q_1 \leq q \leq q_2$).

D_0, D_1	q	p_e	F_2	p'_e	F_2
$(-20), (20)$	$71.29(q_1 = q_2)$	1	-100.89	1	-100.89
$(-25), (25)$	$71.30(q_1)$	0.8143	-100.76	1	-91.78
	71.33	0.8571	-99.87	0.9500	-95.52
	$71.36(q_2)$	0.9000	-98.28	0.9000	-98.28
$(-30), (30)$	$70.51(q_1)$	0.4667	-92.10	1	-66.92
	71	0.5650	-98.98	0.9197	-80.85
	71.15	0.6053	-100.39	0.8864	-85.567
	71.30	0.6842	-100.66	0.8182	-93.25
	$71.34(q_2)$	0.8000	-94.85	0.8000	-94.85

Again, from Tables 1,2 and 3, we can see that F_3 increases as p increases. But the rate of increase decreases as p increases. Here, the social optimum corresponds to $p = 1$ (p_s) in all cases.

Next, we vary values of N and L and see the effect of p on different performance measures. We fix $\alpha = \beta = [1 \ 0]$, $\gamma = [0.8 \ 0.2]$, $T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix}$, $S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}$,

$$U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

Table 9. Effect of p on different performances varying N and L .

(N, L)	p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
(3,2)	0.1	0.5045	1.0248	1.8794	0.6445	-10.2243
	0.2	0.2571	1.0514	1.7601	1.2429	2.1430
	0.3	0.1762	1.0806	1.6421	1.7921	6.1878
	0.4	0.1368	1.1129	1.5254	2.2882	8.1599
	0.5	0.1138	1.1486	1.4102	2.7271	9.3077
(3,3)	0.1	0.4888	1.0241	2.8168	0.6562	-9.4391
	0.2	0.2433	1.0487	2.6346	1.2652	2.8337
	0.3	0.1642	1.0746	2.4528	1.8402	6.7919
	0.4	0.1263	1.1028	2.2713	2.3687	8.6865
	0.5	0.1047	1.1341	2.0905	2.8433	9.7656
(3,4)	0.1	0.4880	1.0240	3.8110	0.6506	-9.4013
	0.2	0.2421	1.0481	3.6104	1.2686	2.8936
	0.3	0.1628	1.0728	3.3952	1.8514	6.8617
	0.4	0.1249	1.0988	3.1640	2.3942	8.7560
	0.5	0.1035	1.1271	2.9182	2.8892	9.8269
(4,3)	0.1	0.7456	1.5143	2.8244	0.4878	-22.2785
	0.2	0.3761	1.5217	2.6469	0.9567	-3.8066
	0.3	0.2556	1.5286	2.4667	1.4062	2.2207
	0.4	0.1968	1.5389	2.2847	1.8296	5.1614
	0.5	0.1624	1.5550	2.1023	2.2174	6.8805
(4,4)	0.1	0.7301	1.5151	3.7633	0.4919	-21.5035
	0.2	0.3626	1.5217	3.5220	0.9736	-3.1325
	0.3	0.2438	1.5282	3.2756	1.4414	2.8079
	0.4	0.1866	1.5391	3.0260	1.8842	5.6683
	0.5	0.1521	1.5585	3.6070	2.3134	7.3953
(5,4)	0.1	0.9844	2.0093	3.7704	0.3916	-34.2183
	0.2	0.4924	2.0047	3.5349	0.7743	-9.6201
	0.3	0.3324	1.9939	3.2917	1.1511	-1.6189
	0.4	0.2546	1.9837	3.0421	1.5163	2.2700
	0.5	0.2093	1.9793	2.7891	1.8599	4.5362
(5,5)	0.1	0.9689	2.011	4.7101	0.3947	-33.4434
	0.2	0.4792	2.0051	4.4102	0.7881	-8.9589
	0.3	0.3210	1.9937	4.0986	1.1806	-1.0519
	0.4	0.2450	1.9853	3.7787	1.5617	2.7517
	0.5	0.2013	1.9855	3.4557	1.9168	4.9373

From Table 9, we can see that when N increases (keeping L fixed), $E(W)$ increases. This happens because as N increases, the number of customers in the system hits N at a slower rate and thus the server switches to service mode at a slower rate. When L increases (keeping N fixed), $E(W)$ decreases. This is because when L increases, more customers get service according to $PH(\gamma, U)$ whose service rate is greater than that of $PH(\beta, S)$. As a result, when N increases (keeping L fixed) F_1 decreases and when L increases (keeping N fixed), F_1 increases.

When N increases (keeping L fixed), $E(S)$ increases as expected. As N increases (keeping L fixed), $E(it)$ increases since lesser number of customers are served in a cycle. When L increases (keeping N fixed), $E(it)$ increases, since more items are processed in a cycle. $E(ipo)$ decreases as N increases since the server switches to service mode at a slower rate and as a result to inventory processing at a slower rate.

Next, we consider the following two sets of matrices for D_0 and D_1 .

1. MAP with positive correlation (MPA)

$$D_0 = \begin{bmatrix} -27.0697 & 27.0697 & 0 \\ 0 & -45.0981 & 0 \\ 0 & 0 & -595.5331 \end{bmatrix}, \quad D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 42.3911 & 0 & 2.7070 \\ 189.4878 & 0 & 406.0453 \end{bmatrix}$$

2. MAP with negative correlation (MNA)

$$D_0 = \begin{bmatrix} -7.9382 & 7.9382 & 0 \\ 0 & -13.2250 & 0 \\ 0 & 0 & -404.0521 \end{bmatrix}, \quad D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.7938 & 0 & 12.4311 \\ 119.0723 & 0 & 284.9797 \end{bmatrix}$$

These two MAP processes are normalized so as to have an arrival rate of 20. The arrival process labeled MNA has correlated arrivals with correlation between two successive interarrival times given by -0.1368 and the arrival process corresponding to the one labelled MPA has a positive correlation with value 0.1368.

We fix other parameters same as in the case of Poisson arrivals.

Table 10. Effect of p on various performance measures, when arrival follows MPA.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
0.1	0.0905	1.0695	1.7309	1.3096	10.4728
0.2	0.0931	1.1486	1.5323	2.1580	10.3428
0.3	0.1002	1.2185	1.3794	2.7405	9.9915
0.4	0.1060	1.2807	1.2585	3.1396	9.7006
0.5	0.1108	1.3371	1.1609	3.4116	9.4579

Table 11. Effect of p on various performance measures, when arrival follows MNA.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
0.1	0.1657	0.7477	1.7519	0.9167	6.7171
0.2	0.1148	0.8068	1.5702	1.3605	9.2616
0.3	0.1116	0.9362	1.4262	1.7000	9.4198
0.4	0.1140	1.0727	1.3100	1.9477	9.2978
0.5	0.1181	1.2100	1.2148	2.1222	9.0960

From Table 10, we can see that the $E(W)$ increases as p increases, different from the case of Poisson arrivals. This is due to the effect of positive correlation. Hence, F_1 decreases as p increases. But in the case of MNA (Table 11), $E(W)$ decreases as p increases upto some p_1 (here $p_1 = 0.3$) and after that it increases, as in the case of Poisson arrivals and hence F_1 increases as p increases upto p_1 and after that it decreases. In both cases, as we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers are served in a cycle. $E(ipo)$ increases as p increases. This is because as p increases, the number of customers in the system hit N more rapidly and hence customers leave the system quickly so that the server can switch on to processing at a faster rate.

5. Special Case: The System in Normal Mode

5.1. Waiting time analysis

To find the waiting time of a customer who joins for service at an epoch in the long run, we have to consider different possibilities depending on the status of server at that time. Let Ev denote the event the system is working in normal mode. Let $W(t|Ev)$ be the conditional waiting time of a customer who arrives at time t and $W^*(s|Ev)$ be the corresponding conditional LST.

Let $w_{h,i,j,k,l}$ denote the probability that the tagged customer finds the system in the state (h,i,j,k,l) immediately after his arrival when the system is in normal mode.

Then

$$w_{h,0,1,k,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{h-1,0,1,k,l'}, \quad 2 \leq h \leq N-1, \text{ or } h \geq N+1, 1 \leq k \leq m_2, \\ 1 \leq l \leq n. \\ w_{N,0,1,k,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{N-1,0,1,k,l'}, \quad 1 \leq k \leq m_2, 1 \leq l \leq n.$$

$$\begin{aligned}
 w_{h,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{kk}} x_{h-1,i,1,k,l'}, \quad 2 \leq h \leq N-1, 1 \leq L-N+h-1, \\
 &\quad 1 \leq k \leq m_3, 1 \leq l \leq n. \\
 w_{h,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{kk}} x_{h-1,i,1,k,l'}, \quad h \geq N+1, 1 \leq i \leq L, 1 \leq k \leq m_3, \\
 &\quad 1 \leq l \leq n. \\
 w_{N,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{N-1,i,1,k,l'}, \quad 1 \leq i \leq L, 1 \leq k_2 \leq m_2, 1 \leq l \leq n.
 \end{aligned}$$

Case I: $L \leq N$

Case (1)

Let Ev_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, 0, 1, k, l)$, where $r \geq 1, 1 \leq k \leq m_2, 1 \leq l \leq n$. In this case, processed item is not available to any customer. Thus waiting time is the sum of residual service time and r service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_1) = e'_u(sI - S)^{-1} S^0 (\beta(sI - S)^{-1} S^0)^r.$$

Case (2)

Let Ev_2 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $1 \leq r \leq N-1, 1 \leq i \leq L-N+r, 1 \leq k \leq m_3, 1 \leq l \leq n$. In this case, processed item is available to i customers. Thus waiting time is the sum of residual service time and $i-1$ service time each following $PH(\gamma, U)$ and $r+1-i$ service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (3)

Let Ev_3 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $r \geq N, 1 \leq i \leq L, 1 \leq k \leq m_3, 1 \leq l \leq n$. In this case, processed item is available to i customers. Thus waiting time is the sum of residual service time and $i-1$ service time each following $PH(\gamma, U)$ and $r+1-i$ service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Thus the conditional LST of the waiting time,

$$\begin{aligned}
 W^*(s | Ev) &= \frac{1}{d_3} \left[\sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n W^*(s | Ev, Ev_1) w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1} \sum_{i=1}^{L-N+r} \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Ev_2) w_{r+1,i,1,k,l} \right. \\
 &\quad \left. + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Ev_3) w_{r+1,i,1,k,l} \right], \tag{10}
 \end{aligned}$$

where

$$d_3 = \sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1} \sum_{i=1}^{L-N+r} \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l} + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l}. \quad (11)$$

Case II: $L > N$

Case (1)

Let Fv_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, 0, 1, k, l)$, where $r \geq 1, 1 \leq k \leq m_2, 1 \leq l \leq n$. In this case, processed item is not available to any customer. Thus waiting time is the sum of residual service time and r service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Fv_1) = e'_u(sI - S)^{-1} S^0 (\beta(sI - S)^{-1} S^0)^r.$$

Case (2)

Let Fv_2 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $1 \leq r \leq N-1, 1 \leq i \leq L-N+r, 1 \leq k \leq m_3, 1 \leq l \leq n$.

Case (i), $1 \leq i < r+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (ii), $r+1 \leq i \leq L-N+r$

In this case, processed item is available to all the $r+1$ customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^r.$$

Case (3)

Let Fv_3 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $r \geq N, 1 \leq i \leq L, 1 \leq k \leq m_3, 1 \leq l \leq n$.

Case (i), $N \leq r \leq L$

Case (a), $1 \leq i < r+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (b), $r+1 \leq i \leq L$

In this case, processed item is available to all the $r+1$ customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^r.$$

Case (ii), $r \geq L+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Thus the conditional LST of the waiting time,

$$W^*(s | Ev) = \frac{1}{d_4} \left[\sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n W^*(s | Ev, Fv_1) w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1L-N+r} \sum_{i=1}^{m_3} \sum_{k=1}^n W^*(s | Ev, Fv_2) w_{r+1,i,1,k,l} \right. \\ \left. + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Fv_3) w_{r+1,i,1,k,l} \right], \quad (12)$$

where

$$d_4 = \sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1L-N+r} \sum_{i=1}^{m_3} \sum_{k=1}^n w_{r+1,i,1,k,l} + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l}. \quad (13)$$

$$\text{We fix } N = 3, L = 2, \alpha = \beta = \begin{bmatrix} 1 & 0 \end{bmatrix}, \gamma = \begin{bmatrix} 0.8 & 0.2 \end{bmatrix}, T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix},$$

$$S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}, U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

Table 12. Effect of p on various performance measures, when $D_0 = (-20)$, $D_1 = (20)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.0485	1.5214	0.6972	19.3813	12.5747	-440.0284
0.2	0.0499	1.5604	0.6564	18.3266	12.5034	-263.5605
0.3	0.0512	1.6081	0.6117	17.2386	12.4386	-86.3546
0.4	0.0524	1.6657	0.5640	16.1240	12.3798	91.4217
0.5	0.0535	1.7341	0.5145	14.9939	12.3259	269.4597
0.6	0.0545	1.8144	0.4645	13.8595	12.2749	447.4200
0.7	0.0555	1.9081	0.4153	12.7308	12.2237	624.9757
0.8	0.0566	2.0174	0.3677	11.6154	12.1681	801.8239
0.9	0.0579	2.1455	0.3224	10.5189	12.1027	977.6669
1.0	0.0596	2.2971	0.2796	9.4448	12.0200	1152.1810

From Table 12, we see that $E(W)$ increases as p increases. This happens since when the system is working in normal mode, the number of customers accumulating in the system increases with increasing value of p . As p increases F_1 decreases consequent to increase in $E(W)$. As we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers get served in a cycle. $E(ipo)$ decreases as p increases. This happens due to the fact that when p increases more customers accumulate in the system and hence customers leave the system slowly so that the server switch on to processing at a slower rate. Also from table 12, we see that F_3 increases as p increases. Thus the social optimum corresponds to $p = 1$.

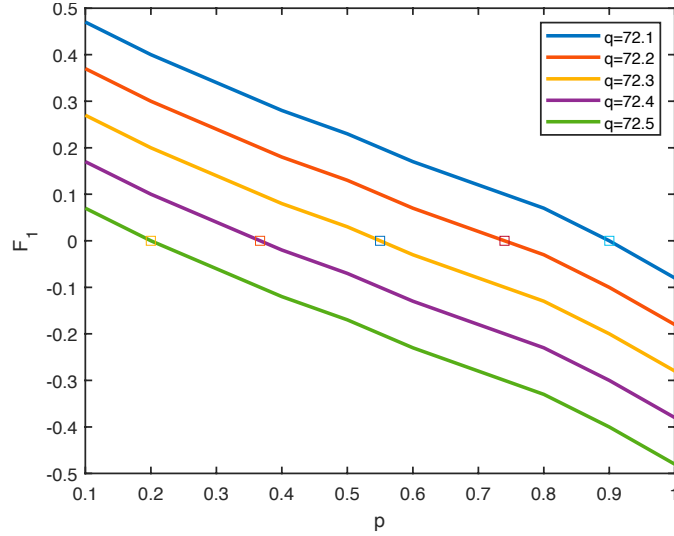


Figure 3. Effect of q on individual equilibrium strategy when $D_0 = (-20), D_1 = (20)$.

Here when $q < 72.02$, the expected net benefit is always positive. When q increases beyond 72.02, (see Table 12), we can find a $p_e \in [0,1]$ such that $F_1(p_e) = 0$ and p_e is decreasing (see Figure 3). Here when the joining probability p adopted by other customers is smaller than p_e , the expected net benefit of an arriving customer is positive provided he joins. Thus the unique best response is 1. Conversely, the unique best response is 0 if $p > p_e$ since, the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individuals best response is a decreasing function of the strategy selected by other customers. Therefore, we can avoid a crowd situation.

Table 13. Effect of q on Revenue function.

q	p_e	F_2
72.1	0.9000	-302.1809
72.2	0.7400	-357.9769
72.3	0.5500	-425.8322
72.4	0.3667	-491.4621
72.5	0.2000	-549.3741

Also, in this case revenue function F_2 decreases as q increases. This happens due to the fact that as q increases, the equilibrium probability p_e decreases and hence $E(ip_o)$ increases (see Table 13).

6. Conclusion

In this paper, we considered a MAP/PH/1 queue with processing of service items under Vacation and N-policy with impatient customers. We studied this system as a Level Dependent QBD. We explained how to find the steady state vectors of the system approximately by finite truncation method. We found the distribution of time until the number of customers hit N . Several system performance characteristics were computed. We computed LST of the waiting time distribution for the case of no reneging. Also we performed some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy for the special case of no reneging. We discussed the special case in which the system is in normal mode. In this case also, we computed LST of the waiting time distribution and performed some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy.

Acknowledgement

This research is supported by the Russian Science Foundation and the Department of Science and Technology (India) via grant No. 16-49-02021 for the joint research project by the V. A. Trapeznikov Institute of Control Science and the CMS College Kottayam.

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**Journal of the Indian Society for
Probability and Statistics**

e-ISSN 2364-9569

J Indian Soc Probab Stat
DOI 10.1007/s41096-020-00081-z



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(M, MAP)/(PH, PH)/1 Queue with Non-preemptive Priority and Working Vacation Under N-Policy

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Accepted: 16 March 2020
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Abstract

In this paper we consider two single server queueing models with non-preemptive priority and working vacation under two distinct N -policies. High priority (type I) customers are served even in vacation mode whereas low priority (type II) customers are served only when the server comes to normal mode of service. Type I customers have only a limited waiting space L whereas type II customers have unlimited capacity. The two distinct N -policies are as described below: In model I, while service of type I customers are in progress in vacation mode (working vacation), if the number of such customers present in the system hits N ($\leq L$) or the vacation timer (clock) expires, whichever occurs first, the server is switched on to normal mode. In model II, switching the server to normal mode from vacation mode occurs as soon as the accumulated number (those served out plus those present in the system) of type I customers during that working vacation hits N or the vacation timer expires, whichever occurs first. Type I customers arrive according to a Poisson process whereas type II customer's arrival is governed by Markovian Arrival Process. Service time of type I and type II customers follow distinct phase type distributions. At a service completion epoch, finding the system empty, server takes an exponentially distributed working vacation. During working vacation, type I customers are served at a reduced rate. On vacation expiration, the service of the type I customer already in service, will start from the beginning in the normal mode of service. We analyze these models in steady state to compute the distribution of duration of service time continuously in slow mode, expected number of returns to 0 type I customer state, starting from 0 type I customer state during vacation mode of service before the arrival of a type II customer, the distribution of a p -cycle in normal mode, LSTs of busy cycle, busy period of type I customers generated during the service time of a type II customer and LSTs of waiting time distributions of type I and type II customers. We compare these models in steady state by numerical experiments to identify the superior model.

Keywords Working vacation · N -policy · Non-preemptive priority

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Published online: 20 April 2020

Springer

1 Introduction

In vacation queueing systems, the server may not be available for a certain duration of time since he has to attend some supplementary jobs or is to undergo maintenance work or by its failure resulting in interruption of current service or simply to take a break. Levi and Yechiali (1975) introduced the concept of server vacation. They considered both single vacation and multiple vacation queueing models. Under a single vacation policy, after taking a vacation at the end of a busy period, the server either serves the waiting customers, if any, else stays idle. Under multiple vacation policy, the server takes vacations until it finds at least one customer waiting in the system at a vacation completion instant. In both cases Laplace–Steiltjes transforms of the occupation period, vacation period and waiting time were derived and generating function of the number of units in the system are calculated.

Considerable number of work in this area upto 1986 were surveyed by Doshi in Doshi (1986). More studies on vacation models could be found in Takagi (1991) and in Tian and Zhang (2006). Servi and Finn (2002) introduced the concept of a working vacation in which the server offers services at a lower rate during vacation if customers are available. They computed explicit formulae for the mean, variance and distribution of the number of customers and time spent by a customer in the system. Kim et al. (2003) considered the $M/G/1$ queue with working vacations and obtained the steady-state queue length distribution. Wu and Takagi (2006) considered $M/G/1$ queue with multiple working vacations and obtained the distribution of the queue size and the time in the system for an arbitrary customer in the steady-state. The concept of vacation interruption was introduced by Li and Tian (2007). They studied the $M/M/1$ queue with working vacations and vacation interruptions. Under the vacation interruption policy, the server can come back from the vacation without completing the vacation. By employing the matrix-geometric method, they obtained the distributions and the stochastic decomposition for the number of customers and the waiting time. Li et al. (2008) analyzed a single server vacation queue with a general arrival process with working vacation and vacation interruption. By matrix manipulations they obtained various performance measures such as mean queue length and waiting time.

In classical queueing models N -policy is used as a control mechanism to start service when the number of customers present in the system hits N starting from the epoch the server becomes idle due to the system becoming empty. Yadin and Naor (1963) introduced the concept of N -policy for $M/M/1$ queueing system without start-up time. Lee et al. (1994) considered an $M^X/G/1$ queueing system with N -policy and multiple vacations. They obtained the system size distribution and showed that the system size could be decomposed into three random variables one of which is the system size of ordinary $M^X/G/1$ queue. They also derived the waiting time distribution, some performance measures and also a condition under which the optimal stationary operating policy is achieved under

a linear cost structure. Kasahara et al. (1996) considered MAP/G/1 queueing systems with and without vacations. For both the cases, they analyzed the stationary queue length and the waiting time distributions, and derived recursive formulas to compute the moments of those distributions. Also they provided a numerical algorithm to obtain the mass function of the stationary queue length.

Zhang and Hou (2011) considered the MAP/G/1 queue with working vacations and vacation interruption and obtained the queue length distributions. Sreenivasan et al. (2013) studied a MAP/PH/1 queueing model with working vacations, vacation interruptions and N-policy. The server takes vacation and offers service at a lower rate during those times. The server returns to normal state whenever a random clock expires or the queue length hits a specific threshold value whichever occurs first. They analyzed the model in steady state using matrix analytic methods. Goswami and Selvaraju (2013) analyzed a working vacation queueing model with priority customers where the service time of customers follows phase-type distributions. They assumed that after serving a customer in working vacation, if the server finds any customer waiting in the queue, the vacation is interrupted and the server switches to normal service mode. They derived distributions of length of busy period, busy cycle, queue length and waiting time for the two types of customers.

Working vacation queueing system provides relief to customers since the server is always available for service, though at a reduced rate at the beginning of a cycle. Thus customer impatience gets reduced through introduction of working vacation in the place of vacation (without service). We have introduced a two-priority system where high priority customers alone are served during working vacation. This is a realistic situation since the system has to take care of impatience of such customers more than that of low priority customers. Further we imposed finite capacity for the High priority queue; this is to ensure “not too large waiting time” for such customers.

In the N -policy introduced by Yadin and Naor (1963), the server waits (or server is not activated) until the number of customers present in the system becomes N to start service in every new cycle. A customer arriving during this time will have to wait until the server is activated. The customers could become impatient while no service is provided. The purpose is to extend the duration of a busy period and thus reduce per unit time cost to the system. In a working vacation queueing model the above definition of N -policy needs modification. In Sreenivasan et al. (2013) the N -policy is introduced as follows: The server goes on vacation when, at the end of a service, no customer is left in the system. However, he starts giving service at a slower mode with the arrival of the first customer to the system. This is called working vacation since the server serves even during vacation. New customers may arrive during that service time. The service continues to be on vacation mode until either the number of customers in the system reaches N or the vacation timer expires, whichever occurs first. In the absence of occurrence of these events, the server goes for another vacation when the system becoming empty again. If the vacation timer has large mean value and arrival rate is much slower than even service rate during

working vacation, it will take a long time for N customers to be present at any given time. In fact, quite often the system becomes empty more often than the service hits normal mode.

We introduce another type of N -policy, in connection with working vacation. The server on vacation serves in working vacation mode customers who arrive after the just concluded busy period. This continues until the vacation timer expires or the number of customers present in the system plus number of customers already served (accumulated number) during the current vacation hits N , whichever occurs first; else the server goes for another vacation since the system is found to be empty immediately after completion of a service. We provide a comparison between the two models to check which is superior under given conditions. During working vacation type I customers alone receive service. This assumption can be justified; type I customers are more impatient than type II, though we have not brought in this paper the customer impatience factor.

In this paper we analyze two single server queueing models with two priority classes of customers where type I customers are assumed to have a non-preemptive priority over type II. In model I, we use N -policy as a control mechanism to end a working vacation, as described: *During a working vacation, either N type I customers should be present in the system at a given epoch or the vacation clock should expire, whichever occurs first in order to switch to normal mode of service.* In model II also we use N -policy as a control mechanism to terminate a working vacation: *During a working vacation, either the number of type I customers present in the system plus number of type I customers already served during that vacation hits N or the vacation clock expires, whichever occurs first in order to switch to normal mode of service.* Type I customers alone are served during working vacation. Thus the idle time of the server in the discussed N -policy is better utilized in working vacation under N -policy. This also helps in reducing impatience of high priority customers, further since the normal mode is realized in model II at a higher rate than in model I, we expect the former to perform better, which is seen to be true through numerical experiments.

The rest of the paper is arranged as follows. The mathematical formulation of model I is given in Sect. 2. Section 3 provides steady state analysis of the model. Waiting time analysis of type I and type II customers are discussed in Sects. 4 and 5 respectively. The mathematical formulation of model II is given in Sect. 6. Section 7 provides steady state analysis of the model. Waiting time analysis of type I and type II customers are in Sects. 8 and 9 respectively. Numerical results and comparison of the models are given in Sect. 10. A detailed description of the infinitesimal generators of models I and II are given in “Appendices 1 and 2” respectively. Proofs of two theorems stated in Sect. 5, are given in “Appendix 1”.

Notations and abbreviations used in the sequel:

- $\mathbf{e}$: Column vector of 1's of appropriate order.
- $\mathbf{e}(a)$: Column vector of 1's of order a
- $CTMC$: Continuous time Markov chain.

- I : identity matrix of appropriate order.
- I_a : identity matrix of order a .
- $\mathbf{e}_a$: column vector of order equal to the number of service phases, with 1 in the a th position and the remaining entries zero.
- $\mathbf{e}_a'$: Transpose of $\mathbf{e}_a$.
- $\mathbf{e}_a(b)$: Column vector of order b with 1 in the a th position and the remaining entries zero.
- $\mathbf{e}_a'(b)$: Transpose of $\mathbf{e}_a(b)$.
- *MAP*: Markovian Arrival Process
- *LST*: Laplace-Steiltjes Transform
- *LIQBD*: Level Independent Quasi-Birth-and-Death
- *WV*: Working Vacation
- $d_{ij}^{(k)}$: entries of D_k , $k = 0$ or 1 .

2 Mathematical Formulation of Model I

We consider a single server queue with two priority classes of customers where type I customers arrive according to a Poisson process with rate λ and type II customer arrival follows a Markovian Arrival Process with representation (D_0, D_1) of order n . Service time of type I customer is assumed to be of Phase type distributed with representation (α, T) of order m and of a type II customer is assumed to be of Phase type distributed with representation (α', T') of order m' . The maximum number of type I customers in the system is restricted to L . They are assumed to have a non-preemptive priority over type II customers. At a service completion epoch, finding the system empty, server takes a WV. The duration of vacation is assumed to be exponentially distributed with parameter η . Type I customers arriving during vacation are served at a lower rate(WV): Phase Type distribution with representation $(\alpha, \theta T)$, $0 < \theta < 1$. Thus the expected service rate in normal mode is $\mu = [\alpha(-T)^{-1}\mathbf{e}]^{-1}$ and $\theta\mu$ is the rate of the vacation mode of service. If on completion of service of a type I customer during WV, no type I is waiting, then the server continues in vacation, even if type II customers are available in the system. The server turns to normal working mode during a WV either when the vacation clock expires or when the number of type I customers in the system hits level N , $1 \leq N \leq L$ whichever occurs first. Type II customers are considered for service only when on completion of vacation, no type I customer is present in the system or on service completion of a type I customer in normal mode none of type I customer is left in the system. The expected service rate of a type II customer is $\mu' = [\alpha'(-T')^{-1}\mathbf{e}]^{-1}$. Also on vacation expiration, the service of the type I customer already in service, starts from the beginning in the normal mode of service.

Let $Q^* = D_0 + D_1$ be the generator matrix of the type II arrival process and π^* be its stationary probability vector. Hence π^* is the unique (positive) probability vector satisfying

$$\pi^* Q^* = 0, \pi^* e = 1$$

The constant $\beta^* = \pi^* D_1 e$, referred to as *fundamental rate*, gives the expected number of type II arrivals per unit of time in the stationary version of the MAP. It is assumed that the two arrival processes are independent of each other and are also independent of the service processes.

2.1 The QBD Process

The model described in Sect. 2 can be studied as a LIQBD process. First we introduce the following notations:

- $N_1(t)$: The number of type II customers in the system at time t ,
 $N_2(t)$: The number of type I customers in the system at time t

At time t :

$$S(t) = \begin{cases} 0, & \text{if the server is on vacation/on WV} \\ 1, & \text{if type I customer in service and service in normal mode at time } t \\ 2, & \text{if type II customer in service at time } t \end{cases}$$

- $J(t)$: The phase of the service process when the server is busy
 $M(t)$: The phase of arrival of the type II customer.

It is easy to verify that $\{(N_1(t), N_2(t), S(t), J(t), M(t)) : t \geq 0\}$ is a LIQBD with state space

$$\Omega = \cup_{i=0}^{\infty} l(i)$$

where

$$\begin{aligned} l(0) &= \{(0, 0, k)/1 \leq k \leq n\} \cup \{(0, i_2, j_1, j_2, k)/1 \leq i_2 \leq N-1; j_1 = 0 \text{ or } 1; 1 \leq j_2 \leq m; 1 \leq k \leq n\} \cup \{(0, i_2, 1, j_2, k)/N \leq i_2 \leq L; 1 \leq j_2 \leq m; 1 \leq k \leq n\} \text{ and for } i_1 \geq 1 \\ l(i_1) &= \{(i_1, 0, 0, k)/1 \leq k \leq n\} \cup \{(i_1, 0, 2, j_2, k)/1 \leq j_2 \leq m'; 1 \leq k \leq n\} \cup \{(i_1, i_2, 0, j_2, k)/1 \leq i_2 \leq N-1; 1 \leq j_2 \leq m; 1 \leq k \leq n\} \cup \{(i_1, i_2, 1, j_2, k)/1 \leq i_2 \leq L; 1 \leq j_2 \leq m; 1 \leq k \leq n\} \cup \{(i_1, i_2, 2, j_2, k)/1 \leq i_2 \leq L; 1 \leq j_2 \leq m'; 1 \leq k \leq n\} \end{aligned}$$

Note that when $N_1(t) = N_2(t) = 0$, server will be on vacation and so $S(t)$ and $J(t)$ need not be considered. Also when $N_2(t) = 0$ and $S(t) = 0$, then $J(t)$ need not be considered. The only other component in the state vector in both cases would be $M(t)$.

The infinitesimal generator of this CTMC is

$$Q_1 = \begin{bmatrix} B_0 & C_0 & & & \\ B_1 & A_1 & A_0 & & \\ & A_2 & A_1 & A_0 & \\ & & \ddots & \ddots & \ddots \end{bmatrix}$$

where B_0 contains transitions within the level 0; C_0 represents transitions from level 0 to level 1; B_1 represents transitions from level 1 to level 0; A_0 represents transitions from level h to level $h + 1$ for $h \geq 1$, A_1 represents transitions within the level h for $h \geq 1$ and A_2 represents transitions from level h to $h - 1$ for $h \geq 2$. The boundary blocks B_0, C_0, B_1 are of orders $n(1 + m(L + N - 1)) \times n(1 + m(L + N - 1))$, $n(1 + m(L + N - 1)) \times n(1 + mN + (L - 1)m + (L + 1)m')$, $n(1 + mN + (L - 1)m + (L + 1)m') \times n(1 + m(L + N - 1))$ respectively. A_0, A_1, A_2 are square matrices of order $n(1 + mN + (L - 1)m + (L + 1)m')$.

The entries in the block matrices are given in details in “Appendix 1”.

3 Steady State Analysis

Let $\pi = (\pi_0, \pi_1, \dots, \pi_L)$ denote the steady state probability vector of the generator

$$A = A_0 + A_1 + A_2 = \begin{bmatrix} F_0 & F_1 & & & & & & & & \\ F_2 & F_3 & \lambda I & & & & & & & \\ & F_4 & F_3 & \lambda I & & & & & & \\ & & \ddots & \ddots & \ddots & & & & & \\ & & & F_4 & F_3 & \lambda I & & & & \\ & & & F_4 & F_3 & F_5 & & & & \\ & & & & F_6 & F_7 & \lambda I & & & \\ & & & & & F_8 & F_7 & \lambda I & & \\ & & & & & & \ddots & \ddots & \ddots & \\ & & & & & & & F_8 & F_7 & \lambda I \\ & & & & & & & & F_8 & F_9 \end{bmatrix}$$

where

$$\begin{aligned}
 F_0(k, l) &= \begin{cases} D_0 + D_1 - (\lambda + \eta)I_n & k = 1, l = 1 \\ \eta(\alpha' \otimes I_n) & k = 1, l = 2 \\ 0 & k = 2, l = 1 \\ \mathbf{T}^0 \alpha' \otimes I_n + T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = 2, l = 2 \end{cases} \\
 F_1(k, l) &= \begin{cases} \lambda(\alpha \otimes I_n) & k = 1, l = 1 \\ \lambda I_{m'n} & k = 2, l = 3 \\ 0 & \text{otherwise} \end{cases}, \quad F_2(k, l) = \begin{cases} \theta \mathbf{T}^0 \otimes I_n & k = 1, l = 1 \\ \mathbf{T}^0 \alpha' \otimes I_n & k = 2, l = 2 \\ 0 & \text{otherwise} \end{cases} \\
 F_3(k, l) &= \begin{cases} \theta T \oplus D_0 - (\lambda + \eta)I_{mn} + I_m \otimes D_1 & k = 1, l = 1 \\ \mathbf{e}(m) \otimes \eta(\alpha \otimes I_n) & k = 1, l = 2 \\ T \oplus D_0 - \lambda I_{mn} + I_m \otimes D_1 & k = l = 2 \\ T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = l = 3 \\ \mathbf{T}^0 \alpha \otimes I_n & k = 3, l = 2 \\ 0 & \text{otherwise} \end{cases} \\
 F_4(k, l) &= \begin{cases} \theta \mathbf{T}^0 \alpha \otimes I_n & k = 1, l = 1 \\ \mathbf{T}^0 \alpha \otimes I_n & k = 2, l = 2 \\ 0 & \text{otherwise} \end{cases}, \quad F_5(k, l) = \begin{cases} \lambda \mathbf{e}(m) \otimes (\alpha \otimes I_n) & k = l = 1 \\ \lambda I_{mn} & k = 2, l = 1 \\ \lambda I_{m'n} & k = 3, l = 2 \\ 0 & \text{otherwise} \end{cases} \\
 F_6(k, l) &= \begin{cases} \mathbf{T}^0 \alpha \otimes I_n & k = 1, l = 2 \\ 0 & \text{otherwise} \end{cases} \\
 F_7(k, l) &= \begin{cases} T \oplus D_0 - \lambda I_{mn} + I_m \otimes D_1 & k = l = 1 \\ T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = l = 2 \\ 0 & k = 1, l = 2 \\ \mathbf{T}^0 \alpha \otimes I_n & k = 2, l = 1 \end{cases} \\
 F_8(k, l) &= \begin{cases} \mathbf{T}^0 \alpha \otimes I_n & k = l = 1 \\ 0 & \text{otherwise} \end{cases}, \quad F_9(k, l) = \begin{cases} T \oplus D_0 + I_m \otimes D_1 & k = l = 1 \\ T' \oplus D_0 + I_{m'} \otimes D_1 & k = l = 2 \\ \mathbf{T}^0 \alpha \otimes I_n & k = 2, l = 1 \end{cases}
 \end{aligned}$$

with dimension of F_0, F_1, F_2 be $n(1 + m') \times n(1 + m')$, $n(1 + m') \times (2m + m')n$, $(2m + m')n \times n(1 + m')$ respectively. F_3, F_4 are square matrices of order $(2m + m')n$, F_5 is of order $(2m + m')n \times (m + m')n$, F_6 is of order $(m + m')n \times (2m + m')n$, F_7, F_8, F_9 are square matrices of order $(m + m')n$. ie,

$$\pi A = 0, \pi \mathbf{e} = 1 \quad (1)$$

The *LIQBD* description of the model indicates that the queueing system is stable (see Neuts (1981)) if and only if the left drift exceeds that of right drift. That is,

$$\pi A_0 \mathbf{e} < \pi A_2 \mathbf{e} \quad (2)$$

The vector π cannot be obtained directly in terms of the parametres of the model. From (1) we get

$$\pi_i = \pi_{i-1} \mathcal{U}_{i-1}, 1 \leq i \leq L \quad (3)$$

where

$$\mathcal{U}_0 = -F_1(F_3 + \mathcal{U}_1 F_4)^{-1}$$

$$\mathcal{U}_i = \begin{cases} -\lambda(F_3 + U_{i+1} F_4)^{-1} & \text{for } 1 \leq i \leq N-3 \\ -\lambda(F_3 + \mathcal{U}_{N-1} F_6)^{-1} & \text{for } i = N-2 \\ -F_5(F_7 + \mathcal{U}_N F_8)^{-1}, & \text{for } i = N-1 \\ -\lambda(F_7 + \mathcal{U}_{i+1} F_8)^{-1} & \text{for } N \leq i \leq L-2 \\ -\lambda F_9^{-1} & \text{for } i = L-1 \end{cases}$$

From the normalizing condition $\pi e = 1$ we have

$$\pi_0 \left(\sum_{j=0}^{L-1} \prod_{i=0}^j \mathcal{U}_i + I \right) e = 1 \quad (4)$$

The inequality (2) gives the stability condition as

$$\pi_0 \left[(I_{(1+m')}) \otimes D_1 \right] e + \sum_{i=0}^{N-2} \prod_{j=0}^i \mathcal{U}_j (I_{(2m+m')}) \otimes D_1 \right] e + \sum_{i=N-1}^{L-1} \prod_{j=0}^i \mathcal{U}_j (I_{(m+m')}) \otimes D_1 \right] e \Bigg]$$

$$< \pi_0 \left[A_{20} + \sum_{i=0}^{N-2} \prod_{j=0}^i \mathcal{U}_j A_{21} + \sum_{i=N+1}^{L-1} \prod_{j=0}^i \mathcal{U}_j A_{22} \right] \quad (5)$$

where, $\mathcal{A}_{20} = \begin{bmatrix} \mathbf{0} \\ (\mathbf{T}'^0 \alpha' \otimes I) e \end{bmatrix}$, $\mathcal{A}_{21} = \mathcal{A}_{22} = \begin{bmatrix} \mathbf{0} \\ (\mathbf{T}'^0 \alpha \otimes I) e \end{bmatrix}$, with $\mathbf{0}$ a zero column vector of order n , $2mn$ and mn for A_{20} , A_{21} and A_{22} respectively.

Let $\mathbf{x}$ be the steady state probability vector of Q . We partition this vector as

$$\mathbf{x} = (\mathbf{x}_0, \mathbf{x}_1, \mathbf{x}_2 \dots),$$

where $\mathbf{x}_0$ is of dimension $n(1 + m(L + N - 1))$, $\mathbf{x}_1, \mathbf{x}_2, \dots$ are of dimension $n(1 + mN + (L - 1)m + (L + 1)m')$. Under the stability condition, we have

$$\mathbf{x}_i = \mathbf{x}_1 R^{i-1}, i \geq 2$$

where the matrix R is the minimal nonnegative solution to the matrix quadratic equation

$$R^2 A_2 + R A_1 + A_0 = 0$$

and the vectors $\mathbf{x}_0$ and $\mathbf{x}_1$ are obtained by solving the equations

$$\mathbf{x}_0 B_0 + \mathbf{x}_1 B_1 = 0 \quad (6)$$

$$\mathbf{x}_0 C_0 + \mathbf{x}_1 (A_1 + R A_2) = 0 \quad (7)$$

subject to the normalizing condition

$$\mathbf{x}_0 e + \mathbf{x}_1 (I - R)^{-1} e = 1 \quad (8)$$

For evaluating the performance of the system we have to compute certain distributions. We proceed to such computations.

3.1 Distribution of Duration of Slow Service Mode

The duration T_{slow} of a slow service mode is defined as the time the server stays in slow service mode (through initiating a WV) until either switching to normal mode through the vacation clock realization or with the number of type I customers in the system hitting the threshold value N or the number of type I customers hitting 0 before expiration of vacation, whichever occurs first. We consider the Markov process $\chi_1(t) = \{N(t), J(t) : t \geq 0\}$ where $N(t)$ is the number of type I customers in the system at time t , $J(t)$ the service phase at time t . Thus the state space of the process is $\{(i, j) : 1 \leq i \leq N-1, 1 \leq j \leq m\} \cup \{0\} \cup \{*_1\} \cup \{*_2\}$ where 0 denotes the absorbing state indicating that there is no type I customer in the system and $*_1$ denotes the absorbing state indicating the vacation expiration by vacation clock realization and $*_2$ denotes the absorbing state indicating the vacation expiration by the number of type I customers in the system hitting N . The initial probability vector is given by

$$\beta_1 = \frac{1}{d_1}(w_1, w_2, \dots, w_m, \mathbf{0})$$

where, for, $1 \leq j \leq m$, $w_j = \sum_{k=1}^n \frac{\lambda \alpha_j}{\lambda + \eta - d_{kk}^{(0)}} x_{0,0,k} + \sum_{i=1}^{\infty} \sum_{k=1}^n \frac{\lambda \alpha_j}{\lambda + \eta - d_{kk}^{(0)}} x_{i,0,0,k}$, with

$$d_1 = \sum_{k=1}^n \frac{\lambda}{\lambda + \eta - d_{kk}^{(0)}} x_{0,0,k} + \sum_{i=1}^{\infty} \sum_{k=1}^n \frac{\lambda}{\lambda + \eta - d_{kk}^{(0)}} x_{i,0,0,k} \text{ and } \mathbf{0} \text{ is a zero matrix of order } 1 \times (N-2)m.$$

The infinitesimal generator S_1 of $\chi_1(t)$ has the form

$$S_1 = \begin{bmatrix} S_1 & S_1^{(0)} & S_1^{(1)} & S_1^{(2)} \\ \mathbf{0} & 0 & 0 & 0 \end{bmatrix}$$

where

$$S_1 = \begin{bmatrix} \theta T - (\lambda + \eta)I & \lambda I & & \\ \theta T^0 \alpha & \theta T - (\lambda + \eta)I & \lambda I & \\ & \ddots & \ddots & \\ & \theta T^0 \alpha & \theta T - (\lambda + \eta)I & \lambda I \\ & & \theta T^0 \alpha & \theta T - (\lambda + \eta)I \end{bmatrix}$$

$$\mathbf{S}_1^{(0)} = \begin{bmatrix} \theta T^0 \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \end{bmatrix}, \mathbf{S}_1^{(1)} = \begin{bmatrix} \eta \mathbf{e}(m) \\ \vdots \\ \eta \mathbf{e}(m) \\ \eta \mathbf{e}(m) \end{bmatrix}, \mathbf{S}_1^{(2)} = \begin{bmatrix} \mathbf{0} \\ \vdots \\ \mathbf{0} \\ \lambda \mathbf{e}(m) \end{bmatrix}.$$

Thus we have the following lemma.

Lemma 1 *The expected duration of time the server stays continuously in WV until the number of type I customers in the system reach 0 is given by $\beta_1(-S_1)^{-2}\mathbf{S}_1^{(0)}$.*

Our objective is to compute the expected number of hits to zero type I customer state until the server returns to normal mode of service before the arrival of a type II customer. Define the random variable M_1 as number of returns to 0 type I customer state starting from 0 type I customer state during vacation mode of service before the arrival of a type II customer.

3.2 Expected Value of M_1

Lemma 2 provides the expected duration of the time starting from the beginning of a vacation until the start of the next vacation, without going to normal mode of service in between, before the arrival of a type II customer. As a first step for computing expected number of such hits, we compute the following distribution. Let T_s denote the duration of slow service until the arrival of a type II customer.

3.2.1 Distribution of T_s

We consider the Markov process $\chi_2(t) = \{(N(t), J(t), M(t)) : t \geq 0\}$ where $N(t)$ is the number of type I customers in the system at time t , $J(t)$ the service phase and $M(t)$ the arrival phase of type II customer at that instant. Thus the state space of the process is $\{(i, j, k) : 1 \leq i \leq N-1, 1 \leq j \leq m, 1 \leq k \leq n\} \cup \{0\} \cup \{*_1\} \cup \{*_2\}$ where 0 denotes the absorbing state indicating that there is no type I customer in the system, $*_1, *_2$ denote the absorbing states indicating the vacation expiration and arrival of a type II customer respectively. The initial probability vector is given by

$$\beta_2 = (1/d_2)(w_{1,1}, \dots, w_{1,n}, \dots, w_{m,1}, \dots, w_{m,n}, \mathbf{0})$$

where, for, $1 \leq j \leq m, 1 \leq k \leq n$,

$$w_{j,k} = \frac{\lambda \alpha_j}{\lambda + \eta - d_{kk}^{(0)}} x_{0,0,k},$$

$d_2 = \sum_{k=1}^n \frac{\lambda}{\lambda + \eta - d_{kk}^{(0)}} x_{0,0,k}$ and $\mathbf{0}$ is a zero matrix of order $1 \times (N-2)mn$. The infinitesimal generator $\mathbf{S}_2$ of $\chi_2(t)$ has the form

$$\mathbf{S}_2 = \begin{bmatrix} S_2 & \mathbf{S}_2^{(0)} & \mathbf{S}_2^{(1)} & \mathbf{S}_2^{(2)} \\ \mathbf{0} & 0 & 0 & 0 \end{bmatrix}$$

where

$$\mathbf{S}_2 = \begin{bmatrix} \theta T \oplus D_0 - (\lambda + \eta)I & \lambda I & \lambda I & \lambda I \\ \theta \mathbf{T}^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \lambda I & \lambda I \\ \ddots & \ddots & \ddots & \ddots \\ \theta \mathbf{T}^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \theta \mathbf{T}^0 \alpha \otimes I & \lambda I \\ \theta \mathbf{T}^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \theta \mathbf{T}^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I \end{bmatrix}$$

$$\mathbf{S}_2^{(0)} = \begin{bmatrix} \theta T^0 \otimes \mathbf{e}(n) \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \end{bmatrix}, \mathbf{S}_2^{(1)} = \begin{bmatrix} \mathbf{e}(m) \otimes \delta \\ \vdots \\ \mathbf{e}(m) \otimes \delta \end{bmatrix}, \mathbf{S}_2^{(2)} = \begin{bmatrix} \eta \mathbf{e}(mn) \\ \vdots \\ \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \end{bmatrix} \text{ where } \mathbf{0} \text{ is a}$$

zero matrix of order $mn \times 1$ and

$$\delta = \begin{bmatrix} \delta_1 \\ \vdots \\ \delta_n \end{bmatrix}, \quad (\text{c1})$$

with δ_i representing the i th rowsum of D_1 .

Thus we have the following lemma.

Lemma 2 *The expected duration of time the server remains continuously in WV until the number of type I customers reach 0 and before the arrival of a type II customer is given by $\beta_2(-S_2)^{-2}\mathbf{S}_2^{(0)}$.*

Next we compute the following distribution. Let T'_s denote the duration of time the server, starting in slow service mode until either he gets back to normal mode through vacation expiration or the arrival of a type II customer, whichever occurs first.

3.2.2 Distribution of T'_s

The distribution of T'_s can be shown to be phase type with representation (β_3, S_3) where the underlying Markov chain has state space $\{(0, k) : 1 \leq k \leq n\} \cup \{(i, j, k) : 1 \leq i \leq N-1, 1 \leq j \leq m, 1 \leq k \leq n\} \cup \{*_1\} \cup \{*_2\}$ i denote the number of type I customers in the system, j the service phase, k , the arrival phase of type II customer, $*_1$, the absorbing state indicating the vacation expiration and $*_2$, the absorbing state indicating the arrival of a type II customer.

The initial probability vector is given by

$$\beta_3 = (1/d_2)(\mathbf{0}, w_{1,1}, \dots, w_{1,n}, \dots, w_{m,1}, \dots, w_{m,n}, \mathbf{0})$$

where, for, $1 \leq j \leq m$, $1 \leq k \leq n$, $w_{j,k}$ and d_2 are defined as in Sect. 3.2.1, the first $\mathbf{0}$ is a zero matrix of order n and the second $\mathbf{0}$ is a zero matrix of order $1 \times (N - 2)mn$.

The infinitesimal generator $\mathbf{S}_3$ of the above process denoted by $\chi_3(t)$ has the form

$$\mathbf{S}_3 = \begin{bmatrix} S_3 & \mathbf{S}_3^{(0)} & \mathbf{S}_3^{(1)} \\ \mathbf{0} & 0 & 0 \end{bmatrix}$$

where

$$\mathbf{S}_3 = \begin{bmatrix} D_0 - \lambda I & \lambda(\alpha \otimes I) & & & \\ \theta T^0 \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \lambda I & & \\ & \theta T^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \lambda I & \\ & & \ddots & \ddots & \\ & & \theta T^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I & \lambda I \\ & & & \theta T^0 \alpha \otimes I & \theta T \oplus D_0 - (\lambda + \eta)I \end{bmatrix},$$

$$\mathbf{S}_3^{(0)} = \begin{bmatrix} \mathbf{0} \\ \eta \mathbf{e}(mn) \\ \vdots \\ \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \end{bmatrix}, \text{ and } \mathbf{S}_3^{(1)} = \begin{bmatrix} \delta \\ \mathbf{e}(m) \otimes \delta \\ \vdots \\ \mathbf{e}(m) \otimes \delta \end{bmatrix} \text{ where } \mathbf{0} \text{ is a zero matrix of order } n \text{ and } \delta \text{ is given by (c1).}$$

Thus we have the following Lemma.

Lemma 3 *The expected duration of time the server remains in WV with or without hitting zero state of type I customer until the arrival of a type II customer is given by $\beta_3(-S_3)^{-2}\mathbf{S}_3^{(1)}$.*

The theorem below provides the expected number of visits to the state “no type I customer”, starting from that state, before the arrival of a type II customer.

Theorem 4 *The expected number of returns to 0 type I customer state during the vacation mode of service starting from that state before the arrival of a type II customer is given by $\left(\frac{1}{\lambda} + \beta_3(-S_3)^{-2}\mathbf{S}_3^{(1)}\right) / \left(\frac{1}{\lambda} + \beta_2(-S_2)^{-2}\mathbf{S}_2^{(0)}\right)$.*

4 Waiting Time Analysis of a Type I Customer

To find the waiting time of a type I customer who arrives at time x , we have to consider different possibilities depending on the status of server at that time. The server may be on vacation, WV, normal mode 1 or in normal mode 2. Let Z_1 be the random variable representing the waiting time of a type I customer in the queue. Define $W_1(x) = \text{Prob}(Z_1 \leq x)$ and $W_1^*(s)$ be the corresponding LST.

Case I

The tagged customer arrives to the system when the server is on vacation. Suppose E_1 denote the event that the system is in the state $(0, 1, 0, u, v)$, $1 \leq u \leq m$, $1 \leq v \leq n$ or in the state $(n_1, 1, 0, u, v)$, $n_1 \geq 1$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. Let $W_1^*(s/E_1)$ denote the corresponding LST. Then

$$W_1^*(s/E_1) = 1.$$

Case II

The tagged type I customer arrives to the system when the server is on WV. Suppose that $a + 1$ is the position of the tagged customer when he arrives the system. For $1 \leq a \leq N - 2$, let E_2 denote the event the system be in the state $(n_1, a + 1, 0, u, v)$, $n_1 \geq 0$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. Let $W_1^*(s/E_2)$ denote the corresponding LST.

Case (i)

Let E denote the event that the server switches to normal mode due to random clock (vacation clock) realization during the slow service. Then $E = \cup_{i=1}^{i=a+1} (E \cap H_i)$ where H_1 denotes the event the random clock expires during the residual service time of the customer in service and for $2 \leq i \leq a$, H_i denotes the event the random clock expire during the i th service. In these cases, the waiting time of an arbitrary type I customer is the sum of time duration, starting from his arrival epoch till random clock expiration, service time of the customer in service at the time of random clock expiration from the beginning in normal mode of service and service time of the remaining customers. Let H_{a+1} denotes the event the random clock expires after the a th service. In this case, the waiting time of an arbitrary customer is the sum of the residual service time of the customer in service when the tagged customer arrives and service time of remaining $a - 1$ type I customers in slow mode. Now,

$$P(E/E_2) = \left(\int_{t=0}^{\infty} (\mathbf{e}'_{a+1}(N-1) \otimes \mathbf{e}'_u(m)) \exp(S_1 t) \mathbf{S}_1^{(1)} dt \right)$$

where S_1 , $\mathbf{S}_1^{(1)}$ are as defined in Sect. 3.1.

Let $p_{a,u} = (\mathbf{e}'_{a+1}(N-1) \otimes \mathbf{e}'_u(m))(-S_1)^{-2} \mathbf{S}_1^{(1)})^{-1}$ be the rate of absorption to $\{*_1\}$ from S_1 and $\mu^{(i)}$ denote the expected rate of sum of i service time distributions, each following $PH(\alpha, T)$ (except $\mu^{(1)}$) (see Breuer and Baum 2005) from the arrival epoch of the tagged customer. Here, $\mu^{(1)} = \theta \mu_u$ which is the rate of residual service time when the server is providing slow service in phase u . Now,

$$\begin{aligned} P(H_1/E, E_2) &= \frac{P_{a,u}}{p_{a,u} + \mu^{(1)}}, \\ P(H_i/E, E_2) &= \frac{P_{a,u}}{p_{a,u} + \mu^{(i)}} - \frac{P_{a,u}}{p_{a,u} + \mu^{(i-1)}} \text{ for } 2 \leq i \leq a, \\ P(H_{a+1}/E, E_2) &= \frac{\mu^{(a)}}{p_{a,u} + \mu^{(a)}} \end{aligned}$$

Then the conditional LSTs are given by

$$\begin{aligned} W_1^*(s/E_2, E, H_1) &= \left(\frac{\eta}{s + \eta} \right) (\alpha(sI - T)^{-1} \mathbf{T}^0)^a, \\ W_1^*(s/E_2, E, H_i) &= \left(\frac{\eta}{s + \eta} \right) (\alpha(sI - T)^{-1} \mathbf{T}^0)^{a-i+1}, \text{ for } 2 \leq i \leq a \end{aligned}$$

and

$$W_1^*(s/E_2, E, H_{a+1}) = (\mathbf{e}'_u(sI - \theta T)^{-1} \theta \mathbf{T}^0) (\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}.$$

Thus conditional LST

$$W_1^*(s/E_2, E) = \sum_{i=1}^{a+1} W_1^*(s/E_2, E, H_i) P(H_i/E_2, E).$$

Case (ii)

Let F denote the event “the server switches to normal mode when the number of type I customers in the system hit N ” during the slow service. Then $F = \cup_{i=1}^{a+1} (F \cap J_i)$ where J_1 denotes the event: the number of type I customers in the system reaches N during the residual service time. For $2 \leq i \leq a$, J_i denotes the event: the number of type I customers in the system reaches N during the i th customer’s service time. In these cases, the waiting time of an arbitrary type I customer is the sum of time duration starting from his arrival epoch till the number of type I customers hit N , service time of the customer in service at the time of switching to normal mode from the beginning in normal mode of service and service time of remaining customers. Let J_{a+1} denote the event “the number of type I customers in the system reaches N after the a th customer’s service”. In this case, the waiting time of an arbitrary customer is the sum of the residual service time of the customer in service when the tagged customer arrives and service time of remaining $a - 1$ type I customers in slow mode.

Now,

$$P(F/E_2) = \left(\int_{t=0}^{\infty} (\mathbf{e}'_{a+1}(N-1) \otimes \mathbf{e}'_u(m)) \exp(S_1 t) \mathbf{S}_1^{(2)} dt \right)$$

where $S_1, \mathbf{S}_1^{(2)}$ are as defined in Sect. 3.1.

Let $q_{a,u} = (\mathbf{e}'_{a+1}(N-1) \otimes \mathbf{e}'_u(m))(-S_1)^{-2} \mathbf{S}_1^{(2)}{}^{-1}$ be the rate of absorption to $\{*_2\}$ from S_1

$$P(J_1/F, E_2) = \frac{q_{a,u}}{q_{a,u} + \mu^{(1)}},$$

$$P(J_i/F, E_2) = \frac{q_{a,u}}{q_{a,u} + \mu^{(i)}} - \frac{q_{a,u}}{q_{a,u} + \mu^{(i-1)}}, \text{ for } 2 \leq i \leq a$$

and

$$P(J_{a+1}/F, A_2) = \frac{\mu^{(a)}}{q_{a,u} + \mu^{(a)}}$$

The conditional LSTs are given by

$$W_1^*(s/E_2, F, J_1) = \left(\frac{\lambda}{s + \lambda}\right)^{N-a-1} (\alpha(sI - T)^{-1} \mathbf{T}^0)^a,$$

$$W_1^*(s/E_2, F, J_i) = \left(\frac{\lambda}{s + \lambda}\right)^{N-a+i-2} (\alpha(sI - T)^{-1} \mathbf{T}^0)^{a-i+1}, \text{ for } 2 \leq i \leq a$$

and

$$W_1^*(s/E_2, F, J_{a+1}) = (\mathbf{e}'_u(sI - \theta T)^{-1} \theta \mathbf{T}^0)(\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}.$$

Thus the conditional LST,

$$W_1^*(s/E_2, F) = \sum_{i=1}^{a+1} W_1^*(s/E_2, F, J_i) P(J_i/E_2, F)$$

Case (iii)

Let G denote the event that the system becomes empty before vacation expiration.

$$P(G/E_2) = \left(\int_{t=0}^{\infty} (\mathbf{e}'_{a+1}(N-1) \otimes \mathbf{e}'_u(m)) \exp(S_1 t) \mathbf{S}_1^{(0)} dt \right)$$

where $S_1, \mathbf{S}_1^{(0)}$ are as defined in Sect. 3.1.

In this case the conditional LST,

$$W_1^*(s/E_2, G) = (\mathbf{e}'_u(sI - \theta T)^{-1} \theta \mathbf{T}^0)(\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}.$$

Thus the conditional LST,

$$W_1^*(s/E_2) = W_1^*(s/E_2, E)P(E/E_2) + W_1^*(s/E_2, F)P(F/E_2) + W_1^*(s/E_2, G)P(G/E_2).$$

Case III

The customer arrives to the system when the server is in normal mode 1 of service. Suppose that $a + 1$ is the position of the tagged customer when he arrives the system. Let E_3 denote the event the system is in the state $(n_1, a + 1, 1, u, v)$, $n_1 \geq 0$, $1 \leq a \leq L - 1$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of residual normal service of the

type I customer in service and $a - 1$ remaining normal service time of type I customers. Let $W_1^*(s/E_3)$ denote the corresponding conditional LST.

Then conditional LST,

$$W_1^*(s/E_3) = (\mathbf{e}'_u(sI - T)^{-1}\mathbf{T}^0)(\boldsymbol{\alpha}(sI - T)^{-1}\mathbf{T}^0)^{a-1}.$$

Case IV

The customer arrives to the system when the server is in normal mode 2 of service. Suppose that $a + 1$ be the position of the tagged customer when he arrives the system. Let E_4 denote the event the system is in the state $(n_1, a + 1, 2, u, v)$, $n_1 \geq 1$, $0 \leq a \leq L - 1$, $1 \leq u \leq m'$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of residual service time of the type II customer in service and a remaining normal service time of type I customers. Let $W_1^*(s/E_4)$ denote the corresponding LST.

Then the conditional LST,

$$W_1^*(s/E_4) = (\mathbf{e}'_u(sI - T)^{-1}\mathbf{T}'^0)(\boldsymbol{\alpha}(sI - T)^{-1}\mathbf{T}^0)^a.$$

Let $w_{i_1, i_2, j, k, l}$ denote the probability that the system is in the state (i_1, i_2, j, k, l) immediately after arrival of the tagged customer. Then,

$$\begin{aligned} w_{0,1,0,u,v} &= \frac{\lambda\alpha_u}{\lambda + \eta - d_{vv}^{(0)}} x_{0,0,v}, \text{ for } 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,1,0,u,v} &= \frac{\lambda\alpha_u}{\lambda + \eta - d_{vv}^{(0)}} x_{n_1,0,0,v}, \text{ for } n_1 \geq 1, 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,0,u,v} &= \frac{\lambda}{\lambda + \eta - \theta T_{uu} - d_{vv}^{(0)}} x_{n_1,a,0,u,v}, \text{ for } n_1 \geq 0, 1 \leq a \leq N - 2, 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,N,1,u,v} &= \sum_{u'=1}^m \frac{\lambda\alpha_u}{\lambda + \eta - \theta T_{u'u'} - d_{vv}^{(0)}} x_{n_1,N-1,0,u',v} + \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,N-1,1,u,v}, \text{ for } n_1 \geq 0, \\ &\quad 1 \leq u, u' \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,1,u,v} &= \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,a,1,u,v}, \text{ for } n_1 \geq 0, 1 \leq a \leq N - 2 \text{ or } N \leq a \leq L - 1, \\ &\quad 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,2,u,v} &= \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,a,2,u,v}, \text{ for } n_1 \geq 1, 0 \leq a \leq L - 1, 1 \leq u \leq m', 1 \leq v \leq n \end{aligned}$$

Thus we have the following theorem.

Theorem 5 *The LST of the waiting time of a type I customer is given by*

$$\begin{aligned} W_1^*(s) = \frac{1}{d} &\left[\sum_{n_1=0}^{\infty} \sum_{v=1}^n w_{n_1,1,0,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{N-2} \sum_{u=1}^m \sum_{v=1}^n W_1^*(s/E_2)(w_{n_1,a+1,0,u,v}) \right. \\ &\left. + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^m \sum_{v=1}^n W_1^*(s/E_3)(w_{n_1,a+1,1,u,v}) + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m'} \sum_{v=1}^n W_1^*(s/E_4)(w_{n_1,a+1,2,u,v}) \right] \quad (9) \end{aligned}$$

where

$$d = \sum_{n_1=0}^{\infty} \sum_{v=1}^n w_{n_1,1,0,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{N-2} \sum_{u=1}^m \sum_{v=1}^n w_{n_1,a+1,0,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^m \sum_{v=1}^n w_{n_1,a+1,1,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m'} \sum_{v=1}^n w_{n_1,a+1,2,u,v}. \quad (10)$$

5 Waiting Time Analysis of Type II Customer

To find the LST of the waiting time distribution of a type II customer, we have to compute certain distributions. We proceed to such computations.

Definition 6 Duration of time with p type I customers in the system at a service commencement epoch of type I customers until the number of type I customers become zero for the first time is defined as a p -cycle denoted by B_p .

5.1 Distribution of a p -Cycle in Normal Mode

This can be studied as a phase type distribution with representation (γ_p, T_1) where the underlying markov chain has state space $\{(i, j) : 1 \leq i \leq L, 1 \leq j \leq m\} \cup \{0\}$ where i denotes the number of type I customers in the system, j the service phase and 0 the absorbing state indicating that the number of type I customers become zero. The infinitesimal generator $\mathbf{T}_1$ of $B_p(t)$ has the form

$$\mathbf{T}_1 = \begin{bmatrix} T_1 & \mathbf{T}_1^0 \\ \mathbf{0} & 0 \end{bmatrix}$$

where

$$T_1 = \begin{bmatrix} T - \lambda I & \lambda I & & & \\ \mathbf{T}^0 \alpha & T - \lambda I & \lambda I & & \\ \ddots & \ddots & \ddots & & \\ & \mathbf{T}^0 \alpha & T - \lambda I & \lambda I & \\ & & \mathbf{T}^0 \alpha & T & \end{bmatrix} \quad (c2)$$

$$\mathbf{T}_1^0 = \begin{bmatrix} \mathbf{T}^0 \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \\ \mathbf{0} \end{bmatrix} \quad (c3)$$

and the initial probability vector is

$$\gamma_p = [\mathbf{0} \ \cdots \ \mathbf{0} \ \alpha \ \mathbf{0} \ \cdots \ \mathbf{0}], 1 \leq p \leq L \quad (\text{c4})$$

where α is in the p th position and $\mathbf{0}$ is a zero matrix of order m .

Thus we have the following theorem.

Theorem 7 *The LST of the length of a p -cycle is given by $\gamma_p(sI - T_1)^{-1}\mathbf{T}_1^0$.*

5.2 LST of the Busy Cycle Generated by Type I Customers Arriving During the Service Time of a Type II Customer

Theorem 8 *The LST of the busy cycle generated by type I customers arriving during the service time of a type II customer is given by*

$$\begin{aligned} \hat{B}_{c_L}(s) &= \alpha' \left[(s + \lambda)I - T' \right]^{-1} \mathbf{T}'^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} \mathbf{T}_1^0 \lambda^p \\ &\quad \alpha' \left[(s + \lambda)I - T' \right]^{-(p+1)} \mathbf{T}'^0 + \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \\ &\quad \alpha' \left[\lambda^{-1}((s + \lambda)I - T') \right]^{-L} \left[I - \lambda \left[(s + \lambda)I - T' \right]^{-1} \right]^{-1} \left[(s + \lambda)I - T' \right]^{-1} \mathbf{T}'^0. \end{aligned} \quad (11)$$

Proof The proof is given in the “Appendix 1”. $\square$

5.3 LST of the Busy Period of Type I Customers Generated During the Service Time of a Type II Customer

Theorem 9 *The LST of the busy period of type I customers generated during the service time of a type II customer is given by*

$$\begin{aligned} \bar{B}_L(s) &= \alpha' \left[\lambda I - T' \right]^{-1} \mathbf{T}'^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} \mathbf{T}_1^0 \lambda^p \alpha' \left[\lambda I - T' \right]^{-(p+1)} \mathbf{T}'^0 \\ &\quad + \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \alpha' \left[\lambda^{-1}(\lambda I - T') \right]^{-L} \\ &\quad \left[I - \lambda \left[\lambda I - T' \right]^{-1} \right]^{-1} \left[\lambda I - T' \right]^{-1} \mathbf{T}'^0. \end{aligned} \quad (12)$$

Proof The proof is given in “Appendix 1”. $\square$

Let T_s'' denote the duration of time the server stays in vacation mode until either he gets back to normal mode through the random clock realization or the WV is interrupted with the number of type I customers in the system hitting N .

5.4 Conditional Distribution of T_s'' Given a Type II Customer Arrives Before the Random Clock Expires

We can study this by a phase type distribution with representation (β_4, S_4) where the underlying markov chain has state space

$\{0\} \cup \{(i, j) : 1 \leq i \leq N-1, 1 \leq j \leq m\} \cup \{*\}$ where i denotes the number of type I customers in the system, j the service phase and $*$ the absorbing state indicating the vacation expiration. The infinitesimal generator S_4 of the above process denoted by $\chi_4(t)$ is given by

$$S_4 = \begin{bmatrix} S_4 & S_4^0 \\ \mathbf{0} & 0 \end{bmatrix}, \text{ where,} \quad (c5)$$

$$S_4 = \begin{bmatrix} -(\lambda + \eta) & \lambda\alpha & & & \\ \theta T^0 & \theta T - (\lambda + \eta)I & \lambda I & & \\ & \theta T^0 \alpha & \theta T - (\lambda + \eta)I & \lambda I & \\ & & \ddots & \ddots & \\ & & & \theta T^0 \alpha & \theta T - (\lambda + \eta)I & \lambda I \\ & & & & \theta T^0 \alpha & \theta T - (\lambda + \eta)I & \lambda I \end{bmatrix}$$

and

$$S_4^0 = \begin{bmatrix} \eta \\ \eta \mathbf{e}(m) \\ \vdots \\ \eta \mathbf{e}(m) \\ (\lambda + \eta) \mathbf{e}(m) \end{bmatrix}. \quad (c6)$$

The initial probability vector is given by

$$\beta_4 = (1, \mathbf{0}), \text{ where } \mathbf{0} \text{ is a zero matrix of order } 1 \times (N-1)m. \quad (c7)$$

Thus we have the following lemma.

Lemma 10 *The expected duration of time the server stays in vacation mode until either the server gets back to normal mode through the random clock expiring or the WV is interrupted as the number of type I customers in the system hits N given a type II customer arrives before the random clock expires, is given by $\beta_4(-S_4)^{-1}\mathbf{e}$*

To find the waiting time of a type II customer who joins for service at time x , we have to consider different possibilities depending on the status of server at that time. The server may be in vacation mode, WV mode, normal mode 1 or in normal mode 2. Let Z_2 be the random variable representing the waiting time of a type II customer in the queue. Define $W_2(x) = \text{Prob}(Z_2 \leq x)$ and $W_2^*(s)$ be the corresponding LST.

Case I

Let F_1 denote the event that the system is in the state $(1, 0, 0, v)$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode and the

time duration of busy period generated by type I customers present at that time, if any. Let $W_2^*(s/F_1)$ denote the corresponding conditional LST of the waiting time.

Then

$$W_2^*(s/F_1) = \beta_4(sI - S_4)^{-1} \mathbf{S}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right]$$

where t_p , $0 \leq p \leq N$ denote the probabaility that there are p type I customers when the vacation expires, which is given by

$$t_p = \begin{cases} \int_{t=0}^{\infty} \beta_4(e^{S_4 t})_p \eta dt & \text{if } p = 0 \\ \int_{t=0}^{\infty} \beta_4(e^{S_4 t})_p \eta \mathbf{e}(m) dt & \text{if } 1 \leq p \leq N-1 \\ \int_{t=0}^{\infty} \beta_4(e^{S_4 t})_{N-1} \lambda \mathbf{e}(m) dt & \text{if } p = N \end{cases}$$

where $(e^{S_4 t})_p$ denote the columns in $e^{S_4 t}$ corresponding to p type I customer states, T_1 , $\mathbf{T}_1^0$, γ_p , S_4 , $\mathbf{S}_4^0$ and β_4 are given by (c2), (c3), (c4), (c5), (c6) and (c7) respectively.

Case II

Let F_2 denote the event the system is in the state $(b+1, 0, 0, v)$, $b \geq 1$, $1 \leq v \leq n$ immedietly after arrival of the tagged customer. In this case the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode, the time duration of busy period generated by type I customers present at that time, if any and the time duration of busy cycles generated by type I customers arriving during the service time of each of the b type II customers. Let $W_2^*(s/F_2)$ denote the corresponding conditional LST of the waiting time.

Then

$$W_2^*(s/F_2) = \beta_4(sI - S_4)^{-1} \mathbf{S}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right] (\hat{B}_{c_L}(s))^b$$

where $\hat{B}_{c_L}(s)$ is given by Theorem 8.

Case III

Let F_3 denote the event the system is in the state $(b+1, a, 0, u, v)$, $b \geq 0$, $1 \leq a \leq N-1$, $1 \leq u \leq m$, $1 \leq v \leq n$ immedietly after arrival of the tagged customer. In this case also the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode, the time duration of busy period generated by type I customers present at that time, if any and the time duration of busy cycles generated by type I customers arriving during the service time of each of the b type II customers. Let $W_2^*(s/F_3)$ denote the corresponding conditional LST.

Then

$$W_2^*(s/F_3) = \beta_a^u(sI - S_4)^{-1} \mathbf{S}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right] (\hat{B}_{c_L}(s))^b$$

where $\beta_a^u = (0, \mathbf{0}, \dots, \mathbf{e}'_u, \dots, \mathbf{0})$, $\mathbf{e}'_u$ is in the $(a+1)^{th}$ position and $\mathbf{0}$ denotes zero matrix of order $1 \times m$.

Case IV

Let F_4 denote the event the system is in the state $(b+1, a, 1, u, v)$, $b \geq 0$, $1 \leq a \leq L$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of time duration of an a-cycle in which the current service phase is u and time duration of busy cycles generated by type I customers arriving during the service time of each of the b type II customers. Let $W_2^*(s/F_4)$ denote the corresponding conditional LST.

Then

$$W_2^*(s/F_4) = (\gamma_a^u(sI - T_1)^{-1} \mathbf{T}_1^0) (\hat{B}_{c_L}(s))^b$$

where $\gamma_a^u = (\mathbf{0}, \dots, \mathbf{e}'_u, \dots, \mathbf{0})$, where $\mathbf{e}'_u$ is in the a^{th} position and $\mathbf{0}$ denotes zero matrix of order $1 \times m$.

Case V

Let F_5 denote the event the system is in the state $(b+1, a, 2, u, v)$, $b \geq 1$, $0 \leq a \leq L$, $1 \leq u \leq m'$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of the residual service time of the type II customer in service, time duration of the busy period of type I customers generated during the service time of type II customer in service when the tagged customer arrives and time duration of busy cycles generated by type I customers arriving during the service time of each of the $b-1$ type II customers. Let $W_2^*(s/F_5)$ denote the corresponding conditional LST.

Then

$$W_2^*(s/F_5) = (\mathbf{e}'_u(sI - T')^{-1} \mathbf{T}'^0) (\bar{B}_L(s)) (\hat{B}_{c_L}(s))^{b-1}$$

where $\bar{B}_L(s)$ is given by Theorem 9.

Let $w_{i_1, i_2, j, k, l}$ denote the probability that the system is in the state (i_1, i_2, j, k, l) immediately after arrival of the tagged customer. Then,

$$\begin{aligned}
 w_{1,0,0,v} &= \frac{d_{v'}^{(1)}}{\lambda + \eta - d_{v'}^{(0)}} x_{0,0,v'}, \text{ for, } 1 \leq v, v' \leq n \\
 w_{b+1,0,0,v} &= \frac{d_{v'}^{(1)}}{\lambda + \eta - d_{v'}^{(0)}} x_{b,0,0,v'}, \text{ for, } b \geq 1, 1 \leq v, v' \leq n \\
 w_{b+1,a,0,u,v} &= \frac{d_{v'}^{(1)}}{\lambda + \eta - \theta T_{uu} - d_{v'}^{(0)}} x_{b,a,0,u,v'}, \text{ for, } b \geq 0, 1 \leq a \leq N-1, 1 \leq v, v' \leq n \\
 w_{b+1,a,1,u,v} &= \frac{d_{v'}^{(1)}}{\lambda - T_{uu} - d_{v'}^{(0)}} x_{b,a,1,u,v'}, \text{ for, } b \geq 0, 1 \leq a \leq L, 1 \leq u \leq m, 1 \leq v, v' \leq n \\
 w_{b+1,a,2,u,v} &= \frac{d_{v'}^{(1)}}{\lambda - T_{uu} - d_{v'}^{(0)}} x_{b,a,2,u,v'}, \text{ for, } b \geq 1, 0 \leq a \leq L, 1 \leq u \leq m', 1 \leq v, v' \leq n
 \end{aligned}$$

Thus we have the following theorem

Theorem 11 *The LST of the waiting time of a type II customer is given by*

$$\begin{aligned}
 W_2^*(s) &= \sum_{v=1}^n W_2^*(s/F_1) w_{1,0,0,v} + \sum_{b=1}^{\infty} \sum_{v=1}^n W_2^*(s/F_2) w_{b+1,0,0,v} \\
 &+ \sum_{b=0}^{\infty} \sum_{a=1}^{N-1} \sum_{u=1}^m \sum_{v=1}^n W_2^*(s/F_3) (w_{b+1,a,0,u,v}) \\
 &+ \sum_{b=0}^{\infty} \sum_{a=1}^L \sum_{u=1}^m \sum_{v=1}^n W_2^*(s/F_4) (w_{b+1,a,1,u,v}) \\
 &+ \sum_{b=1}^{\infty} \sum_{a=0}^L \sum_{u=1}^{m'} \sum_{v=1}^n W_2^*(s/F_5) (w_{b+1,a,2,u,v}).
 \end{aligned} \tag{13}$$

Next we proceed to the analysis of model II.

6 Mathematical Formulation of Model II

Now we consider the case where the server continues to serve at a lower rate until either the vacation clock realizes or the number of type I customers present in the system plus the number of type I customers already served during the current vacation equal to N . All other assumptions are same as in model I.

In this case, $N_1(t)$, $N_2(t)$, $S(t)$, $J(t)$ and $M(t)$ are as defined for model I and we define $K(t)$ to be number of type I customers present in the system + number of type I customers already served during the current vacation at time t .

It is easy to verify that $\{(N_1(t), N_2(t), S(t), K(t), J(t), M(t)) : t \geq 0\}$ is an LIQBD with state space

$$\Omega = \cup_{i=0}^{\infty} l(i)$$

where

$$\begin{aligned}
 l(0) &= \{(0, 0, k/1 \leq k \leq n)\} \cup \{(0, i_2, 0, j_2, j_3, k)/1 \leq i_2 \leq N-1; i_2 \leq j_2 \leq N-1; \\
 &1 \leq j_3 \leq m; 1 \leq k \leq n\} \cup \{(0, i_2, 1, j_3, k)/1 \leq i_2 \leq L; 1 \leq j_3 \leq m; 1 \leq k \leq n\} \text{ and for } i_1 \geq 1, \\
 l(i_1) &= \{(i_1, 0, 0, k)/1 \leq k \leq n\} \cup \{(i_1, 0, 2, j_3, k)/1 \leq j_3 \leq m'; 1 \leq k \leq n\} \cup \{(i_1, i_2, \\
 &0, j_2, j_3, k)/1 \leq i_2 \leq N-1; i_2 \leq j_2 \leq N-1; 1 \leq j_3 \leq m; 1 \leq k \leq n\} \cup \{(i_1, i_2, 1, j_3, k)/1 \\
 &\leq i_2 \leq L; 1 \leq j_3 \leq m'; 1 \leq k \leq n\}
 \end{aligned}$$

Here also we note that when $N_1(t) = N_2(t) = 0$, server will be on vacation and so $S(t)$, $K(t)$ and $J(t)$ need not be considered. When $N_2(t) = 0$ and $S(t) = 0$, then $K(t)$ and $J(t)$ need not be considered. The only other component in the state vector in both cases would be $M(t)$. Also when $S(t) = 1$ or 2 , then $K(t)$ need not be considered.

The infinitesimal generator of the above process is

$$\mathcal{Q}_2 = \begin{bmatrix} G_0 & H_0 & & & \\ H_1 & A_1 & A_0 & & \\ & A_2 & A_1 & A_0 & \\ & & \ddots & \ddots & \ddots \end{bmatrix}.$$

where G_0 contains transitions within the level 0; H_0 represents transitions from level 0 to level 1; H_1 represents transitions from level 1 to level 0; A_0 represents transitions from level h to level $h + 1$ for $h \geq 1$, A_1 represents transitions within the level h for $h \geq 1$ and A_2 represents transitions from level h to level $h - 1$ for $h \geq 2$.

The boundary blocks G_0, H_0, H_1 are of orders $(n + \frac{mn}{2}(N^2 - N + 2L)) \times (n + \frac{mn}{2}(N^2 - N + 2L))$, $(n + \frac{mn}{2}(N^2 - N + 2L)) \times ((1 + m')n + \frac{mn}{2}(N^2 - N + 2L) + Lm'n)$, $((1 + m')n + \frac{mn}{2}(N^2 - N + 2L) + Lm'n) \times (n + \frac{mn}{2}(N^2 - N + 2L))$ respectively. A_0, A_1, A_2 are square matrices of order $(1 + m')n + \frac{mn}{2}(N^2 - N + 2L) + Lm'n$.

7 Steady State Analysis

Let $\pi = (\pi_0, \pi_1, \dots, \pi_L)$ denote the steady state probability vector of the generator

$$A = A_0 + A_1 + A_2 = \begin{bmatrix} B_1 & B_2 & & & & & & & \\ C_1 & E_1 & F_1 & & & & & & \\ & C_2 & E_2 & F_2 & & & & & \\ & & \ddots & \ddots & \ddots & & & & \\ & & & C_{N-1} & E_{N-1} & F_{N-1} & & & \\ & & & & G' & H & J & & \\ & & & & & G & H & J & \\ & & & & & & \ddots & \ddots & \ddots \\ & & & & & & & G & H & J \\ & & & & & & & & G & K \end{bmatrix}$$

where

$$B_1(k, l) = \begin{cases} D_0 + D_1 - (\lambda + \eta)I_n & k = 1, l = 1 \\ \eta(\alpha' \otimes I_n) & k = 1, l = 2 \\ 0 & k = 2, l = 1 \\ \mathbf{T}^0 \alpha' \otimes I_n + T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = 2, l = 2 \end{cases}$$

$$B_2(k, l) = \begin{cases} \lambda(\alpha \otimes I_n) & k = 1, l = 1 \\ \lambda I_{m'n} & k = 2, l = 2 \\ 0 & \text{otherwise} \end{cases}, C_1(k, l) = \begin{cases} \mathbf{e}(N-1) \otimes (\theta \mathbf{T}^0 \otimes I_n) & k = 1, l = 1 \\ \mathbf{T}^0 \alpha' \otimes I_n & k = 2, l = 2 \\ 0 & \text{otherwise} \end{cases}$$

For $2 \leq i \leq N-1$,

$$C_i(k, l) = \begin{cases} I_{N-i} \otimes (\theta \mathbf{T}^0 \alpha \otimes I_n) & k = 1, l = 2 \\ \mathbf{T}^0 \alpha \otimes I_n & k = 2, l = 3 \\ 0 & \text{otherwise} \end{cases}$$

For $1 \leq i \leq N-1$,

$$E_i(k, l) = \begin{cases} I_{N-i} \otimes (\theta T \oplus D_0 - (\lambda + \eta)I_{mn} + I_m \otimes D_1) & k = 1, l = 1 \\ \mathbf{e}((N-i)m) \otimes (\eta(\alpha \otimes I_n)) & k = 1, l = 2 \\ T \oplus D_0 - \lambda I_{mn} + I_m \otimes D_1 & k = 2, l = 2 \\ \mathbf{T}'^0 \alpha \otimes I_n & k = 3, l = 2 \\ T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = 3, l = 3 \\ 0 & \text{otherwise} \end{cases}$$

For $1 \leq i \leq N-2$,

$$F_i(k, l) = \begin{cases} \lambda I_{(N-1-i)mn} & k = 1, l = 1 \\ \lambda \mathbf{e}(m) \otimes (\alpha \otimes I_n) & k = 2, l = 2 \\ \lambda I_{mn} & k = 3, l = 2 \\ \lambda I_{m'n} & k = 4, l = 3 \\ 0 & \text{otherwise} \end{cases}, F_{N-1}(k, l) = \begin{cases} \lambda \mathbf{e}(m) \otimes (\alpha \otimes I_n) & k = 1, l = 1 \\ \lambda I_{mn} & k = 2, l = 1 \\ \lambda I_{m'n} & k = 3, l = 2 \\ 0 & \text{otherwise} \end{cases}$$

$$G'(k, l) = \begin{cases} \mathbf{T}^0 \alpha \otimes I_n & k = 1, l = 2 \\ 0 & \text{otherwise} \end{cases}, G(k, l) = \begin{cases} \mathbf{T}^0 \alpha \otimes I_n & k = 1, l = 1 \\ 0 & \text{otherwise} \end{cases}$$

$$H(k, l) = \begin{cases} T \oplus D_0 - \lambda I_{mn} + I_m \otimes D_1 & k = l = 1 \\ T' \oplus D_0 - \lambda I_{m'n} + I_{m'} \otimes D_1 & k = l = 2 \\ \mathbf{T}'^0 \alpha \otimes I_n & k = 2, l = 1 \\ 0 & \text{otherwise} \end{cases}, J(k, l) = \begin{cases} \lambda I_{mn} & k = 1, l = 1 \\ \lambda I_{m'n} & k = 2, l = 2 \end{cases}$$

$$K(k, l) = \begin{cases} T \oplus D_0 + I_m \otimes D_1 & k = l = 1 \\ T' \oplus D_0 + I_{m'} \otimes D_1 & k = l = 2 \\ \mathbf{T}'^0 \alpha \otimes I_n & k = 2, l = 1 \end{cases}$$

with dimension of B_1, B_2, C_1 be $(n + m'n) \times (n + m'n), (n + m'n) \times (Nmn + m'n), (Nmn + m'n) \times (n + m'n)$ respectively. For $2 \leq i \leq N-1$, C_i be of order

$((N-i+1)mn+m'n) \times ((N-i+2)mn+m'n)$, E_i be a square matrix of order $(N-i+1)mn+m'n$, F_i is of order $((N-i+1)mn+m'n) \times ((N-i)mn+m'n)$, G' is of order $(m+m')n \times (2m+m')n$, G, H, J and K are square matrices of order $(m+m')n$.

ie,

$$\pi A = 0, \pi e = 1. \quad (14)$$

The *LIQBD* description of the model indicates that the queueing system is stable (see Neuts (1981)) if and only if the left drift exceeds that of right drift. That is,

$$\pi A_0 e < \pi A_2 e. \quad (15)$$

The vector π cannot be obtained directly in terms of the parametres of the model. The inequality (15) is simplified in (18) below. From (14) we get

$$\pi_i = \pi_{i-1} \mathcal{U}_{i-1}, 1 \leq i \leq L \quad (16)$$

where

$$\mathcal{U}_0 = -B_2(E_1 + \mathcal{U}_1 C_2)^{-1}$$

$$\mathcal{U}_i = \begin{cases} -F_i(D_{i+1} + \mathcal{U}_{i+1} C_{i+2})^{-1} & \text{for } 1 \leq i \leq N-3 \\ -F_{N-2}(E_{N-1} + \mathcal{U}_{N-1} G)^{-1} & \text{for } i = N-2 \\ -E_{N-1}(H + \mathcal{U}_N G)^{-1}, & \text{for } i = N-1 \\ -\lambda(H + \mathcal{U}_{i+1} G)^{-1} & \text{for } N \leq i \leq L-2 \\ -\lambda J^{-1} & \text{for } i = L-1. \end{cases}$$

From the normalizing condition $\pi e = 1$ we have

$$\pi_0 \left(\sum_{j=0}^{L-1} \prod_{i=0}^j \mathcal{U}_i + I \right) e = 1. \quad (17)$$

The inequality (15) gives the stability condition as

$$\pi_0 \left[(I_{(1+m')} \otimes D_1) e + \sum_{i=0}^{N-2} \prod_{j=0}^i \mathcal{U}_j (I_{((N-i)m+m')} \otimes D_1) e + \sum_{i=N-1}^{L-1} \prod_{j=0}^i \mathcal{U}_j (I_{(m+m')} \otimes D_1) e \right] < \pi_0 \left[A_{20} + \sum_{i=0}^{N-2} \prod_{j=0}^i \mathcal{U}_j A_{2i} + \sum_{i=N+1}^{L-1} \prod_{j=0}^i \mathcal{U}_j A_{2(N-1)} \right] \quad (18)$$

where, $A_{20} = \begin{bmatrix} \mathbf{0} \\ (\mathbf{T}'^0 \alpha' \otimes I) e \end{bmatrix}$, $A_{2i} = \begin{bmatrix} \mathbf{0} \\ (\mathbf{T}'^0 \alpha \otimes I) e \end{bmatrix}$, $1 \leq i \leq N-2$ and $A_{2(N-1)} = \begin{bmatrix} \mathbf{0} \\ (\mathbf{T}'^0 \alpha' \otimes I) e \end{bmatrix}$, with $\mathbf{0}$ a zero column vector of order n , $(N-i)mn$ and mn for A_{20} , A_{2i} , $1 \leq i \leq N-2$ and $A_{2(N-1)}$ respectively.

Let $\mathbf{x}$ be the steady state probability vector of Q . We partition this vector as

$$\mathbf{x} = (\mathbf{x}_0, \mathbf{x}_1, \mathbf{x}_2 \dots),$$

where $\mathbf{x}_0$ is of dimension $n + \frac{mn}{2}(N^2 - N + 2L)$, $\mathbf{x}_1, \mathbf{x}_2, \dots$ are of dimension $(1 + m')n + \frac{mn}{2}(N^2 - N + 2L) + Lm'n$. Under the stability condition, we have

$$\mathbf{x}_i = \mathbf{x}_1 R^{i-1}, i \geq 2$$

where the matrix R is the minimal nonnegative solution to the matrix quadratic equation

$$R^2 A_2 + R A_1 + A_0 = 0$$

and the vectors $\mathbf{x}_0$ and $\mathbf{x}_1$ are obtained by solving the equations

$$\mathbf{x}_0 G_0 + \mathbf{x}_1 H_1 = 0 \quad (19)$$

$$\mathbf{x}_0 H_0 + \mathbf{x}_1 (A_1 + R A_2) = 0 \quad (20)$$

subject to the normalizing condition

$$\mathbf{x}_0 \mathbf{e} + \mathbf{x}_1 (I - R)^{-1} \mathbf{e} = 1. \quad (21)$$

7.1 Distribution of Duration of Slow Service Mode

The duration U_{slow} , in slow service mode is defined as the time the server starts in slow service mode (through initiating a WV) until either switching to normal mode through vacation clock realization or with the number of type I customers in the system plus number of type I customers already served during the current vacation hitting the threshold value N , $1 \leq N \leq L$ or the number of type I customers hitting 0 before expiration of vacation. We consider the Markov process $\kappa_1(t) = \{(N(t), J(t), K(t)) : t \geq 0\}$ where $N(t)$ is the number of type I customers in the system at time t , $J(t)$ the number of type I customers in the system plus number of type I customers already served during the current vacation and $K(t)$, the service phase at the time t . Thus the state space of the process is $\{(i, j, k) : 1 \leq i \leq N - 1, i \leq j \leq N - 1, 1 \leq k \leq m\} \cup \{0\} \cup \{*_1\} \cup \{*_2\}$ where 0 denotes the absorbing state indicating that there is no type I customer in the system and $*_1$ denotes the absorbing state indicating the vacation expiration by vacation clock realization and $*_2$ denotes the absorbing state indicating the vacation expiration by the number of type I customers in the system plus number of type I customers already served during the current vacation hitting N . The initial probability vector is given by

$$\gamma_1 = \frac{1}{d_1}(w_1, w_2, \dots, w_m, \mathbf{0})$$

where, for, $1 \leq j \leq m$, w_j and d_1 are defined as in Sect. 3.1 and $\mathbf{0}$ is a zero matrix of order $1 \times \frac{(N-2)(N+1)}{2}m$.

The infinitesimal generator $\mathbf{U}_1$ of $\kappa_1(t)$ has the form

$$\mathbf{U}_1 = \begin{bmatrix} U_1 & \mathbf{U}_1^{(0)} & \mathbf{U}_1^{(1)} & \mathbf{U}_1^{(2)} \\ \mathbf{0} & 0 & 0 & 0 \end{bmatrix} \text{ where,}$$

$$\mathbf{U}_1 = \begin{bmatrix} L_1 & M_1 & & & \\ K_1 & L_2 & M_2 & & \\ & \ddots & \ddots & \ddots & \\ & & K_{N-3} & L_{N-2} & M_{N-2} \\ & & & K_{N-2} & L_{N-1} \end{bmatrix} \text{ with,}$$

$$K_i = [0 \ I_{N-i-1} \otimes (\theta \mathbf{T}^0 \boldsymbol{\alpha})], \text{ for } 1 \leq i \leq N-2$$

where $\mathbf{0}$ is a zero matrix of order $(N-i-1)m \times m$.

$$L_i = I_{N-i} \otimes (\theta T - (\lambda + \eta)I_m), \text{ for } 1 \leq i \leq N-1$$

$$M_i = \begin{bmatrix} \lambda I_{(N-i-1)m} \\ 0 \end{bmatrix}, \text{ for } 1 \leq i \leq N-2$$

where $\mathbf{0}$ is a zero matrix of order $m \times (N-i-1)m$.

$$\mathbf{U}_1^{(0)} = \begin{bmatrix} \mathbf{e}(N-1) \otimes \theta \mathbf{T}^0 \\ \mathbf{0} \end{bmatrix}$$

where $\mathbf{0}$ is a zero matrix of order $\frac{(N-2)(N-1)}{2}m \times 1$.

$$\mathbf{U}_1^{(1)} = \begin{bmatrix} \eta \mathbf{e}((N-2)m) \\ \eta \mathbf{e}(m) \\ \eta \mathbf{e}((N-3)m) \\ \eta \mathbf{e}(m) \\ \vdots \\ \eta \mathbf{e}(m) \\ \eta \mathbf{e}(m) \\ \eta \mathbf{e}(m) \end{bmatrix}, \mathbf{U}_1^{(2)} = \begin{bmatrix} \mathbf{0} \\ \lambda \mathbf{e}(m) \\ \mathbf{0} \\ \lambda \mathbf{e}(m) \\ \vdots \\ \mathbf{0} \\ \lambda \mathbf{e}(m) \\ \lambda \mathbf{e}(m) \end{bmatrix}$$

where $\mathbf{0}$'s are zero matrices of order $(N-2)m \times 1, (N-3)m \times 1, \dots, m \times 1$ respectively. Thus we have the following lemma.

Lemma 12 *The expected duration of time the server remains in WV until the number of type I customers reach 0 is given by $\gamma_1(-U_1)^{-2}\mathbf{U}_1^{(0)}$.*

Define the random variable M_2 as number of returns to 0 type I customer state starting from 0 type I customer state during vacation mode of service before the arrival of a type II customer.

7.2 Expected Value of M_2

Let U_s denote the duration of slow service until the arrival of a type II customer.

7.2.1 Distribution of U_s

We consider the Markov process $\kappa_2(t) = \{(N(t), J(t), K(t), M(t)) : t \geq 0\}$ where $N(t)$ is the number of type I customers in the system at time t , $J(t)$ the number of type I customers in the system plus number of type I customers already served during the current vacation, $K(t)$ the service phase and $M(t)$ the arrival phase of type II customer at that instant. Thus the state space of the process is $\{(i, j, k, l) : 1 \leq i \leq N-1, 1 \leq j \leq N-1, 1 \leq k \leq m, 1 \leq l \leq n\} \cup \{0\} \cup \{*_1\} \cup \{*_2\}$ where 0 denotes the absorbing state indicating that there is no type I customer in the system, $*_1, *_2$ denote the absorbing states indicating the vacation expiration and arrival of a type II customer respectively. The initial probability vector is given by

$$\gamma_2 = (1/d_2)(w_{1,1}, \dots, w_{1,n}, \dots, w_{m,1}, \dots, w_{m,n}, \mathbf{0})$$

where, for, $1 \leq j \leq m$, $1 \leq k \leq n$, $w_{j,k}$ and d_2 are defined as in Sect. 3.2.1 and $\mathbf{0}$ is a zero matrix of order $1 \times \frac{(N-2)(N+1)}{2}mn$. The infinitesimal generator $\mathbf{U}_2$ of $\kappa_2(t)$ has the form

$$\mathbf{U}_2 = \begin{bmatrix} U_2 & \mathbf{U}_2^{(0)} & \mathbf{U}_2^{(1)} & \mathbf{U}_2^{(2)} \\ \mathbf{0} & 0 & 0 & 0 \end{bmatrix} \text{ where,}$$

$$\mathcal{U}_2 = \begin{bmatrix} L_1 & M_1 & & & \\ K_1 & L_2 & M_2 & & \\ & \ddots & \ddots & \ddots & \\ & & K_{N-3} & L_{N-2} & M_{N-2} \\ & & & K_{N-2} & L_{N-1} \end{bmatrix} \text{ with,}$$

$$K_i = [\mathbf{0} \ I_{N-1-i} \otimes (\theta \mathbf{T}^0 \alpha \otimes I)], \text{ for } 1 \leq i \leq N-2,$$

where $\mathbf{0}$ is a zero matrix of order $(N-i-1)mn \times mn$,

$$M_i = \begin{bmatrix} \lambda I_{(N-i-1)mn} \\ \mathbf{0} \end{bmatrix}, \text{ for } 1 \leq i \leq N-2,$$

where $\mathbf{0}$ is a zero matrix of order $mn \times (N-1-i)mn$.

$$L_i = I_{N-i} \otimes (\theta T \oplus D_0 - (\lambda + \eta)I_{mn}), \text{ for } 1 \leq i \leq N-1$$

$$U_2^{(0)} = \begin{bmatrix} \mathbf{e}(N-1) \otimes (\theta T^0 \otimes \mathbf{e}(n)) \\ \mathbf{0} \end{bmatrix}, U_2^{(1)} = \begin{bmatrix} \eta \mathbf{e}((N-2)mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ \eta \mathbf{e}((N-3)mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ \vdots \\ \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \end{bmatrix},$$

$$U_2^{(2)} = \begin{bmatrix} \mathbf{e}((N-1)m) \otimes \delta \\ \mathbf{e}((N-2)m) \otimes \delta \\ \vdots \\ \mathbf{e}(m) \otimes \delta \end{bmatrix}$$

where $\mathbf{0}$ is a zero matrix of order $\frac{(N-2)(N-1)}{2}mn \times 1$ and δ is given by (c1).

Thus we have the following lemma.

Lemma 13 *The expected duration of time the server remains continuously in WV until the number of type I customers reach 0 and before the arrival of a type II customer is given by $\gamma_2(-U_2)^{-2}U_2^{(0)}$.*

Let U'_s denote the duration of time the server starts in slow service mode until either he gets back to normal mode through the vacation expiration or the arrival of a type II customer.

7.2.2 Distribution of U'_s

The distribution of U'_s can be shown to be phase type with representation (γ_3, U_3) where the underlying Markov chain has state space $\{(0, l): 1 \leq l \leq n\} \cup \{(i, j, k, l): 1 \leq i \leq N-1, i \leq j \leq N-1, 1 \leq k \leq m, 1 \leq l \leq n\} \cup \{*_1\} \cup \{*_2\}$, where, i denotes the number of type I customers in the system, j the number of type I customers in the system plus number of type I customers already served during the current vacation, k , the service phase, l , the arrival phase of type II customer, $*_1$ the absorbing state indicating the vacation expiration and $*_2$ the absorbing state indicating the arrival of a type II customer.

The initial probability vector is given by

$$\gamma_3 = (\lambda/d_2)(\mathbf{0}, w_{1,1}, \dots, w_{1,n}, \dots, w_{m,1}, \dots, w_{m,n}, \mathbf{0})$$

For, $1 \leq j \leq m$, $1 \leq k \leq n$, $w_{j,k}$ and d_2 are defined as in Sect. 7.2.1 and, first $\mathbf{0}$ is a zero matrix of order n and second $\mathbf{0}$ is a zero matrix of order $1 \times \frac{(N-2)(N+1)mn}{2}$.

The infinitesimal generator U_3 of the above process denoted by $\kappa_3(t)$ has the form

$$\mathbf{U}_3 = \begin{bmatrix} U_3 & \mathbf{U}_3^{(0)} & \mathbf{U}_3^{(1)} \\ \mathbf{0} & 0 & 0 \end{bmatrix} \text{ where,}$$

$$\mathcal{U}_3 = \begin{bmatrix} D_0 - \lambda I & M_1 & & & \\ K_1 & L_1 & M_2 & & \\ & \ddots & \ddots & \ddots & \\ & & K_{N-2} & L_{N-2} & M_{N-1} \\ & & & K_{N-1} & L_{N-1} \end{bmatrix} \text{ with,}$$

$$M_1 = [\lambda(\alpha \otimes I) \mathbf{0}], \text{ where } \mathbf{0} \text{ is a zero matrix of order } n \times (N-2)mn.$$

$$K_1 = [\mathbf{e}(N-1) \otimes (\theta \mathbf{T}^0 \otimes I)]$$

For $2 \leq i \leq N-1$,

$$K_i = [\mathbf{0} \ I_{N-i} \otimes (\theta \mathbf{T}^0 \alpha \otimes I)], \text{ where } \mathbf{0} \text{ is a zero matrix of order } (N-i)mn \times mn$$

and

$$M_i = \begin{bmatrix} \lambda I_{(N-i)mn} \\ \mathbf{0} \end{bmatrix}$$

where $\mathbf{0}$ is a zero matrix of order $mn \times (N-i)mn$.

$$L_i = I_{N-i} \otimes (\theta T \oplus D_0 - (\lambda + \eta)I_{mn}), \text{ for } 2 \leq i \leq N-1,$$

$$\mathbf{U}_3^{(0)} = \begin{bmatrix} \mathbf{0} \\ \mathbf{e}(N-2) \otimes \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ \mathbf{e}(N-3) \otimes \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ \vdots \\ \eta \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \\ (\lambda + \eta) \mathbf{e}(mn) \end{bmatrix}, \mathbf{U}_3^{(1)} = \begin{bmatrix} \delta \\ \mathbf{e}((N-1)m) \otimes \delta \\ \mathbf{e}((N-2)m) \otimes \delta \\ \vdots \\ \mathbf{e}(m) \otimes \delta \end{bmatrix}$$

where, $\mathbf{0}$ is a zero matrix of order $n \times 1$ and δ is given by (c1).

Thus we have the following lemma.

Lemma 14 *The expected duration of time the server remains in WV with or without hitting zero state of type I customer until the arrival of a type II customer before hitting normal mode is given by $\gamma_3(-U_3)^{-2} \mathbf{U}_3^{(1)}$.*

Thus we arrive at

Theorem 15 *The expected number of returns to 0 type I customer state during the vacation mode of service before the arrival of a type II customer, is given by $\left(\frac{1}{\lambda} + \gamma_3(-U_3)^{-2} \mathbf{U}_3^{(1)}\right) / \left(\frac{1}{\lambda} + \gamma_2(-U_2)^{-2} \mathbf{U}_2^{(0)}\right)$.*

8 Waiting Time Analysis of a Type I Customer

To find the waiting time of a type I customer who joins for service at time x , we have to consider different possibilities depending on the status of server at that time. The server may be on vacation, WV, normal mode 1 or in normal mode 2. Let Z_1 be the random variable representing the waiting time of a type I customer in the queue. Define $W_1(x) = \text{Prob}(Z_1 \leq x)$ and $W_1^*(s)$ be the corresponding LST.

Case I

The tagged customer arrives to the system when the server is on vacation. Suppose E_1 denote the event that the system is in the state $(0, 1, 0, 1, u, v)$, $1 \leq u \leq m$, $1 \leq v \leq n$ or in the state $(n_1, 1, 0, 1, u, v)$, $n_1 \geq 1$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. Let $W_1^*(s|E_1)$ denote the corresponding LST. Then

$$W_1^*(s|E_1) = 1$$

Case II

The tagged type I customer arrives to the system when the server is on WV. Let $W_1^*(s)$ denote the corresponding LST. Suppose that $a+1$ is the position of the tagged customer when he arrives the system. For $1 \leq a \leq N-2$, let E_2 denote the event the system be in the state $(n_1, a+1, 0, t+1, u, v)$, $n_1 \geq 0$, $a \leq t \leq N-2$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. Let $W_1^*(s|E_2)$ denote the corresponding LST.

Case (i)

Let E denote the event that the server switches to normal mode due to random clock expiration during the slow service. Then $E = \cup_{i=1}^{j=a+1} (E \cap K_i)$ where K_1 denotes the event the random clock expire during the residual service time of the customer in service and for $2 \leq i \leq a$, K_i denotes the event the random clock expire during the i th service. In these cases, the waiting time of an arbitrary type I customer is the sum of time duration, starting from his arrival epoch till random clock expiration, service time of the customer in service at the time of random clock expiration from the beginning in the normal mode of service and service time of the remaining customers. Let K_{a+1} denotes the event the random clock expires after the a th service. In this case, the waiting time of an arbitrary customer is the sum of the residual service time of the customer in service when the tagged customer arrives and service time of remaining $a-1$ type I customers in slow mode.

Now,

$$P(E/E_2) = \left(\int_{t=0}^{\infty} \mathbf{e}_{\frac{a}{2}(2N-a-1)m+(t-a-1)m+u} \left(\frac{(N-1)Nm}{2} \right) \exp(U_1 t) \mathbf{U}_1^{(1)} dt \right)$$

where $U_1, \mathbf{U}_1^{(1)}$ are as defined in Sect. 7.1.

Let $p_{a,u} = (\mathbf{e}_2^a (2N-a-1)m+(r-a-1)m+u (\frac{(N-1)Nm}{2})(-U_1)^{-2} \mathbf{U}_1^{(1)})^{-1}$ be the rate of absorption to $\{*_1\}$ from U_1 and $\mu^{(i)}$ denote the expected rate of sum of i service time distributions, each following $PH(\alpha, T)$ from the arrival epoch of the tagged customer. Here, $\mu^{(1)} = \theta\mu_u$ which is the residual service rate when the server is providing slow service in phase u .

$$\begin{aligned} P(K_1|E, E_2) &= \frac{p_{a,u}}{p_{a,u} + \mu^{(1)}}, \\ P(K_i|E, E_2) &= \frac{p_{a,u}}{p_{a,u} + \mu^{(i)}} - \frac{p_{a,u}}{p_{a,u} + \mu^{(i-1)}}, \text{ for } 2 \leq i \leq a, \\ P(K_{a+1}/E, E_2) &= \frac{\mu^{(a)}}{p_{a,u} + \mu^{(a)}}. \end{aligned}$$

Then the conditional LSTs are given by

$$\begin{aligned} W_1^*(s|E_2, E, K_1) &= \left(\frac{\eta}{s + \eta} \right) (\alpha(sI - T)^{-1} \mathbf{T}^0)^a, \\ W_1^*(s|E_2, E, K_i) &= \left(\frac{\eta}{s + \eta} \right) (\alpha(sI - T)^{-1} \mathbf{T}^0)^{a-i+1}, \text{ for } 2 \leq i \leq a \end{aligned}$$

and

$$W_1^*(s|E_2, E, K_{a+1}) = (\mathbf{e}'_u (sI - \theta T)^{-1} \theta \mathbf{T}^0) (\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}.$$

Thus the conditional LST

$$W_1^*(s|E_2, E) = \sum_{i=1}^{a+1} W_1^*(s|E_2, E, K_i) P(K_i|E_2, E).$$

Case(ii)

Let F denote the event “the server switches to normal mode when the number of type I customers in the system plus number of type I customers already served during the current vacation hits N ” during the slow service. Then $F = \cup_{i=1}^{a+1} (F \cap M_i)$ where M_1 denote the event: the number of type I customers plus number of type I customers already served during the current vacation reaches N during the residual service time. For $2 \leq i \leq a$, M_i denote the event: the number of type I customers in the system plus number of type I customers already served during the current vacation reaches N during the i th customer's service time. In these cases, the waiting time of an arbitrary type I customer is the sum of time duration starting from his arrival epoch till the number of type I customers in the system plus number of type I customers already served during the current vacation hits N , service time of the customer in service at the time of switching to normal mode from the beginning in the normal mode of service and service time of remaining customers. Let M_{a+1} denote the event “the number of

type I customers in the system plus number of type I customers already served during the current vacation reaches N after the a th customer's service". In this case, the waiting time of an arbitrary customer is the sum of the residual service time of the customer in service when the tagged customer arrives and service time of remaining $a - 1$ type I customers in slow mode.

Now,

$$P(F/E_2) = \left(\int_{t=0}^{\infty} \mathbf{e}_{\frac{a}{2}(2N-a-1)m+(t-a-1)m+u} \left(\frac{(N-1)Nm}{2} \right) \exp(U_1 t) \mathbf{U}_1^{(2)} dt \right)$$

where $U_1, \mathbf{U}_1^{(2)}$ are as defined in Sect. 7.1.

Let $q_{a,u} = \left(\mathbf{e}_{\frac{a}{2}(2N-a-1)m+(t-a-1)m+u} \left(\frac{(N-1)Nm}{2} \right) (-U_1)^{-2} \mathbf{U}_1^{(2)} \right)^{-1}$ be the rate of absorption to $\{*_2\}$ from U_1 .

$$P(M_1|F, E_2) = \frac{q_{a,u}}{q_{a,u} + \mu^{(1)}},$$

$$P(M_i|F, E_2) = \frac{q_{a,u}}{q_{a,u} + \mu^{(i)}} - \frac{q_{a,u}}{q_{a,u} + \mu^{(i-1)}}, \text{ for } 2 \leq i \leq a$$

and

$$P(M_{a+1}|F, E_2) = \frac{\mu^{(a)}}{q_{a,u} + \mu^{(a)}}$$

The conditional LSTs,

$$W_1^*(s|E_2, F, M_1) = \left(\frac{\lambda}{s + \lambda} \right)^{N-t-1} (\alpha(sI - T)^{-1} \mathbf{T}^0)^a,$$

$$W_1^*(s|E_2, F, M_i) = \left(\frac{\lambda}{s + \lambda} \right)^{N-t-1} (\alpha(sI - T)^{-1} \mathbf{T}^0)^{a-i+1}, \text{ for } 2 \leq i \leq a$$

and

$$W_1^*(s|E_2, F, M_{a+1}) = (\mathbf{e}'_u (sI - \theta T)^{-1} \theta \mathbf{T}^0) (\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}$$

Thus the conditional LST

$$W_1^*(s|E_2, F) = \sum_{i=1}^{a+1} W_1^*(s|E_2, F, M_i) P(M_i|E_2, F)$$

Case (iii)

Let G denote the event that the system becomes empty before vacation expiration.

$$P(G/E_2) = \left(\int_{t=0}^{\infty} \mathbf{e}_{\frac{a}{2}(2N-a-1)m+(t-a-1)m+u} \left(\frac{(N-1)Nm}{2} \right) \exp(U_1 t) \mathbf{U}_1^{(0)} dt \right)$$

In this case the conditional LST,

$$W_1^*(s/E_2, G) = (\mathbf{e}'_u(sI - \theta T)^{-1} \theta \mathbf{T}^0)(\alpha(sI - \theta T)^{-1} \theta \mathbf{T}^0)^{a-1}.$$

Thus the conditional LST,

$$W_1^*(s/E_2) = W_1^*(s/E_2, E)P(E/E_2) + W_1^*(s/E_2, F)P(F/E_2) + W_1^*(s/E_2, G)P(G/E_2).$$

Case III

Let E_3 denote the event that the customer arrives to the system when the server is in normal mode 1. This case is same as for model I. Let $W_1^*(s/E_3)$ denote the corresponding conditional LST.

Then the conditional LST of the waiting time is given by

$$W_1^*(s|E_3) = (\mathbf{e}'_u(sI - T)^{-1} \mathbf{T}^0)(\alpha(sI - T)^{-1} \mathbf{T}^0)^{a-1}.$$

Case IV

Let E_4 denote the event that the customer arrives to the system when the server is in normal mode 2. This case is also same as for model I. Let $W_1^*(s/E_4)$ denote the corresponding LST.

Then the conditional LST of the waiting time,

$$W_1^*(s|E_4) = (\mathbf{e}'_u(sI - T')^{-1} \mathbf{T}'^0)(\alpha(sI - T')^{-1} \mathbf{T}'^0)^a.$$

Let $w_{i_1, i_2, j_1, j_2, k, l}$ denote the probability that the system is in the state $(i_1, i_2, j_1, j_2, k, l)$ immediately after arrival of the tagged customer. Then,

$$\begin{aligned} w_{0,1,0,1,u,v} &= \frac{\lambda \alpha_u}{\lambda + \eta - d_{vv}^{(0)}} x_{0,0,v}, \text{ for } 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,1,0,1,u,v} &= \frac{\lambda \alpha_u}{\lambda + \eta - d_{vv}^{(0)}} x_{n_1,0,0,v}, \text{ for } n_1 \geq 1, 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,0,t+1,u,v} &= \frac{\lambda}{\lambda + \eta - \theta T_{uu} - d_{vv}^{(0)}} x_{n_1,a,0,t,u,v}, \text{ for } n_1 \geq 0, 1 \leq a \leq N-2, a \leq t \leq N-2, \\ &\quad 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,1,u,v} &= \sum_{u'=1}^m \frac{\lambda \alpha_u}{\lambda + \eta - \theta T_{u'u'} - d_{vv}^{(0)}} x_{n_1,a,0,N-1,u',v} + \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,a,1,u,v}, \\ &\quad \text{for } n_1 \geq 0, 1 \leq a \leq N-1, 1 \leq u \leq m, 1 \leq v \leq n \\ w_{n_1,a+1,1,u,v} &= \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,a,1,u,v}, \text{ for } n_1 \geq 0, N \leq a \leq L-1, 1 \leq u \leq m, \\ &\quad 1 \leq v \leq n \\ w_{n_1,a+1,2,u,v} &= \frac{\lambda}{\lambda - T_{uu} - d_{vv}^{(0)}} x_{n_1,a,2,u,v}, \text{ for } n_1 \geq 1, 0 \leq a \leq L-1, 1 \leq u \leq m', 1 \leq v \leq n \end{aligned}$$

Thus we have the following theorem.

Theorem 16 *The LST of the waiting time of a type I customer is given by*

$$\begin{aligned} W_1^*(s) = \frac{1}{d} \left[\sum_{n_1=0}^{\infty} \sum_{v=1}^n w_{n_1,1,0,1,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{N-2} \sum_{t=a}^{N-2} \sum_{u=1}^m \sum_{v=1}^n W_1^*(s|E_2)(w_{n_1,a+1,0,t+1,u,v}) \right. \\ \left. + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^m \sum_{v=1}^n W_1^*(s|E_3)(w_{n_1,a+1,1,u,v}) + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^m \sum_{v=1}^n W_1^*(s|E_4)(w_{n_1,a+1,2,u,v}) \right] \quad (22) \end{aligned}$$

where

$$d = \sum_{n_1=0}^{\infty} \sum_{v=1}^n w_{n_1,1,0,1,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{N-2} \sum_{t=a}^{N-2} \sum_{u=1}^m \sum_{v=1}^n w_{n_1,a+1,0,t+1,u,v} \\ + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^m \sum_{v=1}^n w_{n_1,a+1,1,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^m \sum_{v=1}^n w_{n_1,a+1,2,u,v}. \quad (23)$$

9 Waiting Time Analysis of Type II Customer

To find the LST of the waiting time distribution of a type II customer, we have to compute the following distribution.

Let U_s'' be the duration of time the server, starting in vacation, until either he gets back to normal mode through the random clock expiring or the WV is interrupted as the number of type I customers in the system plus number of type I customers already served during the current vacation hits N .

9.1 Conditional Distribution of U_s'' Given a Type II Customer Arrives Before the Random Clock Expires

We can study this by a phase type distribution with representation (γ_4, U_4) where the underlying markov chain has state space $\{0\} \cup \{(i, j, k) : 1 \leq i \leq N-1, 1 \leq j \leq N-1, 1 \leq k \leq m\} \cup \{*\}$ where i denotes the number of type I customers in the system, j the number of type I customers in the system plus number of type I customers already served during the current vacation, k , the service phase and $*$ denotes the absorbing state indicating the vacation expiration. The infinitesimal generator U_4 of the above process denoted by $\kappa_4(t)$ is given by

$$U_4 = \begin{bmatrix} U_4 & U_4^0 \\ \mathbf{0} & 0 \end{bmatrix}$$

where,

$$U_4 = \begin{bmatrix} -(\lambda + \eta) & M_1 & & & \\ K_1 & L_1 & M_2 & & \\ & \ddots & \ddots & \ddots & \\ & & K_{N-2} & L_{N-2} & M_{N-1} \\ & & & K_{N-1} & L_{N-1} \end{bmatrix} \quad (c8)$$

with

$$M_1 = [\lambda \alpha \mathbf{0}], \text{ where } \mathbf{0} \text{ is a zero matrix of order } 1 \times (N-2)m.$$

$$K_1 = \mathbf{e}(N-1) \otimes \theta \mathbf{T}^0$$

For $2 \leq i \leq N-1$,

$$K_i = \begin{bmatrix} \mathbf{0} & I_{N-i} \otimes (\theta \mathbf{T}^0 \alpha) \end{bmatrix}, \text{ where } \mathbf{0} \text{ is a zero matrix of order } (N-i)m \times m$$

and

$$M_i = \begin{bmatrix} \lambda I_{(N-i)m} \\ \mathbf{0} \end{bmatrix}$$

where $\mathbf{0}$ is a zero matrix of order $m \times (N-i)m$.

$$L_i = I_{N-i} \otimes (\theta T \oplus D_0 - (\lambda + \eta)I_m), \text{ for } 1 \leq i \leq N-1$$

$$\mathbf{U}_4^0 = \begin{bmatrix} \eta \\ \eta \mathbf{e}((N-2)m) \\ (\lambda + \eta) \mathbf{e}(m) \\ \vdots \\ \eta \mathbf{e}(m) \\ (\lambda + \eta) \mathbf{e}(m) \\ (\lambda + \eta) \mathbf{e}(m) \end{bmatrix}. \quad (\text{c9})$$

The initial probability vector is given by

$$\gamma_4 = (1, \mathbf{0}), \text{ where } \mathbf{0} \text{ is a zero matrix of order } 1 \times \frac{(N-1)N}{2}m. \quad (\text{c10})$$

Thus we have the following lemma.

Lemma 17 *The expected duration of time the server stays in vacation mode until either the server gets back to normal mode through the random clock expiring or the WV is interrupted as the number of type I customers in the system plus the number of type I customers already served during the current vacation hits N given a type II customer arrives before the random clock expires, is given by $\gamma_4(-U_4)^{-1}\mathbf{e}$.*

To find the waiting time of a type II customer who arrives at time x , we have to consider different possibilities depending on the status of server at that time. The server may be in vacation mode, WV mode, normal mode 1 or in normal mode 2. Let Z_2 be the random variable representing the waiting time of a type II customer in the queue. Define $W_2(x) = \text{Prob}(Z_2 \leq x)$ and $W_2^*(s)$ be the corresponding LST.

Case I

Let F_1 denote the event that the system is in the state $(1, 0, 0, v)$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode and the time duration of busy period generated by type I customers present at that time, if any. Let $W_2^*(s|F_1)$ denote the corresponding conditional LST of the waiting time.

Then

Table 1 Mean/Variance of number of type I customers in the system for model I: Effect of η and N

η	N = 1, L = 3		N = 2, L = 4		N = 3, L = 5		N = 4, L = 6		N = 5, L = 7	
	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns
0.01	0.6934	0.7328	0.8170	0.9367	0.8928	1.1001	0.9494	1.2388	0.9947	1.3680
0.02	0.6934	0.7328	0.8169	0.9366	0.8924	1.0996	0.9482	1.2371	0.9924	1.3638
0.03	0.6934	0.7328	0.8168	0.9365	0.8919	1.0991	0.9470	1.2354	0.9901	1.3597
0.04	0.6934	0.7328	0.8166	0.9364	0.8914	1.0986	0.9458	1.2338	0.9878	1.3557
0.05	0.6934	0.7328	0.8165	0.9363	0.8909	1.0981	0.9446	1.2322	0.9857	1.3518

Table 2 Mean/Variance of number of type I customers in the system for model II: Effect of η and N

η	N = 1, L = 3		N = 2, L = 4		N = 3, L = 5		N = 4, L = 6		N = 5, L = 7	
	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns
0.01	0.6934	0.7328	0.8170	0.9367	0.8807	1.0854	0.9200	1.1885	0.9479	1.2653
0.02	0.6934	0.7328	0.8169	0.9366	0.8804	1.0851	0.9194	1.1878	0.9470	1.2640
0.03	0.6934	0.7328	0.8168	0.9365	0.8801	1.0848	0.9188	1.1872	0.9461	1.2627
0.04	0.6934	0.7328	0.8166	0.9364	0.8797	1.0845	0.9183	1.1865	0.9453	1.2615
0.05	0.6934	0.7328	0.8165	0.9363	0.8794	1.0842	0.9177	1.1858	0.9444	1.2602

$$W_2^*(s|F_1) = \gamma_4(sI - U_4)^{-1} \mathbf{U}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right]$$

where t_p , $0 \leq p \leq N$ denote the probability that there are p type I customers when the vacation expires which is given by

$$t_p = \begin{cases} \int_{t=0}^{\infty} \gamma_4(e^{U_4 t})_p \eta dt & \text{if } p = 0 \\ \int_{t=0}^{\infty} \gamma_4(e^{U_4 t})_p (U_4^0)_p dt & \text{if } 1 \leq p \leq N-2 \\ \int_{t=0}^{\infty} \gamma_4(e^{U_4 t})_{N-1} \eta \mathbf{e}(m) dt & \text{if } p = N-1 \\ \int_{t=0}^{\infty} \gamma_4(e^{U_4 t})_{N-1} \lambda \mathbf{e}(m) dt & \text{if } p = N \end{cases}$$

where $(e^{U_4 t})_p$ denote the columns in $e^{U_4 t}$ corresponding to p type I customer states and $(U_4^0)_p$ the absorbing rates corresponding to p type I customers, T_1 , T_1^0 , γ_p , U_4 , U_4^0 and γ_4 are given by (c2), (c3),(c4),(c8), (c9) and (c10) respectively.

Case II

Let F_2 denote the event that the system is in the state $(b+1, 0, 0, v)$, $b \geq 1$ immediately after arrival of the tagged customer. In this case the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode, the time duration of busy period generated by type I customers present at that time, if any and the time duration of busy cycles generated by type I customers arriving during the service time of each of the b type II customers. Let $W_2^*(s|F_2)$ denote the corresponding conditional LST of the waiting time.

Then

$$W_2^*(s|F_2) = \gamma_4(sI - U_4)^{-1} \mathbf{U}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right] (\hat{B}_{c_L}(s))^b$$

where $\hat{B}_{c_L}(s)$ is given by Theorem 8.

Case III

Let F_3 denote the event that the system is in the state $(b+1, a, 0, t, u, v)$, $b \geq 0$, $1 \leq a \leq N-1$, $a \leq t \leq N-1$, immediately after arrival of the tagged customer. In this case also the waiting time is the sum of time duration from his arrival epoch till the server shifts to normal mode, the time duration of busy period generated by type I customers present at that time, if any and the time duration of busy cycles generated by type I customers arriving during the service time of each of the b type II customers. Let $W_2^*(s|F_3)$ denote the corresponding conditional LST.

Then

$$W_2^*(s|F_3) = \gamma_{a,t}^u(sI - U_4)^{-1} \mathbf{U}_4^0 \left[t_0 + \sum_{p=1}^N \gamma_p(sI - T_1)^{-1} \mathbf{T}_1^0 t_p \right] (\hat{B}_{c_L}(s))^b$$

where $\gamma_{a,t}^u = (0, \mathbf{0}, \dots, \mathbf{e}'_{t-a+1}(N-a) \otimes \mathbf{e}'_u, \mathbf{0})$ with $\mathbf{e}'_{t-a+1}(N-a) \otimes \mathbf{e}'_u$ is in the $(a+1)$ th position, where $\mathbf{0}$'s are zero matrices of order $(N-1)m, \dots, (N-a+1)m, (N-a-1)m, \dots, m$ respectively,

$\gamma_p = (0, \mathbf{0}, \dots, \alpha, \mathbf{0}, \dots, \mathbf{0})$ where α is in the p th position and $\mathbf{0}$ denotes zero matrix of order m .

Case IV

Let F_4 denote the event that the system is in the state $(b+1, a, 1, u, v)$, $b \geq 0$, $1 \leq a \leq L$, $1 \leq u \leq m$, $1 \leq v \leq n$ immediately after arrival of the tagged customer. This case is same as for model I. Let $W_2^*(s|F_4)$ denote the corresponding conditional LST.

Then

$$W_2^*(s|F_4) = (\gamma_a^u(sI - T_1)^{-1} \mathbf{T}_1^0) (\hat{B}_{c_L}(s))^b$$

where $\gamma_a^u = (\mathbf{0}, \dots, \mathbf{e}'_u, \dots, \mathbf{0})$ where $\mathbf{e}'_u$ is in the a^{th} position and $\mathbf{0}$ denotes zero matrix of order m .

Case V

Let F_5 denote the event that the system is in the state $(b+1, a, 2, u, v)$, $b \geq 1$, $0 \leq a \leq L$, $1 \leq u \leq m'$, $1 \leq v \leq n$, immediately after arrival of the tagged

Table 3 Effect of η and N on Mean/Variance of number of type II customers in the system for model I

(N, L)	η	EXP		ERA		HEXP		MNA		MPA	
		Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns
(1,3)	0.01	2.95	8.11	2.44	4.30	3.14	9.82	3.17	9.15	16.35	693.94
	0.03	2.93	8.05	2.42	4.25	3.12	9.76	3.15	9.09	16.33	693.59
	0.05	2.91	8.00	2.40	4.21	3.11	9.70	3.13	9.03	16.32	693.26
(2,4)	0.01	5.18	19.55	4.63	13.78	5.39	22.02	5.40	20.89	19.49	804.02
	0.03	5.02	18.46	4.47	12.80	5.22	20.90	5.24	19.79	19.33	800.52
	0.05	4.87	17.54	4.32	11.97	5.08	19.94	5.10	18.85	19.19	797.43
(3,5)	0.01	9.57	68.10	9.01	59.18	9.78	71.77	9.80	69.91	24.23	944.18
	0.03	8.67	55.02	8.10	46.70	8.88	58.46	8.89	56.74	23.33	917.61
	0.05	7.97	45.98	7.40	38.13	8.18	49.25	8.19	47.64	22.63	898.11
(4,6)	0.01	17.20	242.85	16.63	228.76	17.41	248.45	17.43	245.42	31.98	1242
	0.03	13.84	151.36	13.51	139.51	14.05	156.12	14.06	153.60	28.61	1101
	0.05	11.72	105.45	11.15	95.01	11.94	109.69	11.95	107.48	26.50	1023
(5,7)	0.01	28.54	717.64	27.96	695.96	28.75	726.08	28.76	721.32	43.35	1890
	0.03	19.59	321.67	19.02	305.95	19.81	327.88	19.82	324.48	34.41	1360
	0.05	15.22	186.29	14.65	173.48	15.43	191.41	15.44	188.67	30.03	1159

Table 4 Effect of η and N on Mean/Variance of number of type II customers in the system for model II

(N, L)	η	EXP		ERA		HEXP		MNA		MPA	
		Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns	Ens	Vns
(1,3)	0.01	2.95	8.11	2.44	4.30	3.14	9.82	3.17	9.15	16.35	693.94
	0.03	2.93	8.05	2.42	4.25	3.12	9.76	3.15	9.09	16.33	693.59
	0.05	2.91	8.00	2.40	4.21	3.11	9.70	3.13	9.03	16.32	693.26
(2,4)	0.01	5.18	19.55	4.63	13.78	5.39	22.02	5.40	20.89	19.49	804.02
	0.03	5.02	18.46	4.47	12.80	5.22	20.90	5.24	19.79	19.33	800.52
	0.05	4.87	17.54	4.32	11.97	5.08	19.94	5.10	18.85	19.19	797.43
(3,5)	0.01	7.60	41.37	7.04	33.76	7.81	44.54	7.83	42.99	22.26	888.02
	0.03	7.11	36.04	6.55	28.76	7.33	39.10	7.34	37.61	21.77	875.40
	0.05	6.71	32.00	6.14	24.99	6.92	34.95	6.93	33.53	21.37	865.32
(4,6)	0.01	10.24	77.57	9.67	68.12	10.45	81.44	10.46	79.46	25.01	972.90
	0.03	9.20	61.54	8.63	52.78	9.41	65.15	9.43	63.33	23.98	941.38
	0.05	8.41	50.75	7.84	42.52	8.62	54.17	8.63	52.47	23.18	918.76
(5,7)	0.01	13.09	132.02	12.52	120.63	13.31	136.61	13.32	134.19	27.91	1073
	0.03	11.26	94.86	10.68	84.70	11.47	99.00	11.48	96.86	26.07	1009
	0.05	9.96	72.76	9.39	63.45	10.18	76.57	10.19	74.62	24.78	967

customer. This case is also same as for model I. Let $W_2^*(s|F_5)$ denote the corresponding conditional LST.

Then

$$W_2^*(s|F_5) = (\mathbf{e}'_u(sI - T')^{-1}\mathbf{T}'^0)(\bar{B}_L(s)(\hat{B}_{c_L}(s))^{b-1}$$

$\bar{B}_L(s)$ is given by Theorem 9.

Let $w_{i_1, i_2, j_1, j_2, k, l}$ denote the probability that the system is in the state $(i_1, i_2, j_1, j_2, k, l)$ immediately after arrival of the tagged customer. Then,

$$\begin{aligned} w_{1,0,0,v} &= \frac{d_{v,v}^{(1)}}{\lambda + \eta - d_{v,v}^{(0)}} x_{0,0,v}, \text{ for, } 1 \leq v, v' \leq n \\ w_{b+1,0,0,v} &= \frac{d_{v,v}^{(1)}}{\lambda + \eta - d_{v,v}^{(0)}} x_{b,0,0,v}, \text{ for, } b \geq 1, 1 \leq v, v' \leq n \\ w_{b+1,a,0,t,u,v} &= \frac{d_{v,v}^{(1)}}{\lambda + \eta - T_{uu} - d_{v,v}^{(0)}} x_{b,a,0,t,u,v}, \text{ for, } b \geq 0, 1 \leq a \leq N-1, a \leq t \leq N-1, 1 \leq u \leq m, \\ &\quad 1 \leq v, v' \leq n \\ w_{b+1,a,1,u,v} &= \frac{d_{v,v}^{(1)}}{\lambda - T_{uu} - d_{v,v}^{(0)}} x_{b,a,1,u,v}, \text{ for, } b \geq 0, 1 \leq a \leq L, 1 \leq u \leq m, 1 \leq v, v' \leq n \\ w_{b+1,a,2,u,v} &= \frac{d_{v,v}^{(1)}}{\lambda - T_{uu} - d_{v,v}^{(0)}} x_{b,a,2,u,v}, \text{ for, } b \geq 1, 0 \leq a \leq L, 1 \leq u \leq m', 1 \leq v, v' \leq n \end{aligned}$$

We sum up the above discussions in the following.

Theorem 18 *The LST of the waiting time of a type II customer is given by*

$$\begin{aligned} W_2^*(s) &= \sum_{v=1}^n W_2^*(s|F_1)w_{1,0,0,v} + \sum_{b=1}^{\infty} \sum_{v=1}^n W_2^*(s|F_2)w_{b+1,0,0,v} \\ &\quad + \sum_{b=0}^{\infty} \sum_{a=1}^{N-1} \sum_{t=a}^{N-1} \sum_{u=1}^m \sum_{v=1}^n W_2^*(s|F_3)(w_{b+1,a,0,t,u,v}) \\ &\quad + \sum_{b=0}^{\infty} \sum_{a=1}^L \sum_{u=1}^m \sum_{v=1}^n W_2^*(s|F_4)w_{b+1,a,1,u,v} + \sum_{b=1}^{\infty} \sum_{a=0}^L \sum_{u=1}^m \sum_{v=1}^n W_2^*(s|F_5)w_{b+1,a,2,u,v}. \end{aligned} \quad (24)$$

10 Numerical Results

10.1 Comparison of Mean/Variance of Number of Type I and Type II Customers in the System

$$\begin{aligned} \text{We fix } \lambda &= 1, \quad \theta = 0.6, \quad D_0 = (-1), D_1 = (1), \\ \alpha &= \begin{bmatrix} 1 & 0 \end{bmatrix}, T = \begin{bmatrix} -5 & 5 \\ 0 & -5 \end{bmatrix} \text{ and } \alpha' = \begin{bmatrix} 0.8 & 0.2 \end{bmatrix}, T' = \begin{bmatrix} -2.5 & 0 \\ 2.5 & -5 \end{bmatrix}. \end{aligned}$$

In Tables 1 and 2, Ens denote the expected number of type I customers in the system and Vns, its variance. In these tables we look at the values of these measures as

Table 5 Optimal N for EXP type II arrival process

N	Model I			Model II		
	R_s	E_v	Cost	R_s	E_v	Cost
1	0.0296	11.3725	89.2242	0.0296	11.3725	89.2242
2	0.0049	69.0362	18.2221	0.0049	69.0362	18.2221
3	0.0012	278.8977	17.6771	0.0018	194.1026	15.0271
4	6.8644×10^{-4}	508.8820	27.5034	9.8500×10^{-4}	352.2230	20.5662
5	6.0488×10^{-4}	578.3485	30.7321	7.3595×10^{-4}	473.2067	25.8682
6	5.9332×10^{-4}	589.9899	31.2795	6.4776×10^{-4}	538.8852	28.8875
7	5.9171×10^{-4}	591.7079	31.3605	6.1432×10^{-4}	569.0909	30.2975
8	5.9149×10^{-4}	591.9660	31.3728	6.0103×10^{-4}	582.1676	30.9115

Bold values give minimum expected cost as N increases from 1 to 8

Table 6 Optimal N for ERA type II arrival process

N	Model I			Model II		
	R_s	E_v	Cost	R_s	E_v	Cost
1	0.0296	11.4214	89.2608	0.0296	11.4214	89.2608
2	0.0049	69.0862	18.2303	0.0049	69.0862	18.2303
3	0.0012	278.9481	17.6811	0.0018	194.1521	15.0316
4	6.8678×10^{-4}	508.8366	27.5022	9.8536×10^{-4}	352.1463	20.5634
5	6.0530×10^{-4}	578.5717	30.7445	7.3622×10^{-4}	472.8003	25.8487
6	5.9375×10^{-4}	590.2368	31.2931	6.4809×10^{-4}	538.5319	28.8709
7	5.9215×10^{-4}	591.9584	31.3744	6.1472×10^{-4}	568.9850	30.2934
8	5.9193×10^{-4}	592.2169	31.3866	6.0148×10^{-4}	582.2466	30.9168

Bold values give minimum expected cost as N increases from 1 to 8

Table 7 Optimal N for HEXP type II arrival process

N	Model I			Model II		
	R_s	E_v	Cost	R_s	E_v	Cost
1	0.0295	11.3550	89.2110	0.0295	11.3550	89.2110
2	0.0049	69.0182	18.2191	0.0049	69.0182	18.2191
3	0.0012	278.8795	17.6757	0.0018	194.0845	15.0254
4	6.8630×10^{-4}	508.6858	27.4932	9.8486×10^{-4}	352.2043	20.5648
5	6.0476×10^{-4}	578.2960	30.7291	7.3583×10^{-4}	473.1812	25.8666
6	5.9320×10^{-4}	589.9324	31.2762	6.4767×10^{-4}	539.0376	28.8949
7	5.9159×10^{-4}	591.6498	31.3573	6.1420×10^{-4}	569.1643	30.3008
8	5.9137×10^{-4}	591.9077	31.3695	6.0091×10^{-4}	582.1740	30.9114

Bold values give minimum expected cost as N increases from 1 to 8

Table 8 Optimal N for MPA type II arrival process

N	Model I			Model II		
	R_s	E_v	Cost	R_s	E_v	Cost
1	0.0295	11.3028	89.1856	0.0295	11.3028	89.1856
2	0.0049	68.9648	18.2125	0.0049	68.9648	18.2125
3	0.0012	278.8374	17.6726	0.0018	194.0309	15.0213
4	6.8609×10^{-4}	508.7016	27.4933	9.8461×10^{-4}	352.1768	20.5627
5	6.0451×10^{-4}	578.2791	30.7275	7.3563×10^{-4}	473.2641	25.8701
6	5.9292×10^{-4}	589.7552	31.2665	6.4742×10^{-4}	538.9180	28.8882
7	5.9131×10^{-4}	591.4750	31.3477	6.1393×10^{-4}	569.0171	30.2927
8	5.9109×10^{-4}	591.7349	31.3600	6.0062×10^{-4}	582.0134	30.9025

Bold values give minimum expected cost as N increases from 1 to 8

Table 9 Optimal N for MNA type II arrival process

N	Model I			Model II		
	R_s	E_v	Cost	R_s	E_v	Cost
1	0.0295	11.4336	89.1955	0.0295	11.4336	89.1955
2	0.0049	69.0995	18.2198	0.0049	69.0995	18.2198
3	0.0012	278.9731	17.6795	0.0018	194.1663	15.0283
4	6.8611×10^{-4}	508.7373	27.4952	9.8464×10^{-4}	352.2857	20.5682
5	6.0454×10^{-4}	578.2921	30.7282	7.3564×10^{-4}	473.2473	25.8693
6	5.9296×10^{-4}	589.9148	31.2746	6.4746×10^{-4}	539.1406	28.8994
7	5.9135×10^{-4}	591.6300	31.3556	6.1397×10^{-4}	569.2176	30.3028
8	5.9113×10^{-4}	591.8876	31.3678	6.0066×10^{-4}	582.1987	30.9119

Bold values give minimum expected cost as N increases from 1 to 8

functions of N and η . The two models coincide with the classical model in the case $N = 1$. These two models coincide in the case of $N = 2$ also. As expected, in both the models, both mean and variance are non-increasing functions of η (for fixed N) and is also non-decreasing functions of N (for fixed η). The rate of decrease of mean and variance as η grows shows an increasing trend with value of N going up. This happens due to the diminished effect of N as N increases. Also the rate of increase of mean and variance with growth of N decreases as η increases. This is due to the increased effect of η with growth of η . But variance is larger than mean in all cases. When $N \geq 3$, both E_n s and V_n s are comparatively less in model II than in model I.

For the arrival process of type II customers, we consider the following five sets of matrices for D_0 and D_1 .

1. Exponential (EXP) $D_0 = (-1)$, $D_1 = (1)$

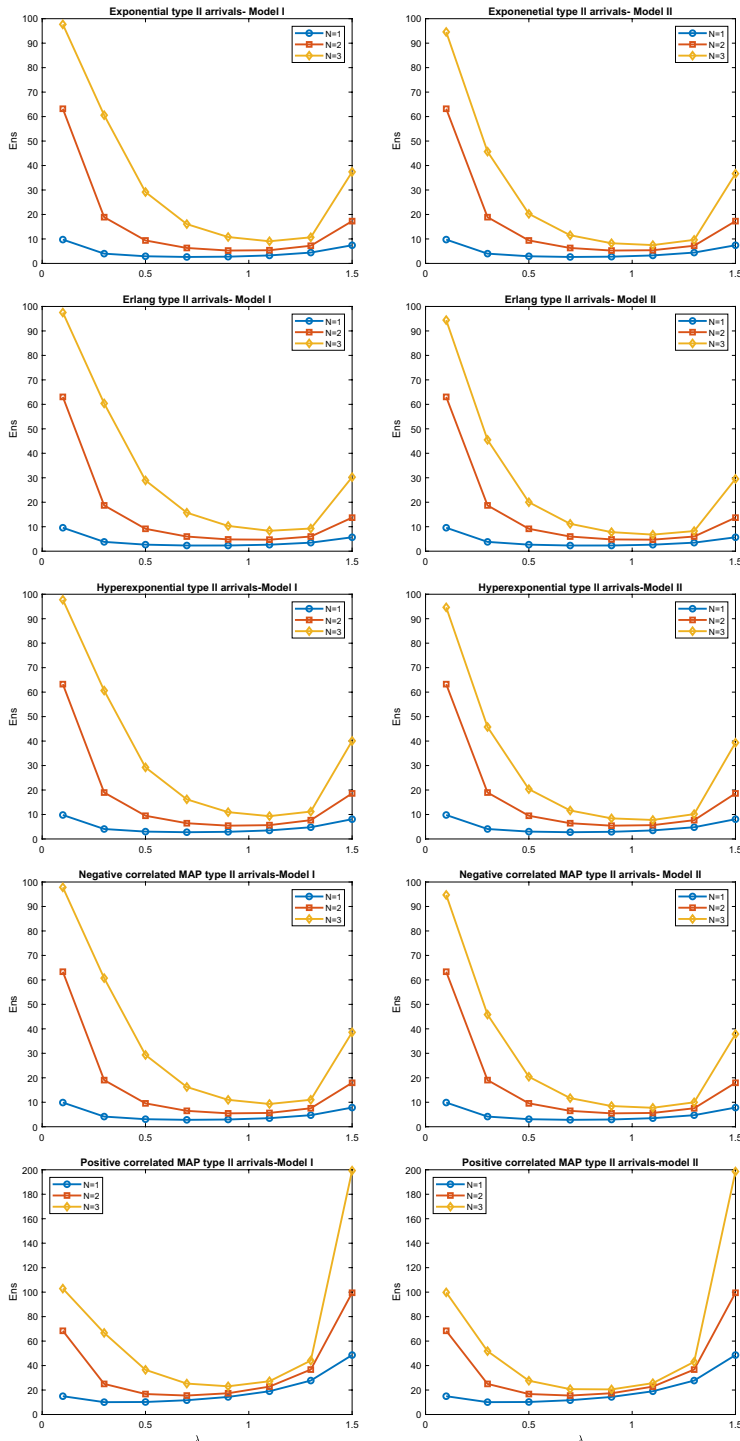


Fig. 1 Effect of λ on expected number of type II customers in the system

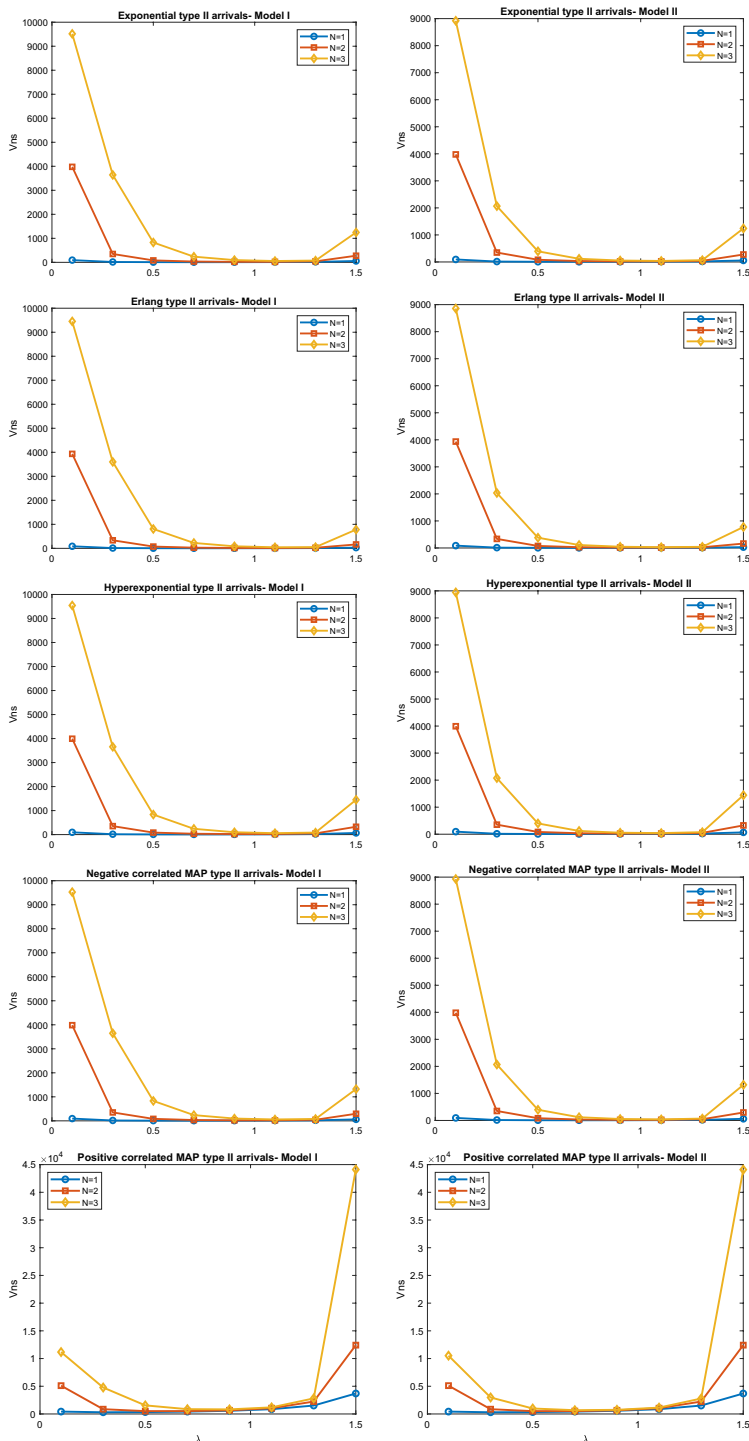


Fig. 2 Effect of λ on Variance of number of type II customers in the system

2. Erlang (ERA) $D_0 = \begin{bmatrix} -3 & 3 & 0 \\ 0 & -3 & 3 \\ 0 & 0 & -3 \end{bmatrix}$, $D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 3 & 0 & 0 \end{bmatrix}$
3. Hyperexponential (HEXP) $D_0 = \begin{bmatrix} -3.4000 & 0 \\ 0 & -0.8500 \end{bmatrix}$, $D_1 = \begin{bmatrix} 0.6800 & 2.7200 \\ 0.1700 & 0.6800 \end{bmatrix}$
4. MAP with negative correlation (MNA) $D_0 = \begin{bmatrix} -0.8101 & 0.8101 & 0 \\ 0 & -1.3497 & 0 \\ 0 & 0 & -40.5065 \end{bmatrix}$, $D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0810 & 0 & 1.2687 \\ 38.0761 & 0 & 2.4304 \end{bmatrix}$
5. MAP with positive correlation (MPA) $D_0 = \begin{bmatrix} -0.8101 & 0.8101 & 0 \\ 0 & -1.3497 & 0 \\ 0 & 0 & -40.5065 \end{bmatrix}$, $D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 1.2687 & 0 & 0.0810 \\ 2.4304 & 0 & 38.0761 \end{bmatrix}$

All these five MAP processes are normalized so as to have an arrival rate of 1. However, these are qualitatively different in that they have different variance and correlation structure. The first three arrival processes, namely EXP, ERA and HEXP correspond to renewal processes and so the correlation is 0. The arrival process labelled MNA has correlated arrivals with correlation between two successive interarrival times given by -0.4211 and the arrival process corresponding to the one labelled MPA has a positive correlation with value 0.4211.

For the service time distributions, we consider phase type distributions, $\alpha = [1 \ 0]$, $T = \begin{bmatrix} -5 & 5 \\ 0 & -5 \end{bmatrix}$ and $\alpha' = [0.8 \ 0.2]$, $T' = \begin{bmatrix} -2.5 & 0 \\ 2.5 & -5 \end{bmatrix}$. We fix $\lambda = 1$ and $\theta = 0.6$.

In this case also, the mean and variance are both non-increasing functions of η (for fixed N) and is a non-decreasing function of N (for fixed η , with reference to Tables 3 and 4), for the input parameters prescribed. This is the case for all combinations of arrival processes of type II customer. But the rate of change in the case of MPA is much smaller compared to other arrivals. Both mean and variance are significantly larger for MPA indicating the role played by (positively) correlated arrivals. We observe that both mean and variance change significantly as functions of η when N becomes large for both the models. When $N \geq 3$, both Ens and Vns are comparatively less for model II compared to model I.

From Figs. 1 and 2, we note that as λ increases both Ens and Vns of type II customers decrease first but increase after a certain stage for all values of N and for all type II arrival processes for both the models. This happens because as λ increases, the rate of hitting N become faster and the queue length decreases. But when λ reaches a specified value the queue length increases due to diminished effect of N . As N increases this λ value becomes larger and larger. This λ value is different for different type II arrival processes and is the smallest for the arrival process MPA.

10.2 Optimal N

Next, we find an optimal N for both the models by constructing a suitable cost function.

Let

C_s : Unit time cost of switching to normal mode

C_h : Holding cost for retaining a type II customer when the server is in vacation/
WV

R_s : Rate of switching to normal mode

E_v : Expected number of type II customers in the system till vacation expires.

Then the expected cost per unit time,

$$C = C_s \times R_s + C_h \times E_v$$

For model I,

$$R_s = \sum_{n_1=1}^{\infty} \sum_{k=1}^n \eta x_{n_1,0,0,k} + \sum_{n_1=0}^{\infty} \sum_{n_2=1}^{N-1} \sum_{j_2=1}^m \sum_{k=1}^n \eta x_{n_1,n_2,0,j_2,k} + \sum_{n_1=0}^{\infty} \sum_{j_2=1}^m \sum_{k=1}^n \lambda x_{n_1,N-1,0,j_2,k}$$

and

$$E_v = \sum_{n_1=1}^{\infty} \sum_{k=1}^n n_1 x_{n_1,0,0,k} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^{N-1} \sum_{j_2=1}^m \sum_{k=1}^n n_1 x_{n_1,n_2,0,j_2,k}$$

For model II,

$$\begin{aligned} R_s &= \sum_{n_1=1}^{\infty} \sum_{l=1}^n \eta x_{n_1,0,0,l} + \sum_{n_1=0}^{\infty} \sum_{n_2=1}^{N-1} \sum_{j_2=n_2}^{N-1} \sum_{k=1}^m \sum_{l=1}^n \eta x_{n_1,n_2,0,j_2,k,l} \\ &+ \sum_{n_1=0}^{\infty} \sum_{n_2=1}^{N-1} \sum_{k=1}^m \sum_{l=1}^n \lambda x_{n_1,n_2,0,N-1,0,k,l} \end{aligned}$$

and

$$E_v = \sum_{n_1=1}^{\infty} \sum_{l=1}^n n_1 x_{n_1,0,0,l} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^{N-1} \sum_{j_2=n_2}^{N-1} \sum_{k=1}^m \sum_{l=1}^n n_1 x_{n_1,n_2,0,j_2,k,l}$$

For both models we fix $L = 15$, $\theta = 0.1$, $\lambda = 0.05$, $\eta = 0.001$, $\alpha = \begin{bmatrix} 1 & 0 \end{bmatrix}$, $T = \begin{bmatrix} -5 & 5 \\ 0 & -5 \end{bmatrix}$ and $\alpha' = \begin{bmatrix} 0.8 & 0.2 \end{bmatrix}$, $T' = \begin{bmatrix} -2.5 & 0 \\ 2.5 & -5 \end{bmatrix}$, $C_s = 3000$ and $C_h = 0.05$.

From Tables 5, 6, 7, 8 and 9, we get the expected cost corresponding to different values of N for different type II arrival processes, for model I and model II. For both the models, R_s decreases and E_v increases as N increases. As expected, R_s is higher and E_v is smaller for model II than model I for all type II arrival processes. In all

cases we see that as N increases, the expected cost first decreases, reaches a minimum value and then increases. This is due to the fact that, R_s decreases and E_v increases, as N increases. The optimal cost is slightly different for different type II arrival processes, but it corresponds to $N = 3$ in all cases (This may vary according to variation in the parameters). Hence model II performs much better than model I for all type II arrival processes. It may be noted that we assigned small values for λ (Poisson arrival rate of type I customers), η (parameter of vacation clock duration) for reducing R_s value and to get clear distinction in the expected cost between models I and II.

11 Conclusion

In this paper, we considered two (M, MAP)/(PH, PH)/1 queues with non-preemptive priority and exponentially distributed working vacation under N-policy. Based on two distinct definitions of N-policies, we studied the distribution of the duration of slow service mode without any break, expected number of returns to 0 type I customer state, starting from 0 type I customer state during vacation mode of service before the arrival of a type II customer and the distribution of p-cycle in normal mode. Also we provided LSTs of busy cycle, busy period of type I customers generated during the service time of a type II customer. For the waiting time distributions of both type I and type II customers, we provided an analysis using LST. We also performed some numerical experiments to find the mean and variance of number of both type I and type II customers in the system and optimal N for both models by constructing a cost function. We compared the two queueing models with non-preemptive service and exponentially distributed working vacations and N-policy. These models were analyzed under the assumption of stability. Numerical experiments were carried out to find the superior one. It is possible to generalize the arrival process of type I customers to the MAP. In a future work we propose to extend the models discussed here to the case in which the type II customers are impatient. This will lead to the problem of finding individual optimal strategy of type II customers, maximum revenue of the server and social optimal strategy. The extension of the models discussed to multi-server case is also proposed to be taken up.

Funding A. Krishnamoorthy: Emeritus Fellow (EMERITUS-2017-18 GEN 10822(SA-II)), University Grants Commission, India; V. Divya: Research is supported by the University Grants Commission, Govt. of India, under Faculty Development Programme (Grant No.F.No.FIP/12th Plan/KLKE008 TF 04) in Department of Mathematics, Cochin University of Science and Technology, Cochin-22.

Appendix 1

The Entries in the Block Matrices of the Infinitesimal Generator of the QBD in Model I

Define the entries of $B_{0_{(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}}$ as transition submatrices which contains transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$. Since none or one event alone could

take place in a short interval of time with positive probability, in general, a transition such as $(i_1, i_2, j, k, l) \rightarrow (i'_1, i'_2, j', k', l')$ has positive rate only for exactly one of i'_1, i'_2, j', k', l' different from i_1, i_2, j, k, l .

$$B_{0(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)} = \begin{cases} \lambda(\alpha \otimes I_n) & i_1 = 0, i_2 = 1; j_1 = j_2 = 0; 1 \leq k_2 \leq m, \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq N-2, i_2 = i_1 + 1; j_1 = j_2 = 0; \\ & 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ \lambda e(m) \otimes (\alpha \otimes I_n) & i_1 = N-1, i_2 = N; j_1 = 0, j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 1; \\ & 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 0, j_2 = 0; 1 \leq k_1 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq N-1, i_2 = i_1 - 1; j_1 = 0, j_2 = 0; \\ & 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \eta e(m) \otimes (\alpha \otimes I_n) & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = 0, j_2 = 1; \\ & 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ D_0 - \lambda I_n & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \\ \theta T \oplus D_0 - (\lambda + \eta) I_{mn} & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $C_{0(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)}$ as transition submatrices which contains transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (1, i_2, j_2, k_2, l_2)$

$$C_{0(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)} = \begin{cases} D_1 & i_1 = 0, i_2 = 0; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $B_{1(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)}$ as transition submatrices which contains transitions of the form $(1, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$.

$$B_{1(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)} = \begin{cases} T^0 \otimes I_n & i_1 = i_2 = 0; j_1 = 2, j_2 = 0; 1 \leq k_1 \leq m'; 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq k_1 \leq m', 1 \leq k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $A_{2(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h-1, i_2, j_2, k_2, l_2)$, where $h > 1$.

$$A_{2(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)} = \begin{cases} T^0 \alpha' \otimes I_n & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq k_1 \leq m', 1 \leq k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $A_{1(i_1 j_1 k_1 l_1)}^{(i_2 j_2 k_2 l_2)}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$, where $h \geq 1$.

$$A_{l_{(i_1 j_1, k_1, l_1)}^{(i_2 j_2, k_2, l_2)}} = \begin{cases} \lambda(\alpha \otimes I_n) & i_1 = 0, i_2 = 1; j_1 = j_2 = 0; 1 \leq k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn} & i_1 = 0, i_2 = 1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn}, & 1 \leq i_1 \leq N-2; i_2 = i_1 + 1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \lambda e(m) \otimes (\alpha \otimes I_n) & i_1 = N-1, i_2 = N; j_1 = 0, j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; \\ & 1 \leq l_1, l_2 \leq n \\ \theta T^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = j_2 = 0; 1 \leq k_1 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T^0 \alpha' \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq m, 1 \leq k_2 \leq m'; \\ & 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq N-1, i_2 = i_1 - 1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ \eta(\alpha' \otimes I_n) & i_1 = i_2 = 0; j_1 = 0, j_2 = 2; 1 \leq k_2 \leq m'; 1 \leq l_1, l_2 \leq n \\ \eta e(m) \otimes (\alpha \otimes I_n) & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = 0, j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ D_0 - (\lambda + \eta) I_n & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \\ T' \oplus D_0 - \lambda I_{mn} & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; 1 \leq l_1, l_2 \leq n \\ \theta T \oplus D_0 - (\lambda + \eta) I_{mn} & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ T' \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; \\ & 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; 1 \leq l_1, l_2 \leq n \\ T' \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; 1 \leq l_1, l_2 \leq n \end{cases}$$

Define the entries of $A_{l_{(i_1 j_1, k_1, l_1)}^{(i_2 j_2, k_2, l_2)}}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h+1, i_2, j_2, k_2, l_2)$, where $h \geq 1$.

$$A_{l_{(i_1 j_1, k_1, l_1)}^{(i_2 j_2, k_2, l_2)}} = \begin{cases} D_1 & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m; \\ & 1 \leq l_1, l_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m'; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

Proof of Theorem 3.1

Proof Let B_{c_L} denote the length of the busy cycle generated by type I customers arriving during the service time of a type II customer, $\hat{B}_{c_L}(s)$ the LST of the length of the busy cycle and l the number of type I customers that arrive during service time of type II customer.

Then $B_{c_L} = X + B_L^1 + \dots + B_L^l$ where X denote the service time of the type II customer in service, B_L^j the busy period generated by j th type I customer that arrive during X , where $1 \leq j \leq l$.

$$\begin{aligned}
 \hat{B}_{c_L}(s) &= E(e^{-sB_{c_L}}) \\
 &= \int_{x=0}^{\infty} E(e^{-sB_{c_L}} / X = x) P(x \leq X < x + dx) \\
 &= \int_{x=0}^{\infty} \sum_{p=0}^{\infty} E(e^{-sB_{c_L}} / X = x, l = p) P(l = p / X = x) P(x \leq X < x + dx) \\
 &= \int_{x=0}^{\infty} \sum_{p=0}^{\infty} E(e^{-sB_{c_L}} / X = x, l = p) \frac{e^{-\lambda x} (\lambda x)^p}{p!} \alpha' e^{T'x} \mathbf{T}'^0 dx \\
 &= \int_{x=0}^{\infty} e^{-(s+\lambda)x} \alpha' e^{T'x} \mathbf{T}'^0 dx + \int_{x=0}^{\infty} \sum_{p=1}^{L-1} e^{-sx} \gamma_p (sI - T_1)^{-1} \mathbf{T}_1^0 \frac{e^{-\lambda x} (\lambda x)^p}{p!} \\
 &\quad \times \alpha' e^{T'x} \mathbf{T}'^0 dx + \int_{x=0}^{\infty} \sum_{p=L}^{\infty} e^{-sx} \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \frac{e^{-\lambda x} (\lambda x)^p}{p!} \alpha' e^{T'x} \mathbf{T}'^0 dx \quad (25) \\
 &= \alpha' [(s + \lambda)I - T']^{-1} \mathbf{T}'^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} \mathbf{T}_1^0 \frac{\lambda^p \alpha'^{p!}}{p!} \\
 &\quad \times \int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - T']x} \mathbf{T}'^0 dx \\
 &\quad + \sum_{p=L}^{\infty} \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \frac{\lambda^p}{p!} \alpha' \int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - T']x} \mathbf{T}'^0 dx
 \end{aligned}$$

We have,

$$\int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - T']x} dx = \frac{p!}{[(s + \lambda)I - T']^{p+1}} \quad (26)$$

Substituting (26) in (25), its third term

$$\begin{aligned}
 &= \sum_{p=L}^{\infty} \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \lambda^p \alpha' [(s + \lambda)I - T']^{-(p+1)} \mathbf{T}'^0 \\
 &= \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \alpha' \sum_{p=L}^{\infty} [\lambda^{-1} [(s + \lambda)I - T']]^{-p} [(s + \lambda)I - T']^{-1} \mathbf{T}'^0 \\
 &= \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \alpha' [\lambda^{-1} [(s + \lambda)I - T']]^{-L} \sum_{q=0}^{\infty} [\lambda^{-1} [(s + \lambda)I - T']]^{-q} [(s + \lambda)I - T']^{-1} \mathbf{T}'^0 \quad (27) \\
 &= \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \alpha' [\lambda^{-1} [(s + \lambda)I - T']]^{-L} [I - \lambda[(s + \lambda)I - T']^{-1}]^{-1} [(s + \lambda)I - T']^{-1} \mathbf{T}'^0
 \end{aligned}$$

Substituting (27) in (25) gives

$$\begin{aligned}
 \hat{B}_{c_L}(s) &= \alpha' [(s + \lambda)I - T']^{-1} \mathbf{T}'^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} \mathbf{T}_1^0 \lambda^p \alpha' [(s + \lambda)I - T']^{-(p+1)} \mathbf{T}'^0 \\
 &\quad + \gamma_L (sI - T_1)^{-1} \mathbf{T}_1^0 \alpha' [\lambda^{-1} [(s + \lambda)I - T']]^{-L} [I - \lambda[(s + \lambda)I - T']^{-1}]^{-1} \\
 &\quad \times [(s + \lambda)I - T']^{-1} \mathbf{T}'^0 \quad (28)
 \end{aligned}$$

□

Proof of Theorem 3.2

Proof Let B_L denote the length of the busy period generated by type I customers arriving during the service time of a type II customer, $\hat{B}_L(s)$ the LST of the length of the busy period and l the number of type I customers that arrive during service time of type II customer.

Then $B_L = B_L^1 + \dots + B_L^l$, where B_L^j denote the busy period generated by j th type I customer that arrive during X , where $1 \leq j \leq l$. Proceeding as in the above proof, we get the required result.

□

Appendix 2

The Entries in the Block Matrices of the Infinitesimal Generator of the QBD in Model II

Define the entries of $G_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(0, i_1, j_1, k_1, l_1, m_1) \rightarrow (0, i_2, j_2, k_2, l_2, m_2)$. Since none or one event alone could take place in a short interval of time with positive probability, in general, a transition such as $(i_1, j_1, k_1, l_1, m_1, n_1) \rightarrow (i_2, j_2, k_2, l_2, m_2, n_2)$ has positive rate only for exactly one of $i_2, j_2, k_2, l_2, m_2, n_2$ different from $i_1, j_1, k_1, l_1, m_1, n_1$.

$$G_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)} = \begin{cases} \lambda(\alpha \otimes I_n) & i_1 = 0, i_2 = 1; j_1 = j_2 = 0; k_2 = 1; \\ & 1 \leq l_2 \leq m, 1 \leq m_1, m_2 \leq n \\ \lambda I_{mn} & 1 \leq l_1 \leq N-2, i_2 = i_1 + 1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-2, \\ & k_2 = k_1 + 1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \lambda e(m) \otimes (\alpha \otimes I_n) & 1 \leq i_1 \leq N-1, i_2 = i_1 + 1; j_1 = 0, j_2 = 1; \\ & k_1 = N-1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 1; \\ & 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \eta e(m) \otimes (\alpha \otimes I_n) & 1 \leq l_1 \leq N-1; j_1 = 0, j_2 = 1; \\ & i_1 \leq k_1 \leq N-1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \theta T^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 0, j_2 = 0; 1 \leq k_1 \leq N-1, \\ & 1 \leq l_1 \leq m; 1 \leq m_1, m_2 \leq n \\ T^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 0; 1 \leq l_1 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq i_1 \leq N-1, i_2 = i_1 - 1; j_1 = 0, j_2 = 0; i_1 \leq k_1 \leq \\ & N-1, k_2 = k_1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ T^0 \alpha \otimes I_n & 2 \leq l_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; \\ & 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ D_0 - \lambda I_n & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq m_1, m_2 \leq n \\ \theta T \oplus D_0 - (\lambda + \eta) I_{mn} & 1 \leq l_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-1, \\ & k_2 = k_1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ T \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ T \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \end{cases}$$

Define the entries of $H_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(0, i_1, j_1, k_1, l_1, m_1) \rightarrow (1, i_2, j_2, k_2, l_2, m_2)$

$$H_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)} = \begin{cases} D_1 & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq m_1, m_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-1, k_2 = k_1; \\ & 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ I_m \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \end{cases}$$

Define the entries of $H_{1(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(1, i_1, j_1, k_1, l_1, m_1) \rightarrow (0, i_2, j_2, k_2, l_2, m_2)$.

$$H_{1(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)} = \begin{cases} T^0 \otimes I_n & i_1 = i_2 = 0; j_1 = 2, j_2 = 0; 1 \leq l_1 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ T^0 \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq l_1 \leq m', 1 \leq l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \end{cases}$$

Define the entries of $A_{2(i_1 j_1, k_1, l_1, m_1)}^{(i_2, j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1, m_1) \rightarrow (h-1, i_2, j_2, k_2, l_2, m_2)$, where $h \geq 1$.

$$A_{2(i_1 j_1, k_1, l_1, m_1)}^{(i_2 j_2, k_2, l_2, m_2)} = \begin{cases} \mathbf{T}^{\alpha'} \alpha' \otimes I_n & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ \mathbf{T}^{\alpha'} \alpha \otimes I_n & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = 2, j_2 = 1; 1 \leq l_1 \leq m', 1 \leq l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \end{cases}$$

Define the entries of $A_{1(i_1 j_1, k_1, l_1, m_1)}^{(i_2 j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1, m_1) \rightarrow (h, i_2, j_2, k_2, l_2, m_2)$, where $h \geq 1$.

$$A_{1(i_1 j_1, k_1, l_1, m_1)}^{(i_2 j_2, k_2, l_2, m_2)} = \begin{cases} \lambda(\alpha \otimes I_n) & i_1 = 0, i_2 = 1; j_1 = j_2 = 0; k_2 = 1; \\ & 1 \leq l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq N-2; i_2 = i_1 + 1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-2, \\ & k_2 = k_1 + 1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \lambda \mathbf{e}(m) \otimes (\alpha \otimes I_n) & 1 \leq i_1 \leq N-1; j_1 = 0, j_2 = 1; k_1 = N-1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ \lambda I_{mn} & 0 \leq i_1 \leq L-1, i_2 = i_1 + 1; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ \eta(\alpha' \otimes I_n) & i_1 = i_2 = 0; j_1 = 0, j_2 = 2; 1 \leq l_2 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ \eta \mathbf{e}(m) \otimes (\alpha \otimes I_n) & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = 0, j_2 = 1; i_1 \leq k_1 \leq N-1; \\ & 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \theta \mathbf{T}^0 \otimes I_n & i_1 = 1, i_2 = 0; j_1 = j_2 = 0; 1 \leq k_1 \leq N-1; \\ & 1 \leq l_1 \leq m; 1 \leq m_1, m_2 \leq n \\ \mathbf{T}^0 \alpha' \otimes I_n & i_1 = 1, i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq l_1 \leq m, 1 \leq l_2 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ \theta \mathbf{T}^0 \alpha \otimes I_n & 2 \leq i_1 \leq N-1, i_2 = i_1 - 1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-1, \\ & k_2 = k_1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ \mathbf{T}^0 \alpha \otimes I_n & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ D_0 - (\lambda + \eta) I_n & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq m_1, m_2 \leq n \\ T' \oplus D_0 - \lambda I_{mn} & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; 1 \leq m_1, m_2 \leq n \\ \theta T \oplus D_0 - (\lambda + \eta) I_{mn} & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-1, \\ & k_2 = k_1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ T \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ T' \oplus D_0 - \lambda I_{mn} & 1 \leq i_1 \leq L-1, i_2 = i_1; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; \\ & 1 \leq m_1, m_2 \leq n \\ T \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ T' \oplus D_0 & i_1 = i_2 = L; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; 1 \leq m_1, m_2 \leq n \end{cases}$$

Define the entries of $A_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2 j_2, k_2, l_2, m_2)}$ as transition submatrices which contains transitions of the form $(h, i_1, j_1, k_1, l_1, m_1) \rightarrow (h+1, i_2, j_2, k_2, l_2, m_2)$, where $h \geq 1$.

$$A_{0(i_1 j_1, k_1, l_1, m_1)}^{(i_2 j_2, k_2, l_2, m_2)} = \begin{cases} D_1 & i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq m_1, m_2 \leq n \\ I_{m'} \otimes D_1 & i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; 1 \leq m_1, m_2 \leq n \\ I_{m'} \otimes D_1 & 1 \leq i_1 \leq N-1, i_2 = i_1; j_1 = j_2 = 0; i_1 \leq k_1 \leq N-1, k_2 = k_1; \\ & 1 \leq l_1, l_2 \leq m; 1 \leq m_1, m_2 \leq n \\ I_{m'} \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq l_1, l_2 \leq m; \\ & 1 \leq m_1, m_2 \leq n \\ I_{m'} \otimes D_1 & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 2; 1 \leq l_1, l_2 \leq m'; 1 \leq m_1, m_2 \leq n \end{cases}$$

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² Department of Mathematics, N.S.S. College, Cherthala 688556, India



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BV 779727

**MEMORANDUM OF UNDERTAKING FOR THE CONDUCT OF
CERTIFIED COURSE IN FINANCIAL ACCOUNTING WITH TALLY**

This Memorandum of Undertaking is made on 28th November 2019 between M/s Priyanka Educational Trust, Cherthala under Priyanka Educational Trust, Cherthala represented by its center head Mr. Somasekharan Nair (hereinafter referred to as PET) on one part.

And

M/s NSS College, Cherthala represented by Dr. B.Gopakumar Principal, NSS College, Cherthala on the other part.



PRINCIPAL
NSS COLLEGE,
CHERTHALA



Nr 20302
22-11-2019

Priyanka
Educational Trust
Cherthala

18/11/2019
CHERTHALA



भारतीय गैर न्यायिक

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കേരളം KERALA

BV 779728

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The PET is affiliated to the TCIL-IT (A Govt of India Enterprise) and is successfully conducting various courses in computer, IT, and other employment-oriented courses including diploma courses in computer application such as DCA, DFA and Tally.

NSS College is an aided college affiliated to the University of Kerala and has conducting various graduate and post graduate courses for many years.

Both the parties have after mutual discussions agreed to execute an agreement for the conduct of classes for Certified Course in Financial Accounting with Tally in the premises of NSS College.

PRINCIPAL
N.S.S. COLLEGE,
CHERTHALA.



NO 30302

23/11/2005

Principals.

Exd. Control Hr. R.

Payable Chertala



Now this Memorandum of Understanding is as under : -

1. PET agrees to conduct classes for Certified Course in Financial Accounting with Tally to the students of NSS College, Cherthala at the latter's premises.
2. The duration of the course is 80 hours (60 hours practical session and 20 hours theory session).
3. PET would send their faculty at their cost to NSS College for completing the committed duration of the course.
4. Only the students from the ongoing batches at NSS College would be admitted to the said course.
5. A fee of Rs. 1,900/- per student will be charged by PET for one course of 80 hours.
6. The said fee has to be collected by the college from the participating pupil.
7. Fifty percent (i.e. Rs. 1000/- per student) of the fee collected will be paid to the PET on completion of first 40 hours of the course and the balance on the completion of 80 hours.
8. The classes will be completed within the period of 80 hours.
9. PET is responsible for all consequences if there is any lapse on its part for the smooth conduct and completion of 80 hours of classes for each batch.
10. The parties have agreed to fix the period of this MOU as one year tentatively extendable from time to time if college feedback system finds that PET is maintaining the required level of standard of quality delivery.
11. This agreement can be modified, altered or terminated by mutual understanding and consent of both parties.





NSS COLLEGE , CHERTHALA
(Re-accredited by NAAC with B++ grade)

INAUGURATION OF CERTIFIED COURSE AND AWARENESS CLASS
BY DEPT OF COMMERCE

Date&Time: 02-Dec-2019, 10 am Venue: Seminar hall

Programme Schedule

Prayer & Acharya Anusmaranam

Welcome Speech:Smt Deepa Divakaran .N (Co-ordinator)

Presidential address &

Inauguration:Dr. B.Gopakumar (Principal, NSS College, Cherthala)

Felicitation:Sri Babu.K.Panicker (IQAC co-ordinator)

Felicitation:Smt Maya Venugopal (PTA Secretary)

Vote of thanks:Dr. Anoop V

Awareness class on certified course (Tally) :Manoj Govind (Executive Vice President, CLAP International School of Business, Bangalore)

December 2019

MUSTER

Name of Establishment NSS College, Chevhalu

Sl. No.	NAME	AADHAAR NUMBER	FATHER'S/HUSBAND'S NAME	SEX	NATURE OF WORK	DATE OF ENTRY INTO SERVICE
1.	Aliswarya A.V			F		
2.	AKShara V.R			"		
3.	Amrutha Prem			"		
4.	Anjitha K P			"		
5.	Azathy Raveendran			"		
6.	Aysbamel A.R			"		
7.	Meera.B			"		
8.	Parvathy S Nair			"		
9.	Princy P			"		
10.	Radhika Krishna S			"		
11.	Sabari Gireesh M			M		
12.	Sivapriya A R			F		
13.	Vaishnavi V Pai			"		
14.	Aathira R			"		
15.	Amrutha T.A			"		
16.	Adarika J			"		
17.	Aparna CA			"		
18.	ASwathy PS			"		
19.	ASwini Madhu			"		
20.	Athulya KB			"		
21.	chandhini G Nair			"		
22.	Gopika V R			"		
23.	Megha MS			"		
24.	Mekha Aravind			"		
25.	Midhun Bhaskar			M		
26.	parvathy SS			F		
27.	Renjomon Baby			M		
28.	Reshma Raj S			F		
29.	Sirni Sebastian			"		
30.	Veena lakshmi KS			"		
31.	Reshma Ramesan			"		
32.	Harinam Anitta			"		
33.	Abhisha KB			"		
34.	parvathy Anil			"		
35.	Haripriya			"		

EDUCATION DEPARTMENT
K.R. P. U. J.
CHEVHALU - 5552
N.S.S. COLLEGE

RULE 29 (5)

ROLL

Total 80 hours (including theory & practical of Communcial - total)

Date of Commencement - 19/12/2019

For The Period Ending

2hrs 5hs 5hrs

Place: Cherthala

Date: 2 hrs 5 hrs 5 hrs																		Place: 1 hr 1 hr 1 hr														Remarks
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31		
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January 2020

Name of Establishment NBS College Cherthala

Sl. No.	NAME	AADHAAR NUMBER	FATHER'S/HUSBAND'S NAME	SEX	NATURE OF WORK	DATE OF ENTRY INTO SERVICE
1.	Aishwarya A.V			F		
2.	Akshara VR			"		
3.	Amrutha prem			"		
4.	Anjitha KP			"		
5.	Azathy Raveendran			"		
6.	Ayshamol AR			"		
7.	Meera B			"		
8.	Pazvathy S Nair			"		
9.	Priory P			"		
10.	Radhika Kalishna S			"		
11.	Sabari Gireesh H			M		
12.	Sivapriya AR			F		
13.	Vaishnavi V Pai			"		
14.	Aathira R			"		
15.	Amrutha TA			"		
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19.	Aswini Madhu			"		
20.	Athulya KB			"		
21.	Chandhini G Nair			"		
22.	Gopika VR			"		
23.	Megha HS			"		
24.	Mekha Aravind			"		
25.	Midhun Bhaskar			"		
26.	Pazvathy SS			M		
27.	Regjomon Baby			F		
28.	Reshma Raj S			M		
29.	Simi Sebastian			F		
30.	Veena Lakshmi K S			"		
31.	Reshma Ramesan			"		
32.	Haziyam Anitha			"		
33.	Abhishta KB			"		
34.	Pazvathy Anil			"		
35.	Hazipriya			"		



RULE 29 (5)

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For The Period Ending 5 hrs

4 hrs

Place: Chevphala

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February 2020

Name of Establishment NSS College, Chevhalak

Sl. No.	NAME	AADHAAR NUMBER	FATHER'S/HUSBAND'S NAME	SEX	NATURE OF WORK	DATE OF ENTRY INTO SERVICE
1.	Aiswarya. A.V			F		
2.	Akshasa. V.R			F		
3.	Amsutha Prem			"		
4.	Anjitha. K.P			"		
5.	Asathy Raveendran			"		
6.	Ayshamol A.R			"		
7.	Meesa. B			"		
8.	Parvathy S. Naix			"		
9.	Princy P			"		
10.	Radhika Krishna S			"		
11.	Sabasi Giseesh M			"		
12.	Sivapriya. A.R			M		
13.	Vaishnavi. V. Pai			F		
14.	Aathisa R			"		
15.	Amsutha. T.A			"		
16.	Anamika. J			"		
17.	Aparna. C.A			"		
18.	Aswathy D.S			"		
19.	Aswini Madhu			"		
20.	Athulya K.B			"		
21.	Chandhini G. Naix			"		
22.	Gopika V.R			"		
23.	Megha. M.S			"		
24.	Mekha Asavind			"		
25.	Midhun Bhaskar			"		
26.	Parvathy S.S			"		
27.	Renjuman. Baby			M		
28.	Reshma Raj S			F		
29.	Simi Sebastian			M		
30.	Veena Lakshmi K.S			F		
31.	Reshma Ramesan			"		
32.	Maziyam Anitha			"		
33.	Abhisha K.B			"		
34.	Parvathy Anil			"		
35.	Hari priya			"		



ROLL

Shrs Shrs
For The Period Ending Shrs Shrs
Place: Shrs

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March

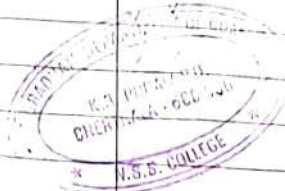
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FORM NO. VI

MUSTER

Name of Establishment NSS College Cherthala

Sl. No.	NAME	AADHAAR NUMBER	FATHER'S/HUSBAND'S NAME	SEX	NATURE OF WORK	DATE OF ENTRY INTO SERVICE
1	Discooraya . D.V			F		
2	Nkshara . V.R			"		
3	Amrutha . Prem			"		
4	Anjitha . K.P			"		
5	Axathy Raveendran			"		
6	Ayshamol . A.R			"		
7	Meera . B			"		
8	Parvathy S Naix			"		
9	Princy . P			"		
10	Radhika Krishna S			"		
11	Subaxi Gireesh . M			"		
12	Siva priya . A.R			M		
13	Vaishnavi V. Pai			F		
14	Aathila . R			"		
15	Amrutha T.A			"		
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17	Aparna . CA			"		
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20	Athulya . K.B			"		
21	Chandhini G. Naix			"		
22	Gopika . V.R			"		
23	Megha . M.S			"		
24	Mekha Aravind			"		
25	Midhun Bhaskar			"		
26	Parvathy . S.S			M		
27	Renjuman Baby			F		
28	Reshma Raj . S			M		
29	Simi Sebastian			F		
30	Veena Lakshmi . K.S			"		
31	Reshma Ramesan			"		
32	Maxiyam Anitta			"		
33	Abhisha . K.B			"		
34	Parvathy Anil			"		
35	Haripriya			"		



ROLL

Sheet For The Period Ending

Place: Chennai

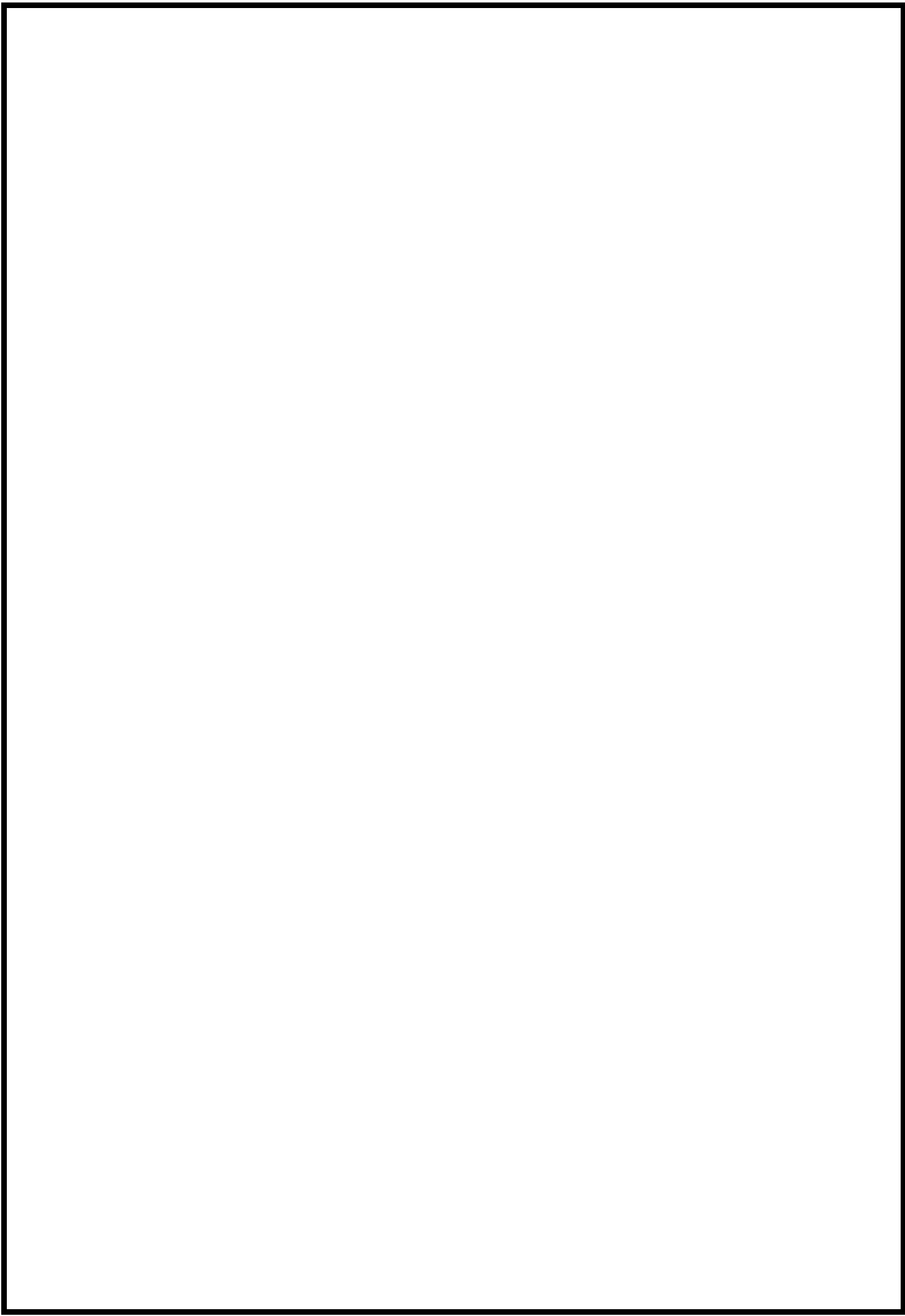
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LINKAGES



(M, MAP)/(PH, PH)/1 queue with Nonpreemptive Priority, Working Interruption and Protection

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Abstract

In this paper we consider a (M,MAP)/(PH,PH)/1 queue with nonpreemptive priority, working interruption and protection. Two types of priority classes of customers where type I customers arrive according to a Poisson process and type II customers arrive according to Markovian Arrival Process are considered. Service time of both type I and type II customers follow mutually independent phase type distributions. The number of type I customers in the system is restricted to a maximum of L. Also type I customers are assumed to have a non-preemptive priority over type II customers. Customer services are subject to interruption by a self induced mechanism. The interruptions occur according to Poisson process. Instead of stopping service completely, the service continues at slower rate during interruption. Also we assume that an interruption occurring while customer is already under interruption will not affect the customer. The server continues to serve at this lower rate until interruption is fixed. The duration of interruption is assumed to be exponentially distributed. A protection mechanism to diminish the effect of interruptions on type I customers service is arranged. The protection for the service of type I customers is provided at the epoch of realization of the clock which starts ticking up the moment a type I customer is taken for service. Type II customers are not provided protection against interruption during their service. Also we assume that type I customers get service at a faster rate starting from the epoch of providing service protection. We analyse the distribution of service time duration of both type I and type II customers and the distribution of a p-cycle. Also we provide LSTs of busy cycle, busy period of type I customers generated during the service time of a type II customer and LSTs of waiting time distributions of type I and type II customers. Also we compute the expected number of interruptions during a type I and a type II service. We perform numerical computations to evaluate important system characteristics and also optimal system cost using a cost function .

Keywords: (M,MAP)/(PH,PH)/1 queue, nonpreemptive priority, working interruption, protection

1 Introduction

Queues with interruption play an important role in day to day life. We encounter different kinds of interruptions in various activities like internet browsing, banking, medical check ups, in supermarkets etc. The works so far reported in the literature discuss about interruptions such as server induced, customer induced, environment dependent service interruptions, server vacations,

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² E-mail: divyavelayudhannair@gmail.com. ⁴ Research is supported by the University Grants Commission, Govt. Of India, under Faculty Development Programme(Grant No.F.No.FIP/12th Plan/KLKE008 TF 04) in Department of Mathematics, Cochin University of Science and Technology, Cochin-22

vacation interruptions and arrival of a priority customer. The first reported work on queues with service interruption is by White and Christie in 1958 in which they considered a two-priority single server system with the low priority customer in service pre-empted on arrival of a high priority customer. Even in the case of single class customer system, the customer in service has to wait whenever a system breakdown occurs. The interrupted service starts from the very beginning (repeat) or from where it got interrupted (resumption) on completion of interruption. These two cases are separately considered in Keilson [2], Gaver [4] and by several other researchers. Fiems et al. [3] introduced probability measures for repeat/resumption on completion of interruption without assigning any rule. Krishnamoorthy et al. [6] are the first to give a specific rule for resumption/repetition of service. We refer the review paper by Krishnamoorthy et al. [5] for details on queueing models with system induced service interruption (priority queues not included).

Varghese et al. [12] introduced a new type of interruption called customer induced interruption in which a customer interrupts own service. They considered an infinite capacity queueing system with a single server in which customers arrive according to a Poisson process with the service time following an exponential distribution. The interruptions occur according to a Poisson process and the duration of each interruption follows an exponential distribution. The self-interrupted customers enter into a finite buffer of size K . Any interrupted customer, finding the buffer full, is considered lost. Those interrupted customers who complete their interruptions move into another buffer of same size and are given a nonpreemptive priority over new customers. They evaluated several performance measures. Numerical illustrations of the system behavior are also provided and also discussed an optimization problem through an illustrative example. Krishnamoorthy et al. [7] extended this to a multi-server queueing system. They investigated the behavior of the queueing system, several performance measures are evaluated and numerical illustrations of the system behavior are provided. Also an optimization problem to maximize the revenue with respect to number of servers is employed and optimal buffer size for the self-interrupted customers are discussed through two illustrative examples. Dudin et al. [13] extended these to MMAP/PH(PH)/c queue with negative arrivals. Varghese and Krishnamoorthy [8] considered a single-server retrial queue with infinite capacity of the primary buffer and finite capacity of the orbit to which customers arrive according to a Poisson process, and the service time follows phase-type distribution. The customer-induced interruption occurs according to a Poisson process. The self-interrupted customers enter into orbit. Any interrupted customer, finding the orbit full, is considered lost. The interrupted customers retries for service after the interruption is completed. Several performance measures were evaluated and some numerical illustrations of the system behavior were provided.

In this paper we consider a single server queueing model with two priority classes of customers where the type I customers are assumed to have a non-preemptive priority over type II customers. We consider customer induced interruption during own service. Instead of stopping service completely, the service continues at slower rate during interruption. The protection for the service of type I customers is provided at the epoch of realization of the clock which starts at the epoch at which the type I customer is taken for service. The rest of the paper is arranged as follows. The mathematical formulation is given in section 2. Section 3 provides steady state analysis of the model. Waiting time analysis of type I and type II customers are discussed in sections 4. Expected number of interruptions during type I and type II services are discussed in sections 5 and 6 respectively. Some other performance measures are discussed in section 7. A related cost function is discussed in section 8. Some numerical results are discussed in section 9. Proofs of two theorems stated in section 4, are given in appendix.

Notations and abbreviations used in the sequel:

- $\mathbf{e}(a)$ = Column vector of 1's of order a
- $\mathbf{e}$ = Column vector of 1's of appropriate order.

- *CTMC*: Continuous time Markov chain.
- I_a = identity matrix of order a .
- $\mathbf{e}_a(b)$ = column vector of order b with 1 in the a th position and the remaining entries zero.
- *MAP*: Markovian Arrival Process
- *LST*: Laplace-Steiltges Transform
- *LIQBD*: Level independent Quasi-Birth and-Death
- *WI*: Working Interruption
- Parameters: λ -arrival rate of type I customers, γ - arrival rate of interruptions, η - parameter of exponential duration of interruption, δ - parameter of exponential protection clock.

2 Mathematical formulation

We consider a single server queue with two priority classes of customers type I and type II with the former arriving according to a Poisson process of rate λ and the latter according to Markovian Arrival Process with representation (D_0, D_1) . Service time of both types follow distinct phase type distributions with representations $\text{PH}(\alpha, T)$ of order m_1 and $\text{PH}(\beta, S)$ of order m_2 respectively. The number of type I customers in the system is restricted to a maximum of L . Also type I customers are assumed to have a non-premptive priority over type II customers. Customer services are subject to interruption by a self induced mechanism. While in interruption arrival of another interruption doesnot affect the customer. The interruptions occur according to Poisson process with rate γ . Instead of stopping the service of that customer completely, it continues at slower rate during interruption. That is, the service time of type I and type II ,during an interruption follow phase type distributions with representation $\text{PH}(\alpha, \theta T)$ and $\text{PH}(\beta, \theta' S)$, $0 < \theta, \theta' < 1$ respectively. Thus $\mu = [\alpha(-T)^{-1}\mathbf{e}]^{-1}$ is the normal service rate and $\theta\mu$ is the interrupted service rate of type I customers and $\mu' = [\beta(-S)^{-1}\mathbf{e}]^{-1}$ and $\theta'\mu'$ are respectively the corresponding rates of normal and interrupted services of type II customers. The server continues to serve at this lower rate until a random clock expires. The duration of interruption is assumed to be exponentially distributed with parameter η . A protection mechanism to diminish the effect of interruptions on type I customers service is arranged. An exponential random clock with mean $\frac{1}{\delta}$ is started simultaneously with each type I service. The protection for the service of type I customers is provided at the epoch of realization of this clock. Type II customers are not provided protection against interruption during their service. Also we assume that the service time of type I customers on activation of protection clock, follows phase type distribution with representation $\text{PH}(\alpha, \phi T)$, $\phi > 1$ and finite.

Let $Q^* = D_0 + D_1$ be the generator matrix of the type II arrival process and π^* be its stationary probability vector. Hence π is the unique (positive) probability vector satisfying $\pi^* Q^* = 0$, $\pi^* \mathbf{e} = 1$. The constant $\beta^* = \pi^* D_1 \mathbf{e}$, referred to as *fundamental rate*, gives the expected number of type II arrivals per unit of time in the stationary version of the MAP. It is assumed that the two arrival processes are mutually independent and are also independent of the service time distributions.

2.1 The QBD process

The model described in section 1 can be studied as a LIQBD process. First we introduce the following notations:

At time t :

$N_1(t)$: number of type II customers in the system,

$N_2(t)$: number of type I customers in the system

$$J(t) = \begin{cases} 0, & \text{if the type I customer in service is unprotected/type II customer is in service} \\ 1, & \text{if the type I customer in service is protected} \end{cases}$$

$$K(t) = \begin{cases} 0, & \text{if the server provides service to type I customer in WI} \\ 1, & \text{if the server provides service to type II customer in WI} \\ 2, & \text{if the server provides normal service to type I customer} \\ 3, & \text{if the server provides normal service to type II customer} \end{cases}$$

$S(t)$: the phase of service when the server is busy

$M(t)$: the phase of arrival of the type II customer.

It is easy to verify that $\{(N_1(t), N_2(t), J(t), K(t), S(t), M(t)): t \geq 0\}$ is a LIQBD with state space

$$l(0) = \{(0, k)/1 \leq k \leq n\} \cup \{(0, i_2, 0, j_2, k_1, k_2)/1 \leq i_2 \leq L, j_2 = 0 \text{ or } 2, 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq n\} \cup \{(0, i_2, 1, 2, k_1, k_2)/1 \leq i_2 \leq L, 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq n\}$$

For $i_1 \geq 1$,

$$\{(i_1, 0, 0, j_2, k_1, k_2)/j_2 = 1 \text{ or } 3, 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq n\} \cup \{(i_1, i_2, 0, j_2, k_1, k_2): 1 \leq i_2 \leq L, j_2 = 0 \text{ or } 2, 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq n\} \cup \{(i_1, i_2, 0, j_2, k_1, k_2): 1 \leq i_2 \leq L, j_2 = 1 \text{ or } 3, 1 \leq k_1 \leq m_2, 1 \leq k_2 \leq n\} \cup \{(i_1, i_2, 1, 2, k_1, k_2)/1 \leq i_2 \leq L, 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq n\}$$

The infinitesimal generator of this CTMC is

$$Q_1 = \begin{bmatrix} B_0 & C_0 & & & \\ B_1 & A_1 & A_0 & & \\ & A_2 & A_1 & A_0 & \\ & & \ddots & \ddots & \ddots \end{bmatrix}$$

where B_0 contains transitions within the level 0; C_0 represents transitions from level 0 to level 1; B_1 represents transitions from level 1 to level 0; A_0 represents transitions from level g to level $g + 1$ for $g \geq 1$, A_1 represents transitions within the level g for $g \geq 1$ and A_2 represents transitions from level g to $g - 1$ for $g \geq 2$. The boundary blocks B_0, C_0, B_1 are of orders $n(1 + 3m_1L) \times n(1 + 3m_1L)$, $n(1 + 3m_1L) \times (2m_2n + (3m_1 + 2m_2)nL)$, $(2m_2n + (3m_1 + 2m_2)nL) \times n(1 + 3m_1L)$ respectively. A_0, A_1, A_2 are square matrices of order $2m_2n + (3m_1 + 2m_2)nL$. Define the entries of $B_0^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$, $C_0^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$, $B_1^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$ as transition submatrices which contains transitions of the form

$(0, h_1, i_1, j_1, k_1, l_1) \rightarrow (0, h_2, i_2, j_2, k_2, l_2)$, $(0, h_1, i_1, j_1, k_1, l_1) \rightarrow (1, h_2, i_2, j_2, k_2, l_2)$ and $(1, h_1, i_1, j_1, k_1, l_1) \rightarrow (0, h_2, i_2, j_2, k_2, l_2)$ respectively. Define the entries of $A_0^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$, $A_1^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$, $A_2^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)}$ as transition submatrices which contains transitions of the form $(g, h_1, i_1, j_1, k_1, l_1) \rightarrow (g + 1, h_2, i_2, j_2, k_2, l_2)$, where $g \geq 1$, $(g, h_1, i_1, j_1, k_1, l_1) \rightarrow (g, h_2, i_2, j_2, k_2, l_2)$, where $g \geq 1$, $(g, h_1, i_1, j_1, k_1, l_1) \rightarrow (g - 1, h_2, i_2, j_2, k_2, l_2)$, where $g \geq 2$ respectively. Since none or one event alone could take place in a short interval of time with positive probability, in general, a transition such as $(g_1, h_1, i_1, j_1, k_1, l_1) \rightarrow (g_2, h_2, i_2, j_2, k_2, l_2)$ has positive rate only for exactly one of $g_1, h_1, i_1, j_1, k_1, l_1$ different from $g_2, h_2, i_2, j_2, k_2, l_2$.

$$B_0^{(h_2, i_2, j_2, k_2, l_2)}_{(h_1, i_1, j_1, k_1, l_1)} =$$

$$\left\{ \begin{array}{ll} \lambda(\alpha \otimes I_n) & h_1 = 0, h_2 = 1; i_2 = 0; j_2 = 2, 1 \leq k_2 \leq m_1, \\ & 1 \leq l_1, l_2 \leq n \\ \lambda I_{m_1 n} & 1 \leq h_1 \leq L-1, h_2 = h_1 + 1; i_1 = i_2 = 0; j_1 = j_2, \\ & j_1 = 0 \text{ or } 2; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \lambda I_{m_1 n} & 1 \leq h_1 \leq L-1, h_2 = h_1 + 1; i_1 = i_2 = 1; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \otimes I_n & h_1 = 1, h_2 = 0; i_1 = 0; j_1 = 0; 1 \leq k_1 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n & h_1 = 1, h_2 = 0; i_1 = 0; j_1 = 2; 1 \leq k_1 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ \phi T^0 \otimes I_n & h_1 = 1, h_2 = 0; i_1 = 1; j_1 = 2; 1 \leq k_1 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = i_2 = 0; j_1 = 0, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = i_2 = 0; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \phi T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = 1, i_2 = 0; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \eta I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2, i_1 = i_2 = 0; j_1 = 0, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \gamma I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 2, j_2 = 0; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \delta I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = 0, i_2 = 1; j_1 = 0 \text{ or } 2, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ D_0 - \lambda I_n & h_1 = h_2 = 0; 1 \leq l_1, l_2 \leq n \\ \theta T \oplus D_0 - (\lambda + \eta + \delta) I_{m_1 n} & 1 \leq h_1 \leq L-1, h_1 = h_2; i_1 = i_2 = 0; \\ & j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T \oplus D_0 - (\lambda + \gamma + \delta) I_{m_1 n} & 1 \leq h_1 \leq L-1, h_1 = h_2; i_1 = i_2 = 0; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \phi T \oplus D_0 - \lambda I_{m_1 n} & 1 \leq h_1 \leq L-1, h_1 = h_2; i_1 = i_2 = 1; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \end{array} \right.$$

$$C_{0(h_1, i_1, j_1, k_1, l_1)}^{(h_2, i_2, j_2, k_2, l_2)} = \left\{ \begin{array}{ll} \beta \otimes D_1 & h_1 = h_2 = 0; i_2 = 0; j_2 = 3; 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_1} \otimes D_1 & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ I_{m_1} \otimes D_1 & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ I_{m_1} \otimes D_1 & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \end{array} \right.$$

$$B_{1(h_1, i_1, j_1, k_1, l_1)}^{(h_2, i_2, j_2, k_2, l_2)} = \left\{ \begin{array}{ll} \theta' S^0 \otimes I_n & h_1 = h_2 = 0; i_1 = 0; j_1 = 1; 1 \leq k_1 \leq m_2, 1 \leq l_1, l_2 \leq n \\ S^0 \otimes I_n & h_1 = h_2 = 0; i_1 = 0; j_1 = 3; 1 \leq k_1 \leq m_2, 1 \leq l_1, l_2 \leq n \\ \theta' S^0 \alpha \otimes I_n & h_1 = h_2, 1 \leq h_1 \leq L; i_1 = i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq m_2, \\ & 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S^0 \alpha \otimes I_n & h_1 = h_2, 1 \leq h_1 \leq L; i_1 = i_2 = 0; j_1 = 3, j_2 = 2; 1 \leq k_1 \leq m_2, \\ & 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \end{array} \right.$$

$$A_{0(h_1, i_1, j_1, k_1, l_1)}^{(h_2, i_2, j_2, k_2, l_2)} = \begin{cases} I_{m_2} \otimes D_1 & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_2} \otimes D_1 & i_1 = i_2 = 0; j_1 = j_2 = 3; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_1} \otimes D_1 & 1 \leq h_1 \leq L; h_1 = h_2; i_1 = i_2 = 0; j_1 = j_2 = 0 \text{ or } 2; 1 \leq k_1, k_2 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ I_{m_1} \otimes D_1 & 1 \leq h_1 \leq L; h_1 = h_2; i_1 = i_2 = 1; j_1 = j_2 = 2; 1 \leq k_1, k_2 \leq m_1; \\ & 1 \leq l_1, l_2 \leq n \\ I_{m_2} \otimes D_1 & 1 \leq h_1 \leq L; h_1 = h_2; i_1 = i_2 = 0; j_1 = j_2 = 1 \text{ or } 3; 1 \leq k_1, k_2 \leq m_2; \\ & 1 \leq l_1, l_2 \leq n \end{cases}$$

$$A_{2(h_1, i_1, j_1, k_1, l_1)}^{(h_2, i_2, j_2, k_2, l_2)} = \begin{cases} \theta' S^0 \beta \otimes I_n & h_1 = h_2 = 0; i_1 = i_2 = 0; j_1 = 1, j_2 = 3; 1 \leq k_1, k_2 \leq m_2; \\ & 1 \leq l_1, l_2 \leq n \\ S^0 \beta \otimes I_n & h_1 = h_2 = 0; i_1 = i_2 = 0; j_1 = j_2 = 3; 1 \leq k_1, k_2 \leq m_2; \\ & 1 \leq l_1, l_2 \leq n \\ \theta' S^0 \alpha \otimes I_n & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 1, j_2 = 2; 1 \leq k_1 \leq m_2, \\ & 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S^0 \alpha \otimes I_n & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 3, j_2 = 2; 1 \leq k_1 \leq m_2, \\ & 1 \leq k_2 \leq m_1, 1 \leq l_1, l_2 \leq n \end{cases}$$

$$\begin{aligned} & A_{1(h_1, i_1, j_1, k_1, l_1)}^{(h_2, i_2, j_2, k_2, l_2)} = \\ \left\{ \begin{array}{ll} \lambda I_{m_1 n} & 1 \leq h_1 \leq L-1, h_2 = h_1 + 1; i_1 = i_2 = 0; j_1 = j_2 = 0 \text{ or } 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \lambda I_{m_2 n} & 0 \leq h_1 \leq L-1, h_2 = h_1 + 1; i_1 = i_2 = 0; j_1 = j_2 = 1 \text{ or } 3; \\ & 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ \lambda I_{m_1 n} & 1 \leq h_1 \leq L-1, h_2 = h_1 + 1; i_1 = i_2 = 1; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \beta \otimes I_n & h_1 = 1, h_2 = 0; i_1 = i_2 = 0; j_1 = 0, j_2 = 3; 1 \leq k_1 \leq m_1, \\ & 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ T^0 \beta \otimes I_n & h_1 = 1, h_2 = 0; i_1 = i_2 = 0; j_1 = 2, j_2 = 3; 1 \leq k_1 \leq m_1, \\ & 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ \phi T^0 \beta \otimes I_n & h_1 = 1, h_2 = 0; i_1 = 1, i_2 = 0; j_1 = 2, j_2 = 3; 1 \leq k_1 \leq m_1, \\ & 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ \theta T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = i_2 = 0; j_1 = 0, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = i_2 = 0; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \phi T^0 \alpha \otimes I_n & 2 \leq h_1 \leq L, h_2 = h_1 - 1; i_1 = i_2 = 1; j_1 = j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \eta I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 0, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \eta I_{m_2 n} & 0 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 1, j_2 = 3; \\ & 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ \gamma I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 2, j_2 = 0; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \gamma I_{m_2 n} & 0 \leq h_1 \leq L, h_1 = h_2; i_1 = i_2 = 0; j_1 = 3, j_2 = 1; \\ & 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ \delta I_{m_1 n} & 1 \leq h_1 \leq L, h_1 = h_2; i_1 = 0, i_2 = 1; j_1 = 0 \text{ or } 2, j_2 = 2; \\ & 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \theta' S \oplus D_0 - (\lambda + \eta) I_{m_2 n} & h_1 = h_2 = 0; i_1 = i_2 = 0; j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ S \oplus D_0 - (\lambda + \gamma) I_{m_2 n} & h_1 = h_2 = 0; i_1 = i_2 = 0; j_1 = j_2 = 3, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ (\theta T \oplus D_0 - (\lambda + \eta + \delta) I_{m_1 n} & 1 \leq h_1 \leq L-1, h_1 = h_2; i_1 = i_2 = 0, j_1 = j_2 = 0, 1 \leq k_1, k_2 \leq m_1, \end{array} \right. \end{aligned}$$

3 Steady State Analysis

Let $\pi = (\pi_0, \pi_1, \dots, \pi_L)$ denote the steady state probability vector of the generator

$$A = A_0 + A_1 + A_2 = \begin{bmatrix} F_0 & F_1 & & & \\ F_2 & F_3 & \lambda I & & \\ & F_4 & F_3 & \lambda I & \\ & & \ddots & \ddots & \ddots \\ & & & F_4 & F_3 & \lambda I \\ & & & & F_4 & F_5 \end{bmatrix} \text{ ie,} \quad \pi A = 0, \pi e = 1. \quad (1)$$

In the above,

$$F_0(k, l) = \begin{cases} \theta' S \oplus D_0 - (\lambda + \eta) I_{m_2 n} + I_{m_2} \otimes D_1 & k = 1, l = 1 \\ \eta I_{m_2 n} + \theta' S^0 \beta \otimes I_n & k = 1, l = 2 \\ \gamma I_{m_2 n} & k = 2, l = 1 \\ S \oplus D_0 - (\lambda + \gamma) I_{m_2 n} + S^0 \beta \otimes I_n + I_{m_2} \otimes D_1 & k = 2, l = 2 \end{cases}, F_1(k, l) = \begin{cases} \lambda I_{m_2 n} & k = 1, l = 2 \\ \lambda I_{m_2 n} & k = 2, l = 4 \\ 0 & \text{otherwise,} \end{cases}$$

$$F_2(k, l) = \begin{cases} \theta T^0 \beta \otimes I_n & k = 1, l = 2 \\ T^0 \beta \otimes I_n & k = 3, l = 2 \\ \phi T^0 \beta \otimes I_n & k = 5, l = 2 \\ 0 & \text{otherwise,} \end{cases}, F_3(k, l) = \begin{cases} \theta T \oplus D_0 - (\lambda + \eta + \delta) I_{m_1 n} + I_{m_1} \otimes D_1 & k = 1, l = 1 \\ \eta I_{m_1 n} & k = 1, l = 3 \\ \delta I_{m_1 n} & k = 1, l = 5 \\ \theta' S \oplus D_0 - (\lambda + \eta) I_{m_2 n} + I_{m_2} \otimes D_1 & k = 2, l = 2 \\ \theta' S^0 \alpha \otimes I_n & k = 2, l = 3 \\ \eta I_{m_2 n} & k = 2, l = 4 \\ \gamma I_{m_1 n} & k = 3, l = 1 \\ T \oplus D_0 - (\lambda + \gamma + \delta) I_{m_1 n} + I_{m_1} \otimes D_1 & k = 3, l = 3 \\ \delta I_{m_1 n} & k = 3, l = 5 \\ \gamma I_{m_2 n} & k = 4, l = 2 \\ S^0 \alpha \otimes I_n & k = 4, l = 3 \\ S \oplus D_0 - (\lambda + \gamma) I_{m_2 n} + I_{m_2} \otimes D_1 & k = 4, l = 4 \\ \phi T \oplus D_0 - \lambda I_{m_1 n} + I_{m_1} \otimes D_1 & k = 5, l = 5 \\ 0 & \text{otherwise} \end{cases}$$

$$F_4(k, l) = \begin{cases} \theta T^0 \alpha \otimes I_n & k = 1, l = 3 \\ T^0 \alpha \otimes I_n & k = 3, l = 3 \\ \phi T^0 \alpha \otimes I_n & k = 5, l = 3 \\ 0 & \text{otherwise} \end{cases}, F_5(k, l) =$$

$$\left\{ \begin{array}{ll} \theta T \oplus D_0 - (\eta + \delta)I_{m_1 n} + I_{m_1} \otimes D_1 & k = 1, l = 1 \\ \eta I_{m_1 n} & k = 1, l = 3 \\ \delta I_{m_1 n} & k = 1, l = 5 \\ \theta' S \oplus D_0 - \eta I_{m_2 n} + I_{m_2} \otimes D_1 & k = 2, l = 2 \\ \theta' S^0 \alpha \otimes I_n & k = 2, l = 3 \\ \eta I_{m_2 n} & k = 2, l = 4 \\ \gamma I_{m_1 n} & k = 3, l = 1 \\ T \oplus D_0 - (\gamma + \delta)I_{m_1 n} + I_{m_1} \otimes D_1 & k = 3, l = 3 \\ \delta I_{m_1 n} & k = 3, l = 5 \\ \gamma I_{m_2 n} & k = 4, l = 2 \\ S^0 \alpha \otimes I_n & k = 4, l = 3 \\ S \oplus D_0 - \gamma I_{m_2 n} + I_{m_2} \otimes D_1 & k = 4, l = 4 \\ \phi T \oplus D_0 + I_{m_1} \otimes D_1 & k = 5, l = 5 \\ 0 & otherwise \end{array} \right.$$

with dimensions of F_0, F_1, F_2 be $2m_2 n \times 2m_2 n$, $2m_2 n \times (3m_1 + 2m_2)n$, $(3m_1 + 2m_2)n \times 2m_2 n$ respectively. F_3, F_4 and F_5 are square matrices of order $(3m_1 + 2m_2)n$.

The *LIQBD* description of the model indicates that the queueing system is stable (see Neuts [9]) if and only if the left drift exceeds that of right drift. That is,

$$\pi A_0 e < \pi A_2 e. \quad (2)$$

The vector π cannot be obtained directly in terms of the parametres of the model. The inequality (2) is simplified in (5) below. From (1) we get

$$\pi_i = \pi_{i-1} \mathcal{U}_{i-1}, 1 \leq i \leq L \quad (3)$$

where

$$\mathcal{U}_0 = -F_1(F_3 + \mathcal{U}_1 F_4)^{-1}$$

$$\mathcal{U}_i = \begin{cases} -\lambda(F_3 + \mathcal{U}_{i+1} F_4)^{-1} & \text{for } 1 \leq i \leq L-2 \\ -\lambda F_5^{-1} & \text{for } i = L-1. \end{cases}$$

From the normalizing condition $\pi e = 1$ we have

$$\pi_0 \left(\sum_{j=0}^{L-1} \prod_{i=0}^j \mathcal{U}_i + I \right) e = 1. \quad (4)$$

The inequality (2) gives the stability condition as

$$\begin{aligned} & \pi_0 \left[(I_{(2m_2)} \otimes D_1) e + \sum_{i=0}^{L-1} \prod_{j=0}^i \mathcal{U}_j (I_{3m_1+2m_2} \otimes D_1) e \right] < \\ & \pi_0 \left[[e_1(2)(\theta' S^0 \beta \otimes I) + e_2(2) S^0 \beta \otimes I] e(m_2 n) + \sum_{i=0}^{L-1} \prod_{j=0}^i \mathcal{U}_j [e_2(5) \theta' S^0 \alpha \otimes I) + \right. \\ & \left. e_4(5) (S^0 \alpha \otimes I) e(m_2 n) \right]. \end{aligned} \quad (5)$$

Let $\mathbf{x}$ be the steady state probability vector of Q . We partition this vector as $\mathbf{x} = (\mathbf{x}_0, \mathbf{x}_1, \mathbf{x}_2 \dots)$, where $\mathbf{x}_0$ is of dimension $n(1 + 3m_1 L)$ and $\mathbf{x}_1, \mathbf{x}_2, \dots$ are each of dimension $n(2m_2 + (3m_1 + 2m_2)L)$. Under the stability condition, we have $\mathbf{x}_i = \mathbf{x}_1 R^{i-1}, i \geq 2$, where the matrix R is the minimal nonnegative solution to the matrix quadratic equation

$$R^2 A_2 + R A_1 + A_0 = 0$$

and the vectors $\mathbf{x}_0$ and $\mathbf{x}_1$ are obtained by solving the equations

$$\mathbf{x}_0 B_0 + \mathbf{x}_1 B_1 = 0 \quad (6)$$

$$\mathbf{x}_0 C_0 + \mathbf{x}_1 (A_1 + R A_2) = 0 \quad (7)$$

subject to the normalizing condition

$$\mathbf{x}_0 e + \mathbf{x}_1 (I - R)^{-1} e = 1 \quad (8)$$

3.1 Analysis of service time of a type I customer

The duration of service of a type I customer is a phase type distribution with

representation (α', S_1) where the underlying MC has state space $\{(i, j, k): i = 0, j = 0 \text{ or } 2, 1 \leq k \leq m_1\} \cup \{(i, 2, k): i = 1, 1 \leq k \leq m_1\} \cup \{*\}$ where i denotes the status of the protection clock, j , the status of the server, k , the service phase and $*$, the absorbing state indicating service completion. The infinitesimal generator is

$$S_1 = \begin{bmatrix} S_1 & S_1^0 \\ \mathbf{0} & 0 \end{bmatrix}, \text{ where, } S_1 = \begin{bmatrix} \theta T - (\eta + \delta)I_{m_1} & \eta I_{m_1} & \delta I_{m_1} \\ \gamma I_{m_1} & T - (\gamma + \delta)I_{m_1} & \delta I_{m_1} \\ \mathbf{0} & \mathbf{0} & \phi T \end{bmatrix} \text{ and } S_1^0 = \begin{bmatrix} \theta T^0 \\ T^0 \\ \phi T^0 \end{bmatrix}$$

The initial probability vector is $\alpha' = [\mathbf{0} \quad \alpha \quad \mathbf{0}]$, where $\mathbf{0}$ is a zero matrix of order $1 \times m_1$.

Thus the service time distribution of a type I customer is $Ph(\alpha', S_1)$ of order $3m_1n$.

3.2 Analysis of service time of a type II customer

The duration of service of a type II customer turn out to be a phase type distribution (β', S_2) where the underlying MC has state space $\{(i, j): i = 1 \text{ or } 3, 1 \leq j \leq m_2\} \cup \{*\}$ where i denotes the status of the server, j , the service phase and $*$, the absorbing state indicating service completion. The infinitesimal generator is

$$S_2 = \begin{bmatrix} S_2 & S_2^0 \\ \mathbf{0} & 0 \end{bmatrix}, \text{ where, } S_2 = \begin{bmatrix} \theta' S - \eta I_{m_2} & \eta I_{m_2} \\ \gamma I_{m_2} & S - \gamma I_{m_2} \end{bmatrix} \text{ and } S_2^0 = \begin{bmatrix} \theta' S^0 \\ S^0 \end{bmatrix}$$

The initial probability vector is $\beta' = [\mathbf{0} \quad \alpha]$, where $\mathbf{0}$ is a zero matrix of order $1 \times m_2$. Thus we have the service time distribution of a type II customer is $Ph(\beta', S_2)$ of order $2m_2n$.

4 Waiting time analysis

4.1 Type I Customer

To find the waiting time of a type I customer who joins for service at time t , we have to consider different possibilities depending on the status of server at that time. Let $W(t)$ be the waiting time of a type I customer who arrives at time t and $W^*(s)$ be the corresponding LST.

Case I

Suppose that E_1 denote the event the system is in the state $(0, v), 1 \leq v \leq n$ when the tagged type I customer arrives. Let $W^*(s/E_1)$ denote the corresponding LST. Then

$$W^*(s/E_1) = 1$$

Case II

E_2 be the event that the system is in the state $(n_1, a, 0, 0, u, v), n_1 \geq 0, 1 \leq a \leq L-1, 1 \leq u \leq m_1, 1 \leq v \leq n$, when the tagged customer arrives the system. In this case the waiting time is the sum of the residual service time of the type I customer in service when the tagged customer arrives and service time of $a-1$ remaining type I customers. Let $W^*(s/E_2)$ represent the corresponding conditonal LST. Then

$$W^*(s/E_2) = (e_u(3m_1)(sI - S_1)^{-1}S_1^0)(\alpha'(sI - S_1)^{-1}S_1^0)^{a-1}.$$

Case III

E_3 denotes the event: the system is in the state $(n_1, a, 0, 2, u, v), n_1 \geq 0, 1 \leq a \leq L-1, 1 \leq u \leq m_1, 1 \leq v \leq n$, when the tagged customer arrives the system. In this case the waiting time is the sum of the residual service time of the type I customer in service when the tagged customer arrives and service times of $a-1$ remaining type I customers. With $W^*(s/E_3)$ as the corresponding conditonal LST, we have

$$W^*(s/E_3) = (e_{m_1+u}(3m_1)(sI - S_1)^{-1}S_1^0)(\alpha'(sI - S_1)^{-1}S_1^0)^{a-1}.$$

Case IV

E_4 denotes the event: the system is in the state $(n_1, a, 1, 2, u, v)$, $n_1 \geq 0, 1 \leq a \leq L-1, 1 \leq u \leq m_1, 1 \leq v \leq n$, when the tagged customer arrives the system. In this case the waiting time is the sum of the residual service time of the type I customer in service when the tagged customer arrives and service times of $a-1$ remaining type I customers. Let $W^*(s/E_4)$ represent the corresponding conditonal LST. Then

$$W^*(s/E_4) = (e_{2m_1+u}(3m_1)(sI - S_1)^{-1}S_1^0)(\alpha'(sI - S_1)^{-1}S_1^0)^{a-1}.$$

Case V

E_5 denotes the event: the system is in the state $(n_1, a, 0, 1, u, v)$, $n_1 \geq 1, 0 \leq a \leq L-1, 1 \leq u \leq m_2, 1 \leq v \leq n$, when the tagged customer arrives the system. In this case the waiting time is the sum of the residual service time of the type II customer in service when the tagged customer arrives and service times of a remaining type I customers. Let $W^*(s/E_5)$ represent the corresponding conditonal LST. Then

$$W^*(s/E_5) = (e_u(2m_2)(sI - S_2)^{-1}S_2^0)(\alpha'(sI - S_1)^{-1}S_1^0)^a.$$

Case VI

E_6 denotes the event: the system is in the state $(n_1, a, 0, 3, u, v)$, $n_1 \geq 1, 0 \leq a \leq L-1, 1 \leq u \leq m_2, 1 \leq v \leq n$, when the tagged customer arrives the system. In this case the waiting time is the sum of the residual service time of the type II customer in service when the tagged customer arrives and service times of a remaining type I customers. Let $W^*(s/E_6)$ represent the corresponding conditonal LST. Then

$$W^*(s/E_6) = (e_{m_2+u}(2m_2)(sI - S_2)^{-1}S_2^0)(\alpha'(sI - S_1)^{-1}S_1^0)^a.$$

Thus the LST of the waiting time

$$\begin{aligned} W^*(s) = & \frac{1}{d} [\sum_{v=1}^n x_{0,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/E_2)x_{n_1,a,0,0,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/E_3) \\ & x_{n_1,a,0,2,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/E_4)x_{n_1,a,1,2,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m_2} \sum_{v=1}^n W^*(s/E_5)x_{n_1,a,0,1,u,v} \\ & + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m_2} \sum_{v=1}^n W^*(s/E_6)x_{n_1,a,0,3,u,v}] \end{aligned} \quad (9)$$

where, $d =$

$$\begin{aligned} & \sum_{v=1}^n x_{0,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1,a,0,0,u,v} + \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1,a,0,2,u,v} + \\ & \sum_{n_1=0}^{\infty} \sum_{a=1}^{L-1} \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1,a,1,2,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m_2} \sum_{v=1}^n x_{n_1,a,0,1,u,v} + \sum_{n_1=1}^{\infty} \sum_{a=0}^{L-1} \sum_{u=1}^{m_2} \sum_{v=1}^n x_{n_1,a,0,3,u,v} \end{aligned}$$

4.2 Type II customer

To find the LST of the waiting time distribution of a type II customer, we have to compute certain distributions. We proceed to such computations.

Definition 1 Consider the duration of time with p type I customers in the system at a service commencement epoch of type I customers until the number of type I customers become zero for the first time, we call this a p -cycle, denoted by B_p .

4.2.1 Distribution of a p -cycle

This is a phase type distribution with representation (γ_p, T_1) where the underlying Markov chain has state space $\{(i, j, k, l): 1 \leq i \leq L, j = 0, k = 0 \text{ or } 2, 1 \leq l \leq m_1\} \cup \{(i, j, k, l): 1 \leq i \leq L, j = 1, k = 2, 1 \leq l \leq m_1\} \cup \{*\}$ and i, j, k, l and $*$ respectively denote the number of type I customers in the system, the status of the protection clock, the status of the server, the service phase and the absorbing state indicating that the number of type I customers become zero. The infinitesimal generator T_1 of $B_p(t)$ has the form

$$T_1 = \begin{bmatrix} T_1 & T_1^0 \\ 0 & 0 \end{bmatrix}, \text{ where } T_1 =$$

$$\begin{bmatrix} E_1 & \lambda I_{m_1} & & & \\ E_2 & E_1 & \lambda I_{m_1} & & \\ & \ddots & \ddots & \ddots & \\ & & E_2 & E_1 & \lambda I_{m_1} \\ & & & E_2 & E_3 \end{bmatrix},$$

$$T_1^0 = \begin{bmatrix} E^0 \\ \mathbf{0} \\ \vdots \\ \mathbf{0} \end{bmatrix}$$

where

$$E_1 = \begin{bmatrix} \theta T - (\lambda + \eta + \delta)I_{m_1} & \eta I_{m_1} & \delta I_{m_1} \\ \gamma I & T - (\lambda + \gamma + \delta)I_{m_1} & \delta I_{m_1} \\ 0 & 0 & \phi T - \lambda I_{m_1} \end{bmatrix}$$

$$\begin{bmatrix} 0 & \theta T^0 \alpha & 0 \\ 0 & T^0 \alpha & 0 \\ 0 & \phi T^0 \alpha & 0 \end{bmatrix}.$$

$$E_3 = \begin{bmatrix} \theta T - (\eta + \delta)I_{m_1} & \eta I_{m_1} & \delta I_{m_1} \\ \gamma I_{m_1} & T - (\gamma + \delta)I_{m_1} & \delta I_{m_1} \\ 0 & 0 & \phi T \end{bmatrix}$$

$$\text{and } E^0 = \begin{bmatrix} \theta T^0 \\ T^0 \\ \phi T^0 \end{bmatrix}.$$

The initial probability vector is

$$\gamma_p = \begin{bmatrix} 0 & \dots & 0 & \gamma' & 0 & \dots & 0 \end{bmatrix}, 1 \leq p \leq L$$

where $\mathbf{0}$ is a zero matrix of order $1 \times 3m_1$, with $\gamma' = \begin{bmatrix} \mathbf{0} & \alpha & \mathbf{0} \end{bmatrix}$, $1 \leq p \leq L$ is in the p th position and $\mathbf{0}$ is a zero matrix of order $1 \times m_1$.

We can compute the LST of the length of the busy period as $\gamma_p(sI - T_1)^{-1}T_1^0$

4.2.2 LST of the busy cycle generated by type I customers arriving during the service time of a type II customer

Theorem 1

The LST of the busy cycle generated by type I customers arriving during the service time of a type II customer is given by

$$\hat{B}_{c_L}(s) = \beta'[(s + \lambda)I - S_2]^{-1}S_2^0 + \sum_{p=1}^{L-1} \gamma_p(sI - T_1)^{-1}T_1^0 \lambda^p \beta'[(s + \lambda)I - S_2]^{-(p+1)}S_2^0 + \gamma_L(sI - T_1)^{-1}T_1^0$$

$$\beta'[\lambda^{-1}((s + \lambda)I - S_2)]^{-L}[I - \lambda[(s + \lambda)I - S_2]^{-1}]^{-1}[(s + \lambda)I - S_2]^{-1}S_2^0 \quad (10)$$

Proof.

The proof is given in the appendix.

4.2.3 LST of the busy period of type I customers generated during the service time of a type II customer

Theorem 2

The LST of the busy period generated by type I customers arriving during the service time of a type II customer is given by

$$\hat{B}_L(s) = \beta'[\lambda I - S_2]^{-1}S_2^0 + \sum_{p=1}^{L-1} \gamma_p(sI - T_1)^{-1}T_1^0\lambda^p\beta'[\lambda I - S_2]^{-(p+1)}S_2^0 + \gamma_L(sI - T_1)^{-1}T_1^0 \beta'[\lambda^{-1}(\lambda I - S_2)]^{-L}[I - \lambda[\lambda I - S_2]^{-1}]^{-1}[\lambda I - S_2]^{-1}S_2^0 \quad (11)$$

Proof.

The proof is given in the appendix.

Now, to find the waiting time of a type II customer who joins for service at time t , we have to consider different possibilities depending on the status of server at that time. Let $W(t)$ be the waiting time of a type II customer who arrives at time t and $W^*(s)$ be the corresponding LST.

Case I

Suppose that F_1 denotes the event the system is in the state $(0, v), 1 \leq v \leq n$ when the tagged customer arrives. Let $W^*(s/F_1)$ denote the corresponding LST. Then

$$W^*(s/F_1) = 1$$

Case II

F_2 be the event that the system is in one of the states $(b, a, 0, 0, u, v), b \geq 0, 1 \leq a \leq L, 1 \leq u \leq m_1, 1 \leq v \leq n$ when the tagged customer arrives. In this case, the waiting time is the length of the busy cycle generated by a type I customers starting from his arrival epoch plus lengths of busy cycles of type I customers generated during service times of each of the b type II customers. Let $W^*(s/F_2)$ denote the corresponding LST. Then

$$W^*(s/F_2) = e_{(a-1)3m_1+u}(3Lm_1)(sI - T_1)^{-1}T_1^0(\hat{B}_{c_L}(s))^b$$

Case III

F_3 denotes the event the system is in one of the states $(b, a, 0, 2, u, v), b \geq 0, 1 \leq a \leq L, 1 \leq u \leq m_1, 1 \leq v \leq n$ when the tagged customer arrives. In this case, the waiting time is the length of the busy cycle generated by a type I customers starting from his arrival epoch plus lengths of busy cycles of type I customers generated during service times of each of the b type II customers. Let $W^*(s/F_3)$ denote the corresponding LST. Then

$$W^*(s/F_3) = e_{(a-1)3m_1+m_1+u}(3Lm_1)(sI - T_1)^{-1}T_1^0(\hat{B}_{c_L}(s))^b$$

Case IV

F_4 denotes the event the system is in one of the states $(b, a, 1, 2, u, v), b \geq 0, 1 \leq a \leq L, 1 \leq u \leq m_1, 1 \leq v \leq n$ when the tagged customer arrives. In this case, the waiting time is the length of the busy cycle generated by a type I customers starting from his arrival epoch plus lengths of busy cycles of type I customers generated during service times of each of the b type II customers. Let $W^*(s/F_4)$ denote the corresponding LST. Then

$$W^*(s/F_4) = e_{(a-1)3m_1+2m_1+u}(3Lm_1)(sI - T_1)^{-1}T_1^0(\hat{B}_{c_L}(s))^b$$

Case V

F_5 denotes the event the system is in one of the states $(b, a, 0, 1, u, v), b \geq 1, 0 \leq a \leq L, 1 \leq u \leq m_2, 1 \leq v \leq n$ when the tagged customer arrives. In this case, the waiting time is the length of residual service time of the type II customer in service plus length of the busy period generated by type I customers arriving during the service time of the type II customer in service plus lengths of busy cycles of type I customers generated during service time of each of the $b-1$ type II customers. Let $W^*(s/F_5)$ denote the corresponding LST. Then

$$W^*(s/F_5) = e_u(2m_2)(sI - S_2)^{-1}S_2^0\hat{B}_L(s)(\hat{B}_{c_L}(s))^{b-1}$$

Case VI

F_6 denotes the event the system is in one of the states $(b, a, 0, 3, u, v)$ when the tagged customer arrives. In this case the waiting time is the length of residual service time of the type II customer in service plus the length of the busy period generated by type I customers arriving during the service time of the type II customer in service plus lengths of busy cycles of type I

customers generated during service time of each of the $b - 1$ type II customers. Let $W^*(s/F_6)$ denote the corresponding LST. Then

$$W^*(s/F_6) = e_{m_2+u}(2m_2)(sI - S_2)^{-1}S_2^0\hat{B}_L(s)(\hat{B}_{c_L}(s))^{b-1}$$

Thus the LST of the waiting time

$$\begin{aligned} W^*(s) = & \sum_{v=1}^n x_{0,v} + \sum_{b=0}^{\infty} \sum_{a=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/F_2) x_{b,a,0,0,u,v} + \sum_{b=0}^{\infty} \sum_{a=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/F_3) \\ & x_{b,a,0,2,u,v} + \sum_{b=0}^{\infty} \sum_{a=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n W^*(s/F_4) x_{b,a,1,2,u,v} + \sum_{b=1}^{\infty} \sum_{a=0}^L \sum_{u=1}^{m_2} \sum_{v=1}^n W^*(s/ \\ & F_5) x_{b,a,0,1,u,v} \\ & + \sum_{b=1}^{\infty} \sum_{a=0}^L \sum_{u=1}^{m_2} \sum_{v=1}^n W^*(s/F_6) x_{b,a,0,3,u,v} \end{aligned} \quad (12)$$

5 Expected number of interruptions during a single type I service

5.1 Distribution of duration of time till interruptions occur during a single type I service

Consider the Markov process, $\chi_1 = (N(t), J(t), K(t))$, where $N(t)$ denotes the number of interruptions upto time t , $J(t)$, status of the server (providing normal or interrupted service) and $K(t)$, the service phase at time t . The state space of the process is given by $\{(0, 2, k)/1 \leq k \leq m_1\} \cup \{(i, j, k)/i \geq 1, j = 0 \text{ or } 2, 1 \leq k \leq m_1\} \cup \{*_1\} \cup \{*_2\}$ where $*_1$ denotes the absorbing state indicating the service completion and $*_2$ denotes the absorbing state indicating the realization of protection. The infinitesimal generator of the process is given by

$$\mathcal{U} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & \dots \\ \delta e(m_1) & T^0 & T - (\gamma + \delta)I_{m_1} & \gamma I_{m_1} & 0 & 0 & 0 & \dots \\ \delta e(m_1) & \theta T^0 & 0 & \theta T - (\eta + \delta)I_{m_1} & \eta I_{m_1} & 0 & 0 & \dots \\ \delta e(m_1) & T^0 & 0 & 0 & T - (\gamma + \delta)I_{m_1} & \gamma I_{m_1} & 0 & \dots \\ \delta e(m_1) & \theta T^0 & 0 & 0 & 0 & \theta T - (\eta + \delta)I_{m_1} & \eta I_{m_1} & \dots \\ \dots & \dots & \dots & \dots & \dots & \dots & \dots & \dots \end{bmatrix}$$

5.2 Distribution of number of interruptions during a single type I service

Let y_k be the probability that the number of interruptions during a single type I service is k . Then y_k is the probability that the absorption occurs from the level k for the process χ_1 . Hence y_k are given by

$$y_0 = -\alpha(T - (\gamma + \delta)I)^{-1}(T^0 + \delta e)$$

and for $k = 1, 2, 3, \dots$

$$y_k = \alpha(T - (\gamma + \delta)I)^{-1}\gamma I((\theta T - (\eta + \delta)I)^{-1}\eta I(T - (\gamma + \delta)I)^{-1}\gamma I)^{k-1}(\theta T - (\eta + \delta)I)^{-1}((\theta T^0 + \delta e) - \eta I(T - (\gamma + \delta)I)^{-1}(T^0 + \delta e)) \quad (13)$$

Therefore, the expected number of interruptions during any particular type I customer service,

$$E(i) = \sum_{k=0}^{\infty} k y_k = \alpha(T - (\gamma + \delta)I)^{-1}\gamma I((I - (\theta T - (\eta + \delta)I)^{-1}\eta I(T - (\gamma + \delta)I)^{-1}\gamma I))^{-2}(\theta T - (\eta + \delta)I)^{-1}((\theta T^0 + \delta e) - \eta I(T - (\gamma + \delta)I)^{-1}(T^0 + \delta e)). \quad (14)$$

6 Expected number of interruptions during a single type II service

6.1 Distribution of duration of time till interruptions occur during a single type II service

Consider the Markov process, $\chi_2 = (N(t), J(t), K(t))$, where $N(t)$ denotes the number of

interruptions, $J(t)$, status of the server (providing normal or interrupted service) and $K(t)$, the service phase at time t . The state space of the process of the process is given by $\{(0,3,k)/1 \leq k \leq m_2\} \cup \{(i,j,k)/i \geq 1, j = 1 \text{ or } 3, 1 \leq k \leq m_2\} \cup \{*\}$ where $*$ denotes the absorbing state indicating the service completion. The infinitesimal generator of the process is given by

$$\mathcal{U} = \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & \dots \\ S^0 & S - \gamma I_{m_2} & \gamma I_{m_2} & 0 & 0 & 0 & \dots \\ \theta' S^0 & 0 & \theta' S - \eta I_{m_2} & \eta I_{m_2} & 0 & 0 & \dots \\ S^0 & 0 & 0 & S - \gamma I_{m_2} & \gamma I_{m_2} & 0 & \dots \\ \theta' S^0 & 0 & 0 & 0 & \theta' S - \eta I_{m_2} & \eta I_{m_2} & \dots \\ \dots & \dots & \dots & \dots & \dots & \dots & \dots \end{bmatrix}$$

6.2 Distribution of number of interruptions during a single type II service

Let z_k be the probabaility that the number of interruptions during a single type II service is k . Then z_k is the probabaility that the absorption occurs from the level k for the process χ_2 . Hence z_k are given by

$$z_0 = -\alpha(S - \gamma I)^{-1} S^0$$

and for $k = 1, 2, 3, \dots$

$$z_k = \alpha(S - \gamma I)^{-1} \gamma I ((\theta' S - \eta I)^{-1} \eta I (S - \gamma I)^{-1} \gamma I)^{k-1} (\theta' S - \eta I)^{-1} (\theta' S^0 - \eta I ((S - \gamma I)^{-1} S^0)) \quad (15)$$

Therefore, the expected number of interruptions during any particular type II customer service,

$$E(i) = \sum_{k=0}^{\infty} k z_k = \alpha(S - \gamma I)^{-1} \gamma I (I - (\theta' S - \eta I)^{-1} \eta I (S - \gamma I)^{-1} \gamma I)^{-2} (\theta' S - \eta I)^{-1} (\theta' S^0 - \eta I (S - \gamma I)^{-1} S^0). \quad (16)$$

7 Other Performance Measures

- The probability that the server is idle:

$$p_{idle} = \sum_{v=1}^n x_{0,v}.$$

- Mean number of type I customers in the system:

$$E_{nsh} = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n n_2 x_{n_1, n_2, 0, 0, u, v} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_2} \sum_{v=1}^n n_2 x_{n_1, n_2, 0, 1, u, v} + \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n n_2 x_{n_1, n_2, 0, 2, u, v} + \sum_{n_1=1}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_2} \sum_{v=1}^n n_2 x_{n_1, n_2, 0, 3, u, v} + \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n n_2 x_{n_1, n_2, 1, 2, u, v}$$

- Mean number of type II customers in the system:

$$E_{nsl} = \sum_{n_1=0}^{\infty} n_1 x_{n_1} \mathbf{e}$$

- The fraction of time during which the system is protected:

$$T_p = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1, n_2, 1, 2, u, v}$$

- The fraction of time the server is providing service to type I customers during WI:

$$T_{ih} = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1, n_2, 0, 0, u, v}$$

- The fraction of time the server is providing service to type II customers during WI:

$$T_{il} = \sum_{n_1=1}^{\infty} \sum_{n_2=0}^L \sum_{u=1}^{m_2} \sum_{v=1}^n x_{n_1, n_2, 0, 1, u, v}$$

- The fraction of time the server is providing service to type I customers in normal mode:

$$T_{nh} = \sum_{n_1=0}^{\infty} \sum_{n_2=1}^L \sum_{u=1}^{m_1} \sum_{v=1}^n x_{n_1, n_2, 0, 2, u, v}$$

- The fraction of time the server provides service to type II customers in normal mode:

$$T_{nl} = \sum_{n_1=1}^{\infty} \sum_{n_2=0}^L \sum_{u=1}^{m_2} \sum_{v=1}^n x_{n_1, n_2, 0, 3, u, v}$$

8 Analysis of a cost function

We construct a cost function based on the above performance measures.

Let

C_h : Holding cost for retaining a type I customer

C_l : Holding cost for retaining a type II customer

C_p : Unit time cost of providing service with protection

C_{ih} : Unit time cost of providing service when the server is providing service to type I customer in WI

C_{il} : Unit time cost of providing service when the server is providing service to type II customer in WI

C_{nh} : Unit time cost of providing service when the server is providing service to type I customer in normal mode

C_{nl} : Unit time cost of providing service when the server is providing service to type II customer in normal mode

Then the expected cost per unit time,

$$C = E_{nsh} \times C_h + E_{nsl} \times C_l + T_p \times \phi C_p + T_{ih} \times \theta C_{ih} + T_{il} \times \theta' C_{il} + T_{nh} \times C_{nh} + T_{nl} \times C_{nl}$$

9 Numerical Results

For the arrival process of type II customers, we consider the following two sets of matrices for D_0 and D_1 :

1. MAP with negative correlation (MNA)

$$D_0 = \begin{bmatrix} -0.8101 & 0.8101 & 0 \\ 0 & -1.3497 & 0 \\ 0 & 0 & -40.5065 \end{bmatrix}, D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.0810 & 0 & 1.2687 \\ 38.0761 & 0 & 2.4304 \end{bmatrix}$$

2. MAP with positive correlation (MPA)

$$D_0 = \begin{bmatrix} -0.8101 & 0.8101 & 0 \\ 0 & -1.3497 & 0 \\ 0 & 0 & -40.5065 \end{bmatrix}, D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 1.2687 & 0 & 0.0810 \\ 2.4304 & 0 & 38.0761 \end{bmatrix}$$

These two MAP processes are normalized so as to have an arrival rate of 1. The arrival process labeled MNA has correlated arrivals with correlation between two successive interarrival times given by -0.4211 and the arrival process corresponding to the one labelled MPA has a positive correlation with value 0.4211.

θ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.3493	1.2748	1.2194	1.1774	1.1450	1.1193	1.0985	1.0815	1.0672	1.0552
E_{nsl}	49.9733	19.8907	13.2051	10.3241	8.7368	7.7382	7.0548	6.5593	6.1843	5.8910
T_p	0.0334	0.0324	0.0318	0.0313	0.0308	0.0305	0.0302	0.0300	0.0298	0.0296
T_{ih}	0.1298	0.1104	0.0955	0.0838	0.0746	0.0672	0.0611	0.0559	0.0516	0.0479
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.3924	0.3988	0.4032	0.4063	0.4086	0.4103	0.4116	0.4126	0.4134	0.4141
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	33.7635	31.2805	30.9839	30.9648	31.0063	31.0595	31.1111	31.1575	31.1982	31.2335

Table 1: Effect of θ : Fix $L = 3, \theta' = 0.6, \lambda = 2, \eta = 0.5, \delta = 1, \gamma = 0.6$ and $\phi = 4$

Tables 1 to 6 contain the effect of different parameters on various performance measures and on the cost function when the arrival process of type II customer is MNA and tables 7 to 12 contain the effect of different parameters on various performance measures and on the cost function when the arrival process of type II customer is MPA.

Table 1 indicates the effect of the parameter θ on various performance measures and the cost function. As θ increases, type I customers get faster service during WI and hence E_{nsh} decreases. Then more number of type II customers also get service and hence E_{nsl} also decreases. T_p and T_{ih} also decreases since the expected number of type I customers during WI decreases. As θ increases, T_{il} and T_{nl} remains fixed due to the diminished effect of θ on type II customers and T_{nh} increases due to the fact that the system stays in WI serving type I customers for lesser time and hence it stays more in normal mode serving type I customers. As θ increases, the system cost first decreases, reach an optimal value(30.9648) corresponding to $\theta = 0.4$ and then increases.

ϕ	1	1.5	2	2.5	3	3.5	4	4.5	5
E_{nsh}	1.3572	1.1902	1.1112	1.0658	1.0366	1.0162	1.0013	0.9898	0.9808
E_{nsl}	1.1787×10^4	12.1182	7.6530	6.1872	5.4634	5.0334	4.7491	4.5473	4.3968
T_p	0.1581	0.1087	0.0826	0.0665	0.0557	0.0479	0.0420	0.0374	0.0337
T_{ih}	0.0482	0.0497	0.0504	0.0507	0.0509	0.0511	0.0512	0.0513	0.0513
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.3591	0.3702	0.3751	0.3778	0.3795	0.3806	0.3814	0.3820	0.3824
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	1.2112×10^3	34.4147	34.2737	34.2923	34.3216	34.3469	34.3673	34.3837	34.3969

Table 2: Effect of ϕ : Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \delta = 1.5$ and $\gamma = 0.6$

Table 2 indicates the effect of the parameter ϕ on various performance measures and the cost function. As ϕ increases, the type I customers in protected mode get faster service and hence

E_{nsh} decreases. As a result, E_{nsl} also decreases. As expected T_p also decreases. As ϕ increases, T_{ih} and T_{nh} increase since T_p decreases. T_{il} and T_{nl} remains unchanged since ϕ has only a small effect on low priority customers. As ϕ increases, the system cost first decreases, reach an optimal value(34.2737) corresponding to $\phi = 2$ and then increases.

δ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.3590	1.3225	1.2883	1.2562	1.2260	1.1975	1.1706	1.1452	1.1212	1.0985
E_{nsl}	1071.6	57.1361	29.5220	19.9883	15.1618	12.2491	10.3021	8.9100	7.8661	7.0548
T_p	0.0035	0.0069	0.0102	0.0133	0.0164	0.0193	0.0222	0.0250	0.0276	0.0302
T_{ih}	0.0865	0.0831	0.0798	0.0767	0.0737	0.0709	0.0683	0.0657	0.0633	0.0611
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.4750	0.4674	0.4599	0.4526	0.4454	0.4384	0.4315	0.4247	0.4181	0.4116
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	129.7496	29.2871	27.4764	27.4443	27.8543	28.4282	29.0719	29.7453	30.4286	31.1111

Table 3: Effect of δ : Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \gamma = 0.6$ and $\phi = 4$

Table 3 indicates the effect of the parameter δ on various performance measures and the cost function. As δ increases, protection clock realizes quickly and hence T_p increases, so T_{ih} and T_{nh} decreases. But T_{il} and T_{nl} remains unchanged since δ has only a small effect on low priority customers. In this case also, as δ increases, the system cost first decreases, reach an optimal value(27.4443) corresponding to $\delta = 0.4$ and then increases.

η	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.1161	1.1112	1.1067	1.1025	1.0985	1.0948	1.0913	1.0880	1.0848	1.0819
E_{nsl}	7.7025	7.5160	7.3475	7.1944	7.0548	6.9270	6.8096	6.7013	6.6012	6.5083
T_p	0.0302	0.0302	0.0302	0.0302	0.0302	0.0302	0.0302	0.0303	0.0303	0.0303
T_{ih}	0.0663	0.0649	0.0636	0.0623	0.0611	0.0599	0.0587	0.0576	0.0566	0.0555
T_{il}	0.0994	0.0958	0.0924	0.0893	0.0863	0.0836	0.0810	0.0785	0.0762	0.0740
T_{nh}	0.4058	0.4074	0.4089	0.4103	0.4116	0.4129	0.4141	0.4153	0.4165	0.4175
T_{nl}	0.3403	0.3425	0.3445	0.3464	0.3482	0.3499	0.3514	0.3529	0.3543	0.3556
C	31.6461	31.5012	31.3642	31.2344	31.1111	30.9939	30.8823	30.7759	30.6743	30.5772

Table 4: Effect of η : Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \gamma = 0.6$ and $\phi = 4$

Table 4 indicates the effect of the parameter η on various performance measures and the cost function. As η increases, the server turns to normal mode quickly. Hence T_{nh} and T_{nl} increase and E_{nsh}, E_{nsl}, T_{ih} and T_{il} decrease. η has only a very small effect on T_p . The cost function decreases as η increases.

θ'	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
E_{nsh}	1.3367	1.1562	1.0535	0.9894	0.9467	0.9166	0.8945	0.8779	0.8649
E_{nsl}	56.6142	10.2087	6.2053	4.7515	4.0095	3.5628	3.2658	3.0547	2.8973
T_p	0.0464	0.0490	0.0504	0.0512	0.0517	0.0521	0.0523	0.0525	0.0527
T_{ih}	0.0369	0.0389	0.0400	0.0406	0.0410	0.0413	0.0415	0.0417	0.0418
T_{il}	0.2089	0.1576	0.1260	0.1049	0.0898	0.0785	0.0697	0.0627	0.0569
T_{nh}	0.3182	0.3357	0.3452	0.3508	0.3544	0.3568	0.3586	0.3599	0.3608
T_{nl}	0.3791	0.3685	0.3622	0.3580	0.3551	0.3529	0.3512	0.3499	0.3488
C	38.4471	35.5315	36.0777	36.5074	36.8103	37.0278	37.1887	37.3113	37.4072

Table 5: Effect of θ' : Fix $L = 3, \theta = 0.7, \lambda = 2, \eta = 0.8, \delta = 2, \gamma = 0.6$ and $\phi = 4$

Table 5 indicates the effect of the parameter θ' on various performance measures and the cost function. As expected, T_{il} decreases and hence E_{nsl} and E_{nsh} decrease, T_{ih} , T_{nh} and T_p increase since type I customers have high priority. As a result, T_{nl} decreases. As θ' increases, the system cost first decreases, reach an optimal value(35.5315) corresponding to $\theta' = 0.2$ and then increases.

γ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	0.9997	1.0204	1.0407	1.0604	1.0797	1.0985	1.1169	1.1349	1.1525	1.1697
E_{nsl}	4.4562	4.8646	5.3190	5.8279	6.4019	7.0548	7.8046	8.6747	9.6973	10.9167
T_p	0.0301	0.0301	0.0302	0.0302	0.0302	0.0302	0.0302	0.0303	0.0303	0.0303
T_{ih}	0.0113	0.0220	0.0324	0.0423	0.0519	0.0611	0.0699	0.0784	0.0866	0.0945
T_{il}	0.0160	0.0313	0.0459	0.0599	0.0734	0.0863	0.0988	0.1107	0.1222	0.1333
T_{nh}	0.4586	0.4485	0.4378	0.4293	0.4203	0.4116	0.4033	0.3953	0.3876	0.3801
T_{nl}	0.3904	0.3812	0.3725	0.3640	0.3560	0.3482	0.3407	0.3336	0.3267	0.3200
C	27.0694	27.9294	28.7606	29.5661	30.3486	31.1111	31.8570	32.5901	33.3148	34.0369

Table 6: Effect of γ : Fix $L = 3, \theta = 0.7, \lambda = 2, \eta = 0.8, \delta = 2, \gamma = 0.6$ and $\phi = 4$

Table 6 indicates the effect of the parameter γ on various performance measures and the cost function. As γ increases, more interruptions occur during service and hence both E_{nsh} and E_{nsl} increases. T_p also increases in a slow rate. As γ increases T_{ih} and T_{il} increase and T_{nh} and T_{nl} decrease since the system stays more time in interruption mode. As γ increases, the cost function increases. Note the sharpness in decrease of the value of E_{nsl} is quite pronounced. However the trend is not seen in table 4 which gives the effect of η .

θ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.3471	1.2716	1.2167	1.1761	1.1451	1.1208	1.1013	1.0853	1.0721	1.0609
E_{nsl}	343.0679	141.3074	96.1158	76.4713	65.5556	58.6331	53.8616	50.3784	47.7263	45.6412
T_p	0.0334	0.0324	0.0318	0.0313	0.0308	0.0305	0.0302	0.0300	0.0298	0.0296
T_{ih}	0.1298	0.1104	0.0955	0.0838	0.0746	0.0672	0.0611	0.0559	0.0516	0.0479
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.3924	0.3988	0.4032	0.4063	0.4086	0.4103	0.4116	0.4126	0.4134	0.4141
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	63.0719	43.4206	39.2737	37.5789	36.6882	36.1497	35.7932	35.5413	35.3548	35.2114

Table 7: Effect of θ : Fix $L = 3, \theta' = 0.6, \lambda = 2, \eta = 0.5, \delta = 1, \gamma = 0.6$ and $\phi = 4$

Table 7 indicates the effect of the parameter θ on various performance measures and the cost function. In this case also E_{nsh} and E_{nsl} decreases as θ increases. But the values of E_{nsl} is much high when the arrival process of type II customer is MPA. All other values are same as in the case of MNA. But the cost function decreases as θ increases.

ϕ	1	1.5	2	2.5	3	3.5	4	4.5	5
E_{nsh}	1.3571	1.1900	1.1141	1.0711	1.0436	1.0244	1.0104	0.9996	0.9911
E_{nsl}	4.4374×10^4	90.9874	58.6062	47.8674	42.5211	39.3245	37.1995	35.6852	34.5516
T_p	0.1581	0.1087	0.0826	0.0665	0.0557	0.0479	0.0420	0.0374	0.0337
T_{ih}	0.0482	0.0497	0.0504	0.0507	0.0509	0.0511	0.0512	0.0513	0.0513
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.3591	0.3702	0.3751	0.3778	0.3795	0.3806	0.3814	0.3820	0.3824
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	4.4699×10^3	42.3015	39.3705	38.4629	38.0309	37.7801	37.6169	37.5023	37.4175

Table 8: Effect of ϕ : Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \delta = 1.5$ and $\gamma = 0.6$

Table 8 indicates the effect of the parameter ϕ on various performance measures and the cost function. Both E_{nsh} and E_{nsl} decrease as ϕ increases. The cost function and E_{nsl} decreases sharply as ϕ increases from 1 to 1.5. However, with further increase in ϕ value does not produce that decrease in values of cost function and E_{nsl} .

δ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.3589	1.3214	1.2867	1.2545	1.2244	1.1965	1.1703	1.1458	1.1229	1.1013
E_{nsl}	7.5197 $\times 10^3$	411.6330	214.5271	146.4434	111.9502	91.1156	77.1729	67.1914	59.6955	53.8616
T_p	0.0035	0.0069	0.0102	0.0133	0.0164	0.0193	0.0222	0.0250	0.0276	0.0302
T_{ih}	0.0865	0.0831	0.0798	0.0767	0.0737	0.0709	0.0683	0.0657	0.0633	0.0611
T_{il}	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863	0.0863
T_{nh}	0.4750	0.4674	0.4599	0.4526	0.4454	0.4384	0.4315	0.4247	0.4181	0.4116
T_{nl}	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482	0.3482
C	774.5584	64.7362	45.9761	40.0889	37.5324	36.3143	35.7588	35.5737	35.6123	35.7932

Table 9: Effect of δ : Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \gamma = 0.6$ and $\phi = 4$

Table 9 indicates the effect of the parameter δ on various performance measures and the cost function. Both E_{nsh} and E_{nsl} decrease as δ increases. In this case, as δ increases, the system cost first decreases, reaches an optimal value(35.5737) corresponding to $\delta = 0.8$ and then increases. Both E_{nsl} and the cost show sharp decrease in their values when δ moves from 0.1 to 0.2. Thereafter the decrease is not that pronounced.

η	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.1184	1.1136	1.1093	1.1051	1.1013	1.0976	1.0942	1.0910	1.0880	1.0851
E_{nsl}	58.6679	57.2868	56.0367	54.8999	53.8616	52.9096	52.0337	51.2250	50.4761	49.7807
T_p	0.0302	0.0302	0.0302	0.0302	0.0302	0.0302	0.0302	0.0303	0.0303	0.0303
T_{ih}	0.0663	0.0649	0.0636	0.0623	0.0611	0.0599	0.0587	0.0576	0.0566	0.0555
T_{il}	0.0994	0.0958	0.0924	0.0893	0.0863	0.0836	0.0810	0.0785	0.0762	0.0740
T_{nh}	0.4058	0.4074	0.4089	0.4103	0.4116	0.4129	0.4141	0.4153	0.4165	0.4175
T_{nl}	0.3403	0.3425	0.3445	0.3464	0.3482	0.3499	0.3514	0.3529	0.3543	0.3556
C	36.7438	36.4795	36.2344	36.0062	35.7932	35.5936	35.4062	35.2298	35.0634	34.9061

Table 10: Effect of η Fix $L = 3, \theta = 0.7, \theta' = 0.6, \lambda = 2, \eta = 0.5, \gamma = 0.6$ and $\phi = 4$

Table 10 indicates the effect of the parameter η on various performance measures and the cost function. Both E_{nsh} and E_{nsl} decrease as η increases. The cost function decreases as η increases.

θ'	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.3380	1.1630	1.0642	1.0027	0.9616	0.9325	0.9111	0.8947	0.8819	0.8716
E_{nsl}	417.6867	79.1550	49.1289	37.9315	32.0828	28.4903	26.0604	24.3078	22.9843	21.9498
T_p	0.0464	0.0490	0.0504	0.0512	0.0517	0.0521	0.0523	0.0525	0.0527	0.0528
T_{ih}	0.0369	0.0389	0.0400	0.0406	0.0410	0.0413	0.0415	0.0417	0.0418	0.0419
T_{il}	0.2089	0.1576	0.1260	0.1049	0.0898	0.0785	0.0697	0.0627	0.0569	0.0521
T_{nh}	0.3182	0.3357	0.3452	0.3508	0.3544	0.3568	0.3586	0.3599	0.3608	0.3616
T_{nl}	0.3791	0.3685	0.3622	0.3580	0.3551	0.3529	0.3512	0.3499	0.3488	0.3479
C	74.5558	42.4295	40.3754	39.8320	39.6251	39.5285	39.4764	39.4450	39.4244	39.4098

Table 11: Effect of θ' : Fix $L = 3, \theta = 0.7, \lambda = 2, \eta = 0.8, \delta = 2, \gamma = 0.6$ and $\phi = 4$

Table 11 indicates the effect of the parameter θ' on various performance measures and the cost function. Both E_{nsh} and E_{nsl} decrease as θ' increases. The cost function decreases as θ' increases, as it is to be expected. However, there is a sharp decrease in value of E_{nsl} when θ' moves from 0.1 to 0.2. For higher values of θ' , the initial sharpness in decrease is not seen.

γ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
E_{nsh}	1.0050	1.0251	1.0448	1.0640	1.0829	1.1013	1.1193	1.1369	1.1542	1.1711
E_{nsl}	34.0325	37.1338	40.5924	44.4740	48.8618	53.8616	59.6115	66.2942	74.1569	83.5428
T_p	0.0301	0.0301	0.0302	0.0302	0.0302	0.0302	0.0302	0.0303	0.0303	0.0303
T_{ih}	0.0113	0.0220	0.0324	0.0423	0.0519	0.0611	0.0699	0.0784	0.0866	0.0945
T_{il}	0.0160	0.0313	0.0459	0.0599	0.0734	0.0863	0.0988	0.1107	0.1222	0.1333
T_{nh}	0.4586	0.4485	0.4378	0.4293	0.4203	0.4116	0.4033	0.3953	0.3876	0.3801
T_{nl}	0.3904	0.3812	0.3725	0.3640	0.3560	0.3482	0.3407	0.3336	0.3267	0.3200
C	30.0298	31.1586	32.2900	33.4325	34.5961	35.7932	37.0389	38.3530	39.7616	41.3003

Table 12: Effect of γ : Fix $L = 3, \theta = 0.7, \lambda = 2, \eta = 0.8, \delta = 2, \gamma = 0.6$ and $\phi = 4$

Table 12 indicates the effect of the parameter γ on various performance measures and the cost function. Both E_{nsh} and E_{nsl} increase as γ increases. As expected, the cost increases as γ increases.

Conclusion

In this paper, we considered a (M,MAP)/(PH,PH)/1 queue with non preemptive priority, exponentially distributed working interruptions and protection. We analysed the distribution of service time of type I and type II customers and the distribution of a p-cycle. Also we provided LSTs of busy cycle, busy period of type I customers generated during the service time of a type II customer. For the waiting time distributions of type I and type II customers, we provided an analysis using LST and the matrix analytic method. We also performed some numerical experiments to evaluate some performance measures and also found optimal values using a cost function. Extension of the model discussed to multi-server is proposed to be taken up in a future study.

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Appendix

Proof of Theorem 1

Proof. Let B_{c_L} denote the length of the busy cycle generated by type I customers arriving during the service time of a type II customer, $\hat{B}_{c_L}(s)$ the LST of the length of the busy cycle and l the number of type I customers that arrive during service time of type II customer.

Then $B_{c_L} = X + B_L^1 + \dots + B_L^l$ where X denote the service time of the type II customer in service, B_L^j the busy period generated by j th type I customers that arrive during X , where $1 \leq j \leq l$.

$$\begin{aligned}\hat{B}_{c_L}(s) &= E(e^{-sB_{c_L}}) \\ &= \int_{x=0}^{\infty} E(e^{-sB_{c_L}}/X=x)P(x \leq X < x+dx) \\ &= \int_{x=0}^{\infty} \sum_{p=0}^{\infty} E(e^{-sB_{c_L}}/X=x, l=p)P(l=p/X=x)P(x \leq X < x+dx) \\ &= \int_{x=0}^{\infty} \sum_{p=0}^{\infty} E(e^{-sB_{c_L}}/X=x, l=p) \frac{e^{-\lambda x}(\lambda x)^p}{p!} \beta' e^{S_2 x} S_2^0 dx \\ &= \int_{x=0}^{\infty} e^{-(s+\lambda)x} \beta' e^{S_2 x} S_2^0 dx + \int_{x=0}^{\infty} \sum_{p=1}^{L-1} e^{-sx} \gamma_p (sI - T_1)^{-1} T_1^0 \frac{e^{-\lambda x}(\lambda x)^p}{p!} \beta' e^{S_2 x} S_2^0 dx \\ &\quad \beta' e^{S_2 x} S_2^0 dx + \int_{x=0}^{\infty} \sum_{p=L}^{\infty} e^{-sx} \gamma_L (sI - T_1)^{-1} T_1^0 \frac{e^{-\lambda x}(\lambda x)^p}{p!} \beta' e^{S_2 x} S_2^0 dx \\ &= \beta' [(s+\lambda)I - S_2]^{-1} S_2^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} T_1^0 \frac{\lambda^p \beta'}{p!} \int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - S_2]x} S_2^0 dx \\ &\quad + \sum_{p=L}^{\infty} \gamma_L (sI - T_1)^{-1} T_1^0 \frac{\lambda^p}{p!} \beta' \int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - S_2]x} S_2^0 dx\end{aligned}\tag{17}$$

We have,

$$\int_{x=0}^{\infty} x^p e^{-[(s+\lambda)I - S_2]x} dx = \frac{p!}{[(s+\lambda)I - S_2]^{p+1}}\tag{18}$$

Substituting (18) in (17), its third term

$$\begin{aligned}&= \sum_{p=L}^{\infty} \gamma_L (sI - T_1)^{-1} T_1^0 \lambda^p \beta' [(s+\lambda)I - S_2]^{-(p+1)} S_2^0 \\ &= \gamma_L (sI - T_1)^{-1} T_1^0 \beta' \sum_{p=L}^{\infty} [\lambda^{-1}[(s+\lambda)I - S_2]]^{-p} [(s+\lambda)I - S_2]^{-1} S_2^0 \\ &= \gamma_L (sI - T_1)^{-1} T_1^0 \beta' [\lambda^{-1}[(s+\lambda)I - S_2]]^{-L} \sum_{q=0}^{\infty} [\lambda^{-1}[(s+\lambda)I - S_2]]^{-q} \\ &\quad [(s+\lambda)I - S_2]^{-1} S_2^0 \\ &= \gamma_L (sI - T_1)^{-1} T_1^0 \beta' [\lambda^{-1}[(s+\lambda)I - S_2]]^{-L} [I - \lambda[(s+\lambda)I - S_2]^{-1}]^{-1} \\ &\quad [(s+\lambda)I - S_2]^{-1} S_2^0\end{aligned}\tag{19}$$

Substituting (19) in (17) gives

$$\begin{aligned}\hat{B}_{c_L}(s) &= \beta' [(s+\lambda)I - S_2]^{-1} S_2^0 + \sum_{p=1}^{L-1} \gamma_p (sI - T_1)^{-1} T_1^0 \lambda^p \beta' [(s+\lambda)I - S_2]^{-(p+1)} S_2^0 + \\ &\gamma_L (sI - T_1)^{-1} T_1^0 \beta' [\lambda^{-1}[(s+\lambda)I - S_2]]^{-L} [I - \lambda[(s+\lambda)I - S_2]^{-1}]^{-1} [(s+\lambda)I - S_2]^{-1} S_2^0\end{aligned}\tag{20}$$

Proof of theorem 2

Proof. Let B_L denote the length of the busy period generated by type I customers arriving during the service time of a type II customer, $\hat{B}_L(s)$ the LST of the length of the busy period and l the number of type I customers that arrive during service time of type II customer.

Then $B_L = B_L^1 + \dots + B_L^l$, where B_L^j denote the busy period generated by j th type I customers that arrive during X , where $1 \leq j \leq l$. Proceeding as in the above proof, we get the required result.



On a Queueing System with Processing of Service Items under Vacation and N-policy with Impatient Customers

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(Received March 2019 ; accepted April 2020)

Abstract: We assume that customers arrive at a single server queueing system according to Markovian Arrival process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level permitted is L . The inventory processing time follows phase type distribution. The server returns from vacation when there are N customers in the system. Service time follows two distinct phase type distributions depending on whether the processed item is available or not at service commencement epoch. Customers join the queue with probability p and balk with probability $1-p$. Also customers waiting for service become impatient and renege after a random time period which is exponentially distributed. We find the distribution of time until the number of customers hit N . Several system performance characteristics are computed. Also we computed Laplace Stieltjes Transform of the waiting time distribution for the case of no reneging. For the special case of no reneging, some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy are also discussed.

Keywords: Balking and reneging of customers, individual and social optimal strategy, L - and N - policies, Laplace-Stieltjes transform, Markovian arrival process, phase type service and inventory processing, server vacation to produce inventory, waiting time distribution.

1. Introduction

In vacation queueing systems, the server may not be available for a period of time due to working on some supplementary jobs, doing some maintenance work or server's failure that interrupt customer service or simply taking a break. Levy and Yechiali [11] introduced the concept of server vacation. Doshi [5] gives an excellent review of work done in vacation queueing models until 1985. Further developments could be accessed from Takagi [13] and Tian and Zhang [14].

In most of the work reported in queueing theory it is implicitly assumed that if the

server is ready to serve and the customers are available to receive service then the service process proceeds. Either availability of "additional" items required to provide service is not taken into consideration/ignored or its abundance is taken for granted. In the latter case the holding cost incurred is completely ignored. Sometimes the item(s) required for service may not be available. In such cases service cannot be provided even when the server is readily available and customer(s) are waiting. Typical example in medical case is operation theatre. In the absence of 'stent' for a heart patient in need of it, surgery cannot be performed. In a vehicle repair shop a vehicle requiring a specific part replacement, cannot be serviced if spares are not available.

(Expansions of various abbreviations are given in 'Notations and abbreviations' below the introduction.)

Thus in several service systems, availability of both customers and servers cannot guarantee service. This will be explained in detail later. This naturally leads to the investigation of availability of additional item(s) required to provide service. As a consequence some optimization problems arise— how much of additional item(s) to be processed at a stretch (optimal value of L) and what should be the optimal value of N ? This leads to the consideration of holding cost, shortage cost and associated revenue loss. Kazimirisky [10] seems to be the first to introduce 'additional items needed for service'. He considered a single server queue with Batch Markovian Arrival and General Service time distribution denoted as BMAP/G/1 queue, with the server engaged in producing additional items whenever the customers are not waiting. Exactly one processed item is required for each customer. The customer service time distribution depends on whether processed item is available or not. Thus there are two distinct service time distributions.

Baek *et al.* [2] considered MMAP (Marked Markovian Arrival Process) of customers of two types— type I (high priority) and type II (low priority). Both type of customers require a certain minimum number of additional items to start their service. Type I customers do not have a waiting space. If a type I customer is in service while another type I customer arrives, the latter leaves the system. On the other hand if a type II customer is in service, the former is pushed out of the system by the type I arrival provided the number of additional items available is at least equal to the minimum number required to start its service. Else, it leaves the system without changing the status. Type II customers have an infinite capacity waiting space. Additional items arrive to the system according to MAP. Dhanya *et al.* [3] extend the above to retrial queueing set up.

Hanukov *et al.* [8] analyze a simple queueing system where again additional items are needed for service of a customer (one item for each customer). Arrival process is Poisson and service time is exponentially distributed. Accumulation of N customers (N -policy) in queue to commence service, once server becomes idle, was introduced by Yadin and Naor [18]. This has the advantage that the length of a busy period becomes larger when server is

activated on accumulation of N or more customers, thereby bringing down the expected cost incurred per unit time. Divya *et al.* [4] considered single server queue in which the customers arrive according to Markovian Arrival Process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level permitted is L . The inventory processing time follows phase type distribution [12]. The server returns from vacation when there are N customers in the system. The service time follows two distinct phase type distributions depending on whether the processed item is available or not at service commencement epoch. They analysed the distribution of time until the number of customers hit N or the inventory level reaches L , distribution of idle time, the distribution of time until the number of customers hit N and also the distribution of number of inventory processed before the arrival of first customer. They also provided Laplace Stieltjes Transform of busy cycles in which no item is left in the inventory and atleast one item is left in the inventory. They performed some numerical computations to evaluate the expected idle time, standard deviation and coefficient of variation of idle time of the server.

In real life, people become impatient while waiting for service. Hence to model reality, we should take into consideration customers' impatience. To characterize customers' impatience, some terminologies like balking, reneging and retrials are employed in queueing system. Balking customers decide not to join the queue if it is too long and reneging customers leave the queue if they have waited too long for service. Retrial queues study systems where the customers do not wait in a line when server is found to be busy; instead they keep repeating their attempts to access the server at random time points (see Falin and Templeton [6], Artalejo and Gomaz-Corral [1]). Wang *et al.* [16] has presented a review on queueing systems with impatient customers.

Yechiali [19] considers an M/M/c queue, where $c = 1$, or $1 < c < \infty$, or $c = \infty$. At random epochs, the system as a whole gets shock and as a consequence it breaks down. This results in the loss of all customers who are either on the wait for service or undergoing service. When the system is down and undergoing repair process, newly arriving customers become impatient. Their impatience is reflected through a timer of random duration—each customer has his own timer. If a timer expires before the system is repaired, that customer abandons the queue. He analyzed the above described model and derived various quality of service measures such as mean sojourn time of a served customer; proportion of customers served; rate of lost customers due to disasters; and rate of abandonments due to impatience.

Wang and Zhang [17] consider a single-server service-inventory system where customers arrive according to a Poisson process and service times are independent and exponentially distributed. A customer takes exactly one item from the inventory upon service completion. A continuous review policy is adopted to replenish the inventory. With two different information levels, i.e. the fully unobservable case and the partially observable

case, arriving customers decide whether to join or to balk the system. They investigated customers individually optimal and socially optimal strategies, and further consider the optimal pricing issue that maximises the servers revenue. Some numerical experiments were carried out to show that the individually optimal joining probability (or threshold) is not always greater than that of socially optimal one. It was observed that, to maximise the servers revenue, concealing some system information from customers may be more profitable. Conversely, to maximise the social welfare, customers need more system information. Finally, numerical results in the fully unobservable case illustrate a reasonable phenomenon that the revenue maximum is equal to social optimum in most cases.

The present paper extends the work of Hanukov *et al.* [8] to a single server, Markovian Arrival, Phase Type distributed processing time and Phase Type distributed service time (Two distinct types—one when the processed item is available and the other when processed is not available) which we denote as MAP/(PH,PH)/1 queue under N -policy with requirement of one additional item to each customer. At service completion epoch, if the server becomes idle for want of customers, he starts processing additional items required to serve customers. A maximum of L items are processed at a stretch. However during this process, if number of customers in the system hits N , then the server immediately commences service. Processing time of items have Phase type distribution. Service time also follows Phase type distribution; however two distinct distributions are considered—one when an additional item is available to start service and the other when no additional item is available. This is already discussed in Divya *et al.* [4]. In this paper we extend that to the case where the customers are impatient. In addition we investigate the individual, social and system optimal strategies by introducing appropriate costs associated with certain system parameters.

Next we turn to further details of the present work. We consider a single server queueing system in which the customers arrive according to Markovian Arrival process. When the system is empty, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level is L . The inventory processing time follows phase type distribution. The server returns from vacation when there are N customers in the system. Service time follows two distinct phase type distributions according as there is no processed item or there are processed items at the beginning of service. Customers join the queue with probability p and balk with probability $1 - p$. Also the customers waiting for service may become impatient and renege after a random time period which is exponentially distributed. Whereas Wang and Zhang [17] follow replenishment policy through external sources in the context of queueing-inventory, we investigate the system in which the item is processed by the server himself. Further, in Wang and Zhang model, the server has to stay idle when inventory level drops to zero; in the present model the server processes the item and serves customer if at a service

commencement epoch the item is not available.

In all papers cited above that deal with additional items for service/inventory /processed item, it is assumed that service time duration for a customer in the presence of additional items are shorter than that starts in the absence of such an item. Thus the availability of such an item reduces the waiting time of customers in the system. This additional items are in some cases reusable. In this paper we consider the case of non-reusable items.

The rest of the paper is arranged as follows. The model description and mathematical formulation are given in section 2. Section 3 provides steady state analysis of the model, the distribution of time until the number of customers hit N and some other performance measures. For the special case of no reneging some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy are discussed in section 4. Section 5 contains a special case in which the system is working in normal mode.

Notations and abbreviations used in the sequel:

- $e(a)$ = Column vector of 1s' of order a .
- e'_a : Transpose of e_a .
- e = Column vector of 1s' of appropriate order.
- $CTMC$: Continuous time Markov chain.
- I_a = identity matrix of order a .
- $e_a(b)$ = column vector of order b with 1 in the a th position and the remaining entries zero.
- PH : Phase Type
- MAP : Markovian Arrival Process
- LST : Laplace-Steiltjes Transform
- $LDQBD$: Level Dependent Quasi-Birth and-Death
- $d_{ij}^{(k)}$: entries of D_k , $k = 0$ or 1
- δ_l : l^{th} entry of $D_1 e$.

Highlights of this paper are:

- Extends Hanukov *et al.* [8] to the case of Markovian arrival process. In addition, we set an upper bound (L) on the maximum number items processed by the server at a stretch. Further we introduce the N -policy for the number of customers to accumulate, in order to start a fresh service cycle.

- The inventory processing time and service time distributions follow more general distributions (phase type).
- Extends Divya *et.al.* [4] by introducing the two types of customer impatience: balking and reneging. Thus the system turns out to be a level dependent QBD.
- For the special case of no reneging, some numerical experiments to compute individual optimal strategy, maximum revenue to the server and social optimal strategy are also discussed.

2. Model Description and Mathematical formulation

We assume that the customers arrive at a single server queueing system according to MAP with representation (D_0, D_1) of order n . At the end of a service if the system is left with no customer, the server goes for vacation and produces inventory for future use during this period. The maximum inventory level is restricted to L . Processing time for each item in the inventory follows phase type distribution $PH(\alpha, T)$ of order m_1 . Server returns from vacation when there are N customers in the system. Service time follows $PH(\beta, S)$ of order m_2 when there is no processed item and it follows $PH(\gamma, U)$ of order m_3 when there are processed items. Customers join the queue with probability p and balk with probability $1 - p$. Also the customers waiting for service may become impatient and renege after a random time period which is exponentially distributed with parameter $(n-1)\phi$, $n \geq 1$, where n is the number of customers in the system.

Let $Q^* = D_0 + D_1$ be the generator matrix of arrival process and π^* be its stationary probability vector. Hence π^* is the unique (positive) probability vector satisfying

$$\pi^* Q^* = 0, \pi^* e = 1.$$

The constant $\beta^* = \pi^* D_1 e$, referred to as *fundamental rate*, gives the expected number of arrivals per unit of time in the stationary version of the MAP. It is assumed that arrival process is independent of the inventory processing and service process.

The model described in above can be studied as a level dependent quasi-birth-and-death (LDQBD) process. First we introduce the following notations:

At time t :

$N(t)$: the number of customers in the system at time t ,

$I(t)$: the number of processed inventory,

$$J(t) = \begin{cases} 0, & \text{when the server is on vacation,} \\ 1, & \text{when the server is busy serving a customer.} \end{cases}$$

$K(t)$: the phase of the inventory processing/service process,

$M(t)$: the phase of arrival of customer.

It is easy to verify that $\{(N(t), I(t), J(t), K(t), M(t)) : t \geq 0\}$ is a LDQBD with state space

$$l(0) = \{(0, i, 0, k_1, l) : 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \quad \{(0, L, 0, l) : 1 \leq l \leq n\}.$$

For $1 \leq h \leq N-1$,

$$l(h) = \{(h, i, 0, k_1, l) : 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \quad \{(h, L, 0, l) : 1 \leq l \leq n\} \\ \{(h, 0, 1, k_2, l) : 1 \leq k_2 \leq m_2, 1 \leq l \leq n\} \quad \{(h, i, 1, k_3, l) : 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n\}$$

and for $h \geq N$,

$$l(h) = \{(h, 0, 1, k_2, l) : 1 \leq k_2 \leq m_2, 1 \leq l \leq n\} \quad \{(h, i, 1, k_3, l) : 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n\}.$$

Note that when $0 \leq N(t) \leq N-1$ and $I(t) = L$, the server will be idle as the inventory level reaches its maximum level 'L' and the number of customers doesnot hit 'N'. So $K(t)$ neednot be considered.

The infinitesimal generator of this CTMC is

$$_1 = \begin{bmatrix} B_0 & C_0 & & & & & & & \\ B_1 & E_1 & I \otimes pD_1 & & & & & & \\ & B_2 & E_2 & I \otimes pD_1 & & & & & \\ & & & & B_{N-2} & E_{N-2} & I \otimes pD_1 & & \\ & & & & & B_{N-1} & E_{N-1} & F & \\ & & & & & & B'_N & A_1^{(N)} & A_0^{(N)} \\ & & & & & & & A_2^{(N+1)} & A_1^{(N+1)} & A_0^{(N+1)} \end{bmatrix}.$$

The boundary blocks B_0, C_0, B_1 are of orders $(Lm_1 + 1)n \times (Lm_1 + 1)n$, $(Lm_1 + 1)n \times ((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n)$, $((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n) \times (Lm_1 + 1)n$, respectively. For $2 \leq h \leq N-1$, B_h and for $1 \leq h \leq N-1$, E_h are square matrices of order $(m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n$. F and B'_N are of orders $((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n) \times (m_2 + Lm_3)n$ and $(m_2 + Lm_3)n \times ((m_1 + m_2)n + (L-1)(m_1 + m_3)n + (1 + m_3)n)$, respectively. For $h \geq N$, $A_0^{(h)}$, $A_1^{(h)}$ and for $h \geq N+1$, $A_2^{(h)}$ are square matrices of order $(m_2 + Lm_3)n$. Define the entries $B_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, $C_{0(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ and $B_{1(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$ as transition submatrices which contain transitions of the form $(0, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$, $(0, i_1, j_1, k_1, l_1) \rightarrow (1, i_2, j_2, k_2, l_2)$ and $(1, i_1, j_1, k_1, l_1) \rightarrow (0, i_2, j_2, k_2, l_2)$, respectively. Define $E_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, $B_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)}$, F and B'_N as transition

submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$, where $1 \leq h \leq N-1$, $(h, i_1, j_1, k_1, l_1) \rightarrow (h-1, i_2, j_2, k_2, l_2)$, where $2 \leq h \leq N-1$, $(N-1, i_1, j_1, k_1, l_1) \rightarrow (N, i_2, j_2, k_2, l_2)$ and $(N, i_1, j_1, k_1, l_1) \rightarrow (N-1, i_2, j_2, k_2, l_2)$, respectively. Define the entries $A_2^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$, $A_1^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$ and $A_0^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)}$ as transition submatrices which contain transitions of the form $(h, i_1, j_1, k_1, l_1) \rightarrow (h-1, i_2, j_2, k_2, l_2)$, where $h \geq N+1$, $(h, i_1, j_1, k_1, l_1) \rightarrow (h, i_2, j_2, k_2, l_2)$ and $(h, i_1, j_1, k_1, l_1) \rightarrow (h+1, i_2, j_2, k_2, l_2)$, where $h \geq N$, respectively. Since none or one event alone could take place in a short interval of time with positive probability, in general, a transition such as $(h_1, i_1, j_1, k_1, l_1) \rightarrow (h_2, i_2, j_2, k_2, l_2)$ has positive rate only for exactly one of h_1, i_1, j_1, k_1, l_1 different from h_2, i_2, j_2, k_2, l_2

$$B_0^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} T^0 \alpha \otimes I_n & i_2 = i_1 + 1, 0 \leq i_1 \leq L-2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n & i_1 = L-1, i_2 = L; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T \oplus \Delta & i_1 = i_2, 0 \leq i_1 \leq L-1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ \Delta & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \end{cases}$$

where

$$\Delta = D_0 + (1-p) \begin{bmatrix} \delta_1 & & \\ & \delta_2 & \\ & & \ddots \\ & & & \delta_n \end{bmatrix},$$

$$C_0^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} I_{m_1} \otimes pD_1, & 0 \leq i_1 \leq L-1; i_1 = i_2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ pD_1, & i_2 = i_1 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$B_1^{(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S^0 \alpha \otimes I_n, & i_1 = i_2 = 0; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq m_2, 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ U^0 \alpha \otimes I_n, & 1 \leq i_1 \leq L; i_2 = i_1 - 1; j_1 = 1, j_2 = 0; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_1; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $1 \leq h \leq N-1$,

$$E_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} T^0 \alpha \otimes I_n, & 0 \leq i_1 \leq L-2, i_2 = i_1 + 1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T^0 \otimes I_n, & i_1 = L-1, i_2 = L; j_1 = j_2 = 0; 1 \leq k_1 \leq m_1; 1 \leq l_1, l_2 \leq n \\ T \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2, 0 \leq i_1 \leq L-1; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2 = 0, j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ U \oplus \Delta - (h-1)\phi I_{m_1 n}, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_3, 1 \leq l_1, l_2 \leq n \\ \Delta - (h-1)\phi I_n, & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $2 \leq h \leq N-1$,

$$B_{h(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} (h-1)\phi I_{m_1 n}, & 0 \leq i_1 \leq L-1, i_1 = i_2; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_n, & i_1 = i_2 = L; j_1 = j_2 = 0; 1 \leq k_1, k_2 \leq m_1; 1 \leq l_1, l_2 \leq n \\ S^0 \beta \otimes I_n + (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_{m_3 n}, & 1 \leq i_1 \leq L, i_1 = i_2; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$F_{(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} e(m_1) \otimes (\beta \otimes pD_1), & i_1 = i_2 = 0; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_2} \otimes pD_1, & i_2 = i_1 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ I_{m_3} \otimes pD_1, & i_2 = i_1, 1 \leq i_1 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ e(m_1) \otimes (\gamma \otimes pD_1), & 1 \leq i_1 \leq L-1; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ \gamma \otimes pD_1, & i_1 = i_2 = L; j_1 = 0, j_2 = 1; 1 \leq k_1 \leq m_1, 1 \leq k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \end{cases}$$

$$B'_{N(i_1, j_1, k_1, l_1)}^{(i_2, j_2, k_2, l_2)} = \begin{cases} S^0 \beta \otimes I_n + (N-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & 2 \leq i_1 \leq L, i_2 = i_1 - 1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ (N-1)\phi I_{m_3 n}, & 1 \leq i_1 \leq L, i_2 = i_1; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $h \geq N+1$,

$$A_2^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S^0 \beta \otimes I_n + (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \beta \otimes I_n, & i_1 = 1, i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1 \leq m_3, 1 \leq k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ U^0 \gamma \otimes I_n, & i_2 = i_1 - 1, 2 \leq i_2 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n \\ (h-1)\phi I_{m_3 n}, & i_2 = i_1, 1 \leq i_2 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

For $h \geq N$,

$$A_1^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} S \oplus \Delta - (h-1)\phi I_{m_2 n}, & i_1 = i_2 = 0, j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_2, 1 \leq l_1, l_2 \leq n \\ U \oplus \Delta - (h-1)\phi I_{m_3 n}, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1, 1 \leq k_1, k_2 \leq m_3, 1 \leq l_1, l_2 \leq n. \end{cases}$$

$$A_0^{(h)(i_2, j_2, k_2, l_2)}_{(i_1, j_1, k_1, l_1)} = \begin{cases} I_{m_2} \otimes pD_1, & i_1 = i_2 = 0; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_2; 1 \leq l_1, l_2 \leq n \\ I_{m_3} \otimes pD_1, & i_1 = i_2, 1 \leq i_1 \leq L; j_1 = j_2 = 1; 1 \leq k_1, k_2 \leq m_3; 1 \leq l_1, l_2 \leq n. \end{cases}$$

Remarks: When $L = 0$ (that is, no item processed during vacation) the problem discussed reduces to classical N -policy.

3. Steady State Analysis

3.1. Stability condition

Lemma 3.1. *The system under consideration is stable.*

Proof. We use the following result to prove this(see Tweedie [15]).

Result (Tweedie [15]) Let $\{X(t)\}$ be a Markov process with discrete state space and rates of transition q_{sr} , $s, r \in S$, $\sum_r q_{sr} = 0$. Assume that there exist

1. a function $\psi(s)$, $s \in S$, which is bounded from below (this function is said to be a Lyapunov or test function);
2. a positive number ϵ such that:

- variables $y_s = \sum_{r \neq s} q_{sr} (\psi(r) - \psi(s)) < \infty$ for all $s \in S$;
- $y_s \leq -\epsilon$ for all $s \in S$ except perhaps a finite number of states.

Then the process $\{X(t)\}$ is regular and ergodic.

For the model under discussion, we consider the following test function:

$$\psi(s) = \psi(h, i, j, k, l) = h.$$

The mean drifts

$$\begin{aligned} y_s &= \sum_{r \neq s} q_{sr} (\psi(r) - \psi(s)) \\ &= q_{s,s+1} - q_{s,s-1}. \end{aligned} \tag{1}$$

We have $q_{s,s+1} = r_1$, say (a constant) and $q_{s,s-1} = r_2 + (s-1)\phi$, where r_2 is a constant. Hence from (1), $y_s = r_1 - r_2 - (s-1)\phi$, which depends only on the level s . Now,

$$\lim_{s \rightarrow \infty} y_s = -\infty.$$

Thus the assumptions of Tweedie's result hold and hence the Markov process under consideration is regular and ergodic. Hence the system is stable.

3.2. Steady state vector

By finite truncation method we get steady state vectors of the LDQBD approximately. In this method, we truncate the infinitesimal generator at a finite level K . The level K is chosen in such a way that probability of customer loss due to truncation is small. To get an appropriate level, say K_f , we start with an initial value for K and increasing it in unit steps until a properly chosen cut-off criterion is satisfied. Here, we use the algorithm by Artalejo *et al.* [1], the steps of which are explained below.

With K as cut-off level, the modified generator is

$$\bar{Q}(K) = \begin{bmatrix} B_0 & C_0 & & & & & & & & \\ B_1 & E_1 & I \otimes pD_1 & & & & & & & \\ & B_2 & E_2 & I \otimes pD_1 & & & & & & \\ & & & & & & & & & \\ & & & B_{N-2} & E_{N-2} & I \otimes pD_1 & & & & \\ & & & B_{N-1} & E_{N-1} & F & & & & \\ & & & & B'_N & A_1^{(N)} & A_0^{(N)} & & & \\ & & & & & A_2^{(N+1)} & A_1^{(N+1)} & A_0^{(N+1)} & & \\ & & & & & & & & & \\ & & & & & & A_2^{(K-1)} & A_1^{(K-1)} & A_0^{(K-1)} & \\ & & & & & & & A_2^{(K)} & \theta^{(K)} & \end{bmatrix},$$

where $\theta^{(K)} = A_1^{(K)} + A_0^{(K)}$. Let $\bar{\pi}$ be the stationary distribution of $\bar{Q}(K)$ which satisfies

$$\begin{aligned} \bar{\pi} \bar{Q}(K) &= 0, \\ \bar{\pi} e &= 1, \end{aligned} \quad (2)$$

where $\bar{\pi} = [\bar{\pi}(0), \bar{\pi}(1), \dots, \bar{\pi}(K)]$. Define $\mathbf{y} = [\mathbf{y}_0(K), \mathbf{y}_1(K)]$ with

$$\begin{aligned} \mathbf{y}_0(K) &= [\bar{\pi}(0), \bar{\pi}(1), \dots, \bar{\pi}(K-1)], \\ \mathbf{y}_1(K) &= \bar{\pi}(K). \end{aligned}$$

Now $\mathbf{y}(K, i) = \bar{\pi}(i)$, $0 \leq i \leq K$. Here $\mathbf{y}_0(K)$ is a row vector of dimension $m = (Lm_1 + 1)n + (N-1)[m_2n + L(m_1 + m_3)n + n] + (K-N)(m_2 + Lm_3)n$ and $\mathbf{y}_1(K)$ is a row vector of dimension $(m_2 + Lm_3)n$. Now from (2), we have

$$[\mathbf{y}_0(K), \mathbf{y}_1(K)] \begin{bmatrix} H_{00}(K) & H_{01}(K) \\ H_{10}(K) & H_{11}(K) \end{bmatrix} = [\mathbf{0}_m, \mathbf{0}_{(m_2 + Lm_3)n}], \quad (3)$$

where $H_{00}(K)$ is obtained from $\bar{Q}(K)$ by deleting the last column matrices and last row matrices. $H_{01}(K) = [0, 0, \dots, 0, A_0^{(K-1)}]^T$, $H_{10}(K) = [0, 0, \dots, 0, A_2^{(K)}]$ and $H_{11}(K) = \theta^{(K)}$. These are block structured matrices with $K \times K$, $K \times 1$, $1 \times K$ and 1×1 blocks respectively. $\mathbf{0}_m$ and $\mathbf{0}_{(m_2 + Lm_3)n}$ are row vectors of dimensions m and $(m_2 + Lm_3)n$, respectively, with all entries equal to zero. From (3), we get

$$\mathbf{y}_1(K) H_{10}(K) H_{00}^{-1}(K) = -\mathbf{y}_0(K), \quad (4)$$

$$\mathbf{y}_1(K)[H_{11}(K) - H_{10}(K)H_{00}^{-1}(K)H_{01}(K)] = \mathbf{0}_{(m_2+Lm_3)n}. \quad (5)$$

Also we have

$$H_{00}(K) = \begin{bmatrix} H_{00}(K-1) & H_{01}(K-1) \\ J_0(K-1) & J_1(K-1) \end{bmatrix},$$

where

$$\begin{aligned} J_0(K-1) &= [0, \quad, 0, A_2^{(K-1)}], \\ J_1(K-1) &= A_1^{(K-1)}. \end{aligned}$$

The inverse of matrix $H_{00}(K)$ can be determined using theorem 4.2.4 in Hunter [9] as

$$H_{00}^{-1}(K) = \begin{bmatrix} M_{00}(K) & M_{01}(K) \\ M_{10}(K) & M_{11}(K) \end{bmatrix},$$

where

$$\begin{aligned} M_{00}(K) &= [H_{00}(K-1) - H_{01}(K-1)J_1^{-1}(K-1)J_0(K-1)]^{-1}, \\ M_{01}(K) &= -J_1^{-1}(K-1)J_0(K-1)M_{00}(K), \\ M_{11}(K) &= [J_1(K-1) - J_0(K-1)H_{00}^{-1}(K-1)H_{01}(K-1)]^{-1}, \\ M_{10}(K) &= -H_{00}^{-1}(K-1)H_{01}(K-1)M_{11}(K). \end{aligned}$$

Now we can see that the structure of the block matrices $H_{01}(K-1)$ and $J_0(K-1)$ simplify the above set of equations. We have

$$H_{00}^{-1}(K-1)H_{01}(K-1) = \begin{bmatrix} M_{01}(K-1) \\ M_{11}(K-1) \end{bmatrix} A_0^{(K-2)}.$$

$$\text{Also } J_0(K-1)H_{00}^{-1}(K-1)H_{01}(K-1) = A_2^{(K-1)}M_{11}(K-1)A_0^{(K-2)}.$$

By example 4.2.2 (Hunter [9]), we have $(X + AYB)^{-1} = X^{-1} - X^{-1}A(Y^{-1} + BX^{-1}A)^{-1}BX^{-1}$.

Then we have

$$(X + AYB)^{-1} = [I - X^{-1}A(Y^{-1} + BX^{-1}A)^{-1}B]X^{-1}.$$

Here, we have $X = H_{00}(K-1)$, $A = -H_{01}(K-1)$, $Y = J_1^{-1}(K-1)$ and $B = J_0(K-1)$.

Finally, we get

$$\begin{aligned} M_{00}(K) &= [I - M_{01}(K)J_0(K-1)]H_{00}^{-1}(K-1), \\ M_{11}(K) &= [J_1(K-1) - A_2^{(K-1)}M_{11}(K-1)A_0^{(K-2)}]^{-1}, \\ M_{01}(K) &= -\begin{bmatrix} M_{01}(K-1) \\ M_{11}(K-1) \end{bmatrix} A_0^{(K-2)}M_{11}(K), \\ M_{10}(K) &= -J_1^{-1}(K-1)J_0(K-1)M_{00}(K). \end{aligned}$$

Thus the computation of the vector $\mathbf{y}_1(K)$ reduces to solving the system of equations (5) subject to the normalizing condition

$$\bar{\pi}(K)[\mathbf{e} - H_{10}(K)H_{00}^{-1}(K)\mathbf{e}] = 1.$$

The vector $\mathbf{y}_0(K)$ can be solved by substituting $\mathbf{y}_1(K)$ in (4). To get the cut-off value, successive increments of K are made, starting from $N+2$ and we stop at the point $K = K_c$ when

$$\max_{0 \leq i \leq K_c} \|\mathbf{y}(K_c, i) - \mathbf{y}(K_c - 1, i)\|_\infty < \epsilon,$$

where $\epsilon > 0$ is infinitesimal quantity and $\|\cdot\|_\infty$ is the infinity norm (see Goswami and Selavaraju [7]).

Now we suppose that $x_{h,i,j,k,l}$ represent the steady state probability that system is in state (h, i, j, k, l) .

3.3. Distribution of time until the number of customers hit N

We show that this is a phase type distribution where the underlying Markov process has state space

$$\{(h, i, j, k) : 0 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq j \leq m_1, 1 \leq k \leq n\}$$

$$\cup \{(h, L, k) : 0 \leq h \leq N-1, 1 \leq k \leq n\} \cup \{*\},$$

where $*$ denotes the absorbing state indicating the number of customers reaching N . The infinitesimal generator is

$$\mathbf{V}_1 = \begin{bmatrix} V_1 & V_1^{(0)} \\ \mathbf{0} & \mathbf{0} \end{bmatrix}, \text{ where}$$

$$\mathbf{V}_1 = \begin{bmatrix} H_0 & I_{Lm_1+1} \otimes pD_1 & & & \\ & H_0 & I_{Lm_1+1} \otimes pD_1 & & \\ & G_2 & H_2 & I_{Lm_1+1} \otimes pD_1 & \\ & \ddots & \ddots & \ddots & \\ & & G_{N-2} & H_{N-2} & I_{Lm_1+1} \otimes pD_1 \\ & & & G_{N-1} & H_{N-1} \end{bmatrix},$$

$$V_1^{(0)} = \begin{bmatrix} \mathbf{0} \\ \mathbf{0} \\ \mathbf{e}(Lm_1+1) \otimes p\delta \end{bmatrix}, \text{ with } H_0 = \begin{bmatrix} T \oplus \Delta & T^0 \alpha \otimes I_n & 0 & 0 \\ 0 & T \oplus \Delta & T^0 \alpha \otimes I_n & 0 \\ 0 & 0 & T \oplus \Delta & T^0 \otimes I_n \\ 0 & 0 & 0 & \Delta \end{bmatrix}.$$

For $2 \leq h \leq N-1$,

$$G_h = (h-1)\phi I_{(Lm_1+1)n}$$

and

$$H_h = \begin{bmatrix} T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \alpha \otimes I_n & 0 & 0 \\ 0 & T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \alpha \otimes I_n & 0 \\ 0 & 0 & T \oplus \Delta - (h-1)\phi I_{m_1n} & T^0 \otimes I_n \\ 0 & 0 & 0 & \Delta - (h-1)\phi I_{m_1n} \end{bmatrix}.$$

The initial probability vector is

$$\psi_1 = \left(\frac{1}{d_1}\right)(w_{0,0,1,1}, \dots, w_{0,0,1,n}, \dots, w_{0,0,m_1,1}, \dots, w_{0,0,m_1,n}, \dots, w_{0,L-1,m_1,1}, \dots, w_{0,L-1,m_1,n}, 0),$$

where

$$w_{0,0,k,l} = \sum_{k'=1}^{m_2} \frac{\sigma_{k'} \alpha_k}{-d_{ll}^{(0)} - S_{k'k'}} x_{1,0,1,k',l} + \sum_{k'=1}^{m_3} \frac{\tau_{k'} \alpha_k}{-d_{ll}^{(0)} - U_{k'k'}} x_{1,1,1,k',l},$$

$$w_{0,i,k,l} = \sum_{k'=1}^{m_3} \frac{\tau_{k'} \alpha_k}{-d_{ll}^{(0)} - U_{k'k'}} x_{1,i+1,1,k',l}, \quad \text{with } 1 \leq i \leq L-1,$$

and

$$d_1 = \sum_{l=1}^n \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} w_{0,i,k,l}, \text{ where } \mathbf{0} \text{ is a zero matrix of order } 1 \times ((N-1)Lm_1n + n).$$

Here, $\sigma_{k'}$ represents the absorption rate to phase k' from $PH(\beta, S)$, $\tau_{k'}$ represents the absorption rate to phase k' from $PH(\gamma, U)$, $S_{k'k'}$ represent the $k'k'$ th entry of S , $U_{k'k'}$ represent the $k'k'$ th entry of U , α_k denote the k th component of the initial distribution of inventory processing and $d_{ll}^{(0)}$ represent the diagonal entry in l th row of D_0 .

3.4. Some other Performance Measures

- Probability that the server is idle,

$$P_{idle} = \sum_{h=0}^{N-1} \sum_{l=1}^n x_{h,L,0,l}.$$

- The fraction of time the server is idle waiting for N th customer,

$$P_{id} = \sum_{l=1}^n x_{N-1,L,0,l}.$$

- The fraction of time the server is busy in inventory production,

$$P_{pro} = \sum_{h=0}^{N-1} \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n x_{h,i,0,k,l}.$$

- The fraction of time the server is busy serving,

$$P_{ser} = \sum_{h=1}^K \sum_{k=1}^{m_2} \sum_{l=1}^n x_{h,0,1,k,l} + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n x_{h,i,1,k,l}.$$

- Expected number of customers in the system,

$$\begin{aligned} E(S) = & \sum_{h=1}^{N-1} \sum_{i=0}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n h x_{h,i,0,k,l} + \sum_{h=1}^{N-1} \sum_{l=1}^n h x_{h,L,0,l} + \sum_{h=1}^K \sum_{k=1}^{m_2} \sum_{l=1}^n h x_{h,0,1,k,l} \\ & + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n h x_{h,i,1,k,l}. \end{aligned} \quad (6)$$

- Expected number of items in the inventory,

$$E(it) = \sum_{h=0}^{N-1} \sum_{i=1}^{L-1} \sum_{k=1}^{m_1} \sum_{l=1}^n i x_{h,i,0,k,l} + \sum_{h=0}^{N-1} \sum_{l=1}^n L x_{h,L,0,l} + \sum_{h=1}^K \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n i x_{h,i,1,k,l}. \quad (7)$$

- Expected rate at which the inventory processing is switched on,

$$E(ipo) = \sum_{k=1}^{m_2} \sum_{l=1}^n \sigma_k x_{1,0,1,k,l} + \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n \tau_k x_{1,i,1,k,l}.$$

4. Special Cases

1. $p = 1, \phi = 0$

In this case, the present model reduces to Divya *et al.* [4]. We see that the model can be studied as a LIQBD process.

2. $\phi = 0$

In this case also, the model can be studied as a LIQBD process with obvious modifications in Divya *et al.* [4].

From now on we concentrate in the case $\phi = 0$.

First, we find the LST of the waiting time distribution.

4.1. Waiting time analysis

To find the waiting time of a customer who joins for service at time t , we have to consider different possibilities depending on the status of server at that time. The server may be on vacation or in normal mode. Let $W(t)$ be the waiting time of a customer in the system who arrives at time t and $W^*(s)$ be the corresponding LST.

Case I. (Vacation mode)

Let Ev_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(h'+1, i', 0, k', l')$ or in the state $(h'+1, L, 0, l')$, where $0 \leq h' \leq N-2$, $0 \leq i' \leq L-1$, $1 \leq k' \leq m_1$, $1 \leq l' \leq n$.

In this case, the waiting time is the time until absorption in a Markov process whose state space is given by $\{(h, i, k_1, l) : 1 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n\} \cup \{(h, L, l) : 1 \leq h \leq N-1, 1 \leq l \leq n\} \cup \{(h^*, 0, k_2) : 1 \leq h^* \leq N-1, 1 \leq k_2 \leq m_2\} \cup \{(h^*, i, k_3) : 1 \leq h^* \leq N-1, 1 \leq i \leq L, 1 \leq k_3 \leq m_3\} \cup \{*\}$ where (h, i, k_1, l) denote the states that correspond to the server being on vacation with h customers in the system, i , items in inventory, k_1 , the processing phase and l , the arrival phase, (h, L, l) denote the state that correspond to the server being on vacation mode with h customers in the system, L items in inventory and l , the arrival phase. $(h^*, 0, k_2)$ denote the states that correspond to the tagged customer being in the position h^* when the server is on normal mode, k_2 , the service phase when there is no processed item, (h^*, i, k_3) denote the states that correspond to the tagged customer being in position h^* when the server is in normal mode with i processed items in the inventory and k_3 denote the service phase and $*$ denote the absorbing state indicating the service completion of the tagged customer. Thus the conditional waiting time can be studied by a phase type distribution with representation $PH(\psi_1, W_1)$ where

$$W_1 = \begin{bmatrix} M_{11} & M_{12} \\ 0 & M_{22} \end{bmatrix}, \quad W_1^0 = \begin{bmatrix} \mathbf{0} \\ M^0 \end{bmatrix},$$

where

$$M^0 = \begin{bmatrix} M^{00} \\ \mathbf{0} \end{bmatrix}, \quad \text{with} \quad M^{00} = \begin{bmatrix} S^0 \\ e(L) \otimes U^0 \end{bmatrix},$$

$$M_{11} = \begin{bmatrix} E & I_{Lm_1+1} \otimes D_1 \\ & E & I_{Lm_1+1} \otimes D_1 \\ & & E \end{bmatrix}, \quad \text{where}$$

$$E = \begin{bmatrix} T \oplus \Delta & T^0 \alpha \otimes I_n & & \\ & T \oplus \Delta & T^0 \alpha \otimes I_n & \\ & & T \oplus \Delta & T^0 \otimes I_n \\ & & & \Delta \end{bmatrix},$$

$M_{12} = e_{N-1}(N-1)e_{h_1}'(N-1) \otimes F$, where

$$F = \begin{bmatrix} e(m_1) \otimes (p\delta \otimes \beta) & & \\ & e(m_1) \otimes (p\delta \otimes \gamma) & \\ & & p\delta \otimes \gamma \end{bmatrix},$$

$$M_{22} = \begin{bmatrix} G & & \\ H & G & \\ & & H & G \end{bmatrix}, \quad \text{where } G = \begin{bmatrix} S & \\ & I_L \otimes U \end{bmatrix}, \quad H = \begin{bmatrix} S^0 \beta & 0 & 0 \\ U^0 \beta & 0 & 0 \\ 0 & I_{L-1} \otimes (U^0 \gamma) & 0 \end{bmatrix}.$$

Thus the conditional LST,

$$W^*(s | Ev_1) = \psi_1(sI - W_1)^{-1}W_1^0,$$

where ψ_1 is the initial probability vector which ensures that the Markov chain always starts from the level h .

Case II. (Normal mode)

Let Ev_2 denote the event that the tagged customer immediately after his joining finds the system in the state $(h'+1, 0, 1, k'', l')$, where $h' \geq 1, 1 \leq k'' \leq m_2, 1 \leq l' \leq n$ or in the state $(h'+1, i', 1, k''', l')$, where $1 \leq h' \leq N-1, 1 \leq i' \leq L-N+h', 1 \leq k''' \leq m_3, 1 \leq l' \leq n$ or in the state $(h'+1, i', 1, k''', l')$, where $h' \geq N, 1 \leq i' \leq L, 1 \leq k''' \leq m_3, 1 \leq l' \leq n$.

In this case, the waiting time is the time until absorption in a Markov process whose state space is given by $\{(h, 0, k) : 2 \leq h \leq K, 1 \leq k \leq m_2\} \cup \{(h, i, k) : 2 \leq h \leq N-1, 1 \leq i \leq L-N+h, 1 \leq k \leq m_3\} \cup \{(h, i, k) : N \leq h \leq K, 1 \leq i \leq L, 1 \leq k \leq m_3\} \cup \{*\}$ where $(h, 0, k)$ denote the states that correspond to the server being in normal mode with h customers in the system, service phase k when there is no processed item, (h, i, k) denote the states that correspond to the server being in normal mode with h customers in the system, service phase k when there are i processed items and $*$ denote the absorbing state indicating the service completion of the tagged customer and K is chosen in such a way that

$P\left(\sum_{h=0}^K x_h e > 1 - \epsilon\right) \rightarrow 0$ for every $\epsilon > 0$. Thus the conditional waiting time can be studied by a truncated phase type distribution with representation $Ph(\psi_2, W_2)$ where

$$W_2 = \begin{bmatrix} G_1 & & & & & \\ H_1 & G_2 & & & & \\ & & H_{N-2} & G_{N-1} & & \\ & & & H_{N-1} & G & \\ & & & & H & G \\ & & & & & H & G \end{bmatrix},$$

$$W_2^0 = \begin{bmatrix} E^0 \\ \mathbf{0} \\ \mathbf{0} \end{bmatrix}, \text{ where } E^0 = \begin{bmatrix} S^0 \\ e(L-N+1) \otimes U^0 \end{bmatrix}.$$

For $1 \leq h \leq N-1$,

$$G_h = \begin{bmatrix} S \\ I_{L-N+h} \otimes U \end{bmatrix}, \quad H_h = \begin{bmatrix} S^0 \beta & 0 \\ U^0 \beta & 0 \\ 0 & I_{L-N+h} \otimes U^0 \gamma \end{bmatrix},$$

$$E = \begin{bmatrix} S \\ I_L \otimes U \end{bmatrix}, \quad F = \begin{bmatrix} S^0 \beta & 0 & 0 \\ U^0 \beta & 0 & 0 \\ 0 & I_{L-1} \otimes U^0 \gamma & 0 \end{bmatrix}.$$

Thus the conditional LST,

$$W^*(s | Ev_2) = \psi_2 (sI - W_2)^{-1} W_2^0,$$

where ψ_2 is the initial probability vector which ensures that the Markov chain always starts from the level h .

Let $w_{h,i,j,k,l}$ and $w_{h,L,0,l}$ denote the probability that the tagged customer finds the system in the state (h,i,j,k,l) and $(h,L,0,l)$ respectively immediately after his arrival. Then

$$w_{h,i,0,k_1,l} = \sum_{l'=1}^n \frac{p d_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1 k_1}} x_{h-1,i,0,k_1,l'},$$

$$1 \leq h \leq N-1, 0 \leq i \leq L-1, 1 \leq k_1 \leq m_1, 1 \leq l \leq n$$

$$w_{h,L,0,l} = \sum_{l'=1}^n \frac{p d_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'}} x_{h-1,L,0,l'}, \quad 1 \leq h \leq N-1, 1 \leq l \leq n$$

$$w_{h,0,1,k_2,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2k_2}} x_{h-1,0,1,k_2,l'}, \quad 2 \leq h \leq N-1, \text{ or } h \geq N+1, \\ 1 \leq k_2 \leq m_2, 1 \leq l \leq n$$

$$w_{N,0,1,k_2,l} = \sum_{l'=1}^n \sum_{k_1=1}^{m_1} \frac{pd_{l'l}^{(1)} \beta_{k_2}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1k_1}} x_{N-1,0,0,k_1,l'} \\ + \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2k_2}} x_{N-1,0,1,k_2,l'}, \quad 1 \leq k_2 \leq m_2, 1 \leq l \leq n$$

$$w_{h,i,1,k_3,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{k_3k_3}} x_{h-1,i,1,k_3,l'}, \\ 2 \leq h \leq N-1, 1 \leq L-N+h-1, 1 \leq k_3 \leq m_3, 1 \leq l \leq n$$

$$w_{h,i,1,k_3,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{k_3k_3}} x_{h-1,i,1,k_3,l'}, \quad h \geq N+1, 1 \leq i \leq L, 1 \leq k_3 \leq m_3, 1 \leq l \leq n$$

$$w_{N,i,1,k_3,l} = \sum_{l'=1}^n \sum_{k_1=1}^{m_1} \frac{pd_{l'l}^{(1)} \gamma_{k_3}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - T_{k_1k_1}} x_{N-1,i,0,k_1,l'} \\ + \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{k_2k_2}} x_{N-1,i,1,k_2,l'}, \quad 1 \leq i \leq L, 1 \leq k_2 \leq m_2, 1 \leq l \leq n.$$

Thus we have the following theorem.

Theorem 4.1. *The LST of the waiting time is given by*

$$W^*(s) = \frac{1}{d_2} \left[\sum_{h'=1}^{N-1} \sum_{i'=0}^{L-1} \sum_{k'=1}^{m_1} \sum_{l'=1}^n \psi_1(sI - W_1)^{-1} W_1^0 w_{h',i',0,k',l'} + \sum_{h'=1}^{N-1} \sum_{l'=1}^n \psi_1(sI - W_1)^{-1} W_1^0 w_{h',L,0,l'} \right. \\ + \sum_{h'=1}^{\infty} \sum_{k''=1}^{m_2} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',0,1,k'',l'} + \sum_{h'=1}^N \sum_{i'=1}^{L-N+h'-1} \sum_{k'''=1}^{m_3} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',i',1,k''',l'} \\ \left. + \sum_{h'=N+1}^{\infty} \sum_{i'=1}^L \sum_{k'''=1}^{m_3} \sum_{l'=1}^n \psi_2(sI - W_2)^{-1} W_2^0 w_{h',i',1,k''',l'} \right], \quad (8)$$

where

$$d_2 = \sum_{h'=1}^{N-1} \sum_{i'=0}^{L-1} \sum_{k'=1}^{m_1} \sum_{l'=1}^n w_{h',i',0,k',l'} + \sum_{h'=1}^{N-1} \sum_{l'=1}^n w_{h',L,0,l'} + \sum_{h'=1}^{\infty} \sum_{k''=1}^{m_2} \sum_{l'=1}^n w_{h',0,1,k'',l'} \\ + \sum_{h'=1}^N \sum_{i'=1}^{L-N+h'-1} \sum_{k'''=1}^{m_3} \sum_{l'=1}^n w_{h',i',1,k''',l'} + \sum_{h'=N+1}^{\infty} \sum_{i'=1}^L \sum_{k'''=1}^{m_3} \sum_{l'=1}^n w_{h',i',1,k''',l'}. \quad (9)$$

Now, we assume that each customer receives a reward of R units after service completion and he has to pay a price q ($0 \leq q < R$) for an item. Let h_w denote the waiting

cost per unit time of a customer in the system.

4.2. Individual equilibrium strategy

Define

$$F_1(p) = R - q - h_w E(W).$$

We have to find an equilibrium strategy according to which the customers join the system.

4.3. Revenue maximization

We have to find an optimal price q to maximize the revenue of the server given by

$$F_2(q) = p_e q \pi^* D_1 e - h_1 E(S) - h_2 E(it) - cE(ipo),$$

where

h_1 : holding cost/unit customer/unit time,

h_2 : holding cost/unit item/unit time,

c : switching on cost of inventory processing/unit time,

p_e : Individual equilibrium strategy corresponding to q .

4.4. Social optimal strategy

Next we consider social optimal strategy. For a given price q and a joining probability p , the surplus of all customers S_1 and the server revenue S_2 are given by

$$S_1 = \pi^* p D_1 e (R - q - h_w E(W)) \text{ and } S_2 = p q \pi^* D_1 e - h_1 E(S) - h_2 E(it) - cE(ipo).$$

Therefore, the expected social welfare per unit time is,

$$F_3(p) = S_1 + S_2 = \pi^* p D_1 e (R - h_w E(W)) - h_1 E(S) - h_2 E(it) - cE(ipo).$$

4.5. Numerical results

$$\text{We fix } N = 3, L = 2, \alpha = \beta = \begin{bmatrix} 1 & 0 \end{bmatrix}, \gamma = \begin{bmatrix} 0.8 & 0.2 \end{bmatrix}, T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix},$$

$$S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}, U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

We find the individual optimum and social optimum corresponding to the above parameters.

Table 1. Effect of p on various performance measures, when $D_0 = (-20)$, $D_1 = (20)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.5045	1.0248	1.8794	0.6445	-10.2243	76.2876
0.2	0.2571	1.0514	1.7601	1.2429	2.1430	238.8498
0.3	0.1762	1.0806	1.6421	1.7921	6.1878	339.5609
0.4	0.1368	1.1129	1.5254	2.2882	8.1599	472.8811
0.5	0.1138	1.1486	1.4102	2.7271	9.3077	607.5573
0.6	0.0991	1.1881	1.2965	3.1041	10.0449	743.7425
0.7	0.0891	1.2316	1.1848	3.4149	10.5461	881.5515
0.8	0.0821	1.2794	1.0752	3.6551	10.8954	1021.0406
0.9	0.0773	1.3325	0.9680	3.8207	11.1356	1162.1853
1.0	0.0743	1.3925	0.8635	3.9081	11.2870	1304.8471

Table 2. Effect of p on various performance measures, when $D_0 = (-25)$, $D_1 = (25)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.4052	1.0312	1.8494	0.7985	-5.2584	108.9862
0.2	0.2084	1.0657	1.7009	1.5239	4.5807	273.3550
0.3	0.1446	1.1045	1.5545	2.1694	7.7701	439.4306
0.4	0.1138	1.1486	1.4102	2.7271	9.3077	607.5573
0.5	0.0962	1.1986	1.2684	3.1882	10.1881	778.0391
0.6	0.0853	1.2549	1.1297	3.5441	10.7364	951.0852
0.7	0.0783	1.3187	0.9946	3.7865	11.0843	1126.7485
0.8	0.0743	1.3925	0.8635	3.9081	11.2869	1304.8471
0.9	0.0728	1.4827	0.7371	3.9027	11.3611	1484.8406
1.0	0.0741	1.6035	0.6156	3.7651	11.2950	1665.6008

Table 3. Effect of p on various performance measures, when $D_0 = (-30)$, $D_1 = (30)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.3392	1.0378	1.8196	0.9496	-1.9586	141.7398
0.2	0.1762	1.0806	1.6421	1.7921	6.1878	339.5609
0.3	0.1240	1.1303	1.4676	2.5151	8.8023	540.0395
0.4	0.0991	1.1881	1.2965	3.1041	10.0449	743.7425
0.5	0.0853	1.2549	1.1297	3.5441	10.7365	951.0857
0.6	0.0773	1.3324	0.9680	3.8207	11.1356	1162.1853
0.7	0.0734	1.4260	0.8124	3.9215	11.3322	1376.6660
0.8	0.0732	1.5501	0.6636	3.8363	11.3401	1593.3114
0.9	0.0777	1.7424	0.5222	3.5570	11.1132	1809.3387
1.0	0.0898	2.1045	0.3886	3.0780	10.5080	2018.3028

In Tables 1,2 and 3, $E(W)$ denotes the expected waiting time of an arbitrary customer. We can see that the $E(W)$ decreases as p increases upto some p_1 (shown in bold letters) and after that it increases. This is due to the effect of N -policy. As p increases (upto p_1), the number of customers in the system hit N more fast so that the server stops processing of service items and start serving customers and hence $E(W)$ decreases. When p becomes p_1 , $E(W)$ starts increasing due to the diminished effect of N . Hence F_1 increases as p increases upto p_1 and after that it decreases. As we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers are served in a cycle. $E(ipo)$ increases upto p_1 , as p increases. This is due to the effect of N -policy. As p increases, the number of customers in the system hit N more rapidly and hence customers leave the system quickly sothat the server can switch on to processing at a faster rate. When p increases beyond p_1 , $E(ipo)$ decreases as p increases due to the diminished effect of N .

From Tables 1, 2 and 3, We can see that F_1 is strictly increasing on $[0, p_1]$ and strictly decreasing on $[p_1, 1]$. Thus,

1. If $F_1(p_1) \leq 0$, then $F_1(p) \leq 0$ for all $p \in [0, 1]$. In this case, the maximum benefit is negative which implies that customers do not join the system even if there is no customer in the system.
2. If $F_1(0) > 0$ and $F_1(1) > 0$, then $F_1(p) > 0$ for all $p \in [0, 1]$. In this case, customers prefer to join the system, because the minimal benefit is positive.
3. If $F_1(p_1) \geq 0$ and $F_1(0) < 0$, $\exists p_e \in [0, p_1]$ such that $F_1(p_e) = 0$.
4. If $F_1(p_1) \geq 0$ and $F_1(1) < 0$, $\exists p_e \in [p_1, 1]$ such that $F_1(p_e) = 0$.
5. If $F_1(p_1) \geq 0$, $F_1(0) < 0$ and $F_1(1) < 0$ then $\exists p_e \in [0, p_1]$ such that $F_1(p_e) = 0$ and $p'_e \in [p_1, 1]$ such that $F_1(p'_e) = 0$.

Hence, if, either of the cases 3,4 and 5 happen, then customers are indifferent between joining and balking the system. Suppose that, case 3 holds. Then the above discussions imply that when the joining probability p adopted by other customers is greater than p_e , the expected net benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p < p_e$ because then the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individual's best response is an increasing function of the strategy selected by other customers. Therefore, we expect a crowd situation in this case due to the effect of N -policy.

Next, suppose that, case 4 holds. Then the above discussions imply that when the joining probability p adopted by other customers is smaller than p_e , the expected net

benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p > p_e$ because then the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individual's best response is a decreasing function of the strategy selected by other customers. Therefore, we can avoid a crowd situation. This is due to the diminished effect of N -policy.

Next, suppose that case 5 holds, then the above discussions imply that when the joining probability p adopted by other customers is greater than p_e and less than p'_e , the expected net benefit of an arriving customer is positive provided he joins, thus the unique best response is 1. Conversely, the unique best response is 0 if $p < p_e$ or $p > p'_e$ because then the expected net benefit is negative. If $p = p_e$ or $p = p'_e$, every strategy is the best response since the expected net benefit is always 0.

Table 4. Individual optimum when $D_0 = (-20)$, $D_1 = (20)$.

$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-50.11	-60.11	-70.11	-80.11	-90.11	-100.11	-110.11	-120.11
0.1	39.78	29.78	19.78	9.78	-0.22	-10.22	-20.22	-30.22
0.2	52.14	42.14	32.14	22.14	12.14	2.14	-7.86	-17.86
0.3	56.19	46.19	36.19	26.19	16.19	6.19	-3.81	-13.81
0.4	58.16	48.16	38.16	28.16	18.16	8.16	-1.84	-11.84
0.5	59.31	49.31	39.31	29.31	19.31	9.31	-0.69	-10.69
0.6	60.04	50.04	40.04	30.04	20.04	10.04	0.04	-9.96
0.7	60.55	50.55	40.55	30.55	20.55	10.55	0.55	-9.45
0.8	60.90	50.90	40.90	30.90	20.90	10.90	0.90	-9.1
0.9	61.14	51.14	41.14	31.14	21.14	11.14	1.14	-8.86
1.0	61.29	51.29	41.29	31.29	21.29	11.29	1.29	-8.71

Table 5. Individual optimum when $D_0 = (-25)$, $D_1 = (25)$.

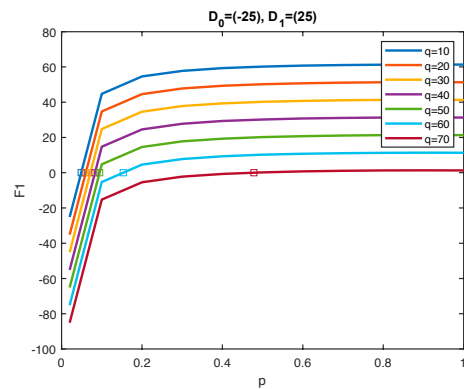
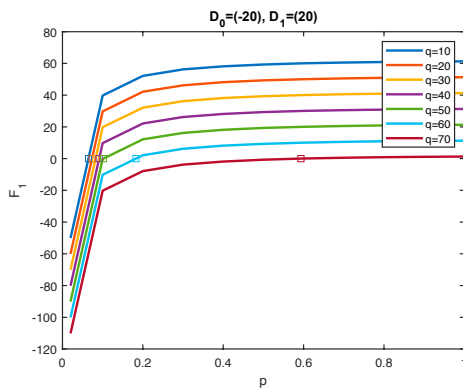
$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-25.12	-35.12	-45.12	-55.12	-65.12	-75.12	-85.12	-95.12
0.1	44.74	34.74	24.74	14.74	4.74	-5.26	-15.26	-25.26
0.2	54.58	44.58	34.58	24.58	14.58	4.58	-5.42	-15.42
0.3	57.77	47.77	37.77	27.77	17.77	7.77	-2.23	-12.23
0.4	59.31	49.31	39.31	29.31	19.31	9.31	-0.69	-10.69
0.5	60.19	50.19	40.19	30.19	20.19	10.19	0.19	-9.81
0.6	60.74	50.74	40.74	30.74	20.74	10.74	0.74	-9.26
0.7	61.08	51.08	41.08	31.08	21.08	11.08	1.08	-8.92
0.8	61.29	51.29	41.29	31.29	21.29	11.29	1.29	-8.71
0.9	61.36	51.36	41.36	31.36	21.36	11.36	1.36	-8.64
1.0	61.30	51.30	41.30	31.30	21.30	11.30	1.30	-8.70

Table 6. Individual optimum when $D_0 = (-30)$, $D_1 = (30)$.

$p \downarrow q \rightarrow$	10	20	30	40	50	60	70	75
0.02	-8.46	-18.46	-28.46	-38.46	-48.46	-58.46	-68.46	-78.46
0.1	48.04	38.04	28.04	18.04	8.04	-1.96	-11.96	-21.96
0.2	56.19	46.19	36.19	26.19	16.19	6.19	-3.81	-13.81
0.3	58.80	48.80	38.80	28.80	18.80	8.80	-1.20	-11.20
0.4	60.05	50.05	40.05	30.05	20.05	10.05	0.05	-9.95
0.5	60.74	50.74	40.74	30.74	20.74	10.74	0.74	-8.26
0.6	61.14	51.14	41.14	31.14	21.14	11.14	1.14	-8.86
0.7	61.33	51.33	41.33	31.33	21.33	11.33	1.33	-8.67
0.8	61.34	51.34	41.34	31.34	21.34	11.34	1.34	-8.86
0.9	61.12	51.12	41.12	31.12	21.12	11.12	1.12	-8.88
1.0	60.51	50.51	40.51	30.51	20.51	10.51	0.51	-9.49

From Tables 4,5 and 6, we get the values of F_1 corresponding to different values of p and q when the arrival rates are 20, 25 and 30 respectively.

In our experiment, $\exists$ a q_1 such that $F_1(p_1) \geq 0$, $F_1(0) < 0$, $F_1(1) \geq 0$ and $\exists$ exactly one equilibrium p_e in $(0, p_1]$ for all $q \in [0, q_1)$ where $0 < q_1 < R$ (in Table 6, $q_1 = 70.51$). Also p_e is strictly increasing for all q in $[0, q_1)$ (in Figure 1, $(p_e, 0)$ corresponding to different q 's are plotted using squares). This is due to the effect of N -policy. Also, $\exists$ a q_2 , where $q_1 \leq q_2 < R$ such that when $q_1 \leq q \leq q_2$, $\exists p_e \in [0, p_1]$ and $p'_e \in [p_1, 1]$ such that p_e is strictly increasing and p'_e is strictly decreasing in $[q_1, q_2]$ (in Table 6, $q_2 = 71.34$). This case is shown in Figure 2. When $q \in (q_2, R]$, $F_1(p) < 0$ for $p \in [0, 1]$ and there is no equilibrium probability. Hence, if q increases (up to q_1), more customers are supposed to join the queue, since the server can start service only if the number of customers in the system hit N . When q increases from q_2 to R , customers do not join the system since the maximum benefit is negative.



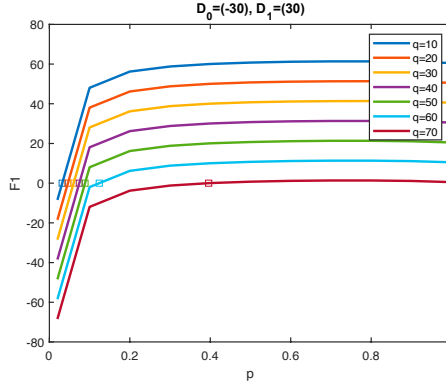


Figure 1. Effect of $q(< q_1)$ on individual equilibrium strategy.

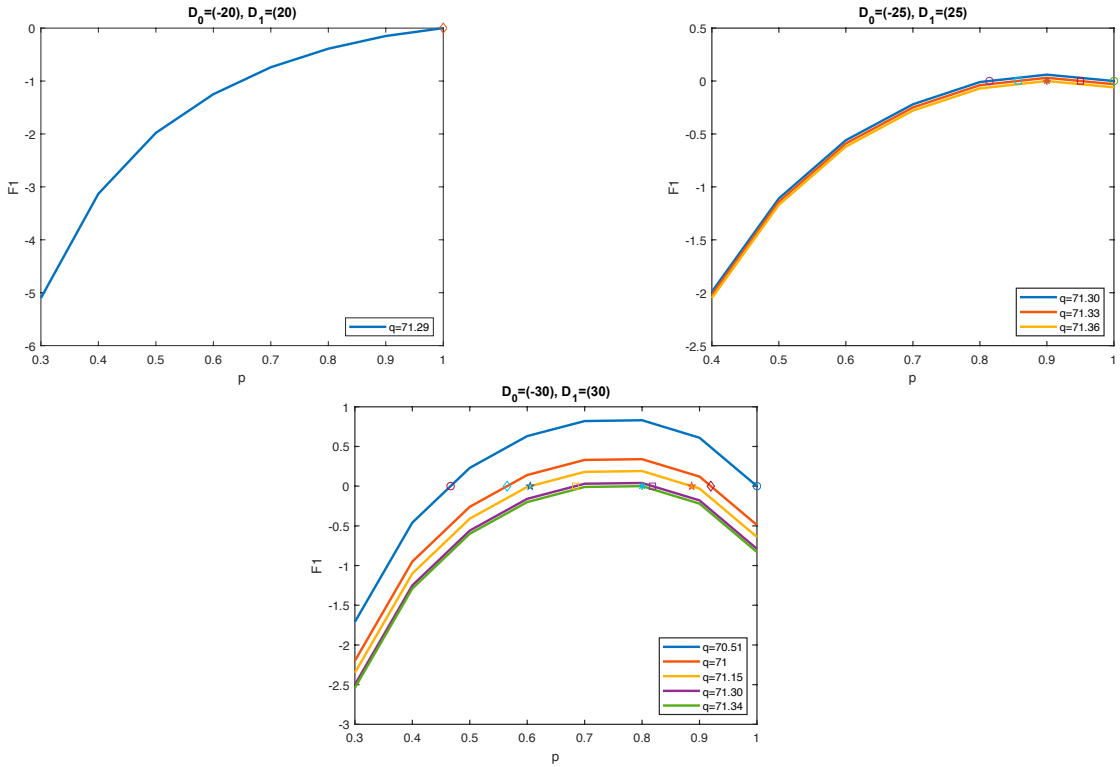


Figure 2. Effect of $q(q_1 \leq q \leq q_2)$ on individual equilibrium strategy.

Figure 1 shows individual equilibrium probabilities p_e as q varies ($0 < q < q_1$), corresponding to different arrival rates. We can see that p_e increases as q increases for the three different arrival rates. But p_e decreases as arrival rate increases. Figure 2 shows individual equilibrium probabilities p_e, p'_e as q varies ($q_1 \leq q \leq q_2$) corresponding to different arrival rates. We see that p_e increases and p'_e decreases as q increases and coincides when $q = q_2$ for three different arrival rates.

Tables 7 and 8 show the effect of q on revenue of the server. Here, we see that F_2

decreases as q increases upto q_1 . This happens because when q increases upto q_1 , p_e increases and hence the rate of hitting N becomes faster so that $E(ip_o)$ increases. But we see that when q increases in $[q_1, q_2]$, after a certain q -value, revenue function increases if p_e is the joining probability. This is due to the diminished effect of N -policy. Here, in all the cases, maximum revenue occur corresponding to $q = 10$ and the revenue decreases if a higher q is levied upto q_1 . But when q increases beyond q_1 , after a certain q -value, revenue increases if a higher q is levied.

Table 7. Revenue Maximization ($0 < q < q_1$).

q	$D_0 = (-20), D_1 = (20)$		$D_0 = (-25), D_1 = (25)$		$D_0 = (-30), D_1 = (30)$	
	p_e	F_2	p_e	F_2	p_e	F_2
10	0.0646	-15.31	0.0327	-11.22	0.0320	-12.45
20	0.0735	-16.82	0.0477	-14.45	0.0461	-16.08
30	0.0824	-18.32	0.0628	-17.67	0.0603	-19.67
40	0.0913	-19.82	0.0778	-20.81	0.0745	-23.20
50	0.1018	-21.56	0.0929	-23.92	0.0886	-26.65
60	0.1827	-34.50	0.1535	-35.91	0.1240	-35.01
70	0.5932	-84.23	0.4784	-84.63	0.3960	-84.29

Table 8. Revenue Maximization ($q_1 \leq q \leq q_2$).

D_0, D_1	q	p_e	F_2	p'_e	F_2
$(-20), (20)$	$71.29(q_1 = q_2)$	1	-100.89	1	-100.89
$(-25), (25)$	$71.30(q_1)$	0.8143	-100.76	1	-91.78
	71.33	0.8571	-99.87	0.9500	-95.52
	$71.36(q_2)$	0.9000	-98.28	0.9000	-98.28
$(-30), (30)$	$70.51(q_1)$	0.4667	-92.10	1	-66.92
	71	0.5650	-98.98	0.9197	-80.85
	71.15	0.6053	-100.39	0.8864	-85.567
	71.30	0.6842	-100.66	0.8182	-93.25
	$71.34(q_2)$	0.8000	-94.85	0.8000	-94.85

Again, from Tables 1,2 and 3, we can see that F_3 increases as p increases. But the rate of increase decreases as p increases. Here, the social optimum corresponds to $p = 1$ (p_s) in all cases.

Next, we vary values of N and L and see the effect of p on different performance measures. We fix $\alpha = \beta = [1 \ 0]$, $\gamma = [0.8 \ 0.2]$, $T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix}$, $S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}$,

$$U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

Table 9. Effect of p on different performances varying N and L .

(N, L)	p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
(3,2)	0.1	0.5045	1.0248	1.8794	0.6445	-10.2243
	0.2	0.2571	1.0514	1.7601	1.2429	2.1430
	0.3	0.1762	1.0806	1.6421	1.7921	6.1878
	0.4	0.1368	1.1129	1.5254	2.2882	8.1599
	0.5	0.1138	1.1486	1.4102	2.7271	9.3077
(3,3)	0.1	0.4888	1.0241	2.8168	0.6562	-9.4391
	0.2	0.2433	1.0487	2.6346	1.2652	2.8337
	0.3	0.1642	1.0746	2.4528	1.8402	6.7919
	0.4	0.1263	1.1028	2.2713	2.3687	8.6865
	0.5	0.1047	1.1341	2.0905	2.8433	9.7656
(3,4)	0.1	0.4880	1.0240	3.8110	0.6506	-9.4013
	0.2	0.2421	1.0481	3.6104	1.2686	2.8936
	0.3	0.1628	1.0728	3.3952	1.8514	6.8617
	0.4	0.1249	1.0988	3.1640	2.3942	8.7560
	0.5	0.1035	1.1271	2.9182	2.8892	9.8269
(4,3)	0.1	0.7456	1.5143	2.8244	0.4878	-22.2785
	0.2	0.3761	1.5217	2.6469	0.9567	-3.8066
	0.3	0.2556	1.5286	2.4667	1.4062	2.2207
	0.4	0.1968	1.5389	2.2847	1.8296	5.1614
	0.5	0.1624	1.5550	2.1023	2.2174	6.8805
(4,4)	0.1	0.7301	1.5151	3.7633	0.4919	-21.5035
	0.2	0.3626	1.5217	3.5220	0.9736	-3.1325
	0.3	0.2438	1.5282	3.2756	1.4414	2.8079
	0.4	0.1866	1.5391	3.0260	1.8842	5.6683
	0.5	0.1521	1.5585	3.6070	2.3134	7.3953
(5,4)	0.1	0.9844	2.0093	3.7704	0.3916	-34.2183
	0.2	0.4924	2.0047	3.5349	0.7743	-9.6201
	0.3	0.3324	1.9939	3.2917	1.1511	-1.6189
	0.4	0.2546	1.9837	3.0421	1.5163	2.2700
	0.5	0.2093	1.9793	2.7891	1.8599	4.5362
(5,5)	0.1	0.9689	2.011	4.7101	0.3947	-33.4434
	0.2	0.4792	2.0051	4.4102	0.7881	-8.9589
	0.3	0.3210	1.9937	4.0986	1.1806	-1.0519
	0.4	0.2450	1.9853	3.7787	1.5617	2.7517
	0.5	0.2013	1.9855	3.4557	1.9168	4.9373

From Table 9, we can see that when N increases (keeping L fixed), $E(W)$ increases. This happens because as N increases, the number of customers in the system hits N at a slower rate and thus the server switches to service mode at a slower rate. When L increases (keeping N fixed), $E(W)$ decreases. This is because when L increases, more customers get service according to $PH(\gamma, U)$ whose service rate is greater than that of $PH(\beta, S)$. As a result, when N increases (keeping L fixed) F_1 decreases and when L increases (keeping N fixed), F_1 increases.

When N increases (keeping L fixed), $E(S)$ increases as expected. As N increases (keeping L fixed), $E(it)$ increases since lesser number of customers are served in a cycle. When L increases (keeping N fixed), $E(it)$ increases, since more items are processed in a cycle. $E(ipo)$ decreases as N increases since the server switches to service mode at a slower rate and as a result to inventory processing at a slower rate.

Next, we consider the following two sets of matrices for D_0 and D_1 .

1. MAP with positive correlation (MPA)

$$D_0 = \begin{bmatrix} -27.0697 & 27.0697 & 0 \\ 0 & -45.0981 & 0 \\ 0 & 0 & -595.5331 \end{bmatrix}, \quad D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 42.3911 & 0 & 2.7070 \\ 189.4878 & 0 & 406.0453 \end{bmatrix}$$

2. MAP with negative correlation (MNA)

$$D_0 = \begin{bmatrix} -7.9382 & 7.9382 & 0 \\ 0 & -13.2250 & 0 \\ 0 & 0 & -404.0521 \end{bmatrix}, \quad D_1 = \begin{bmatrix} 0 & 0 & 0 \\ 0.7938 & 0 & 12.4311 \\ 119.0723 & 0 & 284.9797 \end{bmatrix}$$

These two MAP processes are normalized so as to have an arrival rate of 20. The arrival process labeled MNA has correlated arrivals with correlation between two successive interarrival times given by -0.1368 and the arrival process corresponding to the one labelled MPA has a positive correlation with value 0.1368.

We fix other parameters same as in the case of Poisson arrivals.

Table 10. Effect of p on various performance measures, when arrival follows MPA.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
0.1	0.0905	1.0695	1.7309	1.3096	10.4728
0.2	0.0931	1.1486	1.5323	2.1580	10.3428
0.3	0.1002	1.2185	1.3794	2.7405	9.9915
0.4	0.1060	1.2807	1.2585	3.1396	9.7006
0.5	0.1108	1.3371	1.1609	3.4116	9.4579

Table 11. Effect of p on various performance measures, when arrival follows MNA.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1
0.1	0.1657	0.7477	1.7519	0.9167	6.7171
0.2	0.1148	0.8068	1.5702	1.3605	9.2616
0.3	0.1116	0.9362	1.4262	1.7000	9.4198
0.4	0.1140	1.0727	1.3100	1.9477	9.2978
0.5	0.1181	1.2100	1.2148	2.1222	9.0960

From Table 10, we can see that the $E(W)$ increases as p increases, different from the case of Poisson arrivals. This is due to the effect of positive correlation. Hence, F_1 decreases as p increases. But in the case of MNA (Table 11), $E(W)$ decreases as p increases upto some p_1 (here $p_1 = 0.3$) and after that it increases, as in the case of Poisson arrivals and hence F_1 increases as p increases upto p_1 and after that it decreases. In both cases, as we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers are served in a cycle. $E(ipo)$ increases as p increases. This is because as p increases, the number of customers in the system hit N more rapidly and hence customers leave the system quickly so that the server can switch on to processing at a faster rate.

5. Special Case: The System in Normal Mode

5.1. Waiting time analysis

To find the waiting time of a customer who joins for service at an epoch in the long run, we have to consider different possibilities depending on the status of server at that time. Let Ev denote the event the system is working in normal mode. Let $W(t|Ev)$ be the conditional waiting time of a customer who arrives at time t and $W^*(s|Ev)$ be the corresponding conditional LST.

Let $w_{h,i,j,k,l}$ denote the probability that the tagged customer finds the system in the state (h,i,j,k,l) immediately after his arrival when the system is in normal mode.

Then

$$w_{h,0,1,k,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{h-1,0,1,k,l'}, \quad 2 \leq h \leq N-1, \text{ or } h \geq N+1, 1 \leq k \leq m_2, \\ 1 \leq l \leq n.$$

$$w_{N,0,1,k,l} = \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{N-1,0,1,k,l'}, \quad 1 \leq k \leq m_2, 1 \leq l \leq n.$$

$$\begin{aligned}
 w_{h,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{kk}} x_{h-1,i,1,k,l'}, \quad 2 \leq h \leq N-1, 1 \leq L-N+h-1, \\
 &\quad 1 \leq k \leq m_3, 1 \leq l \leq n. \\
 w_{h,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - U_{kk}} x_{h-1,i,1,k,l'}, \quad h \geq N+1, 1 \leq i \leq L, 1 \leq k \leq m_3, \\
 &\quad 1 \leq l \leq n. \\
 w_{N,i,1,k,l} &= \sum_{l'=1}^n \frac{pd_{l'l}^{(1)}}{-d_{l'l'}^{(0)} - (1-p)\delta_{l'} - S_{kk}} x_{N-1,i,1,k,l'}, \quad 1 \leq i \leq L, 1 \leq k_2 \leq m_2, 1 \leq l \leq n.
 \end{aligned}$$

Case I: $L \leq N$

Case (1)

Let Ev_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, 0, 1, k, l)$, where $r \geq 1, 1 \leq k \leq m_2, 1 \leq l \leq n$. In this case, processed item is not available to any customer. Thus waiting time is the sum of residual service time and r service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_1) = e'_u(sI - S)^{-1} S^0 (\beta(sI - S)^{-1} S^0)^r.$$

Case (2)

Let Ev_2 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $1 \leq r \leq N-1, 1 \leq i \leq L-N+r, 1 \leq k \leq m_3, 1 \leq l \leq n$. In this case, processed item is available to i customers. Thus waiting time is the sum of residual service time and $i-1$ service time each following $PH(\gamma, U)$ and $r+1-i$ service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (3)

Let Ev_3 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $r \geq N, 1 \leq i \leq L, 1 \leq k \leq m_3, 1 \leq l \leq n$. In this case, processed item is available to i customers. Thus waiting time is the sum of residual service time and $i-1$ service time each following $PH(\gamma, U)$ and $r+1-i$ service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Ev_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Thus the conditional LST of the waiting time,

$$\begin{aligned}
 W^*(s | Ev) &= \frac{1}{d_3} \left[\sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n W^*(s | Ev, Ev_1) w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1} \sum_{i=1}^{L-N+r} \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Ev_2) w_{r+1,i,1,k,l} \right. \\
 &\quad \left. + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Ev_3) w_{r+1,i,1,k,l} \right], \tag{10}
 \end{aligned}$$

where

$$d_3 = \sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1} \sum_{i=1}^{L-N+r} \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l} + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l}. \quad (11)$$

Case II: $L > N$

Case (1)

Let Fv_1 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, 0, 1, k, l)$, where $r \geq 1, 1 \leq k \leq m_2, 1 \leq l \leq n$. In this case, processed item is not available to any customer. Thus waiting time is the sum of residual service time and r service time each following $PH(\beta, S)$.

$$W^*(s | Ev, Fv_1) = e'_u(sI - S)^{-1} S^0 (\beta(sI - S)^{-1} S^0)^r.$$

Case (2)

Let Fv_2 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $1 \leq r \leq N-1, 1 \leq i \leq L-N+r, 1 \leq k \leq m_3, 1 \leq l \leq n$.

Case (i), $1 \leq i < r+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (ii), $r+1 \leq i \leq L-N+r$

In this case, processed item is available to all the $r+1$ customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_2) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^r.$$

Case (3)

Let Fv_3 denote the event that the tagged customer immediately after his arrival finds the system in the state $(r+1, i, 1, k, l)$, where $r \geq N, 1 \leq i \leq L, 1 \leq k \leq m_3, 1 \leq l \leq n$.

Case (i), $N \leq r \leq L$

Case (a), $1 \leq i < r+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Case (b), $r+1 \leq i \leq L$

In this case, processed item is available to all the $r+1$ customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^r.$$

Case (ii), $r \geq L+1$

In this case, processed item is available to i customers. Thus the conditional LST,

$$W^*(s | Ev, Fv_3) = e'_u(sI - U)^{-1} U^0 (\gamma(sI - U)^{-1} U^0)^{i-1} (\beta(sI - S)^{-1} S^0)^{r+1-i}.$$

Thus the conditional LST of the waiting time,

$$W^*(s | Ev) = \frac{1}{d_4} \left[\sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n W^*(s | Ev, Fv_1) w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1L-N+r} \sum_{i=1}^{m_3} \sum_{k=1}^n W^*(s | Ev, Fv_2) w_{r+1,i,1,k,l} \right. \\ \left. + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n W^*(s | Ev, Fv_3) w_{r+1,i,1,k,l} \right], \quad (12)$$

where

$$d_4 = \sum_{r=1}^{\infty} \sum_{k=1}^{m_2} \sum_{l=1}^n w_{r+1,0,1,k,l} + \sum_{r=1}^{N-1L-N+r} \sum_{i=1}^{m_3} \sum_{k=1}^n w_{r+1,i,1,k,l} + \sum_{r=N}^{\infty} \sum_{i=1}^L \sum_{k=1}^{m_3} \sum_{l=1}^n w_{r+1,i,1,k,l}. \quad (13)$$

$$\text{We fix } N = 3, L = 2, \alpha = \beta = \begin{bmatrix} 1 & 0 \end{bmatrix}, \gamma = \begin{bmatrix} 0.8 & 0.2 \end{bmatrix}, T = \begin{bmatrix} -50 & 50 \\ 0 & -50 \end{bmatrix},$$

$$S = \begin{bmatrix} -80 & 80 \\ 0 & -80 \end{bmatrix}, U = \begin{bmatrix} -150 & 150 \\ 0 & -150 \end{bmatrix}, R = 75, q = 60, h_w = 50, h_1 = 2, h_2 = 1, c = 30.$$

Table 12. Effect of p on various performance measures, when $D_0 = (-20)$, $D_1 = (20)$.

p	$E(W)$	$E(S)$	$E(it)$	$E(ipo)$	F_1	F_3
0.1	0.0485	1.5214	0.6972	19.3813	12.5747	-440.0284
0.2	0.0499	1.5604	0.6564	18.3266	12.5034	-263.5605
0.3	0.0512	1.6081	0.6117	17.2386	12.4386	-86.3546
0.4	0.0524	1.6657	0.5640	16.1240	12.3798	91.4217
0.5	0.0535	1.7341	0.5145	14.9939	12.3259	269.4597
0.6	0.0545	1.8144	0.4645	13.8595	12.2749	447.4200
0.7	0.0555	1.9081	0.4153	12.7308	12.2237	624.9757
0.8	0.0566	2.0174	0.3677	11.6154	12.1681	801.8239
0.9	0.0579	2.1455	0.3224	10.5189	12.1027	977.6669
1.0	0.0596	2.2971	0.2796	9.4448	12.0200	1152.1810

From Table 12, we see that $E(W)$ increases as p increases. This happens since when the system is working in normal mode, the number of customers accumulating in the system increases with increasing value of p . As p increases F_1 decreases consequent to increase in $E(W)$. As we expect, $E(S)$ increases as p increases. As p increases, $E(it)$ decreases, since larger number of customers get served in a cycle. $E(ipo)$ decreases as p increases. This happens due to the fact that when p increases more customers accumulate in the system and hence customers leave the system slowly so that the server switch on to processing at a slower rate. Also from table 12, we see that F_3 increases as p increases. Thus the social optimum corresponds to $p = 1$.

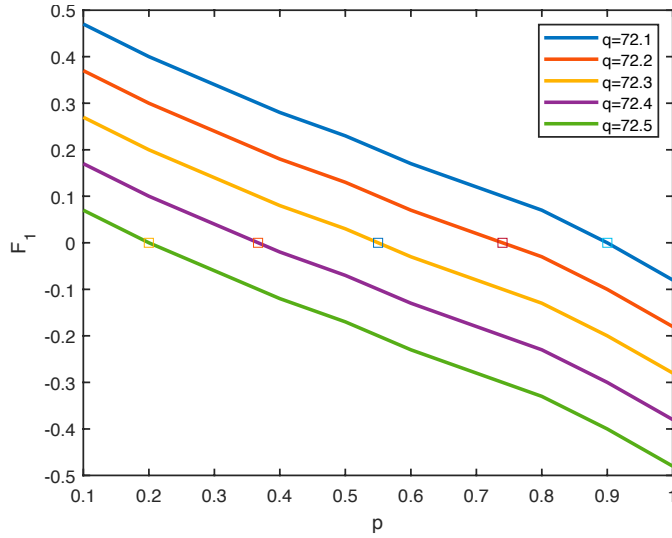


Figure 3. Effect of q on individual equilibrium strategy when $D_0 = (-20), D_1 = (20)$.

Here when $q < 72.02$, the expected net benefit is always positive. When q increases beyond 72.02, (see Table 12), we can find a $p_e \in [0,1]$ such that $F_1(p_e) = 0$ and p_e is decreasing (see Figure 3). Here when the joining probability p adopted by other customers is smaller than p_e , the expected net benefit of an arriving customer is positive provided he joins. Thus the unique best response is 1. Conversely, the unique best response is 0 if $p > p_e$ since, the expected net benefit is negative. If $p = p_e$, every strategy is the best response since the expected net benefit is always 0. This behaviour illustrates a situation that an individual's best response is a decreasing function of the strategy selected by other customers. Therefore, we can avoid a crowd situation.

Table 13. Effect of q on Revenue function.

q	p_e	F_2
72.1	0.9000	-302.1809
72.2	0.7400	-357.9769
72.3	0.5500	-425.8322
72.4	0.3667	-491.4621
72.5	0.2000	-549.3741

Also, in this case revenue function F_2 decreases as q increases. This happens due to the fact that as q increases, the equilibrium probability p_e decreases and hence $E(ip_o)$ increases (see Table 13).

6. Conclusion

In this paper, we considered a MAP/PH/1 queue with processing of service items under Vacation and N-policy with impatient customers. We studied this system as a Level Dependent QBD. We explained how to find the steady state vectors of the system approximately by finite truncation method. We found the distribution of time until the number of customers hit N . Several system performance characteristics were computed. We computed LST of the waiting time distribution for the case of no reneging. Also we performed some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy for the special case of no reneging. We discussed the special case in which the system is in normal mode. In this case also, we computed LST of the waiting time distribution and performed some numerical experiments for computing individual optimal strategy, maximum revenue to the server and social optimal strategy.

Acknowledgement

This research is supported by the Russian Science Foundation and the Department of Science and Technology (India) via grant No. 16-49-02021 for the joint research project by the V. A. Trapeznikov Institute of Control Science and the CMS College Kottayam.

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Spectra of a New Join in Duplication Graph

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Abstract: The *duplication graph* $D_G(G)$ of a graph G is obtained by inserting new vertices corresponding to each vertex of G and making the vertex adjacent to the neighborhood of the corresponding vertex of G and deleting the edges of G . Let G_1 and G_2 be two graph with vertex sets $V(G_1)$ and $V(G_2)$ respectively. The D_G -vertex join of G_1 and G_2 is denoted by $G_1 \sqcup G_2$ and it is the graph obtained from $D_G(G_1)$ and G_2 by joining every vertex of $V(G_1)$ to every vertex of $V(G_2)$. The DG-add vertex join of G_1 and G_2 is denoted by $G_1 \bowtie G_2$ and is the graph obtained from $D_G(G_1)$ and G_2 by joining every additional vertex of $D_G(G_1)$ to every vertex of $V(G_2)$. In this paper we determine the A-spectra and L-spectra of the two new joins of graphs G_1 and G_2 when G_1 is a regular graph and G_2 is an arbitrary graph. As an application we give the number of spanning tree, the Kirchhoff index and Laplace energy like invariant of the new join. Also we obtain some infinite family of new class of integral graphs.

Key Words: Spectrum, cospectral graphs, Join of graphs, spanning tree, Kirchhoff index, Laplace-energy like invariant.

AMS(2010): 05C50.

§1. Introduction

All graphs described in this paper are simple and undirected. Let G be a graph with vertex set $V(G) = \{v_1, v_2, \dots, v_n\}$. The adjacency matrix of G , denoted by $A(G) = (a_{ij})_{n \times n}$ is an $n \times n$ symmetric matrix with

$$a_{ij} = \begin{cases} 1 & \text{if } v_i \text{ and } v_j \text{ are adjacent} \\ 0 & \text{otherwise} \end{cases}$$

Let d_i be the degree of the vertex v_i in G and $D(G) = \text{diag}(d_1, d_2, \dots, d_n)$ be the diagonal matrix of G . The Laplacian matrix is defined as $L(G) = D(G) - A(G)$. The characteristic polynomial of $A(G)$ is defined as $f_G(A : x) = \det(xI_n - A)$, where I_n is the identity matrix of order n . The roots of the characteristic equation of $A(G)$ are called the *eigenvalues* of G . It is

¹Received January 23, 2018, Accepted November 26, 2018.

denoted by $\lambda_1(G) \geq \lambda_2(G) \geq \cdots \geq \lambda_n(G)$. It is called the *A - Spectrum* of G . The eigen values of $L(G)$ is denoted by $0 = \mu_1(G) \leq \mu_2(G), \cdots \leq \mu_n(G)$ and it is called the *L - Spectrum* of G . Since $A(G)$ and $L(G)$ are real and symmetric, their eigen values are all real numbers. A graph is *A - integral*, if the A - spectrum consists only of integers [4,14]. Two graphs are said to be *A - Cospectral* if they have the same A - spectrum.

The characteristic polynomial and spectra of graphs help to investigate some properties of graphs such as energy [8,16], number of spanning trees [18, 9,1], the Kirchhoff index [2, 5, 11], Laplace energy like invariants [7] etc.

The first result on Laplacian matrix, which was discovered by Kirchhoff, appeared in a paper published in the year 1847 is related to electrical network. There exists a vast literature that studies the Laplacian eigen values and their relationship with various properties of graphs [12,13]. Most of the studies of the Laplacian eigen values has naturally concentrated on external non trivial eigen values. Gutman et al. [16] discovered the connection between photoelectron spectra of standard hydrocarbons and the Laplacian eigen values of the underlying molecular graphs.

In a recent paper Reji Kumar and Renny P. Varghese [18] introduced subdivision graph vertex join of two given graphs and studies its spectral properties. They also studied [19] the spectral properties of some classes of hypergraphs.

In the next section we define DG - vertex join and DG - add vertex join of two graphs and discuss some important results, which are found essential to prove the results given in the subsequent sections. In the third section we find the A - spectrum and the L - spectrum of the new join and prove some related results. As an application, we find the number of spanning trees, Kirchhoff index and Laplacian - energy like invariant. Fourth section contains a discussion on some infinite family of integral graphs.

§2. Preliminaries

In a paper published in 1973 on duplicate graphs, which appeared in the *Journal of Indian Mathematical Society*, Sampathkumar [10] defined duplicate graphs. Let G be a graph with vertex set $V(G) = \{v_1, v_2, \cdots, v_n\}$. Take another set $U = \{u_1, u_2, \cdots, u_n\}$. Make u_i adjacent to all the vertices in $N(v_i)$, the neighbourhood set of v_i , in G for each i and remove all edges of G . The resulting graph is called the *duplication graph* of G and is denoted by $D(G)$. The following result tells us an easy way to find the determinant of a bigger matrix using the determinant of relatively smaller matrices.

Proposition 2.1 *Let M_1, M_2, M_3, M_4 be respectively $p \times p, p \times q, q \times p, q \times q$ matrix with M_1 and M_4 are invertible then*

$$\begin{aligned} \det \begin{pmatrix} M_1 & M_2 \\ M_3 & M_4 \end{pmatrix} &= \det(M_1) \det(M_4 - M_3 M_1^{-1} M_2) \\ &= \det(M_4) \det(M_1 - M_2 M_4^{-1} M_3), \end{aligned}$$

where $M_4 - M_3M_1^{-1}M_2$ and $M_1 - M_2M_4^{-1}M_3$ are called the Schur complements of M_1 and M_4 respectively.

Let G be a graph on n vertices, with the adjacency matrix A . The characteristic matrix $xI - A$ of A has determinant $\det(xI - A) = f_G(A : x) \neq 0$, so is invertible. The A - coronal ([6]), $\Gamma_A(x)$ of G is defined to be the sum of the entries of the matrix $(xI - A)^{-1}$. This can be calculated as

$$\Gamma_A(x) = \mathbf{1}_n^T (xI - A)^{-1} \mathbf{1}_n.$$

The A - coronal of some classes of graphs are given here.

Lemma 2.2([6]) *Let G be r - regular on n vertices. Then*

$$\Gamma_A(x) = \frac{n}{x - r}.$$

Since for any graph G with n vertices, each row sum of the Laplacian matrix $L(G)$ is equal to 0, we have $\Gamma_L(x) = \frac{n}{x}$.

Lemma 2.3([6]) *Let G be the bipartite graph K_{pq} , where $p + q = n$. Then*

$$\Gamma_A(x) = \frac{nx + 2pq}{x^2 - pq}.$$

The following results on an $n \times n$ real matrix is useful in this context.

Proposition 2.4([15]) *Let A be an $n \times n$ real matrix, and $J_{s \times t}$ denote the $s \times t$ matrix with all entries equal to one. Then*

$$\det(A + \alpha J_n \times n) = \det(A) + \alpha \mathbf{1}_n^T \text{adj}(A) \mathbf{1}_n.$$

Here α is a real number and $\text{adj}(A)$ is the adjugate matrix of A .

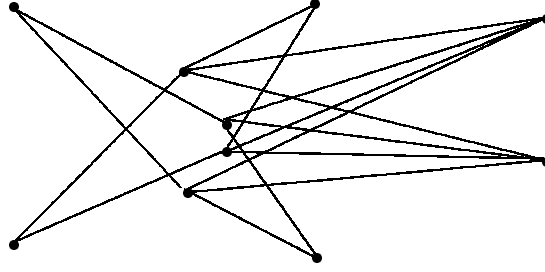
Corollary 2.5([15]) *Let A be an $n \times n$ real matrix. Then*

$$\det(xI_n - A - \alpha J_{n \times n}) = (1 - \alpha \Gamma_A(x)) \det(xI_n - A).$$

Next we proceed to define the DG - vertex join and the DG - advertex join of two graphs.

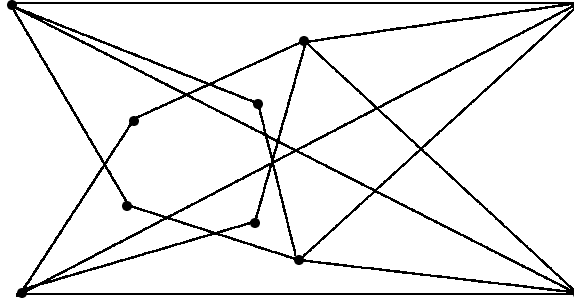
Definition 2.6 *Let G_1 be a graph on n_1 vertices and m_1 edges. G_2 be an arbitrary graph on n_2 vertices. The DG - vertex join of G_1 and G_2 is denoted by $G_1 \sqcup G_2$ and is the graph obtained from $D(G_1)$ and G_2 by joining every vertex of $V(G_1)$ to every vertex of $V(G_2)$. Where $D(G_1)$ is the duplication graph of G_1 .*

In Figure 1 an example of DG - vertex join of the graphs C_4 and K_2 is given.

Figure 1 $C_4 \sqcup K_2$

Definition 2.7 The DG – addvertex join of G_1 and G_2 is denoted by $G_1 \bowtie G_2$ and is the graph obtained from $D(G_1)$ and G_2 by joining the additional vertices of $D(G_1)$ corresponding to the vertices of G_1 with every vertex of $V(G_2)$.

In Figure 2 an example of DG - advertex join of the graphs C_4 and K_2 is given.

Figure 2 $C_4 \bowtie K_2$

§3. Spectrum of $G_1 \sqcup G_2$ for Some Classes of Graphs G_1 and G_2

In this section we study the spectrum of DG - vertex join of some classes of graphs G_1 and G_2 . We prove the following results in this connection.

Theorem 3.1 Let G_1 be an r_1 - regular graph on n_1 vertices and m_1 edges. G_2 be an arbitrary graph on n_2 vertices. Then, the Characteristic polynomial of $G_1 \sqcup G_2$ is

$$f_{G_1 \sqcup G_2}(A : x) = (x^2 - n_1 x \Gamma_{A_2}(x) - r_1^2) \prod_{i=2}^{n_2} (x - \lambda_i(G_2)) \prod_{i=2}^{n_1} (x^2 - \lambda_i(G_1)^2).$$

Proof The adjacency matrix of $G_1 \sqcup G_2$ is

$$A = \begin{bmatrix} 0 & A_1 & J_{n_1 \times n_2} \\ A_1 & 0_{n_1} & 0_{n_1 \times n_2} \\ J_{n_2 \times n_1} & 0_{n_2 \times n_1} & A_2 \end{bmatrix}$$

where A_1 and A_2 are the adjacency matrix of G_1 and G_2 respectively and J is a matrix with each entries 1.

The characteristic polynomial of $G_1 \sqcup G_2$ is

$$\begin{aligned} f_{G_1 \sqcup G_2}(A : x) &= \begin{vmatrix} xI_{n_1} - A_1 & -J \\ -A_1 & xI_{n_1} & 0 \\ -J & 0 & xI_{n_2} - A_2 \end{vmatrix} \\ &= \det(xI_{n_2} - A_2) \det S, \end{aligned}$$

where

$$\begin{aligned} S &= \begin{pmatrix} xI_{n_1} & -A_1 \\ -A_1 & xI_{n_1} \end{pmatrix} - \begin{pmatrix} -J_{n_1 \times n_2} \\ 0 \end{pmatrix} (xI_{n_2} - A_2)^{-1} \begin{pmatrix} -J_{n_2 \times n_1} & 0 \end{pmatrix} \\ &= \begin{pmatrix} xI_{n_1} & -A_1 \\ -A_1 & xI_{n_1} \end{pmatrix} - \begin{pmatrix} \Gamma_{A_2}(x)J_{n_1 \times n_1} & 0 \\ 0 & 0 \end{pmatrix} \\ &= \begin{pmatrix} xI - \Gamma_{A_2}(x)J_{n_1 \times n_1} & -A_1 \\ -A_1 & xI \end{pmatrix} \end{aligned}$$

Whence,

$$\begin{aligned} \det S &= \det(xI) \det \left((xI - \Gamma_{A_2}(x)J - \frac{A_1^2}{x}) \right) \\ &= x^{n_1} \det \left(xI - \Gamma_{A_2}(x)J - \frac{A_1^2}{x} \right) \\ &= x^{n_1} \det \left(xI - \frac{A_1^2}{x} - \Gamma_{A_2}(x)J \right) \\ &= x^{n_1} \det \left(xI - \frac{A_1^2}{x} \right) \left(1 - \Gamma_{A_2}(x) \Gamma_{\frac{A_1^2}{x}}(x) \right), \end{aligned}$$

Notice that G_1 is r_1 - regular and the row sum of A_1^2 is r_1^2 . We get

$$\Gamma_{\frac{A_1^2}{x}} = \frac{n_1}{x - \frac{r_1^2}{x}} = \frac{n_1 x}{x^2 - r_1^2}$$

and

$$\begin{aligned} \det S &= x^{n_1} \det \left(xI - \frac{A_1^2}{x} \right) \left(1 - \frac{n_1 x}{x^2 - r_1^2} \Gamma_{A_2}(x) \right) \\ &= \det(x^2 I - A^2) \left(\frac{x^2 - r_1^2 - n_1 x \Gamma_{A_2}(x)}{x^2 - r_1^2} \right). \end{aligned}$$

Hence

$$\det(xI - A) = (x^2 - n_1 x \Gamma_{A_2}(x) - r_1^2) \prod_{i=1}^{n_2} (x - \lambda_i(G_2)) \prod_{i=2}^{n_1} (x^2 - \lambda_i(G_1)^2). \quad \square$$

Corollary 3.2 *Let G_1 be an r_1 - regular graph on n_1 vertices, G_2 be r_2 - regular graph on n_2 vertices. Then the A - Spectrum of $G_1 \sqcup G_2$ consists of*

- (i) $\lambda_i(G_2)$, for $i = 2, 3, \dots, n_2$;
- (ii) $\pm \lambda_i(G_1)$, for $i = 2, 3, \dots, n_1$;
- (iii) Three roots of the equation

$$x^3 - r_2 x^2 - (n_1 n_2 + r_1^2) x + r_1^2 r_2.$$

Proof If G_2 is r_2 - regular then

$$\Gamma_{A_2}(x) = \frac{n_2}{x - r_2}.$$

We get

$$\begin{aligned} \det(xI - A) &= (x^3 - r_2 x^2 - (n_1 n_2 + r_1^2) x + r_1^2 r_2) \\ &\quad \times \prod_{i=2}^{n_2} (x - \lambda_i(G_2)) \prod_{i=2}^{n_1} (x^2 - \lambda_i(G_1)^2). \quad \square \end{aligned}$$

Corollary 3.3 *Let G_1 be an r_1 - regular graph on n_1 vertices, A - Spectrum of $G_1 \sqcup \overline{K_n}$ consists of*

- (i) 0, repeats n_2 times;
- (ii) $\pm \lambda_i(G_1)$, for $i = 2, 3, \dots, n_1$;
- (iii) $\pm \sqrt{n_1 n_2 + r_1^2}$.

Corollary 3.4 *Let G_1 be an r_1 - regular graph on n_1 vertices. A - Spectrum of $G_1 \sqcup K_{pq}$ consists of*

- (i) 0, repeats $p + q - 2$ times;
- (ii) $\pm \lambda_i(G_1)$, for $i = 2, 3, \dots, n_1$;
- (iii) Four roots of the equation

$$x^4 - (pq + r_1^2 + n_1 p + n_1 q) x^2 - 2pq n_1 x + r_1^2 pq.$$

3.1 Laplacian Spectrum of $G_1 \sqcup G_2$ for Some Classes of Graphs G_1 and G_2

Theorem 3.5 *Let G_1 be an r_1 - regular graph on n_1 vertices and m_1 edges. G_2 be an arbitrary graph on n_2 vertices. then,*

$$\begin{aligned} f_{G_1 \sqcup G_2}(L : x) &= x(x^2 - (n_1 + n_2 + 2r_1)x + r_1(2n_1 + n_2)) \\ &\quad \times \prod_{i=2}^{n_2} (x - n_1 - \mu_i(G_2)) \prod_{i=2}^{n_1} (x^2 - (2r_1 + n_2)x + n_2r_1 + r_1^2 - \lambda_i(G_1)^2). \end{aligned}$$

Proof The Laplace adjacency matrix of $G_1 \sqcup G_2$ is

$$L = \begin{bmatrix} (r_1 + n_2)I & -A_1 & J_{n_1 \times n_2} \\ -A_1 & r_1I & 0_{n_1 \times n_2} \\ -J_{n_2 \times n_1} & 0_{n_1 \times n_1} & n_1I_{n_2} + L_2 \end{bmatrix}$$

where L_2 is the Laplacian adjacency matrix of G_2

The Laplacian characteristic polynomial of $G_1 \sqcup G_2$ is

$$f_{G_1 \sqcup G_2}(L : x) = \begin{vmatrix} (x-r_1-n_2)I_{n_1} & A_1 & J \\ A_1 & (x-r_1)I_{n_1} & 0 \\ J & 0 & (x-n_1)I_{n_2}-L_2 \end{vmatrix}.$$

Using proposition 2.2 we get

$$f_{G_1 \sqcup G_2}(L : x) = \det((x - n_1)I_{n_2} - L_2) \det S,$$

where

$$\begin{aligned} S &= \begin{pmatrix} (x - r_1 - n_2)I_{n_1} & A_1 \\ A_1 & (x - r_1)I_{n_1} \end{pmatrix} - \begin{pmatrix} J \\ 0 \end{pmatrix} ((x - n_1)I_{n_1} - L_2)^{-1} \begin{pmatrix} J & 0 \end{pmatrix} \\ &= \begin{pmatrix} (x - r_1 - n_2)I & A_1 \\ A_1 & (x - r_1)I \end{pmatrix} - \begin{pmatrix} \Gamma_{L_2}(x - n_1)J_{n_1 \times n_1} & 0 \\ 0 & o \end{pmatrix} \\ &= \begin{pmatrix} (x - r_1 - n_2)I - \Gamma_{L_2}(x - n_1)J & A_1 \\ A_1 & (x - r_1)I \end{pmatrix} \end{aligned}$$

Therefore,

$$\det S = (x - r_1)^{n_1} \det \left((x - r_1 - n_2)I - \Gamma_{L_2}(x - n_1)J - \frac{A_1^2}{x - r_1} \right).$$

By Corollary 2.7

$$\begin{aligned}
\det S &= (x - r_1)^{n_1} \det \left((x - r_1 - n_2)I - \frac{A_1^2}{x - r_1} \right) \\
&\quad \times \left(1 - \Gamma_{L_2}(x - n_1) \Gamma_{\frac{A_1^2}{x - r_1}}(x - r_1 - n_2) \right) \\
&= \det \left((x - r_1 - n_2)(x - r_1)I - A^2 \right) \left(1 - \Gamma_{L_2}(x - n_1) \Gamma_{\frac{A_1^2}{x - r_1}}(x - r_1 - n_2) \right).
\end{aligned}$$

Since G_1 is r_1 regular graph, the row sum of $\frac{A_1^2}{x - r_1}$ is $\frac{r_1^2}{x - r_1}$. Therefore,

$$\begin{aligned}
\Gamma_{\frac{A_1^2}{x - r_1}}(x - r_1 - n_2) &= \frac{n_1(x - r_1)}{x^2 - (2r_1 + n_2)x + n_2r_1}, \\
1 - \Gamma_{L_2}(x - n_1) \Gamma_{\frac{A_1^2}{x - r_1}}(x - r_1 - n_2) &= \frac{x(x^2 - (n_1 + n_2 + 2r_1)x + r_1(2n_1 + n_2))}{(x - n_1)(x^2 - (2r_1 + n_2)x + n_2r_1)}.
\end{aligned}$$

Hence

$$\begin{aligned}
f_{G_1 \sqcup G_2}(L : x) &= x(x^2 - (n_1 + n_2 + 2r_1)x + r_1(2n_1 + n_2)) \\
&\quad \times \prod_{i=2}^{n_2} (x - n_1 - \mu_i(G_2)) \prod_{i=2}^{n_1} (x^2 - (2r_1 + n_2)x + n_2r_1 + r_1^2 - \lambda_i(G_1)^2). \quad \square
\end{aligned}$$

Let $t(G)$ denote the number of spanning tree of the graph G , the total number of distinct spanning subgraphs of G that are trees. The number of spanning trees of the graph describe the network which is one of the natural characteristics of its reliability. If G is a connected graph with n vertices and the Laplacian spectrum $0 = \mu_1(G) \leq \mu_2(G), \dots, \mu_n(G)$ then ([17])

$$t(G) = \frac{\mu_2(G)\mu_3(G) \cdots \mu_n(G)}{n}$$

Corollary 3.6 *Let G_1 be an r_1 - regular graph on n_1 vertices and G_2 be an arbitrary graph on n_2 vertices. Then*

$$t(G_1 \sqcup G_2) = \frac{r_1(2n_1 + n_2) \prod_{i=2}^{n_1} (n_1 + \mu_i(G_2)) \prod_{i=2}^{n_2} (r_1^2 + n_2r_1 - \lambda_i^2(G_1))}{2n_1 + n_2}.$$

Proof By Theorem 3.5 the roots of $f_{G_1 \sqcup G_2}(L : x)$ are as follows:

- (i) 0;
- (ii) $n_1 + \mu_i(G_2)$ for $i = 2, 3, \dots, n_2$;
- (iii) Two roots say x_1 and x_2 of the equation $x^2 - (n_1 + n_2 + 2r_1)x + r_1(2n_1 + n_2)$;
- (iv) Two roots say x_{i1} and x_{i2} of the equation $x^2 - (2r_1 + n_2)x + n_2r_1 + r_1^2 - \lambda_i(G_1)^2$ for $i = 2, 3, \dots, n_2$.

For Case (iii), $x_1x_2 = r_1(2n_1 + n_2)$, and for Case (iv), $x_{i1}x_{i2} = n_2r_1 + r_1^2 - \lambda_i(G_1)^2$,

$i = 2, 3, \dots, n_2$. Then, we get that

$$t(G_1 \sqcup G_2) = \frac{r_1(2n_1 + n_2) \prod_{i=2}^{n_1} (n_1 + \mu_i(G_2)) \prod_{i=2}^{n_2} (r_1^2 + n_2 r_1 - \lambda_i^2(G_1))}{2n_1 + n_2}. \quad \square$$

Another Laplacian spectrum based on graph invariant was defined by Liu and Liu [3] called the Laplacian - energy - like invariant. The Laplacian - energy - like invariant(LEL) of a graph G of n vertices is defined as

$$LEL(G) = \sum_{i=2}^n \sqrt{\mu_i}.$$

Corollary 3.7 *Let G_1 be an r_1 - regular graph on n_1 vertices and G_2 be an arbitrary graph on n_2 vertices. Then Laplace - energy - like invariant*

$$\begin{aligned} LEL &= \left(n_1 + n_2 + 2r_1 + 2\sqrt{r_1(2n_1 + n_2)} \right)^{1/2} + \sum_{i=2}^{n_2} (n_1 + \mu_i(G_1)^2)^{1/2} \\ &\quad + \sum_{i=2}^{n_1} \left(\frac{2r_1 + n_2 + \sqrt{r_1^2 + n_2 r_1 - \lambda_i(G_1)^2}}{r_1^2 + n_2 r_1 - \lambda_i(G_1)^2} \right)^{1/2}. \end{aligned}$$

Proof Using Theorem 3.5 and Corollary 3.6 we have

$$\begin{aligned} \sqrt{x_1} + \sqrt{x_2} &= (x_1 + x_2 + 2\sqrt{x_1 x_2})^{1/2} \\ &= \left(n_1 + n_2 + 2\sqrt{r_1(2n_1 + n_2)} \right)^{1/2}, \\ \frac{1}{\sqrt{x_{i1}}} + \frac{1}{\sqrt{x_{i2}}} &= \frac{\sqrt{x_{i1}} + \sqrt{x_{i2}}}{2\sqrt{x_{i1} x_{i2}}} \\ &= \left(\frac{x_1 + x_2 + \sqrt{x_1 x_2}}{x_{i1} x_{i2}} \right)^{1/2} \\ &= \left(\frac{2r_1 + n_2 + \sqrt{r_1^2 + n_2 r_1 - \lambda_i(G_1)^2}}{r_1^2 + n_2 r_1 - \lambda_i(G_1)^2} \right)^{1/2}. \end{aligned}$$

Hence the required result is obtained using the formula for LEL. $\square$

Klein [5] propounder of *resistance distance* defined electric resistance in network corresponding to the considered graph as the resistance distance between any two adjacent nodes is 1 ohm. The sum of the resistance distance between all pairs of the vertices of a graph is conceived as a new graph invariant. The electric resistance is calculated by means of the Kirchhoff laws called *kirchhoff index*.

Kirchhoff index of a connected graph G with $n(n \geq 2)$ vertices is defined as

$$Kf(G) = n \sum_{i=1}^{n-1} \frac{1}{\mu_i}$$

Corollary 3.8 *Let G_1 be an r_1 - regular graph on n_1 vertices. G_2 be an arbitrary graph on n_2*

vertices. Then

$$Kf(G_1 \sqcup G_2) = (2n_1 + n_2) \left[\frac{n_1 + n_2 + 2r_1}{r_1(2n_1 + n_2)} + \sum_{i=2}^{n_2} \frac{1}{n_1 + \mu_i(G_2)} + \sum_{i=2}^{n_1} \frac{2r_1 + n_2}{r_1^2 + n_2r_1 - \lambda_i(G_1)^2} \right].$$

Proof Using Theorem 3.5, Corollary 3.7 and the formula for Kirchhoff index we obtain the required result. $\square$

3.2 Spectra of DG - add Vertex Graph of Some Classes of Graphs

Next we discuss some spectral properties of the *DG* - add vertex graph of some classes of graphs.

Proposition 3.9 *Let G_1 be an r_1 - regular graph on n_1 vertices and G_2 be an arbitrary graph on n_2 vertices. Then $G_1 \sqcup G_2$ and $G_1 \bowtie G_2$ are A - cospectral*

Proof Notice that the characteristic polynomials of $G_1 \sqcup G_2$ and $G_1 \bowtie G_2$ are same. Hence we get the result. $\square$

Proposition 3.10 *Let G_1 be an r_1 - regular graph on n_1 vertices and G_2 be an arbitrary graph on n_2 vertices then $G_1 \sqcup G_2$ and $G_1 \bowtie G_2$ are L - cospectral.*

§4. Infinite Families of Integral Graphs

The following properties give a necessary and sufficient condition for *DG* - vertex join and *DG* - add vertex join of G_1 and G_2 to be integral.

Proposition 4.1 *Let G_1 be r_1 - regular graph on n_1 vertices and G_2 be r_2 - regular graph on n_2 vertices. $G_1 \sqcup G_2$ (respectively $G_1 \bowtie G_2$) is an integral graph if and only if G_1 and G_2 are integral graphs and the roots of $x^3 - r_2x^2 - (n_1n_2 + r_1^2)x + r_1^2r_2$ are integers.*

In particular if $G_2 = \overline{K_n}$ (totally disconnected) then $r_2 = 0$ then $G_1 \sqcup G_2$ (respectively $G_1 \bowtie G_2$) is integral iff G_1 is an integral graph and $n_1n_2 + r_1^2$ is a perfect square.

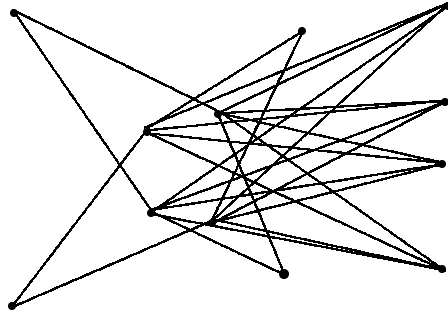


Figure 3 $K_4 \sqcup \overline{K_4}$ with spectrum $\{-5, -1^3, 0^4, 1^3, 5\}$

Proposition 4.2 *Let G_1 be r_1 - regular graph on n_1 . $G_1 \sqcup K_{pq}$ (respectively $G_1 \bowtie K_{pq}$) is an integral graph if and only if G_1 is an integral graph and the roots of $x^4 - (pq + r_1^2 + n_1p + n_1q)x^2 - 2pqn_1x + r_1^2pq$ are integers.*

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Collaborations of Indian institutions which conduct mathematical research: A study from the perspective of social network analysis

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Abstract

Expansion of knowledge in the realm of higher mathematics is highly important when progress of human society is concerned. As a fast developing country, in India we need a monitoring system which would tell us the exact nature of changes taking place in the field of mathematics. This is the motivation behind this paper. In this paper we present an analysis of the network formed by institutions which conduct research and publish articles in the field of Mathematics. Collaboration between a member of one institute and a member of another institute make a connection between the institutions. We make a comparative study of the network formed in consecutive years over a period of time giving emphasis to importance of institutions in the research network.

Suggested Citation

 K. Reji Kumar & Shibu Manuel, 2018. "Collaborations of Indian institutions which conduct mathematical research: A study from the perspective of social network analysis," *Scientometrics*, Springer/Akadémiiai Kiadó, vol. 117(2), pages 1041-1051, November.

Handle: *RePEc:spr:scient:v:117:y:2018:i:2:d:10.1007_s11192-018-2898-0*

DOI: 10.1007/s11192-018-2898-0

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Intuitionistic Fuzzy Soft Multiset and its Application

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Abstract: This paper explains extension of fuzzy soft Multi sets. The extension explain on intuitionistic form of fuzzy soft multi set. We here define intuitionistic fuzzy soft multi set relations, its various type including equivalence relation. Composition of relations and the equivalence of the inverse relation when relation satisfies the equivalence property are discussed. The notion of intuitionistic fuzzy soft multi sets are tried to apply in a decision making problem which study the spread of a plant known as Makaniya Micrantha. Finally we use normalized Euclidean distance formula for intuitionistic fuzzy soft multi set, which lead to a conclusion to which area has maximum and minimum spread of this plant there by giving a result, to which area the destruction of the plant is require and in which area its to be promoted. We establish several important results with adequate examples.

Key Words: Fuzzy sets, soft sets, multisets, fuzzy topology.

AMS(2010): 03E72, 54A40.

§1. Introduction

Uncertainties rule the human mind and perceptions. In most of the engineering, physics, chemical, computer sciences, biological economics, social sciences and medical sciences problems human mind deal with these uncertainties. Classical or ordinary set theory, which is based on the crisp and we can see exact case may not be fully suitable for handling such kind of problems of uncertainty.

A number of mathematical tools like probability theory, fuzzy sets [1], soft sets Molodtsov [3] rough sets [2] are well known and are efficient and effective models for dealing with vagueness and uncertainties. Then also each of them has distinguished advantages and certain limitations. Parametrization was not done before 1990s. This inadequacy of parametrization tools was perfectly cleared by Molodtsov [3] by introducing a new concept named soft set theory.

He applied soft set theory in many fields, game theory, smoothness of a function, the Riemann integral, Perron integral and measurement theory. Maji [4] presented an application

¹Proceedings of the *International Conference on Discrete Mathematics and its Applications*, Manonmaniam Sundaranar University, January 18-20, 2018.

²Received March 30, 2018, Accepted September 24, 2018, Edited by K. Selvakumar.

of soft sets in decision making problems for the first time, that was based on the reduction of parameters to keep the optimal choice of objects. The concept of soft set relations are introduced as a sub soft set of the cartesian product of the soft sets in [10]. Combining soft sets with fuzzy sets, Maji et al [6] defined fuzzy soft sets which are used for solving decision making problems. As a generalization of Molodtsov soft set Alkhazaleh [7] presented the definition of a soft multi set and he defined basic operations such as complement, union, and intersection etc. Salleh and Alkhazaleh presented the application of soft multi set in decision making problem. AND, OR operator on soft multiset and DeMorgans laws was proved by [11] Babitha. In 2011 Salleh gave a brief literature survey from soft sets to intuitionistic fuzzy soft sets. In 2012 Alkhazaleh and Salleh [8] introduced the concept of fuzzy soft multi set theory and studied the application of the set. Mukherjee and Das [14] in 2014 introduced the concepts of intuitionistic fuzzy soft multi sets and studied intuitionistic fuzzy soft multi topological spaces in details. While going through the above references it seems to be important to go through the non membership of the relations of fuzzy soft multi sets.

This paper can be considered as an extension of the paper [12] 'Interior exterior and boundary of fuzzy soft multi set topology on decision making.

§2. Preliminaries

Definition 2.1 ([3]) *Let U be an initial universe set and E be set of parameters. Let $P(U)$ denotes the power set of U and $A \subseteq U$. A pair (F, A) is called a soft set over U , where F is a mapping given by $F : A \rightarrow P(U)$.*

Definition 2.2 ([9]) *A multiset M drawn from the set X is represented by a function Count M or C_M defined as $C_M : X \rightarrow N$, where N represents the set of non negative integers.*

Definition 2.3 ([4]) *Let U be an initial universal set and let E be a set of parameters. Let I^U denote the power set of all fuzzy subsets of U . Let $A \subseteq E$. A pair (F, E) is called a fuzzy soft set over U , where F is a mapping given by $F : A \rightarrow I^U$.*

Definition 2.4 ([8]) *Let $\{U_i : i \in I\}$ be a collection of universes such that $\cap_{i \in I} U_i = \phi$ and let $\{E_{U_i} : i \in I\}$ be a collection of sets of parameters. Let $U = \pi_{i \in I} P(U_i)$ where $P(U_i)$ denotes the power set of U_i , $E = \pi_{i \in I} E_{U_i}$ and $A \subseteq E$. A pair (F, A) is called a fuzzy soft multiset over U , where F is a mapping given by $F : A \rightarrow U$, for all e in A $F(e) = (\{\frac{u}{\mu_{F(e)}(u)}\}; i \in I)$.*

Definition 2.5 ([8]) *A soft multiset (F, A) over U is called a seminull soft multiset, denoted by $(F, A) \approx \phi$, if at least one of the soft multiset parts of (F, A) equals ϕ .*

A soft multiset (F, A) over U is called a null soft multiset, denoted by $(F, A) \phi$, if all of the soft multiset parts of (F, A) equals ϕ .

Definition 2.6 ([8]) *A soft multiset (F, A) over U is called an absolute soft multiset, denoted by $(F, A)_A$, if $(e_{U_i, j}, F_{e_{U_i, j}}) = U_i, \forall i$.*

Definition 2.7 ([8]) *The union of two soft multisets (F, A) and (G, B) over U , denoted by*

$(F, A) \cup (G, B)$ is the soft multiset (H, C) where $C = A \cup B$, and $\forall \epsilon \in C$,

$$\begin{aligned} H(\epsilon) &= F(\epsilon), \text{ if } \epsilon \in A - B, \\ &= G(\epsilon), \text{ if } \epsilon \in B - A, \\ &= F(\epsilon) \cup G(\epsilon), \text{ if } \epsilon \in A \cap B. \end{aligned}$$

Definition 2.8 ([8]) The intersection of two soft multisets (F, A) and (G, B) over U , denoted by $(F, A) \cap (G, B)$ is the soft multiset (H, C) where $C = A \cup B$, and $\forall \epsilon \in C$,

$$\begin{aligned} H(\epsilon) &= F(\epsilon), \text{ if } \epsilon \in A - B, \\ &= \{G(\epsilon), \text{ if } \epsilon \in B - A\}, \\ &= \{F(\epsilon) \cap G(\epsilon), \text{ if } \epsilon \in A \cap B\}. \end{aligned}$$

Definition 2.9 ([8]) The union of two fuzzy soft multisets (F, A) and (G, B) over U , denoted by $(F, A) \cup (G, B)$ is the soft multiset (H, C) where $C = A \cup B$, and $\forall \epsilon \in C$,

$$\begin{aligned} H(\epsilon) &= F(\epsilon), \text{ if } \epsilon \in A - B, \\ &= G(\epsilon), \text{ if } \epsilon \in B - A, \\ &= \cup(F(\epsilon), G(\epsilon)) \text{ if } \epsilon \in A \cap B, \end{aligned}$$

where

$$\bigcup(F(\epsilon), G(\epsilon)) = s(F_{\epsilon_{U_i, j}}, G_{\epsilon_{U_i, j}}),$$

for all $i \in \{1, 2, \dots, m\}$ with s as an s -norm.

Definition 2.10 ([8]) The intersection of two fuzzy soft multisets (F, A) and (G, B) over U , denoted by $(F, A) \cap (G, B)$ is the soft multiset (H, C) where $C = A \cup B$, and for all $\epsilon \in C$,

$$\begin{aligned} H(\epsilon) &= F(\epsilon), \text{ if } \epsilon \in A - B, \\ &= G(\epsilon), \text{ if } \epsilon \in B - A, \\ &= \cap(F(\epsilon), G(\epsilon)) \text{ if } \epsilon \in A \cap B, \end{aligned}$$

where

$$\bigcap(F(\epsilon), G(\epsilon)) = t(F_{\epsilon_{U_i, j}}, G_{\epsilon_{U_i, j}}),$$

for all $i \in \{1, 2, \dots, m\}$ with t as an t -norm.

Definition 2.11 ([13]) The normalized Euclidean distance d_{n-H} between two intuitionistic fuzzy set A and B is defined as

$$d_{n=H}(A, B) = \left(\frac{1}{2n} \sum_n^{i=1} \left[(\mu_A(x_i) - \mu_B(x_i))^2 + (\nu_A(x_i) - \nu_B(x_i))^2 + (\pi_A(x_i) - \pi_B(x_i))^2 \right] \right)^{\frac{1}{2}},$$

where, $X = \{x_1, x_2, \dots, x_n\}$ for $i = 1, 2, \dots, n$.

§3. Intuitionistic Fuzzy Soft Multiset Relations

Definition 3.1 Let (F, A) and (G, B) be two intuitionistic fuzzy soft multi sets over U . Then the Cartesian product of (F, A) and (G, B) is defined as $(F, A) \times (G, B) = \{(a_i, b_j) : \min((\eta_{F(a)}(a_k), \eta_{G(b)}(a_k)), \max(\mu_{F(a)}(a_k), \mu_{G(b)}(a_k)))/u \in U_i\}$.

Example 3.2 Consider two intuitionistic fuzzy soft multi sets (F, A) and (F_2, B) as follows

$$\begin{aligned} (F_1, A) &= \{(a_1, (\{\frac{h_1}{0.5, 0.3}, \frac{h_2}{0.4, 0.3}, \frac{h_3}{0.1, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.8, 0.1}, \frac{c_2}{0.5, 0.3}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.8, 0.1}, \frac{p_2}{0, 0}\}), \\ &\quad (a_2, (\{\frac{h_1}{0.9, 0.1}, \frac{h_2}{0.5, 0.4}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.7, 0.2}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})\}, \\ (F_2, B) &= \{(a_1, (\{\frac{h_1}{0.7, 0.3}, \frac{h_2}{0.7, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.8, 0.1}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.3}, \frac{p_2}{0, 0}\}), \\ &\quad (a_2, (\{\frac{h_1}{0.9, 0.1}, \frac{h_2}{0.5, 0.4}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0, 0}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.8, 0.2}, \frac{p_2}{0, 0}\})\}, \\ (F_1, A) \times (F_2, B) &= \{((a_1, b_1), (\{\frac{h_1}{0.2, 0.3}, \frac{h_2}{0.2}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.8, 0.2}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \\ &\quad \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((a_1, b_2), (\{\frac{h_1}{0.2, 0.3}, \frac{h_2}{0.2, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0, 0}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \\ &\quad \{\frac{p_1}{0.8, 0.2}, \frac{p_2}{0, 0}\})), ((a_2, b_1), (\{\frac{h_1}{0.7, 0.2}, \frac{h_2}{0.5, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.7, 0.2}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \\ &\quad \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((a_2, b_2), (\{\frac{h_1}{0.9, 0.1}, \frac{h_2}{0.5, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0, 0}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \\ &\quad \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})))\}. \end{aligned}$$

Definition 3.3 Consider two intuitionistic fuzzy soft multi sets (F, A) and (G, B) over U . Then the intuitionistic fuzzy soft multi set relation R on (F, A) and (G, B) is a fuzzy soft multi subset of the set $(F, A) \times (G, B)$. Where R is given by $R: A \times B \rightarrow U$.

Definition 3.4 An intuitionistic fuzzy soft multi relation R is said to be reflexive if and only if $\mu_{R(a,a)}(u) = 1$ for all $u \in U$ and $a \in A$.

Example 3.5 Consider a relation R in (F, A) as $\{((a_1, a_1), (\{\frac{h_1}{1, 0}, \frac{h_2}{1, 0}, \frac{h_3}{1, 0}, \frac{h_4}{1, 0}\}), \{\frac{c_1}{1, 0}, \frac{c_2}{1, 0}, \frac{c_3}{1, 0}\}, \{\frac{p_1}{1, 0}, \frac{p_2}{1, 0}\})), ((a_1, a_2), (\{\frac{h_1}{0.2, 0.3}, \frac{h_2}{0.2, 0.5}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0, 0}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.8, 0.2}, \frac{p_2}{0, 0}\})), ((a_2, a_1), (\{\frac{h_1}{0.7, 0.2}, \frac{h_2}{0.5, 0.4}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.7, 0.3}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.3}, \frac{p_2}{0, 0}\})), ((a_2, a_2), (\{\frac{h_1}{1, 0}, \frac{h_2}{1, 0}, \frac{h_3}{1, 0}, \frac{h_4}{1, 0}\}), \{\frac{c_1}{1, 0}, \frac{c_2}{1, 0}, \frac{c_3}{1, 0}\}, \{\frac{p_1}{1, 0}, \frac{p_2}{1, 0}\})))\}$, then R is a reflexive relation.

Definition 3.6 Consider an intuitionistic fuzzy soft multi sets (F, A) , then a relation R on (F, A) is said to be symmetric if and only if $(a, b) \in R$ then $(b, a) \in R$.

Example 3.7 Consider a relation R in (F, A) as

$\{((a_1, b_1), (\{\frac{h_1}{0.2, 0.4}, \frac{h_2}{0.2, 0.5}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.8, 0.1}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((a_1, b_2), (\{\frac{h_1}{0.2, 0.6}, \frac{h_2}{0.6, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.8, 0.1}, \frac{p_2}{0, 0}\})), ((b_1, a_1), (\{\frac{h_1}{0.2, 0.7}, \frac{h_2}{0.2, 0.7}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.8, 0.1}, \frac{p_2}{0, 0}\})), ((a_2, b_2), (\{\frac{h_1}{0.9, 0.1}, \frac{h_2}{0.5, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}), \{\frac{c_1}{0, 0}, \frac{c_2}{0, 0}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})))\}$, then R is a Symmetric relation.

Definition 3.8 Consider two intuitionistic fuzzy soft multi set relations R_1, R_2 in (F, A) , the composition of R_1 and R_2 is defined as $R_1 \circ R_2: A \times A \rightarrow U$. That is

$$\mu_{R_1 \circ R_2(a,b)}(u) = \max_k \{ \min(\eta_{R_1(a,k)}(u), \eta_{R_2(k,b)}(u)), \max(\mu_{R_1(a,k)}(u), \mu_{R_2(k,b)}(u)) \},$$

for all $u \in U_i, a, b, k \in A$.

Definition 3.9 Consider an intuitionistic fuzzy soft multi sets (F, A) , then a relation R on (F, A) is said to be transitive if and only if $RoR \subseteq R$.

Example 3.10 Consider a relation R in (F, A) as

$\{((a_1, b_1), (\{\frac{h_1}{0.3, 0.4}, \frac{h_2}{0.7, 0.2}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.8, 0.2}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((a_1, b_2), (\{\frac{h_1}{0.2, 0.5}, \frac{h_2}{0.6, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.2}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.3}, \frac{p_2}{0, 0}\})), ((a_2, b_1), (\{\frac{h_1}{0.3, 0.5}, \frac{h_2}{0.6, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.1}, \frac{p_2}{0, 0}\})), ((a_2, b_2), (\{\frac{h_1}{0.9, 0.1}, \frac{h_2}{0.6, 0.2}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.6, 0.3}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})))\}$, then R is a transitive relation.

Definition 3.11 Consider a relation R in an intuitionistic fuzzy soft multi set (F, A) . Then R is said to be an equivalence relation if R is reflexive, symmetric, and transitive.

Definition 3.12 Consider a relation R in an intuitionistic fuzzy soft multi set (F, A) . Then the inverse of R , denoted by R^{-1} is defined as $R^{-1}(a, b) = R(b, a)$ for all $a, b \in A$.

Example 3.13 Consider a relation R in (F, A) as

$\{((a, a), (\{\frac{h_1}{0.3, 0.5}, \frac{h_2}{0.7, 0.2}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.8, 0.2}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((a, b), (\{\frac{h_1}{0.2, 0.7}, \frac{h_2}{0.6, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((b, a), (\{\frac{h_1}{0.2, 0.7}, \frac{h_2}{0.6, 0.3}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.3, 0.5}, \frac{c_2}{0.8, 0.1}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5, 0.4}, \frac{p_2}{0, 0}\})), ((b, b), (\{\frac{h_1}{0.9}, \frac{h_2}{0.6}, \frac{h_3}{0, 0}, \frac{h_4}{0, 0}\}, \{\frac{c_1}{0.6}, \frac{c_2}{0.8}, \frac{c_3}{0, 0}\}, \{\frac{p_1}{0.5}, \frac{p_2}{0, 0}\})))\}$, here (a, b) is the inverse of (b, a) .

Theorem 3.14 A relation R is a symmetric intuitionistic fuzzy soft multi relation on (F, A) if and only if the inverse of R , R^{-1} is symmetric.

Proof Consider the relation R as a symmetric intuitionistic fuzzy soft multi relation on (F, A) . Then $\mu_{R^{-1}(a, b)} = \mu_{R(b, a)} = \mu_{R(a, b)} = \mu_{R^{-1}(b, a)}$. Therefore $\mu_{R^{-1}(a, b)} = \mu_{R^{-1}(b, a)}$. Therefore R^{-1} is symmetric.

Conversely assume that R^{-1} is a symmetric fuzzy soft multi set on (F, A) . Then $\mu_{R(a, b)} = \mu_{R^{-1}(b, a)} = \mu_{R^{-1}(a, b)} = \mu_{R(b, a)}$. Since $\mu_{R(a, b)} = \mu_{R(b, a)}$, R is a symmetric intuitionistic fuzzy soft multi relation on (F, A) . $\square$

Theorem 3.15 A relation R is a reflexive intuitionistic fuzzy soft multi relation on (F, A) if and only if the inverse of R , R^{-1} is reflexive.

Proof Consider the relation R as a reflexive intuitionistic fuzzy soft multi relation on (F, A) . Then $\mu_{R^{-1}(a, a)} = \mu_{R(a, a)}$. Thus R^{-1} is reflexive. Conversely let R^{-1} is a reflexive relation on (F, A) . Then $\mu_{R(a, a)} = \mu_{R^{-1}(a, a)}$ Therefore R is reflexive. $\square$

Theorem 3.16 A relation R is a transitive intuitionistic fuzzy soft multi relation on (F, A) if and only if the inverse of R , R^{-1} is transitive.

Proof Consider the relation R as a transitive intuitionistic fuzzy soft multi relation on (F, A) . For every $a, b \in A$

$$\begin{aligned} \mu_{R^{-1}(a, b)} &= \mu_{R(b, a)} \geq \mu_{RoR(b, a)} \\ &= \max_k \{ \min(\eta_{R(b, k)}, \eta_{R(k, a)}), \max(\mu_{R(b, k)}, \mu_{R(k, a)}) \} \end{aligned}$$

$$\begin{aligned}
&= \max_k \{ \min(\eta_{R(k,a)}, \eta_{R(b,k)}), \max(\mu_{R(k,a)}, \mu_{R(b,k)}) \} \\
&= \max_k \{ \min(\eta_{R^{-1}(a,k)}, \eta_{R^{-1}(k,b)}), \max(\mu_{R^{-1}(a,k)}, \mu_{R^{-1}(k,b)}) \} \\
&= \mu_{R^{-1} \circ R^{-1}(a,b)}.
\end{aligned}$$

Hence R^{-1} is a transitive fuzzy soft multi relation on (F, A) .

Conversely assume that R^{-1} is a transitive fuzzy soft multi relation on (F, A) . For any $(a, a) \in A \times A$,

$$\begin{aligned}
\mu_{R(a,b)} &= \mu_{R^{-1}(b,a)} \geq \mu_{R^{-1} \circ R^{-1}} \\
&= \max_k \{ \min(\eta_{R^{-1}(b,k)}, \eta_{R^{-1}(k,a)}), \max(\mu_{R^{-1}(b,k)}, \mu_{R^{-1}(k,a)}) \} \\
&= \max_k \{ \min(\eta_{R^{-1}(k,a)}, \eta_{R^{-1}(b,k)}), \max(\mu_{R^{-1}(k,a)}, \mu_{R^{-1}(b,k)}) \} \\
&= \max_k \{ \min(\eta_{R(a,k)}, \eta_{R(k,b)}), \max(\mu_{R(a,k)}, \mu_{R(k,b)}) \} \\
&= \mu_{RoR(a,b)}. \quad \square
\end{aligned}$$

Theorem 3.17 Consider a relation R in an intuitionistic fuzzy soft multi set (F, A) . Then R is transitive if and only if $R^n \subseteq R$, for $n \geq 1$.

Proof Let the relation R be transitive. We have to prove that $R^n \subseteq R$, for $n \geq 1$. For this we use the method of induction.

Suppose $n = 1$. Then $R^1 = R$, therefore it is obviously true.

Assume that $R^n \subseteq R$ for $n \geq 1$. Consider $(x, y) \in R^{n+1}$. We know from the definition that $R^{n+1} = R^n \circ R$, then there must be an $k \in A$ such that $(x, k) \in R$ also $(k, y) \in R^n$. Our assumption was $R^n \subseteq R$, therefore $(k, y) \in R$. As R is transitive $(x, k) \in R$, $(k, y) \in R$ which implies $(x, y) \in R$. Also we know (x, y) is an arbitrary element of R^{n+1} , which shows that $R^{n+1} \subseteq R$.

Now we have to show the converse part, that is if $R^n \subseteq R$ then R is transitive. Assume that $(x, y), (y, z) \in R$, by the definition of composition $(x, z) \in R^2$. But $R^2 \subseteq R$ implies $(x, z) \in R$. Therefore R is transitive. $\square$

Theorem 3.18 Consider a relation R from an intuitionistic fuzzy soft multi set (F, A) to a fuzzy soft multi set (F, B) , then we have the following $R((F, A) \cup (F, B)) \neq R((F, A)) \cup R((F, B))$.

Proof We discuss it with 3 cases as following.

Case 1. Consider the intuitionistic fuzzy soft multi sets (F, A) and (F, B) with same elements. Then it is obvious that $R((F, A) \cup (F, B)) = R((F, A)) \cup R((F, B))$.

Case 2. Let every element of intuitionistic fuzzy soft multi set (F, A) is in the intuitionistic fuzzy soft multi set (F, B) . If $(F, A) = \{a_i, \{\frac{x_k}{k_{pa}}, \frac{y_l}{l_{qa}} \dots\}, i, k, l \in N$, and $k_{pa}, l_{qa} \dots$ are corresponding fuzzy values} and $(F, B) = \{b_i, \{\frac{x_k}{k_{pb}}, \frac{y_l}{l_{qb}} \dots\}, i, k, l \in N$, and $k_{pb}, l_{qb} \dots$ are corresponding fuzzy values}, then

$$\begin{aligned}
(F, A) \cup (F, B) &= \{c_i, \{\frac{x_k}{k_{pab}}, \frac{y_l}{l_{qab}} \dots\}, i, k, l \in N, \\
&\text{where } k_{pab} = \max\{k_{pa}, k_{pb}\}, l_{qab} = \max\{l_{qa}, l_{qb}\} \dots,
\end{aligned}$$

are the corresponding fuzzy values. In k_{pb} , k represents the position of multi content, pb

represents the fuzzy value and it correspond to the intuitionistic fuzzy soft multi set (F, B) . Then $R(F, A) \subseteq R(F, B)$ and therefore $R((F, A) \cup (F, B)) = R(F, B)$.

Case 3. Let some elements in (F, A) are in (F, B) .

If $(F, A) = \{a_i, \{\frac{x_k}{k_{pa}}, \frac{y_l}{l_{qa}}, \dots\}, i, k, l \in N, \text{ and } k_{pa}, l_{qa}, \dots \text{ are corresponding fuzzy values}\}$ and $(F, B) = \{b_i, \{\frac{x_k}{k_{pb}}, \frac{z_m}{m_{rb}}, \dots\}, i, k, m \in N, \text{ and } k_{pb}, m_{rb}, \dots \text{ are corresponding fuzzy values}\}$.

Now $(F, A) \cup (F, B) = \{c_i, \{\frac{x_k}{k_{pab}}, \frac{y_l}{l_{qa}}, \frac{z_m}{m_{rb}}, i, k, l, m \in N, \text{ and } l_{qa}, m_{rb}, \dots \text{ are corresponding fuzzy values}\}, \text{ where } k_{pab} \text{ is the max } \{k_{pa}, k_{pb}\}$.

$R((F, A) \cup (F, B)) = \{(c_i, c_j), \{\frac{x_k}{k_{pab*}}, \frac{y_l}{l_{qa}}, \frac{z_m}{m_{rb}}, i, k, l, m \in N, \text{ and } l_{qa}, m_{rb}, \dots \text{ are corresponding fuzzy values}\}, \text{ where } k_{pab*} \text{ is the minimum of } k_{pab} \text{'s, if } i \neq j. \text{ Now } R(F, A) \text{ and } R(F, B) \text{ are found by taking the minimum of } k_{pa} \text{'s or } k_{pb} \text{'s respectively. Then } R((F, A)) \cup R((F, B)) \text{ will be the maximum } \{k_{pa} \text{'s and } k_{pb} \text{'s. So it is clear that } R((F, A) \cup (F, B)) \neq R((F, A)) \cup R((F, B)). \square$

Theorem 3.19 Let (F, A) , (G, B) , (H, C) be intuitionistic fuzzy soft multi sets in (X, E, τ) and let $(F, A) \subseteq (G, B) \subseteq (H, C)$. Then $(G, B) - (F, A) \subseteq (H, C) - (F, A)$.

Proof Here $(F, A) \subseteq (G, B)$ and $(G, B) \subseteq (H, C) \Rightarrow (F, A) \subseteq (H, C)$. That is

$$\eta_{(F,A)}(x) \leq \eta_{(G,B)}(x) \leq \eta_{(H,C)}(x) \text{ and } \mu_{(F,A)}(x) \geq \mu_{(G,B)}(x) \geq \mu_{(H,C)}(x)$$

for all $x \in X$ As (F, A) is the smallest of other (G, B) and (H, C) subtract (F, A) from both sides of $(G, B) \subseteq (H, C)$. Hence

$$(G, B) - (F, A) \subseteq (H, C) - (F, A). \quad \square$$

§ 4. An Application of Intuitionistic Fuzzy Soft Multiset Theory

Invasion of alien plant species is considered as a serious problem that which different countries have to face. Alien plant species invade and badly affect both natural and semi natural ecosystems. Many weed plants contain a variety of metabolites which are harmful as well as beneficial to biotic stresses, whereas same compounds in plants are beneficial to insects also for their better growth and development to continue their life style. Mikania micrantha is one of the 100 worst alien species. It is among the ten worst exotic species in South-east and South Asia, and one of the 16 exotic species in China. Mikania micrantha, an invasive alien species, is native to Central and South America is one of the serious bio diversity.

Let $S = \{s_1, s_2, s_3, s_4\}$ be the different universes or countries. Consider $C = \{\text{Biological, Medical, Shade, Spread}\}$ as the set of decision parameters. and $S_u = \{\text{Biological property, Medicinal advantage for animals, Medicinal disadvantage for animals, Shade for air fields, quick spread}\}$ are the set of subjects relating to the above parameters. That is Micania Micrantha have many advantages as well as disadvantages. Such as we have many many biological aspects we have to learn from this plant. These plants are good to some extend to some animals, but shows serious kidney decease to many animals. Another thing is it spreads as fast as other plants.

Table 1. Parameters vs Subject

.	Biological	Medical(A)	Medical(D)	Shade	Spread
Biological	(0.81,0.1,0.1)	(0.7,0.21,0.1)	(0.9,0.0,0.11)	(0.6,0.31,0.1)	(0.8,0.11,0.1)
Medical	(0.91,0.1,0.0)	(0.8,0.11,0.1)	(0.8,0.11,0.1)	(0.51, 0.3,0.2)	(0.7,0.21,0.1)
Shade	(0.5,0.31,0.2)	(0.51,0.2,0.3)	(0.9,0.0,0.1)	(0.5,0.41,0.1)	(0.71,0.1,0.2)
Spread	(0.71,0.2,0.1)	(0.5,0.41,0.1)	(0.9,0.11,0.0)	(0.61,0.3, 0.1)	(0.8,0.0,0.21)

Each of these parameter is described by three numbers such as soft multi membership, soft multi non membership, and soft multi hesitation margin. Here the parameter vs subject describes while biological, biological is considered the possibility that the plant is used for biological aspects is 0.8 and its non usage is 0.1 and that of degree of hesitancy at that time is 0.1. Similarly all the other triplets are defined. After going through a serious calculations we have the following table.

Table 2. Universe vs subject

.	Biological	Medical(A)	Medical(D)	Shade	Spread
S_1	(0.61,0.3,0.1)	(0.5,0.41,0.1)	(0.6,0.21,0.2)	(0.5,0.3,0.2)	(0.5,0.51,0.0)
S_2	(0.9,0.11,0.0)	(0.81,0.1,0.1)	(0.8,0.1,0.1)	(0.5, 0.3,0.2)	(0.7,0.2,0.1)
S_3	(0.5,0.3,0.2)	(0.5,0.2,0.3)	(0.9,0.0,0.1)	(0.5,0.4,0.1)	(0.7,0.1,0.2)
S_4	(0.7,0.21,0.1)	(0.51,0.4,0.1)	(0.9,0.1,0.0)	(0.6,0.31, 0.1)	(0.81,0.0,0.2)

Each of four countries different usage and non usage in different subjects are given. Using the definition cited in the preliminary section we have the following table

Table 3. Universe vs Parameter

.	Biological	Medical	Shade	Spread
S_1	0.1007	0.0898	0.0905	0.0806
S_2	0.0739	0.0857	0.0592	0.0955
S_3	0.0783	0.0806	0.0812	0.0858
S_4	0.0832	0.0750	0.0929	0.1015

From the above table, it is clear that the shortest distance gives the proper area determination for each universe. The result is as follows

S_1 needs to take the measure to stop the spread of Macania Micrantha.

S_2 needs to take step to use Micania Micrantha as a shade for air field etc.

S_3 needs to take step to use Micania Micrantha as a research area in biological studies

S_4 needs to take step to take Micania Micrantha as a medicinal plant.

So we have seen that each country have different aspects about the same plant, that is each country will have economic growth if the plant is treated in different way.

§4. Conclusion

Here we establish many properties of intuitionistic fuzzy soft multi set theory. Here we have gone through the properties of fuzzy soft multiset relations. The variation between two intuitionistic fuzzy soft multi set relations are also proved. Szmidt's distance between intuitionistic fuzzy sets was discussed and extended to intuitionistic fuzzy soft multi sets through an application on a decision making problem. The distance formula can be used for various uncertainty problems, and can be used as a perfect tool for finding the apt decision.

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Secondary Metabolites and their Antioxidant Activities: An Overview

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Received: July 14, 2020; **Published:** September 19, 2020

Abstract

Free radicals/reactive oxygen species (ROSs)/reactive nitrogen species (RNSs) are produced by cells through endogenous reactions, various physiochemical events or pathological issues. Antioxidants (AOX) are compounds scavenge or regulate these radicals. The balance between these molecules and AOXs is a pre-requisite for healthy physiological functions of the body. If free radicals/ROS/RNS over expresses in the system results into oxidative stress and other life style disorders i.e., oxidize lipids, proteins, nucleic acids and trigger a number of human diseases. Therefore, inclusion of antioxidants in food can assist in balancing this sort of oxidative bursts. Chemical antioxidants like butylated hydroxy anisole, butylated hydroxy toluene, propyl gallate and tert-butyl hydroquinone were employed in food resulted in to dangerous ill effects. Thus, the search for plant based biomolecules with antioxidative activity has been explored in recent times. The present overview provides an account of plants employed by the local people as antioxidants or functional foods or stress reliever in alleviating human diseases. The secondary metabolites analyzed in the present study as antioxidants include polyphenolics, carotenoids, alkaloids, terpenoids and flavonoids. These molecules function either as free radical scavengers or function by retarding chain initiation or involved in repair of damaged biomolecules. The output of the study will assist in providing knowledge regarding appropriate applications of the natural bioactive components in the regular diet as nutraceuticals.

Keywords: Antioxidant; Free Radicals; Oxidative Stress; Secondary Metabolites; Ethnic People

Introduction

Reactive radicals such as reactive oxygen species (ROSs) and reactive nitrogen species (RNS), are produced inside the living systems through multiple metabolic pathways. The cell system is in built with antioxidants that safe guard the cells from oxidative burst caused by these free radicals. Antioxidants (AOX) like glutathione, thioredoxin and AOX enzymes (superoxide dismutase, glutathione peroxidase, catalase regulates the oxidative stress and protect lipids, proteins, and nucleic acids. Similarly, tocopherols, ascorbate, carotenoids, flavonoids, amino acids are also natural AOXs present in the vegetables, fruits and leaves. There is a high demand for such foods balanced with AOXs and probiotics that may vitalize the human health. AOXs had a growing demand in pharmaceutical field owing to their protective roles in scavenging ROS/RNS against oxidative stress induced pathological event. Exploration of AOX features of herbals requires proper protocols, which address the mode of AOX activity and its kinetics of the reactions. Many reports were correlated about the AOX potentialities of plant based secondary metabolites using wide protocols with human health. Protocols like inhibited autoxidation connected

with termination-enhancing AOXs, for chain-breaking AOXs, and preventive AOXs are unique. The easy protocol is *in vitro* determination of AOX power of food items. Some of the methods are 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate), 2,2-diphenyl-1-picrylhydrazyl radical scavenging, Fe^{3+} - Fe^{2+} transformation method, ferric reducing antioxidant power protocol, cupric ions reducing power, Folin-Ciocalteu reducing power, peroxy radical ($\text{ROO}\cdot$), superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen ($^1\text{O}_2$), nitric oxide radical ($\text{NO}\cdot$) scavenging and chemiluminescence assay. Many phytochemicals from indigenous plants have been found to possess antioxidant activity under *in vivo* and *in vitro* assays. In fact, only a few species have been validated therapeutically useful under *in vivo* conditions due to their interference with physiological processes like absorption, distribution, metabolism, storage and excretion. However, the phytochemicals are being screened only in terms of *in vitro* antioxidant activity and the data are then directly employed to their therapeutic usefulness. In this juncture, the overview of the present study refers selected ethnic plants used by the native people and their applications.

Materials and Methods

The plants were selected based on the traditional usage by the native people or tribes of the locality. The algal species includes *Gracilaria dura*, *Kappaphycus alvarezii* and *Hypnea musciformis*; bryophytes: *Plagiochila beddomei*, *Leucobryum bowringii*, *Octoblepharum albidum*, *Marchantia polymorpha*, *Marchantia linearis*, *Pallavicinia lyellii*, *Thuidium tamariscellum*; angiosperms: *Solanum aculeatissimum*, *Solanum mauritianum*, *Artemisia japonica*, *A. nilagirica*, *Premna serratifolia*, *Orthosiphon aristatus*, *Pouteria campechiana*, *Pogostemon benghalensis*, *P. cablin*, *Thottea siliquosa*, *Tylophora Subramanii*, *Begonia cultivars*, *Osbeckia* species *Clerodendron infortunatum*, *Tectona grandis* and Wild Balsam Species. Protocols used were 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate), 2,2-diphenyl-1-picrylhydrazyl radical scavenging, Fe^{3+} - Fe^{2+} transformation method, ferric reducing antioxidant power protocol, cupric ions reducing power, Folin-Ciocalteu reducing power, peroxy radical ($\text{ROO}\cdot$), superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen ($^1\text{O}_2$) scavenging assay.

Results and Discussion

Sumayya, *et al.* [1] explored the local usage of seaweeds as sources of ailments for curing many human disorders along the coastal belts of Kerala and established that they are proven sources of biologically active metabolites. Many of the active principles produced by marine seaweeds were used in traditional and complementary medicine. Hence, a preliminary phytochemical survey of the seaweeds will be needed to reveal its secondary metabolite constituents and their by channeling towards its therapeutic values. In this study, the methanol extract of *Gracilaria dura* was subjected to phytochemical analysis to know the secondary metabolites present in the extract. The crude extract was purified by column chromatography. The resultant fraction was subject to GC-MS analysis revealed a pool of terpenoids. Four major terpenoids such as dodecanal, hexadecane, tetradecanal, heptadecane and 6, 4, 10-trimethyl-2-pentadecanone (sesquiterpenoid) were noticed. The purified terpenoid extract showed remarkable antioxidant activity in terms of scavenging hydroxyl radicals in a concentration dependent manner. The extract exhibited remarkable profile on the FRAP assay and strong DPPH scavenging potential. The IC_{50} values were significant and comparable with the synthetic antioxidants like ascorbate and quercetin. The obtained results suggest the possible use of red algae, *G. dura* as a good candidate in terms of functional food supplement and also in combating carcinogenesis and inflammatory disorders. Thus, *G. dura* may be considered for future application in medicine, food and cosmetic industries [2].

Similarly, the edible marine algae *Kappaphycus alvarezii* and *Hypnea musciformis*, were evaluated for their phytochemicals followed by its quantification, purification of terpenoids and evaluation of their *in vitro* antioxidant activity in terms of ABTS, FRAP reducing power, superoxide, hydroxyl radical scavenging and hydrogen peroxide radical assay, the metal chelating activities. Qualitative analysis revealed the phytochemicals such as carbohydrates, proteins, alkaloids, glycosides, flavonoids, steroids, saponins, phlobatannins and polyphenolics. The quantified data of *K. alvarezii* were carbohydrate - 38.28 mg/g, protein - 1.06 mg/g and fat - 1.02 mg /g of dry wt., whereas *H. musciformis* revealed 18.02, 1.2 and 0.934 mg/g of dry wt. respectively. Polyphenol forms the major secondary metabolites especially

the terpenoids. The column chromatography purified terpenoids composition from *H. musciformis* and *K. alvarezii* were fractionated by GC-MS analysis. They showed remarkable antioxidant activities in terms of scavenging hydroxyl radicals in a concentration dependent manner. Further, the extract exhibited strong inhibition on the H_2O_2 and DPPH scavenging assay. The IC_{50} values were significant and comparable with ascorbate and quercetin. Thus, the selected seaweeds may be included in daily dietaries to combat energy malnutrition and micronutrient deficiencies [3].

Remesh and Manju [4] documented the ethnobryological usage of bryophytes by the ethnic tribes from Western Ghats, India. In this scenario following bryophytes were evaluated scientifically for their importance.

Methanol extract of *Plagiochila beddomei* the liverwort was evaluated in terms of antioxidant and antimicrobial activities. Total phenolics was fractionated by high performance liquid chromatography showed the phenolic acids such as coumaric, ferulic, gallic, caffeic, protocatechol, cinnamic, sinapate, chlorogenate and hydroxyl benzoate. Methanolic extract displayed broad spectrum of antimicrobial activity against various bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and fungi like *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Aspergillus niger*, *A. flavus*, *A. terreus* and *Mucor indicus*. Poor survivorship curve of bacteria and inhibition of fungal spore germination substantiated the antimicrobial power of the extract. *P. beddomei* displayed significant antioxidant activity in terms of 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging ability, ferric-reducing antioxidant power, ferric thiocyanate assay and hydroxyl radical-scavenging activities. Phenols and flavonoids were noticed in high levels. Total phenol content was showed the highest correlation with FRAP assay (R^2 0.966). MTT assay also revealed the non-toxic nature of the extract [5].

In addition, Manoj, *et al.* [6] screened *Leucobryum bowringii* and *Octoblepharum albidum*, the bryophytes of Western Ghats in terms of total flavonoids, phenolic content, its fractionation by RP-HPLC and HPLC-PAD. Antioxidant potentialities were examined using DPPH, Ferric Reducing Antioxidant Power, Hydroxyl radical assay, FTC assay and DNA nick assay. Phytochemical study revealed the presence of phenols, saponins, flavonoids, glycosides and tannins. Significant antioxidant potentialities in terms of FRAP, DPPH, hydroxyl radical scavenging and FT assay. Phenols and flavonoids showed many phenolic acids and flavonoids such as quercetin, kaempferol and rutin analogues. Significant linear correlation was observed between total phenolic content and FRAP assay. Reduction in the formation of nicked DNA and simultaneously with increased native form of DNA. Thus, it can be summarized that the selected bryophyte extracts could be an important source of phenolic compounds with high antioxidant capacity comparable with red wine or beverages like tea.

Currently, different therapeutic strategies have been designed for the prevention and treatment of reactive oxygen species mediated diseases, with special emphasis on antioxidant therapy, that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. Among the different phytochemicals, phenolic compounds are established free radical scavengers and antioxidants common among plants; they possess many biological effects, mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals. Present investigation on *Marchantia polymorpha* a bryophyte has been an attempt to screen the phytochemicals and their potential roles in antioxidant potentialities. To complete the objectives, several parameters such as solvent extracts using nonpolar to polar, qualitative analysis of the secondary metabolites, estimation of total phenolic content and its fractionation by RP-HPLC. Antioxidant potentialities were examined with aqueous and ethanolic extracts using DPPH*, FRAP, Hydroxyl radical assay, FTC assay and DNA nick assay. The phytochemical study revealed the presence of phenols, saponins, flavonoids, glycosides and tannins. Significant antioxidant potentialities in terms of FRAP, DPPH, hydroxyl radical scavenging and FT assay. Phenols showed the peaks of many phenolic acids such as gallate, vanilate, chlorogenate, cinnamate, protocatechol, coumarate, ferulate, sinapic, caffeate and hydroxyl benzoate. Significant linear correlation was observed between total phenolic content and FRAP assay. Reduction in the formation of nicked DNA and increased native form of DNA. Aqueous and ethanolic extracts of *Marchantia polymorpha* could be a source of polyphenolic compounds with high antioxidant capacity comparable with standard ascorbate and butylated hydroxytoluene [7].

Remya Krishnan and Murugan [8] screened the phytochemicals and evaluated the antioxidant potentials of ethanolic and water extracts of *Marchantia linearis* (Bryophyta). The goals are achieved in terms of evaluating total flavonoids, phenolic content and its fractionation by RP-HPLC and HPLC-PAD. Antioxidant potentialities was validated by accessing free-radical (DPPH*, ABTS*, FRAP, phosphomolybdenum, H_2O_2 and $\cdot OH$) scavenging, iron chelating activity and scavenging of superoxide radicals were examined. RP-HPLC analysis revealed phenolic acids such as coumarate, sinapate, vanillic and gallic acids. Flavonoids noticed were quercetin, luteolin and apigenin. *M. linearis* extracts presented a remarkable capacity to scavenge all the tested reactive species with IC_{50} values being found at the $\mu g/mL$ level. Ethanolic extract was shown to have the highest phenolics while lowest IC_{50} values for the DPPH*, ABTS* radical scavenging capacities, iron chelating scavenging efficiency, significant activities in scavenging of superoxide radicals, hydrogen peroxide and hydroxyl radicals. The obtained results suggest the potential of *M. linearis* as a potent candidate against free-radical-associated oxidative damage.

Lubaina, *et al.* [9] studied the phytochemical constituents and antioxidant potentialities of petroleum ether, ethyl acetate and water extracts of *Pallavicinia lyellii* a thalloid liverwort. Qualitative aqueous extract analysis of phytochemicals showed the presence of glycosides, tannins, coumarins, alkaloids, saponins, flavonoids, phenols, steroids, reducing sugar and terpenoids. Total flavonoids and phenols were revealed significant levels. Total phenols was fractionated by reverse phase high performance liquid chromatography exhibited phenolic acids such as gallate, chlorogenate, cinnamate, protocatechol, hydroxybenzoate, coumarate, caffeate and ferulate. The *in vitro* antioxidant activity determined through 2,2-diphenyl-1-picrylhydrazyl, ferric reducing antioxidant potentiality, hydroxyl radical scavenging activity, superoxide anion radicals quenching effect and metal chelating assay. Among them aqueous extract showed the remarkable free radical quenching activity in terms of FRAP ($478 \pm 1.76 \mu M/g$), DPPH ($89 \pm 0.23\%$) and hydroxyl radical scavenging activity ($83.1 \pm 0.23\%$) whereas ethyl acetate fraction exhibited the highest superoxide anion scavenging and iron reduction ability with inhibition rate of $90 \pm 0.23\%$ and $25.3 \pm 0.96 \mu g/ml$ respectively at $1000 \mu g/ml$. The significant antioxidant potentialities may be contributed by the phenolic constituents. The results suggest that *P. lyelli* has promising antioxidant activity and could serve as potential source of natural antioxidants.

Greeshma, *et al.* [10] field surveyed the ignored medicinal bryophytes of the biological world at Neyyar wildlife sanctuary, Trivandrum, Kerala and subsequently scientifically validated the AOX potentialities of *Thuidium tamariscellum*. The analysis includes the phytochemicals screening and its antioxidant potentialities in terms of DPPH, ABTS, H_2O_2 , FRAP and metal chelating ability. Total terpenoids level was remarkable. Interestingly, a concentration dependent free radical scavenging potential was noticed and was comparable with the synthetic antioxidant ascorbate. Further, the FTIR analysis of petroleum ether, chloroform, ethyl acetate, and methanol extract confirmed the presence of alcohols/phenols, primary, secondary amines, amides, alkanes, aldehydes, saturated aliphatic alkenes aromatics and aromatic amines, nitro compounds, carboxylic acids, esters, ethers, aliphatic amines, alkyl halides and carbonyls in the moss indicating the presence of medicinally important compounds like flavonoids, terpenoids and alkaloids in the various solvent extracts of the moss. Thus, the overall result of the present study showed that the moss is rich in important pharmaceutical compounds and was reflected as its antioxidant potential. Further studies are warranted to isolate, identify and purify the lead terpenoid present in the moss [11].

Ayyanar and Ignacimuthu [12] documented ethno botanical medicinal plants commonly used by Kani tribals in Tirunelveli hills of Western Ghats. Based on the document, Meenu Krishnan, *et al.* [13] analyzed antihaemolytic, anti-lipid peroxidative potential by purified protease inhibitors from the fruits of *Solanum aculeatissimum*. In human erythrocytes against hydrogen peroxide toxicity. Protease inhibitor was isolated and purified from the fruits of *S. aculeatissimum* (SAPI) via four sequential step procedures i.e., salt precipitation to sepharose affinity chromatography. The purity was confirmed by reverse phase HPLC chromatography. The molecular mass was detected using size elution chromatography (22.2 kDa). It has deep roots in history, being one of the major botanicals used in traditional medicine to treat conditions ranging from diabetes, malaria, to snakebites. Subsequently, antihaemolytic and anti-lipid peroxidative potential was carried. Prior to the addition of H_2O_2 to induce haemolysis, different concentrations (50 - 500 $\mu g/ml$) of SAPI was added to 2 ml of 4% erythrocyte suspension and allowed to incubate for 5 minutes at room temperature. The mixture was centrifuged and the colour density

of the supernatant was measured spectro photometrically. Quercetin was used as internal standard. The % haemolysis and IC_{50} values were calculated. The PI was potent against haemolysis of the erythrocyte in concentration dependent manner with $IC_{50} = 368.54 \mu\text{g/ml}$. IC_{50} value of quercetin was $224 \mu\text{g/ml}$. The lower the IC_{50} the more protection offered against haemolysis by SAPI. These results suggest that the SAPI is ideal anti-haemolytic agent and offered significant biological action compared with standard drug employed.

Murugan, *et al.* [14] investigated the *in vivo* genotoxicity of *Artemisia japonica* and *A. nilagirica* and its potential antigenotoxicity against cyclophosphamide (CP) induced DNA damage. Initially, the essential oil (EO) was analyzed by GC-MS. *A. japonica* and *A. nilagirica* (250 and 500 mg/kg b.w.) was administered to Swiss white mice of both sexes for 15 days, and the animals received an injection of saline or CP (0.9% NaCl) 24 h before they were euthanized. The GC-MS analysis revealed the presence of camphor, 8-cineole, borneol, artemisia ketone and β -thujone the major compounds in *A. nilagirica*. Meanwhile *A. japonica* contains linalool, caryophyllene oxide, *trans*-linalool oxide, p-cymene and 1, 8 - cineole. Significant inhibitions were observed in the evaluated parameters, demonstrating the absence of cytotoxic and genotoxic effects of EOs at all tested doses. In liver, kidney, cardiac and bone marrow cells, EO reduced the DNA damage induced by CP. Results showed a dose–response action of EO. Furthermore, the various tissues showed a difference in the potential antigenotoxic effects. In conclusion, EO of *Artemisia* was not genotoxic and inhibited the genotoxicity induced by CP. Further investigations are needed to purify the potential compound from EO and also to analyze the effects over human health.

Jayakumar, *et al.* [15] evaluated the mitigating effect of purified solasodine from *Solanum mauritianum* against hydrogen peroxide induced oxidative damage in human erythrocytes. *Solanum mauritianum* (Solanaceae) is an exotic species from South America. *Solanum* species are known for alkaloid content which marks them medicinally important. Herbal products have received considerable attention in recent years due to their diverse pharmacological properties, including antioxidant and antitumor potential. Solasodine, the nitrogen analogue of diosgenin, has been reported as potential steroidal precursor for the supplementary source of the commercial synthesis of diverse steroidal drugs. Free radicals are formed during the course of normal metabolic process in the biological system. They are highly reactive molecules due to the presence of unpaired electron. In this juncture, present study was aimed to isolate and purify the alkaloid solasodine from fruits and leaf and to evaluate its antioxidant activity via the assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide, hydrogen peroxide, superoxide anion scavenging activities etc. The alkaloid solasodine was isolated from leaves and fruits of the species using non polar to polar solvents and subsequently purified using column chromatography. Remarkable antioxidant activities are shown by the alkaloid solasodine. IC_{50} values were comparable with synthetic antioxidants such as ascorbate, rutin. Moreover, the solasodine exhibited concentration dependent inhibitory activity against the free radicals. Significant scavenging property was noticed especially with DPPH radicals. Antihaemolytic activity of the solasodine was also confirmed from this study. Thus, it can be suggested that solasodine show more protective effects through antioxidant potential against H_2O_2 induced oxidative damage on erythrocytes. So, solasodine can effectively be used as natural antioxidant for the treatment and prevention of lipid peroxidation related disorders.

Similarly, Jayakumar, *et al.* [16] proved the antioxidant and antihemolytic activities of purified caulophyllumine-A from *Solanum mauritianum*. Ripened fruits of *Solanum mauritianum* are used by the local people as vegetable during famine periods and also the fruits and leaves are used to cure various ailments. In this scenario, the present investigation was undertaken to isolate the lead alkaloid molecule from *S. mauritianum* and to evaluate its antioxidant potentialities. In the first phase, the crude alkaloid was isolated, purified by column chromatography yielded a bluish coloured fraction caulophyllumine- A and was further confirmed by NMR. Further, the antioxidant activity was assayed using the DPPH radical scavenging and other assays. The IC_{50} values ranged from 66.5 to $121 \mu\text{g/ml}$. Protective effects of caulophyllumine-A, against H_2O_2 induced oxidative damage in plasmid pBR322 DNA was remarkable at the tested doses ($\mu\text{g/ml}$). Finally, the antihemolytic potential of caulophyllumine- A was analyzed against human blood erythrocytes, whereby the % lysis of RBCs was found to be in the minimal range of 4.5 to 12.4% comparable with the control ascorbate.

Lubaina and Murugan [17] documented the reactive oxygen species and ascorbate–glutathione interplay in signaling and stress responses in *Sesamum orientale* against *Alternaria sesami*. *Sesamum orientale* the wild and cultivar Thilarani exposed to *Alternaria sesami*

infection triggered the signal cascade H_2O_2 content that was positively correlated with lipid peroxidation. The data were also supported by H_2O_2 localization as observed by scanning electron microscopy. Parallely, infection altered chloroplasts marginally and mitochondria effectively in susceptible cultivar than wild sesame. Deformities in the structure of these organelles were accompanied by changes in antioxidant machinery. H_2O_2 can be effectively detoxified via the ascorbate–glutathione cycle. Increases in ascorbate peroxidase, and glutathione reductase activities concomitant with ascorbate (AsA) and glutathione interplay, as well as AsA regeneration ability, function to keep the balance of cellular H_2O_2 under pathogenicity. Dehydroascorbate reductase and monodehydroascorbate reductase are responsible for AsA regeneration. Oxidative damage in Thilarani cultivar compared to wild sesame is attributed by a lower induction of the ascorbate–glutathione cycle as an antioxidant defense system and was not sufficient to protect mitochondria but prevent ultra-structural damage of chloroplasts. Overall, the availability of antioxidants and the induction of antioxidant enzyme activities for detoxifying reactive oxygen species (ROS) are regulated effectively in wild sesame against *A. sesami* induced oxidative stress. The experiments using ROS scavengers demonstrate that the antioxidant defense system is modulated by O_2^{--} or H_2O_2 signals.

Documenting and validating traditional and indigenous system of medicine in India has been continuously increasing from the last few decades. The current study was made to evaluate the phytochemical constituents and antioxidant potentiality of *Premna serratifolia* an aromatic medicinal plant known as ‘Agnimantha’ in Ayurvedic system of medicine. It has immense ethno pharmacological importance in folk medicine for curing various human ailments. The qualitative phytochemical screening of the aqueous and ethanolic leaf extracts of *P. serratifolia* revealed the presence of glycosides, flavonoids, alkaloids, tannins, terpenoids and steroids. Subsequently, the quantification of major secondary metabolites revealed remarkable level of phenols followed by terpenoids and flavonoids. The *in vitro* antioxidant activity was determined by DPPH, FRAP, hydroxyl radical scavenging activity, hydrogen peroxide scavenging, superoxide anion radicals quenching effect and metal chelating assay. The aqueous extracts showed positive correlation between concentrations of the extracts with DPPH radical scavenging capacity. Similarly, the other antioxidant potentials also displayed remarkable activities when comparable with synthetic antioxidants such as ascorbate and BHA. The results suggest that the aqueous extract of *P. serratifolia* are potential source for natural antioxidants due to the presence of polyphenols and that could have a protective role as well as prevention from life style diseases [18].

Oxidative stress plays significant role in pathophysiologic events of acute and chronic diseases. Intracellular biomolecules such as lipids, proteins and nucleic acids are damaged via oxidation by excessive ROS. Protease inhibitor was isolated and purified from the fruits of *Solanum aculeatissimum* (SAPI) via four sequential step procedure i.e. salt precipitation to Sepharose affinity chromatography. Subsequently, the antioxidant power is analysed using DPPH, H_2O_2 , O_2^{--} , ABTS, $OH\cdot$ radicals scavenging activity, reducing power potential, metal chelating ability and FRAP (Ferric reducing antioxidant power) method. SAPI exhibited significant IC_{50} values for most of the AOX assays. DPPH radical scavenging, reducing power, metal chelating ability, ABTS and $OH\cdot$ radical scavenging activities were comparable with the synthetic antioxidants like ascorbate and BHT. Further studies are warranted to trace the molecular mechanism of AOX activity by SAPI using *in vivo* animal models [19].

The use of traditional medicine is expanding to newer horizons and plants still remain as the novel source of structurally important compounds that lead to the development of innovative drugs. It is anticipated that plants can provide potential bioactive compounds for the development of new ‘leads’ to combat various diseases. The systematically performed *in vitro* assays revealed that the tested plant extract may find in therapy as agent with high pharmaceutical value. In this juncture, the present study was undertaken to analyze the antioxidant, antimicrobial and anticancer potentiality of the medicinal herb *Orthosiphon aristatus* Benth. The results reveal marginal antibacterial, anti-inflammatory and anticancer activity than the respective standards. Considering anticancer activity, the extract showed high IC_{50} values for all the cell lines tested. The data on anti-inflammatory activity indicate an inhibition percentage of 19.38, when treated with 10 μ g/ml of extract while indomethacin produced 54.4% inhibition. For antibacterial activity, inhibitory values at higher concentrations were quite good. In general, more activity was obtained when using more concentrated decoctions of the plant extract studied. The best results (60.5% inhibition) were obtained with antioxidant activity. This study provides evidence that the plant extract have an-

tioxidant properties, as tested through the DPPH method. Therefore, the plant may have great relevance in the prevention and therapies of diseases in which oxidant or free radicals are implicated. The ability of the extracts to scavenge the free radical is an indication of the broad spectrum antioxidant potential of *O. aristatus*, which make the plant a candidate for bio prospecting for antioxidant drugs. In addition, the plant is a good candidate for further phytochemical and chromatographic studies to isolate and fully characterize the compound related to this *in vitro* biological activity [20].

Sunila and Murugan [21] aimed to study the changes in the level of phenols and flavonoids during fruit development of *Pouteria campechiana* and its antioxidant potential. The number of experiments conducted was twenty for each case. Polyphenols at different ontological stages may help producers and food technologists to identify which cultivar and/or maturity stage are most adequate for their need. Egg fruits were harvested and classified into six developmental stages based on week after pollination (WAP): stage I (4WAP); stage II (8 WAP); stage III (12WAP); stage IV (16WAP); stage V (20 WAP) and stage VI (24 WAP). The total phenolics and flavonoids of egg fruits at different developmental stages were investigated. The antioxidant capacities of ethanolic and aqueous extracts were determined by different assays such as FRAP, DPPH, ABTS, superoxide anion, hydroxyl radical and H₂O₂ scavenging assays. The total phenolic contents varied from 30.35 to 2.26 mg chlorogenic acid equivalents/g dry weight (DW), and the total flavonoid contents ranged from 0.683 to 3.37 mg rutin equivalents/g DW. Total phenolics showed an initial increase and subsequently decreased during development. In contrast, flavonoids increased from stage I to VI. Antioxidant assays showed varied patterns of inhibition. Significant correlations were observed between antioxidant capacities and total phenolic and flavonoid contents.

Essential oils (Eos) are complex mixtures of volatile lipophilic components and are obtained from leaf, twigs, fruits, flowers of higher plants. These oils are isolated commonly through hydrodistillation. *Pogostemon* species are well known for their essential oils and therapeutic values. Chemical composition and antioxidant activity of Eos from two species viz. *Pogostemon benghalensis* and *P. cablin* were evaluated. Essential oil from both species was extracted by hydrodistillation using Clevenger type apparatus. The essential oil obtained was subjected to GC-Fid analysis followed by GC-MS. The antioxidant activity was studied using DPPH, ABTS, FRAP, metal chelating, hydrogen peroxide, super oxide radical, reducing power, hydroxyl radical scavenging assays using ascorbic acid and butylated hydroxytoluene as standards. 36 to 41 compounds were identified from the essential oil of *Pogostemon* species. α -Cadinol (35.78%) and patchouli alcohol (34.85%) were the major components in these oils. In addition to these compounds 1,8 cineole, aromadendrene, β -patchoulene, α -caryophyllene, β -caryophyllene, α -patchoulene, germacrene A were identified as the other predominant compounds. The antioxidant activity of Eos was significant when compared with standards. 75.3 to 91.5% inhibition activity was noticed against various free radical scavenging assays. The Eos showed remarkable IC₅₀ value which suggests their potency to use as a natural antioxidant in various industries, foods and therapeutics [22].

Ethnic usage reveals that most of the plants possess a wide array of biological and pharmacological potentialities that may protect tissues from oxidative damages. In the present part, various solvent extracts of root and leaves of *Thottea siliquosa* was screened to evaluate their potent *in vitro* antioxidant activity, total phenol, alkaloid, saponins and flavonoid contents in order to find possible sources for future novel antioxidants for pharmaceutical formulations. Thorough study was performed with the petroleum ether and ethyl acetate extracts of root and leaf of the plant by *in vitro* chemical analysis and antioxidant potentialities. Preliminary qualitative analysis revealed the presence of alkaloids, tannins, glycosides, coumarins, flavonoids and saponins. Phenols (2.5 - 4.8 mg/g), alkaloids (43 - 287 μ g/g) and saponins (66-303 μ g/g) represented the highest level both in the petroleum ether and ethyl acetate extracts of root and leaves. The antioxidant potentialities in terms of DPPH radical, hydroxyl radical, superoxide anion and nitric oxide quenching capacity revealed varying responses. The IC₅₀ values are comparable with the standards such as ascorbate and butylhydroxy toluene. Lipid peroxidation level was comparatively inhibited by the extracts. Results from present study suggest that the phytochemicals in the extracts of *T. siliquosa* act as antioxidant agents as it was substantiated by its free radical scavenging activities [23].

Artemisia species are used in traditional medicine for the treatment of various ailments such as indigestion, infection, irregular menstruation, cramp, cold, epilepsy, typhoid, tuberculosis, urinary calculi, colic, fever, asthma, bronchitis, sciatica, flatulence, anaemia, insom-

nia, gout, depression, nervousness, hysteria, measles, bruise, chilblain, leprosy, malaria and cancer. Similarly, most species has been used as antiseptic, antioxidant, anthelmintic, expectorant, diuretic and insect repellent. The biological activity shown by the plants was due to the essential oil present in them. The present study describes the fractionation of essential oil and its antioxidant potentialities in two allied species - *Artemisia japonica* Thunb. and *A. nilagirica* (Clarke) Pamp. The essential oil from *Artemisia japonica* and *A. nilagirica* was isolated by hydrodistillation and analyzed by GC-MS. 30 and 19 compounds were noticed in the oil respectively. The predominant phytochemicals in the essential oil of *A. nilagirica* were camphor (41%), 8-Cineole (17.0%), Borneol (8.6%) Artemisia ketone (6.4%) and β -Thujone (6.0%). Meanwhile *A. japonica* contain Linalool (70.4%), Caryophyllene oxide (6.7%), *trans*-Linalool oxide (4.5%), p-cymene (3.4%) and 1, 8-Cineole (2.3%). Significant antioxidant potentialities was displayed in terms of DPPH scavenging, beta carotene bleaching, total antioxidant activity, soybean oil rancidity and sun flower oil free fatty acids and iodine values. Interestingly, *A. nilagirica* showed remarkable antioxidant potentialities and was comparable with synthetic antioxidants such as ascorbate, butylated hydroxytoluene and BHA. The present study suggests that the tested *Artemisia* species revealed a pool of potential biomolecules in the essential oil and also exerted remarkable antioxidant values. Further studies are warranted to isolate and purify the principle component from the essential oil [24].

Tylophora subramanii, a climbing endemic species of Southern Western Ghats belongs to Asclepiadaceae. The local people use this plant for curing many rheumatic ailments. The objective of this study is to explore the phytochemistry and the antioxidant potential of ethanolic leaf extract of *T. subramanii* which is considered traditionally as an important medicinal plant. The methods adapted includes (a) phytochemical analysis to find out the presence of various bioactive compounds (b) *in vitro* antioxidant analysis of ethanolic leaf extract by 2,2-diphenyl-1-picrylhydrazyl assay, nitric oxide scavenging assay, superoxide scavenging potential, *ex vivo* superoxide dismutase assay, catalase assay. The major outcomes are the following: ethanolic leaf extract showed remarkable levels of the phytochemicals such as flavonoids, tannins, terpenoids, saponins, alkaloids, proteins and carbohydrates. Similarly, it also showed potential antioxidant activities. High catalase and superoxide dismutase activity with low lipid peroxide level, which were comparable with the synthetic antioxidant L-ascorbic acid. Thus, the work concludes that *T. subramanii* display a wide range of pharmacologically useful phytochemicals which exhibited significant antioxidant potentials. Future works are planned to elucidate the lead molecule and its biological potentialities [25].

Aswathy and Murugan [26] documented the ethnobotanical knowledge about herbals used by tribes of Kerala and Tamil Nadu with special reference to *Begonia malabarica*. Anthocyanins from vegetables and flowers have fascinated pharmaceutical industries are proven nutraceutical health. *Begonia* species forms diverse hyper group and are distinguished on the basis of morphological parameters. *Begonia rex-cultorum* (Baby rainbow) and *Begonia malabarica* exhibited the highest antioxidant activities. The anthocyanin concentration positively correlates with the antioxidant potentialities among the cultivars. The IC_{50} value related with DPPH and metal chelating activities of the *B. rex-cultorum* (Baby rainbow) extract are 32.3 μ g/mL and 18.7 μ g/mL respectively. Remarkable scavenging potentialities are displayed against metal chelating, carotene bleaching, ABTS radical, FRAP assays and the results are comparable with synthetic antioxidant like BHT. The diversity in radical scavenging in these assays may be due to factors like stereo selectivity of the radicals or due to the differential solubility of anthocyanin molecules in the crude extract. Further studies are warranted to isolate and fractionate the major anthocyanins in the cultivars [27].

Bosco Lawrence and Murugan [28] reported the folklore use of *Osbeckia* species from Munnar hills, Kerala and subsequently validated the information. Health-benefit properties of natural pigments have been intensely studied, especially the anthocyanins. In the last few decades, research on anthocyanins has attracted biologists by the increasing evidence of their health beneficial effects. *Osbeckia*, belongs to Melastomataceae and is well-known for colouring pigments and other bioactive compounds. In the present study, total anthocyanin and antioxidant capacity indicators were evaluated from 8 *Osbeckia* spp. and anthocyanin was extracted from *in vitro* cultures of *O. aspera* and *O. reticulata*. The antioxidant effect was studied using ABTS (2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay, the FRAP, the scavenging ability of hydroxyl radicals and the superoxide anion scavenging activity. Anthocyanins extracted from *in vitro* cultures were purified and fractionated using column chromatography and LC-MS MS analysis. *In vitro* cultures

of *O. aspera* was obtained in MS medium fortified with various combinations of Benzyl Adenine (BA), Naphthalene acetic acid (NAA) and 2, 4-D. The chromatograms of *O. aspera* revealed the presence of malvidin-3 -diglucoside, peonidin, delphinidin and cyanindin whereas *O. reticulata* cultures accumulated large amounts of malvidin, cyanindin and cyanidin aglycone. Thus, the results tempt to suggest that the *Osbeckia* species are rich in anthocyanin and therefore displayed potential AOX power. *O. aspera* and *O. reticulata* callus was induced for the *in vitro* production of anthocyanins. The pool of anthocyanins was purified and fractionated by LC-MS/MS and AOX assays were performed with the purified anthocyanin which showed higher level activities [29].

Greeshma Murukan and Murugan [30] reported the natural dye yielding plants used by the tribes of Wayanad, Kerala, India. They validated the hepatoprotective and antioxidant activity of purified anthocyanin extracted from the cell suspension culture of *Clerodendron infortunatum*. A protocol has been developed for the induction of callus proliferation from leaf and nodal explants of *C. infortunatum*. The explants were inoculated on murashige and skoog (MS) medium supplemented with diverse combinations of 2, 4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) for triggering callus formation. Subsequently, the green compact callus has been sub-cultured in the medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin for anthocyanin synthesis. Cell suspension culture was also established and the elicitor, salicylic acid was used for triggering anthocyanin synthesis. Three different chromatographic columns (solid phase extraction by Sepharose C18 column, Oasis-MCX and Amberlite XAD 7+Sephadex LH 120 sorbents) were employed to purify the *in vitro* synthesized anthocyanin from cell suspension cultures. For purity evaluation, high-performance liquid chromatography (HPLC) and molar absorptivity assay was used. Further, hepatoprotective and antioxidant activity was evaluated comparing with silymarine, as standard in rats. *In vitro* antioxidant scavenging activity was analysed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay. After 1 month, the leaf explants yielded remarkable green compact callus on murashige and skoog (MS) medium containing 2.0 mg/l benzylaminopurine (BAP) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Salicylic acid enhanced anthocyanin synthesis. The mean purity values obtained by high-performance liquid chromatography (HPLC) were $90.9\% \pm 1.9$ and $80.60\% \pm 2.3$ for Oasis MCX, Amberlite XAD-7+Sephadex LH-20 column respectively. However, the purity calculated by molar absorptivity was found to be less. The highest purity achieved using molar absorptivity analysis was with MCX cartridges i.e., $85.9 \pm 3.8\%$. High-performance liquid chromatography (HPLC) yielded 12 anthocyanin fractions. Remarkable antioxidant scavenging activity was noticed as revealed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay. The hepatoprotective activity (25, 50, 100 mg/100g b. w) was compared with silymarine (25 mg/kg b. w) against carbon tetrachloride (CCl_4) induced toxicity. Anthocyanin extract improved the aspartate aminotransferase (AST), alanine aminotransferase (ALT) and recovered the activity of kidney function by decreasing the urea and creatinine content. In addition, the administration of anthocyanin significantly inhibited the oxidative stress via its scavenging of the reactive oxygen species formed by carbon tetrachloride (CCl_4) stress. Further, a decrease in the malondialdehyde (MDA), hydrogen peroxide (H_2O_2), nitric oxide (NO) accumulation and an increase of glutathione (GSH) content were noticed. Similarly, improved lipid profiles, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were also observed suggesting that anthocyanin significantly suppress the toxicity via its activation of antioxidant enzymes [glutathione transferase (GST), catalase (CAT) and superoxide dismutase (SOD)]. The overall results showed that the purified anthocyanin of *C. infortunatum* functions as an antioxidant and thereby hepatoprotective protection against carbon tetrachloride (CCl_4) induced toxicity in animal models [31].

Greeshma Murukan and Murugan [32] purified and characterized anthocyanin from *in vitro* culture of teak and its antioxidant potential. Anthocyanin was extracted from *in vitro* culture, purified by using amberlite lite XAD column and fractionated by Liquid chromatography mass spectrometry (LC-MS/MS). Various antioxidant assays were carried such as 2,2-diphenyl 1-picryl-hydrazyl-hydrate (DPPH), 2,2'-azino-bis-3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), Oxygen radical absorbance capacity (ORAC), Nitric oxide (NO) and Hydrogen peroxide (H_2O_2). Liquid chromatography mass spectrometry (LC-MS/MS) revealed the major fraction as cyanidin 3-(2-xylosyl-rutinoside) with unknown peaks. The amount of anthocyanin was 15.23 mg/g monomeric anthocyanin. Further, the potential antioxidant capacity of the teak anthocyanin was comparable to common vegetables and fruits. Similarly, high correlations of anthocyanin with

antioxidant activity, such as oxygen radical absorbance capacity (ORAC), 2,2'-azino-bis-3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) ($r = 0.95, 0.93$, and 0.80) were found. The high anthocyanins content and potential antioxidant activity suggests that teak anthocyanin may be applied in the food industry as a good source of natural pigments.

Arathy, *et al.* [33] evaluated the antioxidant activities of purified anthocyanin from wild balsam species. Initially, anthocyanin was extracted from floral leaves of wild balsam species and purified by chromatographic techniques. The major fractions identified were hesperidin, dimethoxy antirrhinin and trimethoxy antirrhinin. Further, the anthocyanin extracts were subjected to *in vitro* protocols like 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, DPPH scavenging assay, ferric reducing antioxidant power (FRAP), beta carotene bleaching assay, metal chelating and H_2O_2 scavenging power. Interestingly, ABTS, FRAP analyzes yielded significant results as compared to others. The data were comparable with that of synthetic antioxidants like ascorbate and catechin. Meanwhile, beta carotene and H_2O_2 scavenging assay showed moderate results. DPPH and metal chelating protocols displayed the values 71% and 64% respectively at 25 $\mu\text{g/ml}$ concentration. The antioxidant results were supported by NMR and LC MS analysis. This study provides model systems for the evaluation of natural antioxidants like anthocyanin. Future *in vivo* clinical studies are warranted to confirm the obtained data.

Conclusion

The overview of the present report infers that these plant extracts possess optimal antioxidant properties. Thus, the extracts can be beneficial in treating stress caused due to multiple of factors. Over the past few years, significant scientific knowledge has been documented regarding plant redox characters and its AOX defense. Plants synthesize and accumulate many non-enzymatic antioxidants like ascorbate, glutathione and polyphenolics. Some of these AOXs occur constitutively, while others are synthesized in response to abiotic and biotic stress events. Almost all plant based phytochemicals exhibit certain level of antioxidant activity under *in vitro* assays. However, for *in vivo* studies, plant antioxidants have to channel through multiple process. However, many pharmaceutical industries tapping such native knowledge of people in to economically important nutraceutical products. In this juncture, the above said species could be further screened for isolating unique antioxidants that can be supplemented to human diet.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgements

The authors thank the study group for their collaboration.

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Volume 8 Issue 10 October 2020

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