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Review Article

Phytochemical Diversity and Pharmacological Perspectives of *Vitex leucoxylon* L.f.: A Comprehensive Review

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Abstract: *Vitex leucoxylon* L.f. (family Lamiaceae) is a large deciduous tree with a spreading crown and trunk, distributed in peninsular India and Sri Lanka. Traditionally, the plant has been used in medicine for the treatment of fever, joint pain, jaundice, anaemia, asthma, cancer, wounds, headache and catarrh, with specific mention in Ayurveda for managing bone disorders. Pharmacological studies have revealed hepatoprotective, antioxidant, anti-inflammatory, antidepressant, antimicrobial and analgesic activities. Phytochemical investigations have identified bioactive compounds including β-sitosterol, dimethyl terephthalate, isovitexin, vitexin, agnuside and aucubin, particularly concentrated in leaves and bark. In the present review, a systematic search was conducted following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines to ensure transparency and reproducibility. A total of 152 records were identified through database searches such as PubMed, Scopus, Elsevier, Springer and Google Scholar using the keywords as "*Vitex leucoxylon* L.f.," "ethnobotany" ands "pharmacology" from the period of 1994 to 2025. After removing duplicates (5 records), 147 studies were screened based on titles and abstracts. Following eligibility assessment of full texts, 107 articles met the inclusion criteria and were synthesized in this review. The identification, screening, eligibility and inclusion of pertinent research are all summarized in the PRISMA flow diagram. Future pharmacological and phytochemical research on *V. leucoxylon* L.f. is highly promising due to its wide therapeutic potential and phytoconstituent profile. This PRISMA-based systematic review reveals despite its enormous pharmacological potential, rigorous standardized, analytical, and clinical studies are needed to translate *Vitex leucoxylon* L.f. into an evidence-based therapeutic resource.

Keywords: Iridoids, *Vitex leucoxylon* L.f., Phytochemistry, Pharmacology, Ethnopharmacology, Systematic Review.

1. INTRODUCTION

The genus *Vitex* (family Lamiaceae) comprises approximately 270 species, characterized by their diverse bioactive constituents and wide range of medicinal properties [1]. These species are traditionally used to treat conditions such as disorders related to inflammation, infections and female reproductive health [2, 3]. *Vitex leucoxylon* L.f. is a deciduous tree with a thick trunk and spreading crown, commonly distributed throughout the Deccan Peninsula of India [4]. It is a riparian species native to peninsular India and Sri Lanka, predominantly growing in alluvial soil types. Ecologically, the plant occurs in mid-storey habitats, is light-dependent and is typically found along river floodplains that undergo periodic inundation. It acts as a natural sand binder and plays an important role in preventing riverbank erosion [5]. The leaves of *V. leucoxylon* L.f. are used in traditional medicine to cure fever, catarrh, headaches, and possess anti-depressant, analgesic, anti-parkinsonian, and anti-

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microbial activities [6]. In Ayurveda, the plant is reported to be beneficial in the treatment of bone disorders, jaundice, asthma, anaemia and malarial fever, with leaf decoctions commonly prescribed for therapeutic purposes [7, 8]. In addition to being applied to wounds, burns, and cuts, leaf paste is said to have antidiabetic qualities [9, 10]. Additionally, the plant is used for medicinal baths in malarial fever [11], as well as for managing joint pain [12], and cancer [13, 14]. It is further documented to promote wound healing, reduce cholesterol levels and exhibit anti-inflammatory, hepatoprotective and insecticidal properties. The root and bark are considered astringent, while the root has also been employed as a febrifuge [15-18]. Phytochemical investigations have identified several compounds from its leaves and bark, such as β-sitosterol, vitexin, dimethyl terephthalate, isovitexin, agnuside and aucubin [19]. HPTLC profiling of *Vitex leucoxylon* L.f. leaves provides a reliable tool for identification and standardization [20]. AgNPs synthesized from *Vitex leucoxylon L.f.* leaf extract are rich in flavonoids, terpenoids, phenols, tannins and saponins, showed stronger antioxidant, anti-inflammatory, cytotoxic and wound-healing activities than the extract alone, with maximal effects against oral cancer and significant *in vitro* wound closure and anti-inflammatory responses [21]. Despite its traditional importance and emerging pharmacological evidence, the available scientific information on *Vitex leucoxylon* L.f. remains fragmented and scattered across different studies. To date, a comprehensive synthesis of its phytochemical constituents, ethnomedicinal applications and experimentally validated pharmacological activities is lacking. This review aims to integrate traditional medicinal knowledge with modern pharmacological findings, thereby establishing a scientific rationale for the therapeutic applications of *Vitex leucoxylon* L.f.. The documentation of its diverse bioactive constituents and experimentally validated biological activities highlights its potential as a valuable source for novel drug leads. Furthermore, the need for thorough research to examine its pharmacological and phytochemical potential is further reinforced by its ecological role and ethnomedicinal significance. *Vitex leucoxylon* L.f. was selected for this review due to its emerging yet limited pharmacological evidence, diverse phytochemical composition, and documented bioactivities, particularly in light of the substantial therapeutic potential demonstrated by related species such as *Vitex negundo* L. and *Vitex trifolia* L. The aim of this systematic review is to comprehensively summarize phytochemical diversity, and pharmacological properties of *Vitex leucoxylon* L.f., identify research gaps and propose directions for future investigation.

2. METHODOLOGY

2.1. Data Sources and Retrieval

A comprehensive literature search was conducted in May 2025 in accordance with PRISMA guidelines, using PubMed, Scopus, Web of Science, and Google Scholar (Figure 1). Grey literature sources, such as books, theses, patents, and regional journals, were also considered. The search strategy used combinations of keywords such as "*Vitex leucoxylon"*, "Phytochemistry", "Pharmacology", "Taxonomy" and "Phytoconstituents". To ensure inclusivity, no date restrictions were applied, and both experimental and clinical studies were considered. Records were restricted to the English language, with duplicates removed through systematic screening. Additionally, backward and forward citation tracking enhanced coverage of relevant literature. The search yielded 152 records, of which 147 remained after duplicate removal. Following the eligibility assessment, 107 studies were included in the qualitative synthesis, comprising 74 original articles, 20 review articles, 6 books, 4 theses, 1 book chapter, 1 patent, and 1 conference paper. These studies formed the basis of the qualitative synthesis. PubMed, Scopus, Web of Science, and Google Scholar and Chemical structures and diagrams were prepared using ChemDraw software, while conceptual illustrations were designed in a mind-map format.

3. TAXONOMIC STATUS

Vitex leucoxylon L.f. was first described by Carl Linnaeus the Younger in *Supplementum Plantarum Systematis Vegetabilium* (1781) from the forests of Ceylon (Sri Lanka) [22]. In the protologue, Linnaeus noted that the leaves resemble five fingers and that the plant bears a monospermic berry (containing a single seed). He further described its oblong, petiolate leaves and dichotomous panicles, remarking that the species closely resembles *Vitex trifolia* L., but differs by possessing glabrous leaves and dichotomous panicles from the first branching

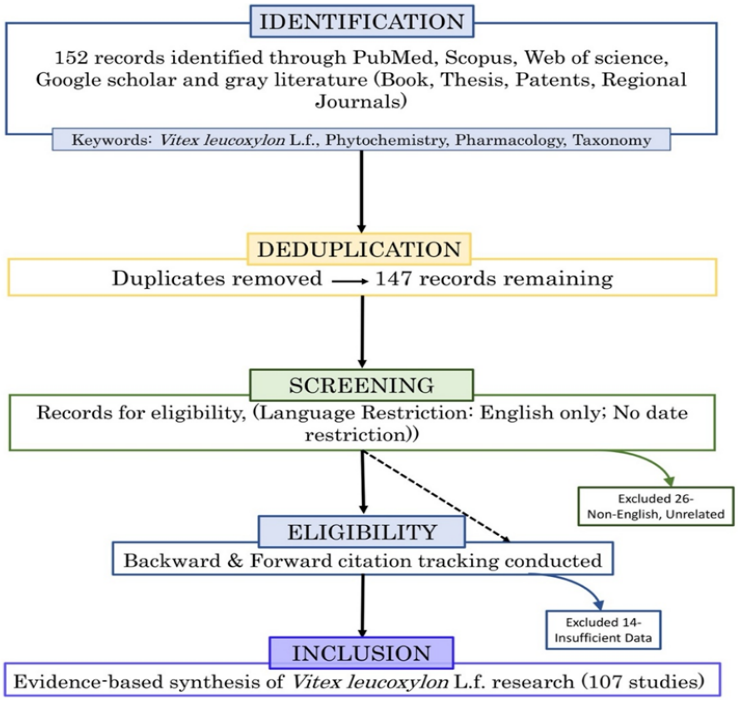

Fig. 1. PRISMA flow diagram of 107 studies on *Vitex leucoxylon* L.f. during May, 2025.

[22]. In 1971, Harold Moldenke described a new variety, *Vitex leucoxylon* L.f. var. *zeylanica* Moldenke, based on smaller leaflets measuring 2.5 - 9 cm in length and 1.3 - 5 cm in width, with margins more or less serrate and surfaces dull grey-green above [23]. Later, in 1977, Moldenke reassigned this taxon to the rank of forma, as *V. leucoxylon* f. *zeylanica* (Moldenke) Moldenke, possibly due to its morphological variation within the species [24]. Earlier, Roxburgh (1832) had described *Vitex saligna* [25]; however, in 1977, Moldenke reduced this taxon to a new status, *V. leucoxylon* L.f. *saligna* (Roxb.) Moldenke, based on leaf morphology. The mature leaflets were described as uniformly narrow, elliptic and thinly chartaceous, being 3 - 4 times longer than wide, with an acute to acuminate apex. Subsequent observations confirmed that these were merely morphological variations within *V. leucoxylon* L.f. Hence, all formae and varieties proposed by Moldenke, are now considered synonyms of *V. leucoxylon* L.f. [24].

3.1. Morphology of *V. leucoxylon* L.f.

Vitex leucoxylon L.f. is a medium-sized deciduous tree, native to India and Sri Lanka, attaining a height of up to 12 m. The stem and branches are obtusely quadrangular. The leaves are compound, with obovate to oblanceolate leaflets that are thin-coriaceous, glabrous, with apices rounded to acute and bases attenuate to cuneate. The margins vary from entire to toothed. Petiolules and petioles are well developed. The inflorescences are axillary or terminal, consisting of loose, spreading panicles or corymbose dichasial cymes. Flowers are bisexual, zygomorphic and hypogynous. The calyx is cupular to campanulate, appressed-pubescent and without teeth. The corolla is cream to purplish in color, with a 5 mm long tube and five lobes, arranged into an obtuse upper lip and lower lip. Stamens are four, didynamous, with paired filaments. The ovary is superior and hairy at the apex, with a long style. The fruit is a drupe, ellipsoid to oblong in shape. Each fruit generally contains four seeds. Flowering occurs from February to April, while fruits and seeds are present throughout the year [25]. The detailed morphological characters are given in Figure 2.

3.2. Geographical Distribution

Vitex leucoxylon L.f. is distributed at altitudes up to 900 m and extending northward to Jhansi and parts of Bihar. The species commonly grows along riverbanks, streams, and ponds, and is well represented in evergreen and semi-evergreen forests. Across Asia, the species occurs in India, Pakistan, Sri Lanka, Myanmar, Indonesia and Malaysia. Within India, it is recorded from several

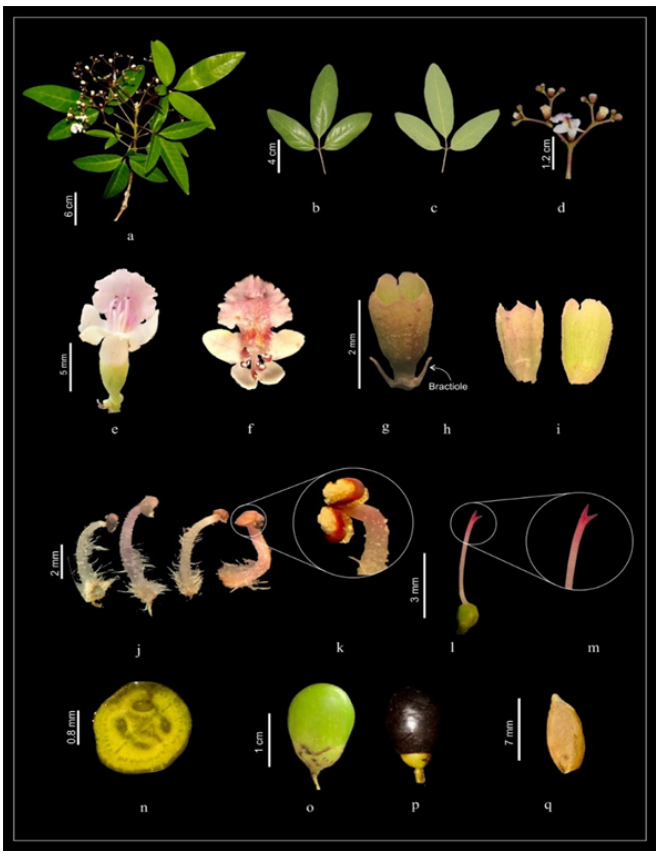

Fig. 2. Morphological features of V. leucoxylon L.f. (a) Habit, (b & c) adaxial and abaxial leaf surfaces, (d) panicle, (e) entire flower, (f) longitudinal section of flower, (g) non-dissected calyx, (h) Bracteole, (i) Dissected calyx, (j) Stamens, (k) Anther lobes enlarged, (l) Style, (m) Stigma, (n) Transverse Section of ovary, (o) Unripe drupe, (p) Ripened drupe, and (q) seed.

states, including Andhra Pradesh, Karnataka, Goa, Kerala, Maharashtra, Madhya Pradesh, Odisha, Uttar Pradesh and Tamil Nadu. In deciduous forests, it is often found scattered along streams, from the Western Ghats to the plains [26].

4. PHYTOCHEMISTRY

Chemical compounds: *Vitex leucoxylon* L.f. serves as a rich reservoir of diverse phytoconstituents represented across different plant parts, as shown in Figure 3 and phytoconstituents diversity displayed in Figure 4. To date, at least 117 compounds have been reported from different parts of the plant, representing a wide spectrum of primary and secondary metabolites. These include 38 miscellaneous compounds, 14 terpenoids, 12 esters, 12 fatty acids, 9 sugars, 8 volatile organic compounds, 7 flavonoids, 7 phenolics, 4 iridoids, 3 flavones and 3 terpenes. For systematic understanding, the identified compounds can be classified into: Basic metabolites (sugars, fatty acids, esters); Derived groups (phenolics, flavones, flavonoids, iridoids); Complex secondary metabolites (terpenes, terpenoids, volatile organic compounds) and Miscellaneous compounds (unique or unclassified phytochemicals). A detailed summary of these phytoconstituents is presented in Supplementary Table 1 (arranged according to their biosynthetic categories, from basic metabolites to complex secondary metabolites), and their structural illustration, given in Figures 5-8 synopsize the chemical makeup of *Vitex leucoxylon* L.f., elucidating a wide range of primary and secondary metabolites that conjointly contribute to its nutritional and pharmacological importance. These chemical structures were redrawn by us using ChemDraw software based on the structures reported in the literature and structural information obtained from PubMed sources. Figure 5 represents sugars and fatty acids/esters representing primary metabolites. The presence of mono- and oligosaccharides such as glucose, mannose, lactose, maltotriose, dextrins and cyclodextrins indicates

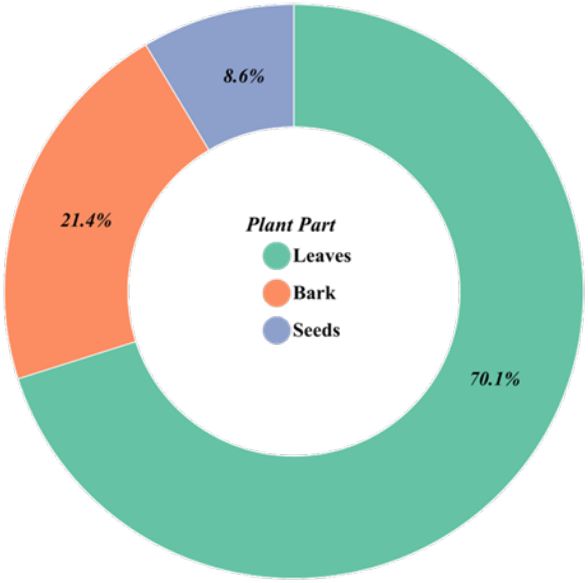

Fig. 3. Distribution pattern of chemical constituents in *Vitex leucoxylon* L.f. plant part.

Fig. 4. Rose plot of phytoconstituent distribution in *Vitex leucoxylon* L.f.

active carbohydrate metabolism and energy storage functions. Saturated and unsaturated fatty acids, including hexadecanoic, octadecadienoic and octadecatrienoic acids and their esters, highlight their role in membrane structure, metabolic regulation and nutritional value. Figure 6 illustrates terpenes, terpenoids, triterpenoids and iridoids, which form a major bioactive group in *V. leucoxylon* L.f.. Compounds such as caryophyllene, phytol, betulinic acid, corosolic acid, β-sitosterol, aucubin and agnuside are widely reported for anti-inflammatory, antioxidant, hepatoprotective and anticancer activities, supporting the medicinal relevance of the species. Figure 7 presents phenolics, flavones and flavonoids associated with antioxidant and protective functions. Phenolic acids, coumarin, cinnamaldehyde and flavonoids such as vitexin, isovitexin and kaempferol glycosides contribute to free radical scavenging and cytoprotective effects. Figure 8 summarizes miscellaneous compounds, including hydrocarbons, long-chain alcohols, aldehydes, amides and sulfur- and nitrogen-containing compounds, reflecting the chemical diversity and potential ecological and biological roles of the plant. Overall, the metabolite profile shown in Figures 5-8 demonstrates that *Vitex leucoxylon* L.f. is chemically rich, containing essential primary metabolites and diverse bioactive secondary compounds, providing a strong basis for its traditional use and pharmacological potential that mentioned in Supplementary Table 1.

5. PHARMACOLOGICAL ACTIVITIES

The pharmacological activities of *Vitex leucoxylon* L.f. have been systematically categorized to provide a clear understanding of its therapeutic potential. These activities are arranged in a streamlined sequence, beginning with general protective effects, followed by metabolic regulation, organ-specific actions, nervous system effects and finally phytotoxicity. The detailed pharmacological activities are presented in Figure 9 and Supplementary Table 2 summarises the pharmacological potential of *Vitex leucoxylon* L.f., providing detailed information on the plant part investigated, type of pharmacological activity, nature of the extract, dose and route of administration, standard drug used, control or assay conditions, experimental method, inducing chemical (if any), biological model or microbial strain tested and the detailed results, thereby offering a comprehensive framework for interpretation and guiding further pharmacological investigations.

5.1. Antimicrobial Activity

In ethnomedicine, several folklore plants used by tribal communities in the Western Ghats of India have been screened for antimicrobial activity. Furthermore, *V. leucoxylon* L.f.-derived biosynthesized silver nanoparticles (AgNPs) demonstrated broad-spectrum antibacterial

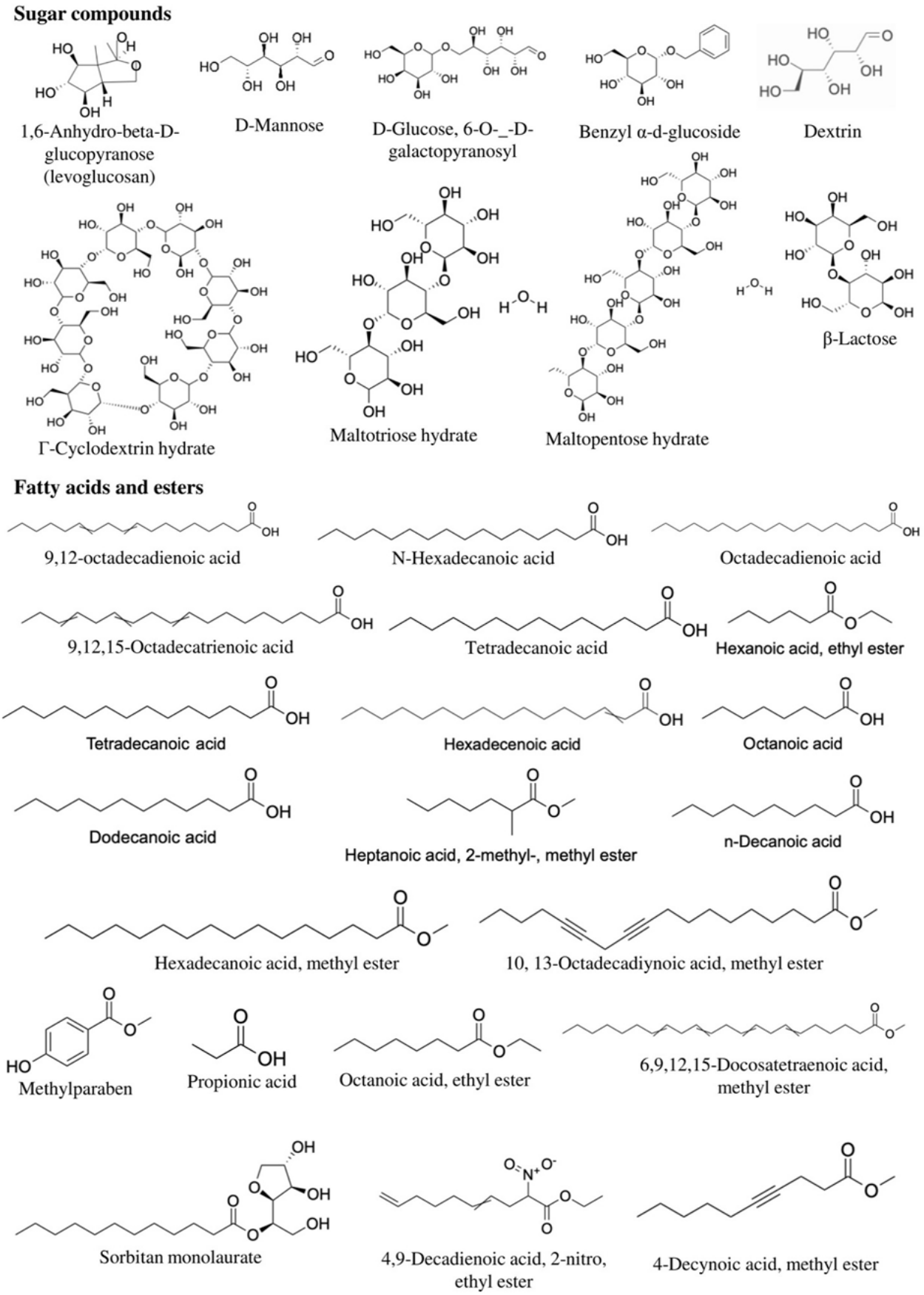

Fig. 5. Structure of phytochemicals characterized from *Vitex leucoxylon* L.f.

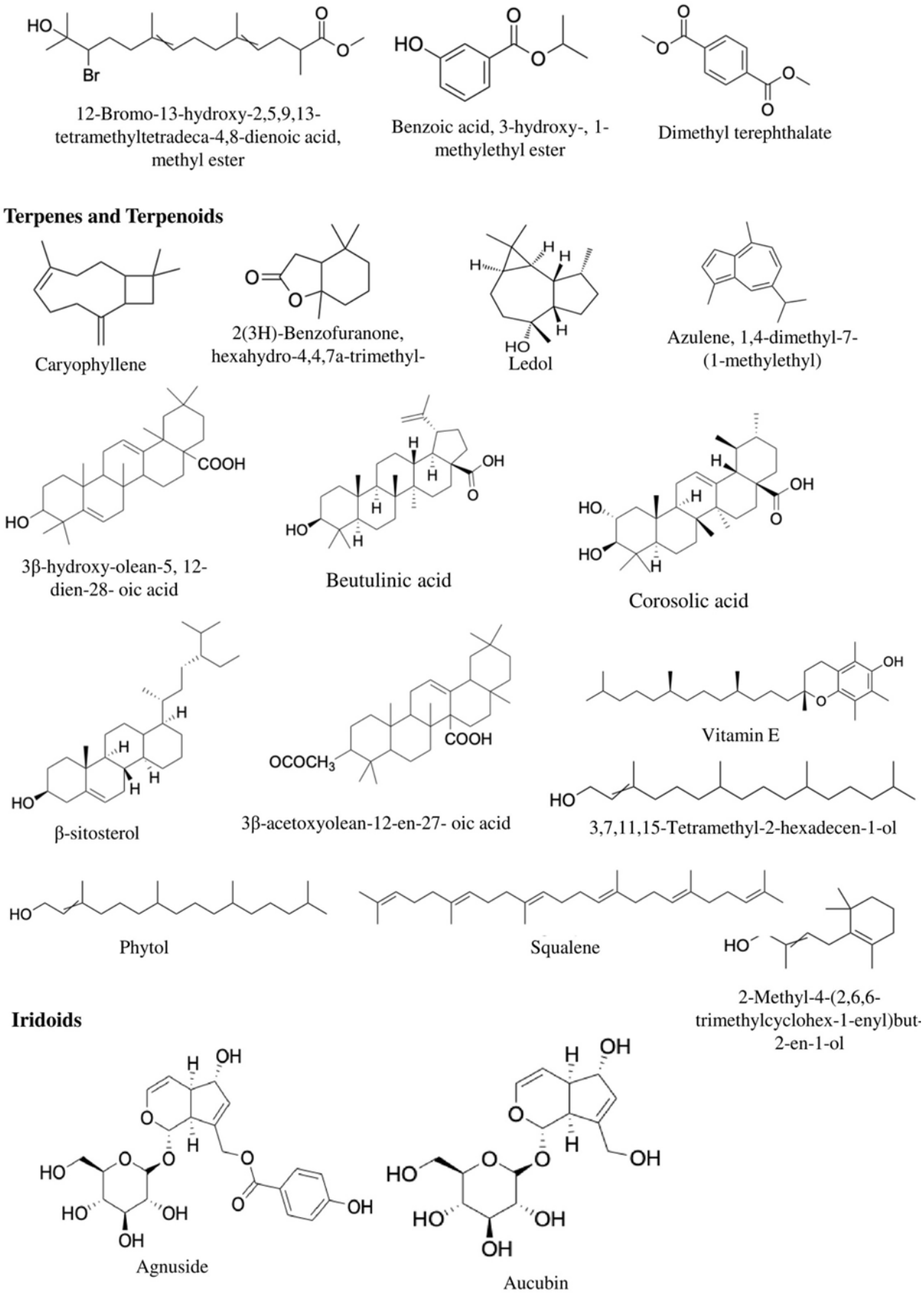

Fig. 6. Structure of phytochemicals characterized from *Vitex leucoxylon* L.f.

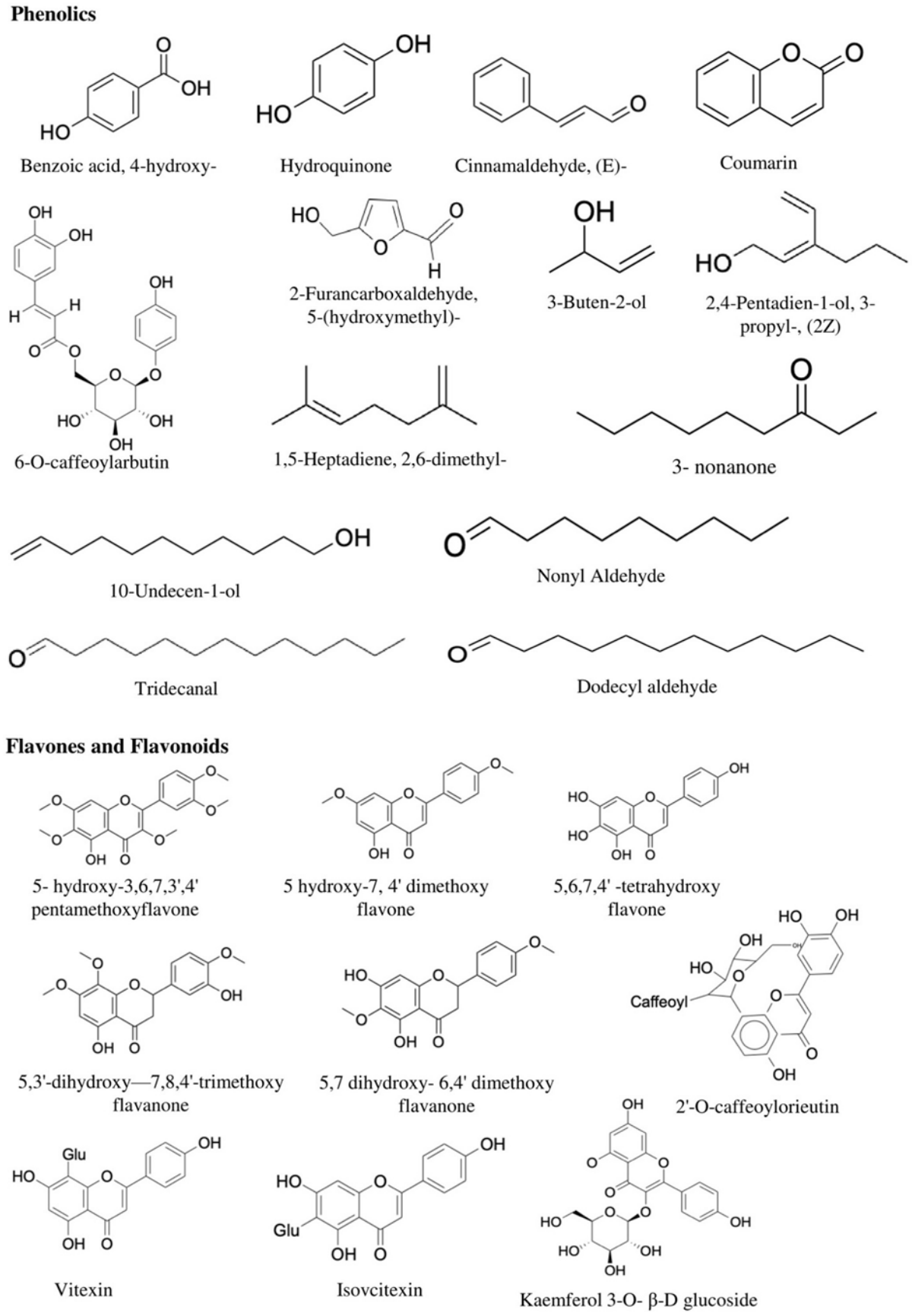

Fig. 7. Structure of phytochemicals characterized from *Vitex leucoxylon* L.f.

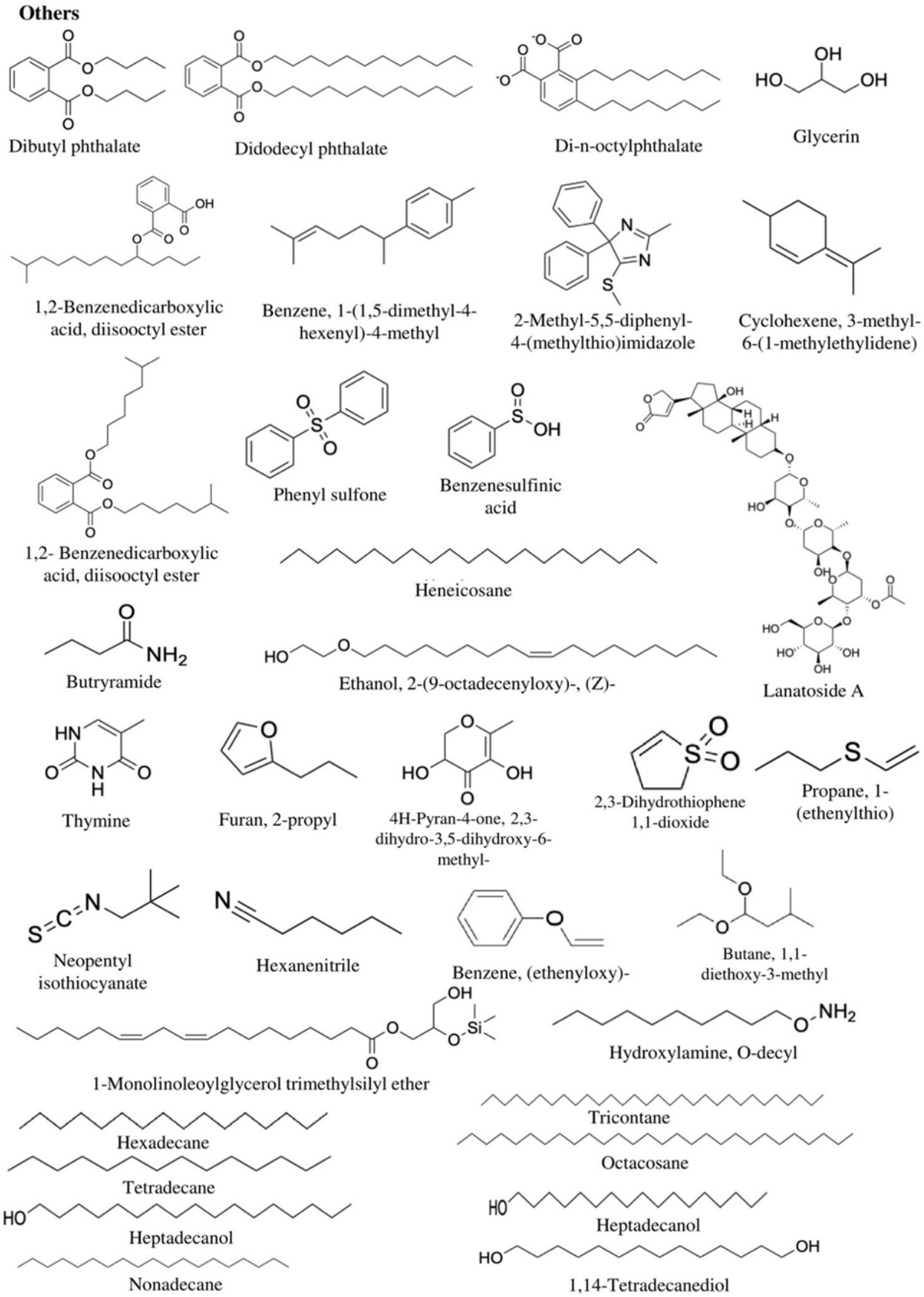

Fig. 8. Structure of phytochemicals characterized from *Vitex leucoxylon* L.f..

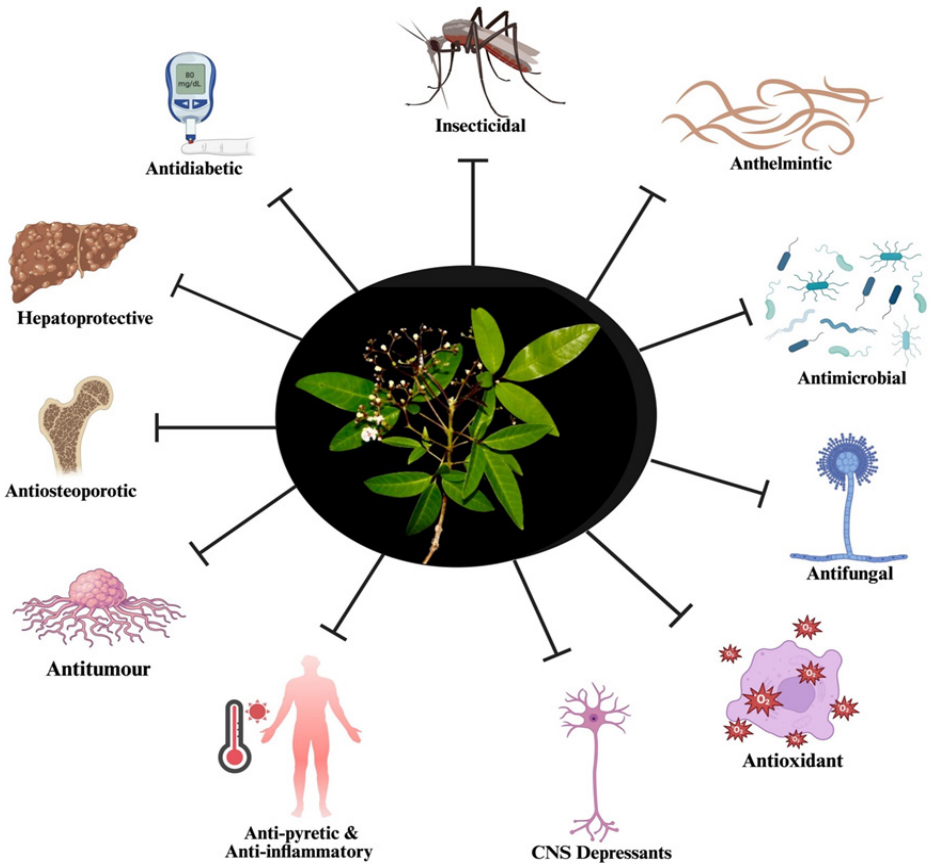

Fig. 9. Pharmacological activities of *Vitex leucoxylon* L.f.

action with potent suppression of Gram-negative bacteria and antifungal activity similar to positive controls. Activity against *Escherichia coli, Bacillus subtilis, Proteus mirabilis, Enterococcus species* and *Klebsiella pneumoniae* was shown by Ananthi. Notably, all five examined pathogens were successfully suppressed by zinc oxide and Mo-doped zinc oxide nanoparticles, which demonstrated the greatest suppression against *Enterococcus*. Additionally, antifungal activity against *Candida albicans, Aspergillus fumigatus* and *Aspergillus niger* was demonstrated using silver and copper nanoparticles produced from *V. leucoxylon* L.f. [21]. Methanolic leaf extracts of *V. leucoxylon* L.f. demonstrated strong antibacterial activity against most tested bacteria except *B. subtilis*. At 50 µg/cup, compounds such as lupeol, betulinic acid, vitexicarpin, vitexin, heptadecanol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid exhibited antibacterial efficacy comparable to chloramphenicol. For antifungal activity, heneicosanoic acid and *p*-hydroxybenzoic acid showed effects similar to nystatin, while vitexicarpin, betulinic acid, vitexin and *p*-hydroxybenzaldehyde produced moderate antifungal activity [27]. Seasonal variation was also observed: ethanol extracts of summer leaves showed the highest inhibition against *Salmonella paratyphi*, *Enterococcus faecalis*, *Vibrio cholerae* and *Staphylococcus aureus*, whereas winter leaf extracts were most effective against *E. coli* and *S. paratyphi* [28]. Leaf extracts of *Vitex leucoxylon* L.f. exhibited notable antibacterial activity against both gram-positive and gram-negative bacteria [29].

5.2. Antioxidant Activity

The antioxidant capacity of *Vitex leucoxylon* L.f. has not been extensively studied, but what little is known about it shows that it is highly active. Ethanolic leaf extracts have demonstrated the highest antioxidant potential, effectively scavenging superoxide, hydroxyl and DPPH radicals in a concentration-dependent manner. Additionally, bark extracts exhibited inhibition of *in vitro* tissue lipid peroxidation [28]. Among solvent fractions, ethyl acetate leaf extracts showed the strongest antioxidant activity across multiple *in vitro* assays

while hexane and methanol extracts displayed no significant effects compared to the standard antioxidant butylated hydroxytoluene (BHT) [30].

5.3. Anti-Inflammatory Activity

Several studies have demonstrated the anti-inflammatory potential of *Vitex leucoxylon* L.f. using experimental models. In carrageenan-induced rat paw oedema, both ethanolic and aqueous bark extracts (500 mg/kg) significantly inhibited paw swelling in albino Wistar rats. Phytochemical screening and acute toxicity assays confirmed safety and the extracts showed comparable efficacy to the reference drug indomethacin [15]. Similarly, ethanolic leaf extract (ETE) and cold aqueous infusion (CAI) suppressed carrageenan-induced oedema and granulation tissue formation in rats, while also reducing acetic acid-induced writhing. Both extracts exhibited analgesic and anti-inflammatory properties, with CAI additionally lowering serum total cholesterol levels and modulating behavioural despair tests in mice [16]. Further evidence supports the anti-arthritic activity of the ethanolic bark extract, which significantly reduced paw oedema in Freund's complete adjuvant (FCA)-induced arthritis. The extract downregulated pro-inflammatory cytokines TNF-α and IL-1β, indicating immunomodulatory action. Based on preclinical safety and efficacy studies, the 5-lipoxygenase (5-LOX) inhibitory fraction of *V. leucoxylon* L.f. bark extract is considered a promising candidate for arthritis management [18]. Leaf and stem bark extracts (chloroform and methanol fractions) were also tested in carrageenan-induced paw oedema models, confirming traditional claims of anti-inflammatory efficacy. Among bioactive constituents, vitexin showed remarkable potency, being ~12.5 times more effective than ibuprofen in suppressing inflammation [27]. Hydroalcoholic and ethanolic extracts further indicated that the anti-inflammatory and analgesic effects are mediated via suppression of prostaglandin biosynthesis [31]. Aqueous stem bark extracts demonstrated benefits in acute inflammation but were associated with reduced wound-breaking strength, reflecting complex effects on tissue repair [32]. Additional *in vivo* studies reported that leaf extracts and cold aqueous infusions significantly reduced carrageenan-induced oedema, granulation tissue formation and serum cholesterol levels [33]. Leaf extracts was evaluated by the HRBC membrane stabilization method using 0.36% NaOH-induced hypotonic haemolysis in vitro. Both hydroalcoholic and ethanolic extracts showed significant membrane stabilization activity. The hydroalcoholic extract showed 96.63%, 90.97%, and 84.16% inhibition at 50, 100, and 1000 µg/mL, respectively, while the ethanolic extract showed 99.03%, 90.02%, and 84.43% inhibition at the same concentrations. The standard drug prednisolone exhibited 99.03%, 97.23% and 98.85% inhibition. The results indicate strong anti-inflammatory potential of the *V. leucoxylon* L.f. leaf extracts, especially the ethanolic extract, which showed activity comparable to prednisolone. [34]

5.4. Anti-Pyretic Activity

The ethyl acetate bark extract of *Vitex leucoxylon* L.f. demonstrated significant antipyretic activity by inhibiting inflammation and reducing fever [35]. No significant variation in baseline temperature was observed at 0 h between treatment groups. However, both the ethyl acetate extract and aspirin markedly reduced the rectal temperature of pyretic rats at the second, third-, and fourth-hours post-treatment. These findings suggest that the ethyl acetate bark extract of *V. leucoxylon* L.f. possesses potent antipyretic properties.

5.5. Analgesic Activity

Cold aqueous infusion and *Vitex leucoxylon* L.f. leaf extract were found to reduce spontaneous motor activity in mice [16]. They also potentiated d-amphetamine-induced stereotypy, enhanced oxotremorine-induced tremors and shortened immobility duration in the behavioural despair test. Furthermore, both the ethanolic extract and the cold aqueous infusion of *V. leucoxylon* L.f. significantly inhibited acetic acid-induced writhing, confirming their analgesic and neuropharmacological activities. *Vitex leucoxylon* L.f. shows promise as a source of novel analgesic and neuroactive agents, warranting further in detail study.

5.6. Anticancer Activity

The antitumour activity of *Vitex leucoxylon* L.f. has been investigated using Dalton's ascitic lymphoma (DAL) in mice [36]. Tumours were induced by intraperitoneal injection of DAL cells and animals were subsequently treated with

ethanol and chloroform extracts of *V. leucoxylon* L.f. (250 and 500 mg/kg) for 14 consecutive days. Oral administration of both extracts prolonged the survival of DAL-bearing mice and restored altered haematological parameters in a dose-dependent manner. The effects were comparable to those of the standard drug 5-fluorouracil (20 mg/kg), with the ethanolic extract demonstrating superior antitumour efficacy relative to the chloroform extract. In another study, *V. leucoxylon* L.f. extract conjugated carboxymethyl cellulose (CMC) membrane is used for bone regeneration, wound healing, and anticancer treatments [37]. The treatment also induced chromatin condensation, nuclear damage and tumour cell death, thereby slowing tumour progression. Additionally, the steroidal fraction improved antioxidant status in treated animals, suggesting a dual role in tumour suppression and oxidative stress reduction.

5.7. Anti-Diabetic Activity

Studies on *Vitex leucoxylon* L.f. suggest that the plant has promising antidiabetic potential [38]. When tested using *in vitro* methods, the aqueous extract stood out by showing notable α-amylase inhibition (54.61 ± 0.46%) and a strong ability to enhance glucose uptake (57.13 ± 0.44%). Compared to extracts prepared with other solvents, the water-based extract performed better, indicating that it contains compounds that are particularly effective in controlling carbohydrate digestion and improving glucose utilization.

5.8. Anti-Helminthic Activity

The anthelmintic potential of *Vitex leucoxylon* L.f. was assessed through *in vitro* studies using *Pheretima posthuma* (Indian earthworm) as the test organism [39]. A distinct dose-dependent response was observed in extracts tested at doses ranging from 50 to 250 mg/20 ml with activity increasing as the concentration increased, among all the solvent extracts tested, the methanolic extract was the most effective for anthelmintic activity. Further studies are required to isolate active compounds and confirm its anthelmintic efficacy *in vivo*.

5.9. Hepato-Protective Activity

Various experimental models have been used to assessed hepatoprotective potential of *Vitex leucoxylon* L.f.. Initially they induce liver damage in albino mice by using carbon tetrachloride (CCl_4), which was ameliorated by alcoholic leaf extracts, as noticed by improvements in key biochemical parameters, including alkaline phosphatase, bilirubin, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) [12]. These outcome corroborated the protective effect of *Vitex leucoxylon* leaf extracts against hepatotoxic compounds. Pre-treatment with silymarin (standard), methanolic and chloroform leaf extracts, and chloroform bark extract produced significant hepatoprotective effects, whereas the methanolic bark extract showed no protective activity [27]. Additionally, ethanolic leaf extract (EVL) was tested in male Wistar rats for antioxidant and anti-hepatocarcinogenic properties against diethylnitrosamine (DEN) and phenobarbital sodium (PB). EVL treatment exhibited a clear dose-response relationship in hepatoprotection, with the high dose gives stronger antioxidant and protective effects [31]. These Results emphasize *Vitex leucoxylon* L.f. as a credible source of hepatoprotective phytochemicals, justifying further investigation.

5.10. Anti-Osteoporosis Activity

Chronic alcohol abuse (CAA) represents a significant risk factor for osteoporosis, associated with impaired bone metabolism and structural deterioration. The ethanolic leaf extract of *Vitex leucoxylon* L.f. (EEVL) has been evaluated in a CAA-induced osteoporosis model using male albino rats [8]. Animals were divided into four groups, six in each group, after grouping the biochemical parameters such as serum calcium, phosphorus, alkaline phosphatase and urinary levels of calcium, phosphorus and creatinine were quantified. Rats treated with EEVL, vitamin D_3 and calcium supplements exhibited a favourable outcome that higher serum calcium and phosphorus levels, along with markedly reduced alkaline phosphatase activity compared to the disease control group. Further, the administration of EEVL resulted in an important reduction in the excretion of calcium, phosphorus, and creatinine in the urine. These biochemical benefits have been proven by radiographic study, which revealed the prevention of bone loss. Each of these results show that EEVL has strong anti-osteoporotic properties, indicating

that it may be used as a phytotherapeutic remedy for osteoporosis brought on by alcohol. Plants rich in flavonoids and saponins are known to support bone formation and reduce bone loss. Similar compounds, including flavonoids, saponins, and iridoid glycosides are reported in *Vitex* species and may help improve calcium metabolism and bone mineralization. However, research on *Vitex leucoxylon* L.f. is still limited compared with *Vitex negundo* and *Vitex trifolia*. Therefore, further studies are needed to identify the active compounds responsible for its anti-osteoporotic effect.

5.11. CNS Depressant Properties

The central nervous system–depressant properties of *Vitex leucoxylon* L.f. have been experimentally studied by Nair [40]. When compared to the control group, animal models' spontaneous and forced locomotor activity were significantly reduced when ethanolic extracts of the plant were taken orally at doses of 250 mg/kg and 500 mg/kg, respectively. The maximum effect was observed at 1hour post-administration. Although the magnitude of the depressant effect was lower than that of the standard reference drug, *Vitex leucoxylon* L.f. extract exhibits a noticeable reduction in aggressive behaviour, with responses closely resembling those of the standard.

5.12. Insecticidal Activity

The insecticidal efficacy of *Vitex* species has been evaluated by Sahayaraj *et al.* [41] against *Corcyra cephalonica* Stainton. Pulverized leaf material of *Vitex leucoxylon* L.f., *V. negundo* Linn., and *V. trifolia* Linn. at concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 g/100 g tested on groundnut seeds. Treatments substantially decreased kernel damage, dry mass loss, larval weight, fertility and survival of the pest. This extract reduced development time, larval weight and fecundity, indicating its potential as an eco-friendly botanical pesticide. Further studies on active compounds and field evaluation are needed to confirm its potential as a sustainable biopesticide.

5.13. Phyto-toxicity

Toxicity studies are important for understanding whether medicinal plants are safe for use. In this study, a combined extract of *Vitex leucoxylon* L.f., *Vitex negundo* L., and *Vitex trifolia* L. was tested for both short-term and repeated-dose toxicity in mice. During the acute toxicity study, animals received a single oral dose of up to 2000 mg/kg and were observed for seven days. No deaths or noticeable changes in behaviour or physical condition were recorded, suggesting good safety at high doses. In the sub-acute study, mice were given the extract orally at doses of 200 and 400 mg/kg for 28 days. Throughout the treatment period, no harmful effects on body weight, behaviour, or survival were seen. Blood and biochemical tests also remained within normal ranges, indicating no systemic toxicity [42]. Overall, these results support the traditional use of *Vitex* species and show that *V. leucoxylon* L.f. extracts are well tolerated at therapeutic levels, but further detailed investigations such as long-term toxicity, chronic toxicity and clinical studies are required to confirm their safety profile.

6. SUMMARY

The phytochemical constitution of *Vitex leucoxylon* L.f. exhibits a rich and diverse spectrum of bioactive compounds present across various plant parts, including leaves, bark and seeds. These main compounds Contain sugars, fatty acids, esters, terpenes, terpenoids, iridoid glycosides, flavonoids, phenolic compounds, volatile components and other secondary metabolites. Notably, leaves has relative higher phytoconstituents than bark and seeds, representing leaves as the main metabolite site. This deviation may elucidate the higher antioxidant and pharmacological activity of leaf extracts reported earlier [1, 3] .

Flavonoids and flavones including vitexin, isovitexin, kaempferol glycosides and methoxylated flavones are strongly associated with antioxidant and anti-inflammatory activities. These compounds scavenge ROS and inhibit lipid peroxidation. earlier studies shown that flavonoid-rich extracts of *Vitex* species possess significant wound healing, anti-inflammatory and hepatoprotective effects [18, 19, 32]. This correlates well with the high total phenolic and flavonoid content observed in antioxidant assays.

Among sugars, the presence of D-mannose is predominately important because it has been noted for anticancer, anti-inflammatory and antiviral activities [43], although maltooligosaccharides

such as maltotriose and maltopentose show immunoregulatory and prebiotic capability [44, 45]. Levoglucosan, D-mannose, maltotriose, maltopentose, gamma-cyclodextrin hydrate, dextrin and lactose indicates an crucial role in energy storage, osmotic equilibrium and precursor functions for secondary metabolism [46]. These compounds may circuitously support plant defence mechanisms and pharmaceutical applications.

Fatty acids stand for a dominant class in leaves and bark. Major compounds such as n-hexadecanoic acid, octanoic acid, linolenic acid (9,12,15-octadecatrienoic acid), oleic acid, dodecanoic acid and octadecanoic acid are well known for antioxidant, antimicrobial, anti-inflammatory and anticancer properties. n-Hexadecanoic acid demonstrate anti-inflammatory activity through enzyme inhibition [47], whereas octanoic acid shows strong antibacterial and biofilm-eradicating properties against *Staphylococcus aureus* [48, 49]. Unsaturated fatty acids such as linolenic and linoleic acid derivatives potent free radical scavengers and membrane stabilizers that supports hepatoprotective and anti-inflammatory activities.

Terpenes and terpenoids has one of the most pharmacologically significant groups in *V. leucoxylon* L.f.. Caryophyllene, phytol, squalene, β-sitosterol, betulinic acid, corosolic acid and vitamin E are major compounds detected. β-Caryophyllene is a well-established sesquiterpene with anticancer, analgesic, anti-inflammatory and antibacterial properties [50]. Squalene is well-known for immunostimulatory and anticancer potential [51, 52]. β-sitosterol has strong antidiabetic and apoptosis-mediated anticancer effects [53 - 55].

Corosolic acid, a pentacyclic triterpenoid (ursane-type) among a carboxylic acid functional group, validates convincing antidiabetic effects by reducing blood glucose levels as well as noticeable anti-inflammatory activity through modulation of inflammatory pathways [56]. The ester component, including hexadecanoic acid methyl ester, methylparaben, benzoic acid esters and various fatty acid esters, indicates preservative, antimicrobial and pesticidal capacity. Hexadecanoic acid methyl ester is prominent source for the antioxidant and hepatoprotective properties [57], while methylparaben and benzoic acid derivatives shows preservative and antimicrobial effects. These esters support infection control and shelf life improvement. Whereas, phytol shows antimicrobial, cytotoxic, antioxidant and anti-inflammatory potential [58].

Iridoid glycosides such as agnuside and aucubin are important chemotaxonomic markers enhancing its medicinal value . Agnuside has been reported for antioxidant, angiogenic and anticancer effects [59, 60], whereas aucubin unveils antioxidant, neuroprotective, hepatoprotective, anti-fibrotic and anticancer potential [61, 62].

Phenolic compounds such as cinnamaldehyde, hydroquinone, coumarin, caffeoylarbutin derivatives and hydroxybenzoic acids further support the antimicrobial, hypoglycemic, anti-inflammatory and anticancer potential of the plant. hydroxybenzoic acid derivatives show strong antibacterial and antioxidant effects [63]. Whereas Coumarin is exceptionally important because of its multi-target anti-inflammatory and anticancer potential [64, 65]. These phenolics are principal contributors to oxidative stress reduction via hydrogen donation and free radical stabilization.

Volatile compounds such as tridecanal, nonyl aldehyde, undecenol derivatives and pentadienol derivatives enhance primarily to antibacterial and antifungal activity. Tridecanal reported for antibacterial, antifungal and antioxidant properties [66, 67], whereas nonanal shows antifungal and antidiarrheal effects [68, 69]. These compounds function as natural defences against pathogens and stress.

Additional compounds such as di-n-octyl phthalate, ethyl iso-allocholate, glycerin, heptacosane, lanatoside A and thymine indicate broader cardioprotective, antidiabetic, antimicrobial and anticancer potential. Heptacosane reported to overcome multidrug resistance and antimalarial activity [70], although ethyl iso-allocholate shows potential antidiabetic and antioxidant activities by insulin signaling pathways [71].

7. CONCLUSIONS

This PRISMA-based systematic review highlights current knowledge on the taxonomy, phytochemistry and pharmacological potential of *Vitex leucoxylon* L.f., emphasizing its rich phytochemical and wide range of experimentally demonstrated bioactivities.

The integrated evidence validates the presence of diverse metabolite classes, including terpenoids, flavonoids, iridoid glycosides, phenolics and fatty acids, which are associated with pharmacological activities such as antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, antidiabetic, anticancer and CNS-related effects. However, the review also reveals that the pre-existing literature is largely preclinical, methodologically heterogeneous and analytically fragmented, with considerable variability in plant part selection, extraction procedures, analytical platforms, experimental models and evaluative metrics. Many phytochemical reports rely predominantly on GC–MS-based characterization without complementary structural validation, while pharmacological studies frequently employ crude extracts with limited mechanistic insight, standardized dosing and toxicity analysis.

Future research should therefore emphasis standardized extraction and analytical methods, consolidation of multi-analytical metabolomic approaches like LC–MS/MS and NMR and quantitative standardization of bioactive markers to facilitate reproducibility and cross-study uniformity. Identifying compound-specific activity correlations, molecular mechanisms of action and structure–activity correlations will be essential for translating ethnomedicinal claims into experimentally validated therapeutic leads. concurrently, systematic toxicological assessments, pharmacokinetic evaluations and well-designed clinical studies are required to evaluate safety, efficacy and bioavailability. Addressing these critical gaps will reinforce integrative synthesis of evidence and improve alignment graphical representations and the synthesized narrative, thereby supporting the systematic progression of *V. leucoxylon* L.f. as a substantiated source of bioactive compounds for pharmaceutical and therapeutic uses. These efforts will not only strengthen its pharmacological relevance but also support its inclusion in evidence-based drug discovery and bioprospecting. Overall, *Vitex leucoxylon* L.f. represents a pharmacologically promising yet under-validated medicinal species that warrants systematic translational research.

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9. AUTHOR CONTRIBUTIONS

SBP: Gathered the data and prepared the manuscript. SPD: Handled formatting. PVP & AVL: contributed as co-authors. SAD: carried out the review and corrected. The final version of the manuscript has been read and approved by all authors..

10. ETHICAL STATEMENT

This work does not include any studies involving human or animal subjects.

11. CONSENT FOR PUBLICATION

All authors have provided their consent for publication of this manuscript.

12. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

A Categorization Based Comparative Study of Food Security and Population in the World's Five Most Populous Countries

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Abstract: This research examines Global Food Security Index (GFSI) along with its components and population changes through comparative analysis for world's top five most populous countries (India, China, USA, Indonesia and Pakistan). This study utilizes innovative Theory of Categorization (ToC) to classify GFSI scores into groups, i.e., poor, weak, normal, good and best. India's GFSI rank slightly dropped from 67th to 68th and categories weak; however, its GFSI scores remains with in normal category. Availability, quality and safety (QS) moved from normal to good, while sustainability and adaptation (SA) improved from weak to normal. India needs to focus on all components with particular attention to affordability and SA. China improved GFSI ranks from 49th to 25th and categories good with significant gains in GFSI scores and its component. USA ranked at 13th in GFSI and categories best. Indonesia ranks 63rd, falls in the weak category. Affordability moved from good to best but sustained efforts are required in other components. Pakistan is ranked at 84th, classified as poor with challenges in SA and QS. China and the USA are managing their populations, whereas unproductive population growth may threaten future food security, particularly for Pakistan, India and Indonesia. This research can assist countries in prioritizing their sectors to achieve food security and in conducting comparable assessments in other countries. Weak countries (Pakistan, Indonesia and India) should enhance food affordability, quality, sustainability, and manage population growth. Strong countries (China and USA) should sustain high standards and invest in sustainable practices and innovations.

Keywords: Global Food Security Index, Comparative Analysis, Theory of Categorization, Top Five Populous Countries.

1. INTRODUCTION

The 1996 World Food Summit defines food security as a fundamental pillar of human well-being [1, 2]. It encompasses the availability, access, utilization and stability of food resources [3-5]. A heightened interest in analyzing and evaluating global food security emerged in the early 21st century. This interest was driven by factors such as environmental changes, growing population, political instability and socio-economic disparities worldwide [6-8]. The Global Food Security Index (GFSI) developed by the research and analysis division of the Economist Group known as Economist Intelligence Unit (EIU) in collaboration with the Barilla Center for Food & Nutrition (BCFN) in 2012 and it is a crucial tool for assessing and comparing the food security situation in various countries [9, 10]. GFSI scores ranging from 0 to 100 and measures the performance, where higher scores reflect superior performance [11-14]. GFSI aims to evaluate a country's food security by assessing affordability, availability, quality and safety (QS), sustainability and adaptation (SA) [15-17]. GFSI serves as an important framework for assessing and comparing food security. It assists policymakers, researchers and other stakeholders in identifying vulnerable areas and supporting the design of focused

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interventions and informed development strategies [18-20]. This index contributes to a broader global understanding of food security by encouraging international collaboration, enabling comprehensive assessment, informing policy decisions and guiding strategic to improve food security worldwide [21]. Islam *et al.* [22] and Hakeem *et al.* [23] indicated that global population was anticipated to reach from 8.9 billion to 10.6 billion by 2050, as opposed to the 7.75 billion recorded in 2020 and these anticipated expansion in population are poised to significantly impact the future food demand. In 1798, economist T.R Malthus (1766-1834) introduced the idea that the ongoing expansion of the population would consistently outpace the availability of food and leading to a global challenge of food scarcity [24, 25]. Malthus was a pioneer in addressing the concern of food scarcity, contending that the expanding global population would ultimately surpass the Earth's ability to sustain it. Organizations such as the World Health Organization (WHO), Food and Agriculture Organization (FAO) and numerous other international bodies are actively engaged in addressing the problem of food insecurity. Islam *et al.* [25] and Kagan [26] indicated that addressing the issue of food insecurity requires a 70% increase in food production within developed countries and a twofold rise in the developing world by the year 2050.

The distinctive characteristics of population as outlined in the United Nations World Population Prospects and the Global Food Security Index, which ranks the top five most populous countries are elaborated as:

India: In the GFSI of 2022, India secures the 68th position. Further examination by specific categories discloses India's standings at 80th, 42nd, 67th and 71st positions for affordability, availability, QS and SA, respectively. India boasts the title of the world's most populous country, with an estimated population of around 1,463.86 million in 2025, contributing a substantial 17.78% to the global population. The 37.61% of India's population resides in urban areas.

China: In the Global Food Security Index 2022 rankings, China secures the 25th position and standings in affordability at 33rd, in availability at 2nd, in QS at 46th and in SA at 55th. Being the second most populous nation, China boasts a population of 1,416.09 million in 2025. The country's population is experiencing steady growth, moderated by the implementation of the "one-child policy" by the government. China's demographic magnitude accounts for a substantial 17.20% of the world's total population. A significant majority about 67.55% resides in urban areas.

United States of America: In the Global Food Security Index of 2022, the USA held the 13th position, scoring 29th, 31st, 3rd and 12th for affordability, availability, QS and SA. in 2025, the USA have a population of 347.27 million, constituting 4.22% of the worldwide population and securing its position as the third most populous country. A significant 82.76% of the USA' population resides in urban areas.

Indonesia: Indonesia is ranked 63rd in the Global Food Security Index for 2022 with specific standings of 44th, 84th, 78th and 83rd for affordability, availability, QS and SA. Indonesia stands as the most densely populated country in Southeast Asia and holds the fourth position globally in terms of population. Indonesia's population in 2025 is 285.72 million, contributing approximately 3.47% to the total world population. The 59.63% of Indonesia's population resides in urban areas.

Pakistan: In the Global Food Security Index 2022, Pakistan holds the 84th position with rankings of 75th, 61st, 97th, and 106th for affordability, availability, QS and SA. Pakistan stands out among Asian nations with population growth. As of 2025, Pakistan's population is at 255.21 million, contributing to 3.10% of the global population. The country holds the 5th position among the world's most populous nations. The 34.39% of Pakistan's population resides in urban areas.

The global population is rapidly increasing, presenting a significant challenge to the world's ability to provide sustenance for nations and ensure food security for everyone. The growing global population has the potential to impact various components of the GFSI, including the affordability of food products, availability of agricultural production, quality and safety of food production practices and sustainable adoption of food production. This study aims to employ a comparative performance analysis (CPA) to examine the complex relationship between population changes and the components of GFSI. The objective is to provide evidence-based insights that may assist policymakers and researchers in developing sustainable and flexible strategies to address the challenges arising from continued global population growth. Such type of CPA has not

been applied previously using the statistical tools, especially for the top five populous countries in the world (India, China, USA, Indonesia, and Pakistan). This study seeks to explore both the favorable and adverse impacts of population growth on the GFSI and its components with a particular emphasis on the world's top five populous countries. The objective is to enhance better understanding of the challenges and opportunities associated with the population changes and food security of these countries.

2. MATERIALS AND METHODS

2.1. Data Collection

The Economist Intelligence Unit (EIU) in collaboration with the Barilla Center for Food & Nutrition (BCFN) developed the Global Food Security Index (GFSI). This tool evaluates and ranks global food security based on factors like affordability, availability, QS, and SA. Data for this study is obtained from the GFSI database, covering the period from 2012 to 2022 and available at https://impact.economist.com/sustainability/project/food-security-index/. GFSI scores are ranging from 0 to 100 and 100 representing the highest score. Data regarding the Annual Population Changes (APC) for the years 2012 to 2025 is gathered from the United Nations World Population Prospects, focusing on the five most populous countries available at https://www.worldometers.info/population/.

2.2. Analysis of Variance

Analysis of Variance (ANOVA) is a statistical method used to assess whether there are any statistically significant differences exist between the means of two or more groups [27-29]. In the current study, ANOVA is applied for six different factors (GFSI, affordability, availability, QS and SA. F- Statistic formula used in ANOVA is defined as:

$$F = \frac{\text{MSE between groups}}{\text{MSE within groups.}} \qquad (1)$$

$$F = \frac{\sum_{i=1}^{m} n_i(\bar{Z}_{i.} - \bar{Z})^2 /(m-1)}{\sum_{i=1}^{m}\sum_{j=1}^{ni}\left(Z_{ij} - \bar{Z}_{i.}\right)^2 /(n-m)} \qquad (2)$$

where *"$Z_{i.}$"* represents the means in the *"i^{th}"* groups, *"n_i"* signifies the number of observations in the *"i^{th}"* group, *"Z"* denotes the overall mean, *"m"* denotes the number of groups, *"Z_{ij}"* is the *"j^{th}"* observations in the *"i^{th}"* out of *"k"* group and *"n"* is sample size. The statistical hypothesis defines as:

Null Hypothesis (H_0): There are no statistically significant differences between the means of the top five populous countries for each factor.

Alternative Hypothesis (H_1): There are statistically significant differences between the means of the top five populous countries for at least one factor.

2.3. Theory of Categorization (ToC)

The GFSI scores and rankings have not yet been classified into distinct groups. GFSI scores range from 1 to 100, where higher scores are deemed better performance and lower scores are considered worse. Similarly, GFSI ranks also span from 1 to 100, where higher ranks are considered worse and lower ranks are considered better. There is a need to categorize GFSI scores and ranks into distinct categories to identify the levels of factors within distinct groups. This study introduced the approach of ToC to identify the GFSI scores and ranks within distinct groups (Table 1) and categorized as poor, weak, normal, good and best as:

2.3.1. GFSI scores categories

- GFSI scores ranging from 0 to 20 indicate a poor level of food security, necessitating rapid and significant improvements.
- GFSI scores ranging from 20.1 to 40 represent weak scores, signaling a subpar performance in terms of food security and substantial enhancements are required.
- GFSI scores ranging from 40.1 to 60 fall into the normal category, suggesting room for improvement, but overall, the performance is deemed satisfactory.
- GFSI scores ranging from 60.1 to 80 are classified as good, indicating effective systems in place, yet there is still potential for refinement.
- GFSI scores ranging from 80.1 to 100 signify the best, indicating high standards of food security.

2.3.2. GFSI ranks categories

- GFSI ranks ranging from 0 to 20 indicate the best category, signify high standards of food security
- GFSI ranks ranging from 21 to 40 represent

Table 1. Theory of categorization of GFSI scores and ranks.

GFSI scores categories range				
Poor	**Weak**	**Normal**	**Good**	**Best**
0-20	20.1- 40	40.1- 60	60.1-80	80.1-100
GFSI ranks categories range				
Poor	**Weak**	**Normal**	**Good**	**Best**
81-100	61-80	41- 60	21- 40	0-20

Source: Author's categorization based on GFSI scores and ranks ranges.

good ranks indicating effective systems in place, yet there is still potential for refinement

- GFSI ranks ranging from 41 to 60 fall into the normal category, suggesting room for improvement, but overall, the performance is deemed satisfactory
- GFSI ranks ranging from 61 to 80 are classified as weak, and substantial enhancements are required.
- Finally, GFSI ranks ranging from 81 to 100 signify the poor level of food security, necessitating rapid and significant improvements.

3. RESULTS AND ANALYSIS

3.1. Analysis of Variance

Table 2 shows ANOVA results assessing significant mean differences in GFSI factors and population growth among the top five populous countries. All the p-value founds less than 0.001 (Sig. = 0.00), leading to accept the "H_1", indicating a statistically significant difference between the groups for six different factors of top five populous countries. It reveals that there exists a statistically significant difference between the means of GFSI score and population growth of top five populous countries in the world. The low P-values consistently suggest a rejection of the null hypothesis emphasizing the impactful nature of these factors on global food security (Table 2).

3.2. Theory of Categorization (ToC)

ToC method outlines GFSI scores and rankings for the top five populous countries. Table 3 shows the Comparative Performance Analysis (CPA) of GFSI ranks and scores along with population changes for India. In 2012, India had a GFSI rank of 67 classified as weak which slightly decreased to 68 in 2022. The change in rank indicates a slight decline (-1) in the overall global food security index. The GFSI score improved from 53.8 in 2012 to 58.9 in 2022, reflecting a positive trend in GFSI scores and classified as normal (Table 3). Components such as availability, QS and SA also show improvements from 56.5, 53.4, 39.9 in 2012 to 62.3, 62.1 and 51.2 in 2022. Availability and QS improved to good from normal, while SA improved to normal from weak. Affordability score decreases to 59.3 in 2022 from 62.5 in 2012. This shows a fall from good to normal for affordability. The population of India increased by 11.47% in 2022 compared to 2012. Despite a marginal drop in GFSI rank, India demonstrated slight improvement in GFSI scores and its components, but it still falls in weak ranks. It gains good category from normal for availability and QS, while attains normal for SA from weak. The decline in affordability may warrant attention to ensure food remains economically accessible to the population.

Table 4 presents a comprehensive overview of China's performance in the GFSI over the span

Table 2. ANOVA for the GFSI score and Population growth for top five populous countries.

	GFSI	**Affordability**	**Availability**	**Quality and safety**	**Sustainability & adaptation**	**Annual population growth**
F-Statistic	155.9	59.4	46.1	292.2	148.9	40.6
P-values	0.00	0.00	0.00	0.00	0.00	0.00

Source: Author's analysis based on GFSI scores and ranks ranges.

Table 3. CPA of GFSI ranks, scores and population changes for India.

Years	GFSI rank of India	GFSI and its components score of India					Yearly % increase in population in 2022 as compared to 2012
		GFSI score	Affordability	Availability	Quality & safety	Sustainability & adaptation	
2012	67 (Weak)	53.8 (Normal)	62.5 (Good)	56.5 (Normal)	53.4 (Normal)	39.6 (Weak)	11.47%
2022	68 (Weak)	58.9 (Normal)	59.3 (Normal)	62.3 (Good)	62.1 (Good)	51.2 (Normal)	

Source: Author's categorization based on GFSI scores and ranks ranges.

of a decade (2012 to 2022) with annual population changes. In 2012, China secured the 49th position in the GFSI rankings with an overall score of 60.5, indicating a classification of normal. The component scores were distributed across the spectrum, with affordability, availability, QS and SA garnering scores of 65 (good), 64.5 (good), 64.8 (good) and 45.8 (normal). China has effectively managed its population growth and reported 4.06% increase in population in 2022 compared to 2012. China recorded significant improvement in its food security, stands at 25th position with an improved GFSI score of 74.2 and categorized as good. A detailed breakdown of GFSI component scores reported advancements in each GFSI components. Affordability score is 86.4 and categories as best, availability found as 79.2 and ranks as good, QS found as 72 and reported as good, while SA found as 54.5 and categories as normal. This shows China's commendable efforts and advancements in enhancing its food security profile over the period spinning from 2012 to 2022. The progress observed in the GFSI and its individual components indicates a favorable trend toward strengthening of accessibility, quality, safety and sustainability of food resources in the country. A steady population growth rate may suggest that food security policies are more effectively aligned with demographic changes.

Table 5 presents the GFSI rankings and scores for the USA, along with the associated components and annual population changes. In 2012, the USA secured the 5th position with an overall GFSI score of 76.7 and it indicating a good level of food security. The components contributing to this score are affordability (87), availability (65.8), QS (88.2) and SA (63.8). Affordability and QS are categories best and all others categories are good. The USA's GFSI rank dropped to 13th place, although it still maintained a good with overall score of 78. The components contributing to the score included affordability (87.1), availability (65.1), QS (88.8) and SA (69.4). Affordability and QS are still categories best and all others are categories good. The yearly increase in population in 2022 as compared to 2012 in USA found 7.70%, which indicates that USA is managing its population

Table 4. CPA of GFSI ranks, scores and population changes for China.

Years	GFSI rank of China	GFSI and its components score of China					Yearly % increase in population in 2022 as compared to 2012
		GFSI score	Affordability	Availability	Quality & safety	Sustainability & adaptation	
2012	49 (Normal)	60.5 (Good)	65 (Good)	64.5 (Good)	64.8 (Good)	45.8 (Normal)	4.06%
2022	25 (Good)	74.2 (Good)	86.4 (Best)	79.2 (Good)	72 (Good)	54.5 (Normal)	

Source: Author's categorization based on GFSI scores and ranks ranges.

Table 5. CPA of GFSI ranks, scores and population changes for USA.

Years	GFSI rank of USA	GFSI and its components score of USA					Yearly % increase in population in 2022 as compared to 2012
		GFSI score	Affordability	Availability	Quality & safety	Sustainability & adaptation	
2012	5 (Best)	76.7 (Good)	87 (Best)	65.8 (Good)	88.2 (Best)	63.8 (Good)	7.70%
2022	13 (Best)	78 (Good)	87.1 (Best)	65.1 (Good)	88.8 (Best)	69.4 (Good)	

Source: Author's categorization based on GFSI scores and ranks ranges.

and account a moderate increase in its population. This comparative analysis suggests that while the United States experienced a slight decline in its GFSI rank from 2012 to 2022, the overall food security remained at a good level. All components are consistent strengths, contributing to the nation's food security. The increase in annual population may indicate a substantial threat for future food security considerations.

Table 6 presents a comparative analysis of the GFSI ranks and scores for Indonesia alongside the annual population changes. In 2012, Indonesia held the 62nd position in the GFSI ranking and it categorizing as weak. The overall GFSI score was 55.4, falling within the normal range. The affordability score is 69 (good), availability score 47 (normal), QS reached 59.1 (normal) and SA scored 43 (normal). Indonesia's GFSI rank slightly declined to 63, still categorized as weak. The overall GFSI score showed improvement, reaching 60.2 and moving into the good category. Examining the components, affordability increased to 81.4 (best), availability reached 50.9 (normal), QS maintained a normal range at 56.2 and SA is 46.3 (normal). The yearly increase in population in 2022 as compared to 2012 in Indonesia found 10.34%. The comparative analysis indicates an overall improvement in Indonesia's GFSI score and it is moving from the normal range in 2012 to the good range in 2022. This positive trend is particularly evident in the affordability component, which shifted from a good to a best rating. The reduction in annual population growth may positively impact food security by easing the pressure on resources. While Indonesia has made progress in enhancing its GFSI score but still ranks weak. There is warrant attention in needed for sustained improvement in all its components except affordability.

Table 7 presents a comparative analysis of the GFSI ranks and scores for Pakistan along with the corresponding annual population changes. In 2012, Pakistan is ranked 94th on the GFSI and it is indicating a relatively poor level of food security. The overall GFSI score for the country is 43.5, categorized as normal. The specific component scores are 47.5 for affordability (normal), 39.2 for availability (weak)

Table 6. CPA of GFSI ranks, scores and population changes for Indonesia.

Years	GFSI rank of Indonesia	GFSI and its components score of Indonesia					Yearly % increase in population in 2022 as compared to 2012
		GFSI score	Affordability	Availability	Quality & safety	Sustainability & adaptation	
2012	62 (Weak)	55.4 (Normal)	69 (Good)	47 (Normal)	59.1 (Normal)	43 (Normal)	10.34%
2022	63 (Weak)	60.2 (Good)	81.4 (Best)	50.9 (Normal)	56.2 (Normal)	46.3 (Normal)	

Source: Author's categorization based on GFSI scores and ranks ranges.

and 52.3 for QS (normal). The SA component scored 34.2 and indicating a weak performance. The yearly increase in population in 2022 as compared to 2012 in Pakistan found 17.35%, which indicates that Pakistan is majorly increasing its population. This trend of unproductive population growth may become a significant indicator of food insecurity in Pakistan. Comparing this to 2022, there has been a slight improvement in Pakistan's GFSI ranking, moving up to 84th place, but still at poor. Slights improvements are observed in the component scores, with affordability rising to 59.9 (normal), availability improving to 58.3 (normal), QS increasing to 49.4 (normal) and SA reaching 37.7 and still weak. This comparative analysis reveals that while there has been slight progress in certain aspects of food security in Pakistan between 2012 and 2022, but the challenges are persist. The slights improvements in affordability and availability are positive indicators, but QS and SA still require more attention. The increases in the population are negatively hitting the food security and ranking it on poor.

4. DISCUSSION

This study explored the relationship between population changes and key components of the Global Food Security Index (GFSI) across the top five populous countries (India, China, USA, Indonesia and Pakistan) using Comparative Performance Analysis (CPA), ANOVA and the Theory of Categorization (ToC). The findings of this study offer important insights into how population changes intersect with the status of GFSI and its components (availability, affordability, QS and SA). The results of the study support the previous literature suggesting that rapid population growth can strain food systems, particularly in low and middle income countries [30, 31]**.** The significant differences found in GFSI scores and population growth rates by ANOVA, validate the hypothesis that population dynamics are non-trivially associated with food security performance [32, 33]. The Theory of Categorization applied innovatively in this study, provided a comparative framework to classify countries and lead to more actionable interpretations for true policy decision. India reflects the complexities of balancing the economic and demographic pressures. The increase in population in India found 11.47% in 2022 as compared to 2012. Improvements in QS and availability suggest the effectiveness of agricultural and regulatory interventions, while the status of affordability is normal. This aligns with previous findings [34, 35], who emphasized that economic access to food remains the most unstable component of food security. China improved its GFSI rank from 49 to 25, stabiles its population growth and reinforced the impact of sustained policy attention on food system resilience. These findings validate the earlier studies by [36, 37], who highlighted that China's strategic investments in food safety, infrastructure and rural development as key to its improved food security system. Although the GFSI ranking of the United States has declined, the country still demonstrates strong performance across all major sectors of GFSI. This suggests that its food system remains resilient and relatively stable even in the face of moderate population changes. However, the downward movement in ranking may indicate the presence of new pressures, potentially linked to climate variability, rising food prices and health related barriers that affecting access to food that have been highlighted in the Global Food Security Report [38, 39]. Indonesia's improvement from a

Table 7. CPA of GFSI ranks, scores and population changes for Pakistan.

Years	GFSI rank of Pakistan	GFSI and its components score of Pakistan					Yearly % increase in population in 2022 as compared to 2012
		GFSI score	Affordability	Availability	Quality & safety	Sustainability & adaptation	
2012	94 (Poor)	43.5 (Normal)	47.5 (Normal)	39.2 (Weak)	52.3 (Normal)	34.2 (Weak)	17.35%
2022	84 (Poor)	52.2 (Normal)	59.9 (Normal)	58.3 (Normal)	49.4 (Normal)	37.7 (Weak)	

Source: Author's categorization based on GFSI scores and ranks ranges.

normal to a good category, particularly through the best sign in affordability signifies progress likely driven by food subsidy reforms and agricultural productivity enhancements. This study aligns with Islam *et al.* [40] and Indriastuti *et al.* [41], that continued progress in all categories of GFSI is needed for long term strategies to attain food security. Pakistan remains in the poor GFSI category and highlighting persistent food security challenges. Despite marginal progress in affordability and availability, the increasing population growth rate is continuously offsetting the gains. The weak performance in sustainability and adaptation reflects structural issues in climate resilience and agricultural resource management practices. This situation aligns with the findings of Aziz [42] and Behera *et al.* [43], who found that food security in Pakistan is increasingly vulnerable due to poor governance and climate variability. The combined application of the CPA and ToC approaches helped to identify key priority areas for policy intervention. China and the USA exemplify relatively successful integration of food policy and demographic control, while Pakistan and Indonesia illustrate the need for comprehensive strategies to address food security threat. In short, these findings suggest that although the rapid population growth remains a fundamental challenge for the food security but its impact varies significantly from county to country based on national policy responses, economic conditions and climate resilience capacities. Therefore, policymakers should adopt localized strategies that prioritize the most lagging components of GFSI informed by periodic CPA assessments and categorization models like ToC.

5. CONCLUSIONS

The rising global population trend poses a significant challenge to achieving food security. This study applied CPA to assess the interaction between population growth and components of the GFSI using Analysis of Variance (ANOVA) and Theory of Categorization **(**ToC**)** for the most five populous countries (India, China, USA, Indonesia and Pakistan). GFSI scores range from 1 to 100 and higher scores are considered better and vice versa, where GFSI ranks also span from 1 to 100 and higher ranks are considered worse and vice versa. The study introduced the innovative approach of ToC that categorize the GFSI scores and ranks into distinct groups such as poor, weak, normal, good and best. ANOVA results indicate that there are statistically significant differences exist between the means of GFSI score and population changes among the top five populous countries. Despite the marginal drop in GFSI rank from 68th to 67th, India has shown slight progress in GFSI scores and its components. While availability and QS have moved from normal to good, SA has improved from weak to normal. However, affordability has declined, highlighting the need to address economic accessibility of food. China has made commendable steps in enhancing its food security profile and ranked 25th from 49th, reflected significant improvements in GFSI scores and its component. The stabilized population growth suggests successful alignment between food security measures and demographic trends. The USA experienced a slight decline in GFSI rank from 5th to 13th. Despite this, overall food security remained at a good level comparing with others countries, with consistent strengths in all components. Indonesia stands at 63rd and still falls in weak category in spite that it has shown overall improvement in its GFSI score, moving from a normal to a good category. The affordability component significantly improved, shifting from good to best. Despite progress, sustained attention is needed for improvement in all components except affordability to strengthen food security in Indonesia. Pakistan stands at 84th position and falls in poor category. Pakistan has seen marginal improvements in GFSI ranking and component scores, particularly in affordability, availability and QS, However, challenges persist as it still falls in normal category and for sustainability and adaptation in weak category. The slight increase in the population growth rate remains serious a concern for food security. Countries should use this study to determine priority sectors for attention in the Global Food Security Index (GFSI) and consider extending similar assessments to other nations.

6. CONFLICT OF INTEREST

All authors declared no conflict of interest.

7. FUNDING STATEMENT

No funding was received to conduct this research.

8. AUTHORSHIP CONTRIBUTION

All authors have equal contributions.

9. ETHICAL STATEMENT

This work does not include any studies involving human or animal subjects.

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Research Article

Dietary Preferences and Their Impact on the Life Cycles of *Bactrocera Zonata* and *Bactrocera Dorsalis*

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Abstract: This study aimed to contribute to the biological control of two economically significant fruit flies species, *Bactrocera zonata* and *Bactrocera dorsalis*. The research examined the effects of different host fruit species and artificial larval diets on their development.. Experiments were conducted under controlled laboratory conditions (28 ± 2 °C, 60-65% relative humidity). Specified five fruit varities as host including apple (*Malus sylvestris*), guava (*Psidium guajava*), mango (*Mangifera indica*), persimmon (*Diospyros kaki*), and pomegranate (*Punica granatum*). Key biological parameters taken into consideration were egg production, larval and pupal development time, survival rate, adult emergence percentages, and sex ratios. Comparisons were made between liquid and solid artificial diets under different host conditions. Results revealed that *P. guajava* was the most favorable host, yielding optimal egg production (297.3 ± 9.3), larval numbers (261 ± 3.2), pupal counts (237.6 ± 27.1), and adult emergence rates (86.9 ± 3.9%). Similarly, *P. granatum* exhibited the longest egg hatching duration (2.3 ± 0.3 days) and larval development time (7.6 ± 0.3 days). While *M. sylvestris* and *D. kaki* showed maximum pupal duration (4.3 ± 0.3 days). Solid artificial diets produced higher egg numbers (408 ± 108.3) and extended developmental periods compared to liquid diets, though liquid diets achieved superior adult emergence rates (80 ± 2.8%). It is concluded that *P. guajava* serves optimally for *Bactr ocera* mass rearing. Moreover, solid diets enhance reproduction while liquid diets improve adult emergence. This can help and inform existing and future biological control programs.

Keywords: Biological Control, Fruit Flies, Mass Rearing, Artificial Diet, Cucurbitaceae, Fruit Preference, Larval Diet.

1. INTRODUCTION

B. zonata is a polyphagous tephritid with its native habitat in tropical Asia before spreading to southern and Southeast Asia, the Middle East, and northern Africa and infesting more than 50 species of wild and cultivated fruits [1, 2]. Similarly, the oriental fruit fly *B. dorsalis* is characterized by a large host range, which covers a wide range of fruits and vegetables, which allows it to spread among territories worldwide and representing a long-lasting challenge to production systems [3, 4]. This broad host specification not only contributes to their pest status but also causes difficulties in their control due to the diversity of nutritional needs required during life cycles [5, 6]. These two species share a specific preference for fleshy fruits like mangoes, guava, citrus, peach, apricot, and figs since these give the best substrates on oviposition and larval development in temperate, subtropical, and tropical zones [7].

The flexibility of adaptation in different agro-climatic conditions and the high reproduction rate make it harder to monitor and control, as the larvae can hide in the fruit pulp impossible to detect at an early stage and treated with chemicals [8]. As a result, their hidden larval growth in the tissues

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of fruits makes them the priority of combined surveillance and management strategies over areas to contain their growth in the tropical and subtropical fruit systems [9]. These qualities explain why area-specific monitoring guidelines are urgently required, because the recent invasion of the *B. dorsalis* on Reunion Island has exacerbated mango infestations alongside *B. zonata* [10].

B. zonata and *B. dorsalis* cause significant economic damages to commercial crops in different countries due to direct decreases in yield and difficult to import fruits due to quarantine against infected hosts [11, 12]. The pests are especially costly since they destroy more than 40 species of fruit crops and require expensive phytosanitary measures, and in the case of fruit flies, crops losses are estimated at about 353.2 million US dollars [13, 14]. The economic losses caused by these infestations are 144.6 million dollars per capita and have caused the host production, which is popular in the southern areas, to be abandoned; this is because of the heavy losses and high management costs [2, 15]. Fruit flies are also difficult to manage because of their behavioral flexibility at different development stages, and possess resistance against several chemical pesticides, thus there is a need of alternative control solutions [16, 17]. As a result, integrated pest management approaches, including the use of fruit bags, protein baits with ammonium-based synergist, and installing traps are promoted to reduce these economic costs and minimize environmental risks of conventional eradication measures [18, 19].

Polyphagous fruit flies such as *B. zonata* and *B. dorsalis* possess distinct ecological characteristics of contrasting developmental times and survival rates of all life cycles, with *B. zonata* completing its cycle comparatively faster than *B. dorsalis* which can confer competitive advantages during comparable hosts [12]. Host fruit preferences also moderate these competitive dynamics with *B. dorsalis* showing different fitness behaviors in guava, papaya, and banana which affect the potential to increase a population in different agroecosystems [20]. This dietary plasticity highlights the underlying evolutionary importance of phenotypic plasticity in such species, in which alterations in gene expression in connection to metabolic and stress-related pathways allow adaptation to changing nutritional qualities of host fruits [21]. Such plasticity is not only beneficial to survive throughout the changes in the availability of resources but also leads to evolutionary trade-offs in the aspects of life-history due to the modulated stress resistance and reproductive plasticity to dietary limitations [21]. As a result, the specified trade-offs are reflected in opposite larval survival and developmental intervals of the species under standardized conditions, which determines their invasion success and interspecific relationships within fruit orchards [12, 21]. The interspecific interactions are also favored by the high variation in the developmental timing of the host fruits, such as the reduction of the third stage of the instar stage and the pupal period of *B. dorsalis* on guava compared with mango and apple; which influence the relative fitness and has the ability to nurture in shared niches [12, 15].

Although there are fundamental gaps in comprehending the research on the pupal growth during different soil regimes, demonstrating how environmental factors affect *B. dorsalis* assessment and stage length, and how all three factors interact with dietary host preferences, and subsequent life cycle fitness are yet to be examined [22]. Additionally, the molecular processes underlying diet-based changes in gene expression and phenotypic plasticity, and their occurrence in related insects, are not yet understood in either *B. zonata* or *B. dorsalis*, which restricts understanding of adaptive responses to nutritional gradients [21]. Moreover, genetic and biotechnological management research is obstructed by the inability to rear both species in mass culture due to their difficulties in culturing at different developmental stages and the absence of standardized artificial diets with diverse nutritional profiles [23].

Artificial diets allow biological parameters in *B. zonata*, which supports parasitoid production to a higher level of pest management [24]. The presence of polypaganthic, high-fecundity, and multivoltine life cycles makes it extremely difficult to mass rear these fruit flies using standard artificial diets, and alternative diets are required to facilitate adaptation in the laboratory and enable advanced pest control programs involving these fruits flies [23]. New artificial diets demonstrated a higher percentage of pupation, adult expression, and ability to fly in mass-reared *B. dorsalis* including liquid diets with mixture of substrates and demonstrated to overcome

the limitations of traditional bran-based media [3, 25]. Such recipes also enhance high flight capacity and longer adult life in *B. dorsalis*, which is well above the international standards of sterile insect releases [3, 26]. These advancements have been extended to *B. zonata*, in which artificial feeding approaches open continuous laboratory culture to overcome seasonal limitations on host availability to research and application of sterile insect techniques [27]. As a result, the development of semi-solid artificial diets with yeast, sucrose, and preservatives has been very effective in mass rearing *B. dorsalis* under controlled laboratory environments [3]. This effectiveness focuses on increasing the use of artificial rearing of *B. doralis* aiding biological control and sterile insect approaches by aiding quality-in-large-scale production proven through a test of larval food substrates in the laboratory [28]. The present study aims to evaluate the ovipositional preference and larval success of *B. zonata* and *B. dorsalis* under choice and no-choice conditions. This information will help to assess the host vulnerability and optimize mass rearing protocols.

2. MATERIALS AND METHODS

2.1. Sample and Sampling

Adult flies of *B. zonata* and *B. dorsalis* were collected from established cultures (originally from a field at Gilgit (35°55′11.36” N, 74°22′47.44” E). Pakistan. Flies were kept in rearing cages (60 × 60 × 48 cm) with cloth sleeves opening at the front for handling. Adults were treated using standard laboratory conditions [29]. Adults were given water-based diet and water on dripping cotton *ad libitum*. For egg laying, adults were offered fresh fruit, and infected fruit were later detached and put into a plastic container with sand at the bottom for pupation. Pupae were separated from sand by using a mesh screen and kept in a jar (15 × 6 × 6 cm) until adults emerged. Temperature was maintained at 25 ℃ - 30 ℃ for 24 hours under light conditions.

2.2. Host Fruits Used

Apple (*Malus sylvestris*), guava (*Psidium guajava*), mango (*Mangifera indica*), persimmon (*Diospyros kaki*), and pomegranate (*Punica granatum*) were available at market during studied period at Gilgit. A no-choice test was used to evaluate host exposure, preference, and progeny of *B. zonata* and *B. dorsalis* separately. Adult *B. zonata* and *B. dorsalis* (5-7 days old) were used separately for additional experiments (male to female ratio 1:1). The experiments were conducted at a research laboratory setup [29]. Each host treatment had three replications. Randomly Fifty pairs of *B. zonata* and *B. dorsalis* adults were kept separately in plastic containers (15 × 6 × 6 cm) and provided 300 g of each host fruit for 48 hours. The exposed host was later exchanged with fresh fruits and infected hosts were checked for oviposition punctures per host for each species. Oviposited fruits were put into plastic boxes with sand substratum. Pupae were sieved, and the number was recorded. The pupae were put into plastic jars to observe adult appearance and the male-to-female ratio.

2.3. Artificial Larval Diet

Liquid artificial larval diet was prepared by mixing 100 mL of distilled water with sugar, sodium benzoate, vitamin B complex, citric acid and streptomycin to make a homogenous solution (Table 1). A beaker containing the mixture of ingredients was put into a freezer. The prepared liquid artificial diet was poured into a container. An egg-laying device instead of a fruit was used. Ten eggs were put into the container with the artificial diet, and the lid was closed to make it airtight. Treatment was replicated three times. The lid of the container was perforated at two days interval. After the third larval instar, the container was opened and pupae were collected [30].

Ingredients of solid artificial larval diet for *B. zonata* and *B. dorsalis* are listed in Table 2. The constituents of group ‘A’ were mixed carefully in a mixer with 550 ml of deionized water. Agar-agar

Table 1. Composition of liquid artificial larval diet.

Ingredient	Quantity
Distilled water	400 ml
Sugar	+48.7 g
Sodium benzoate	0.8 g
Vitamin B complex	+1 ml
Citric acid	+9.2 g
Streptomycin	+1 ml
Baker’s yeast	5 g

Table 2. Composition of solid artificial larval diet.

Group	Ingredient	Quantity (g)
Fraction A (main ingredients)	Wheat germ	26.0
	Kidney bean *Phaseolus vulgaris* flour	51.3
	Chickpea *Cicer arietinum* var. Kabuli flour	56.0
Fraction B	Dried yeast powder	31.6
	Casein	15.2
	L-Ascorbic acid	3.2
	Cholesterol	0.5
	Multivitamin multi-mineral capsule	2 capsules
	Vitamin E capsule USP	1 capsule
	Castor oil USP	1 ml
	ABDEC drops	2 ml
Fraction C	Methyl-p-hydroxybenzoate	1.8
	Sorbic acid	1.3
	Streptomycin sulfate	0.25
	Formaldehyde solution	2 ml
Fraction D	Agar-agar	16.4
Distilled water		820 ml

(Group 'D') weighed dissolved in 270 ml of warm water, heated in a glass beaker, cooled and mixed for about one minute with the other constituents at fast speed in mixer. The components of groups 'B' and 'C' were mixed well. The solution was transferred to disinfected Petri dishes (15-cm diameter). The diet was cooled until it solidified in a refrigerator. The diet was removed from the refrigerator and kept at room temperature for 2-3 hours before use. The solid diet was divided and transferred to plastic rearing containers for development of larvae [31]. Mean percentage of adult fly emergence was calculated using a formula given by Gupta *et al*. [29]:

$$\text{Adult emergence (\%)} = \frac{\text{Number of adults emerged}}{\text{Total number of pupae}} \times 100$$

Sex ratio (female) was calculated using a formula specified by Farooq and Freed [32]:

$$\text{Sex ratio (female)} = \frac{\text{Number of females emerged}}{\text{Total (male + female)}} \times 100$$

Data was subjected to Statistic 8.1 [33]. Mean data on parameters (number of eggs, number of larvae, larval development time, number of pupae, percentage of adults emerged, and sex ratio.

3. RESULTS

Host preferences by life stages of *B. zonata* and *B. dorsalis* are shown in Figures 1 and 2, respectively. Eggs laid by *B. zonata* (297.3 ± 16.1) and *B. dorsalis* (253.6 ± 44.6) were maximum on *P. guajava.* Days for larval development of *B. zonata* and *B. dorsalis* were maximum on *P. granatum* (11.6 ± 0.5 and 12.3 ± 1.1 respectively and fewest on *P. guajava* (7.6 ± 0.5 and 7.3 ± 0.5). The maximum number of larvae produced on *P. guajava.*by *B. zonata* (261 ± 5.5) and by *B. dorsalis* showed (244.3 ± 41.4) were similar compared to other hosts.

Most pupae were produced by *B. zonata* (233 ± 27.6) and *B. dorsalis (*237.6 ± 46.9) on *P. granatum*. Pupal duration was minimum on *P. guajava,* but equally maximum (4.3 ± 0.5) on *P. granatum, M. sylvestris,* and *D. kaki*. *B. dorsalis* had maximum pupal duration (4 ± 1) on *P. granatum* but equally minimum on *P. guajava* and *D. kaki* compared to other hosts. Percentages of adults' emergence of *B. zonata and B. dorsalis* were similar and greatest (82 ± 7.8 and 86.6 ± 6.8, respectively) on *P. guajava.*

P. granatum resulted in the greatest female-to-male ratio (female = 1.2 ± 0.17, male = 0.84 ± 0.13)

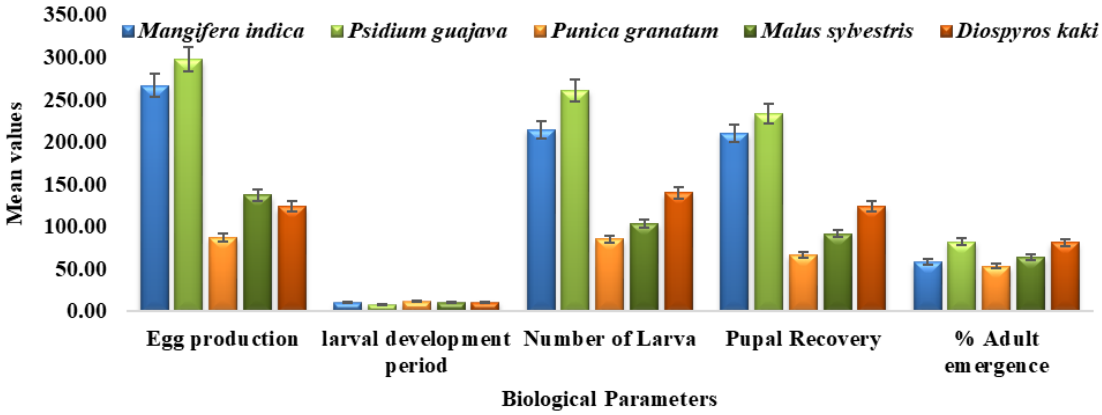

Fig. 1. Biological parameters of *B. zonata* fruit flies on different host fruits.

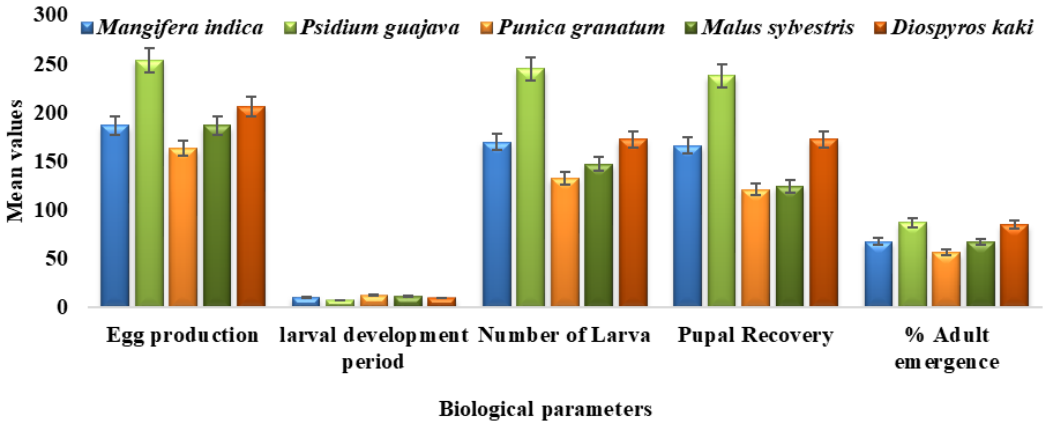

Fig. 2. Biological parameters of *B. dorsalis* fruit flies on different host fruits.

of *B. zonata*, followed by *M. sylvestris* (female = 1.17 ± 0.02, male = 0.86 ± 0.14)). *D. kaki* resulted in the greatest ratio of males to females (male = 1.19 ± 0.23, female = 0.85 ± 0.15), followed by *P. guajava* (male = 1.16 ± 0.03, female = 0.85 ± 0.02).

The greatest female-to-male ratio for *B. dorsalis* was on, *M. indica* (female = 1.61 ± 0.09, male = 0.62 ± 0.06), followed by *P. guajava* (female = 1.18 ± 0.05, male = 0.84 ± 0.04), as shown in Table 3. *P. granatum* resulted in the greatest male-to-male ratio for *B. dorsalis* (male = 1.09 ± 0.39, female = 0.99 ± 0.33), followed by *M. sylvestris* (male = 1.03 ± 1.17, female = 0.98 ± 0.18).

B. zonata produced most eggs (218.3 ± 15.8), as did, *B. dorsalis* (408 ± 108.3) on solid rather than liquid artificial larval diet (Figures 3 and 4). Development duration of *B. zonata* and *B. dorsalis* larvae were similar (20.6 ± 0.57) on solid compared with liquid artificial larval diet. The number of larvae of *B. zonata* and *B. dorsalis* were 182 ± 2 and 258.6 ± 50.1, respectively, on solid compared with liquid artificial larval diet, see Figures 3 and 4.

More pupae of *B. zonata* (142.33 ± 22.5) and *B. dorsalis* (187.6 ± 41.6) developed on solid compared to liquid artificial larval diet (Figures 3 and 4). Larval developmental period was longest for *B. zonata* (9.3 ± 0.57) and *B. dorsalis* (9.67 ± 0.57) on solid artificial larval diet. Percentage of adults emerged *B. zonata* (80 ± 2.8) and *B. dorsalis* (66.6 ± 3.3) was greater on liquid artificial larval diet.

Liquid artificial larval diet resulted in more female than male *B. zonata* adults (female = 1.2 ± 0.02, male = 0.8 ± 7.8), compared with solid artificial larval diet (female = 1.08 ± 0.05, male = 0.9 ± 0.04). For *B. dorsalis*, solid artificial larval diet resulted in a greater female-to-male ratio (female = 1.07 ± 0.14, male = 0.97 ± 1.14) than did liquid artificial larval diet (female = 1.02 ± 0.14, male = 1.0 ± 0.13); these results are presented in Table 4.

Table 3. Ratios of male and female of fruit flies on studied fruits.

Host treatment	*B. zonata*		*B. dorsalis*	
	Male ratio	**Female ratio**	**Male ratio**	**Female ratio**
Mango, *Mangifera indica*	1.08 ± 0.03	0.92 ± 0.04	0.62 ± 0.06	1.61 ± 0.09
Guava, *Psidium guajava*	1.16 ± 0.03	0.85 ± 0.02	0.84 ± 0.04	1.18 ± 0.05
Pomegranate, *Punica granatum*	0.84 ± 0.13	1.2 ± 0.17	1.09 ± 0.39	0.99 ± 0.33
Apple, *Malus sylvestris*	0.86 ± 0.14	1.17 ± 0.02	1.03 ± 1.17	0.98 ± 0.18
Persimmon, *Diospyros kaki*	1.19 ± 0.23	0.85 ± 0.15	0.94 ± 0.04	1.06 ± 0.05

Table 4. Male and female ratio of fruit flies on artificial larval diet.

Treatment given	*B. zonata*		*B. dorsalis*	
	Male ratio	**Female ratio**	**Male ratio**	**Female ratio**
Liquid artificial larval diet	0.8 ± 7.8	1.2 ± 0.02	1.0 ± 0.13	1.02 ± 0.14
Solid artificial larval diet	0.9 ± 0.04	1.08 ± 0.05	0.97 ± 1.14	1.07 ± 0.14

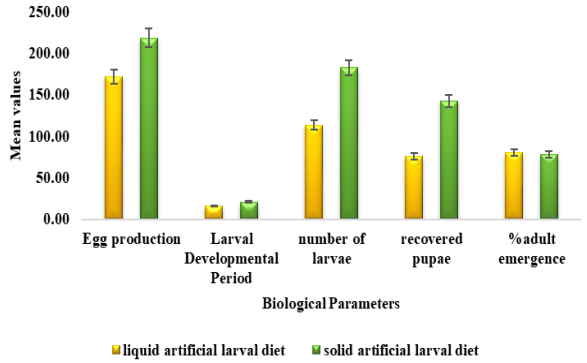

Fig. 3. Biological parameters of *B. zonata* fruit flies on liquid and solid artificial larval diet.

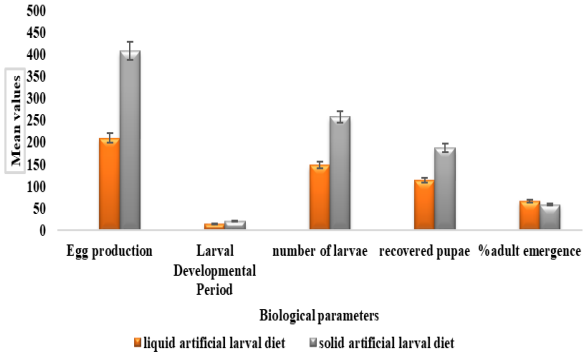

Fig. 4. Biological parameters of *B. dorsalis* fruit flies on liquid and solid artificial larval diets.

4. DISCUSSION

Host preferences of *B. zonata* and *B. dorsalis* fruit flies for five types of fruit apple, guava, mango, persimmon and pomegranate were tested at controlled laboratory conditions. The study showed significant differences among development of immature stages until adults emerged on different host fruits.

Aroma receptors are important for fruit flies from distant areas to find food sources Reisenman and Scott [34]. In present study most pupae were recovered on *P. guajava* and fewest on apple; Rauf *et al.* [31], Kalia and Yadav [35] also found most eggs produced on *P. guajava*. In contrast to our study, *M. indica* was most favorable for egg production in studied by Sarwar *et al.* [36]; but these researchers did not include *P. guajava* in their studies, perhaps because *M. indica* was most preferred by fruit flies. In a current study overall development was maximum on *P. guajava* and minimum on *D. kaki*. In similar study Kalia and Yadav [35] found larval development was longest on *P. guajava* followed by *M. indica*. Most pupae were developed from *P. guajava* and least from *P. granatum.* In contrast to our investigation *M. indica* was most favored, while *M. sylvestris* was least favored for pupae [35, 36].

The impact of guava fruit on longevity, growth and development of fruit fly was established during a similar study as the skin of guava fruit being very thin and soft played vital role in palatability and consumption by fruit flies [15]. A similar pattern was observed in present study. In contrast to our study, *M. indica* was preferred, followed by *M. sylvestris* for percentage emerging reported by Sarwar *et al.* [36]. Kalia and Yadav [35] found most adult emerging on *P. guajava* and lowest by *M. indica*.

During present study two artificial diets (solid and liquid) were tested at laboratory conditions to evaluate biological parameters of *B. zonata* and *B. dorsalis.* Solid artificial larval diet resulted in a greater number of eggs (218.3 ± 15.8) than did liquid diet (171.6 ± 15.8). According to Sookar *et al.* [38] significant eggs were produced on liquid artificial larval diet. Current Study showed solid artificial larval diet resulted in a greater number of larvae (182.3 ± 15.9) than did liquid diet (113 ± 7.5). According to Sookar *et al.* [37], Momen *et al.* [38], and Shinwari *et al.* [30] 95%, 90.98%, and 43.67% of larvae, respectively, developed on artificial larval diets.

Present study showed duration of larval development was longer on solid artificial larval diet (20.6 ± 0.3) than liquid diet (15.6 ± 0.3). According to Abro *et al.* [39], larval duration was 4.95 ± 0.35 days on diet. The present study investigates solid artificial larval diet that resulted in a greater number of pupae (142.3 ± 12.9) compared to liquid larval diet (76 ± 14). According to Gupta *et al.* [29] 89.2% of pupae developed on solid artificial larval diet. According to Sookar *et al.* [37] and Shinwari *et al.* [30] 95%, and 48.07% of pupae developed from artificial liquid larval diet.

In a present research Solid diet resulted in maximum pupal duration (9.3 ± 0.3) compared with liquid larval diet (5.3 ± 0.3). According to Gupta *et al.* [29] pupal duration was 9.8 days on solid larval diet, respectively. According to Abro *et al.* [39], pupal duration was 7.25 ± 0.25 days on larval diet.

Present study, liquid artificial larval diet resulted in greater percentage of adults emerging (80 ± 2.8) than on solid diet (77.6 ± 5.3). According to Gupta *et al.* [29] 97% of adults emerged on solid larval diet, while Shinwari *et al.* [30] and Sookar *et al.* [37] found more than 80% of adults emerged on liquid larval diet. The present study found a greater female-to-male ratio on liquid than solid diet. Rauf *et al.* [31] found a 1:2.3 male-to-female ratio on solid larval diet. Shinwari *et al.* [30] found 43.5:56.5 male-to-female ratio on a liquid larval diet.

The selection of host fruits by *Bactrocera* species is a key factor that affects their reproductive fitness and demographics because nutritional quality differences have a direct effect on oviposition selection and development rates of different geographical strains. *B. dorsalis* frequently prefers oviposition on *P. guajava* as this fruit has a soft and thin skin than other fruits, which is easily penetrated by females [15]. In addition to physical features, volatile organic compounds released by ripe *P. guajava* are effective chemical attractants which act as indication of high nutritional value to gravid females hence it correlates with much greater visits and oviposition punctures as compared to other hosts such as papaya and banana [20].

Methodologies used to evaluate these host preferences include standardization of the fruit condition of the host by selecting disease-free specimens freshly washed, pesticides free and rearing pests under controlled laboratory conditions of 25 ± 1 °C and 65 ± 5% relative humidity [40-41]. Moreover, experimental design needs to use fruits must be same stage of maturity for the consistent volatile profiles and the texture of the fruits because differences in maturity levels can drastically change oviposition behavior and the survival rates of the larvae [42].

These dynamics also become complicated by variations in the geographic strain sensitivity as different populations showing variation in developmental plasticity in response to the same fruit sources or rearing strategies [43, 15]. For instance, comparative study found that developmental stages of larval instar and pupal duration are shorter when larvae fed on *P. guajava* compared to mango or apple, again reflects that the nutritional content of *P. guajava* more effective to supporting the development of larvae and the successful eclosion period [15]. The exceptional nutritional composition of *P. guajava* consisting of unique pH and water content provides an ideal condition to larval growth as compared to other mango varieties such as Kent and apple mango [15, 42].

5. CONCLUSIONS

This investigation revealed that the host selection and larval diet composition influence significantly on the performance of *B. zonata* and *B. dorsalis*. Research showed that *P. guajava* guava was most preferred as a host by *B. zonata* and *B. dorsalis* fruit flies. Guava fruit was preferred for mass rearing fruit flies in a laboratory as well as rearing for biological control agents as larval and pupal parasitoid of fruit flies. Solid diet was preferred over liquid artificial

diet for larval and pupal development of fruit flies during winter due to unavailability of liquid diet for mass rearing in laboratory-controlled conditions. Mass rearing fruit flies under laboratories are useful for developing larva and pupae and their parasitoids on large scale. The larva and pupal parasitoids are released to control fruit flies in fields and farms. Comparative analysis of artificial diets revealed that solid diets enhanced egg production, larval survival, and pupal development. Whereas liquid diets promoted higher adult emergence and more balanced sex ratios. Findings suggest that diet selection can be used deliberately to improve parasitoid production and achieving efficient adult emergence for colony maintenance. In a nutshell, the study provides practical insights for improving mass rearing protocols of *B. zonata* and *B. dorsalis.* These are critical for integrated pest management and biological control programs. The results contribute valuable baseline data for optimizing cost-effective rearing systems and enhancing the effectiveness of area-wide fruit fly management strategies.

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7. CONFLICT OF INTEREST

The authors have no conflicts of interest.

8. ETHICAL STATEMENT

Ethical approval was obtained from the Bio-Ethics committee of Karakoram International University (Approval No. KIU/2023/021).

9. FUNDING

No funding was received to conduct this research.

10. AUTHORSHIP CONTRIBUTION

Chandni Kiran and Maisoor Ahmed Nafees conceived and designed the experiments. Chandni Kiran, Saif Ud Din, and Nasreen performed the experiments. Tika Khan and Akbar Khan analyzed the data, while Saif Ud Din and Akbar Khan wrote the paper.

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Research Article

Spectrophotometric Evaluation of the Sun Protection Factor of Wild *Sideritis raeseri* Extracts under Different Solvent and Temperature Conditions

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Abstract: Plant-origin sunscreen products for skin protection from ultraviolet radiation are preferred over synthetic products as safer with regard to possible toxicity, allergies, and are eco-friendly. This study fills a gap in peer review research for endemic Albanian wild mountain tea (*Sideritis raeseri Boiss & Heldr.*) for its ultraviolet photoprotective properties under various solvent types, incubation days, and temperatures, by utilizing the Mansur method. S. *raeseri* aqueous extracts were centrifuged, filtered, and then mixed in two Erlenmeyer flasks: one with distilled water and the other with ethanol in a 1:4 v/v ratio. Absorbance measurements within the 290-320 nm range were taken at different temperatures over six consecutive days. Neither water nor ethanol absorbs within this range. In extracts at room temperature (29-31 °C), Sun Protection Factor (SPF) value expressed as mean ± standard deviation dropped from 26.426 ± 0.2 on day one to 17.930 ± 0.19 on day six; for refrigerated extracts at 11 °C, SPF dropped from 26.426 ± 0.2 to 22.469 ± 0.19. In the water-ethanolic mixture at room temperature, SPF dropped from 23.281 ± 0.21 to 13.387 ± 0.036, and with refrigeration, from 23.281 ± 0.21 to 21.199 ± 0.21. At refrigerated conditions, the SPF of *S. raeseri* extract was photochemically more stable and showed a longer shelf-life, while SPF values at room temperature incubation were lower. Higher SPF values were found in water solutions than water-ethanol. The refrigerated water-ethanol mixture could be utilized in sunscreen products as a stability factor for SPF values of *S. raeseri* extracts.

Keywords: *Sideritis raeseri*, SPF, UV Protection, Natural Sunscreen, Mansur Method.

1. INTRODUCTION

The pathogenesis of skin cancer is multifactorial (sunburn, time of exposure, protection, etc.) were ultraviolet (UV) radiation is considered to be the main cause and risk factor, as mentioned by Fabris *et al.* [1]. The threat posed by UV radiation has brought along increased awareness among the population, leading to shorter intervals of sun exposure as well as a growing demand for various sun protection products. Increased awareness of UV damage has led to an increased trend of plant-based sunscreen protection products alongside the traditional products. According to Korac and Khambholja [2], conclusive proof suggests that a good quality sunscreen is safe to use when applied correctly; it reduces the risk of skin cancer. To this day, in general, including UV radiation, prevention was and still remains the best and most effective measure for public health protection. . Breitbart *et al*. [3] studied the effect of sun protection on reducing UV-related risks and found that using sun protection products that absorb or block UV radiation, proved to be a highly effective preventive measure. As proved by Raymond and Riskin [4], some synthetic chemical UV filters like oxybenzone, TiO_2, etc., pose a risk to human health, as with the Volatile Organic Components, as proved by Pal *et al*. [5]. Also, Suh *et al*. [6] underline the risk for human health of some of the ingredients of sunscreen creams. According to Wheate *et al* [7], the active pharmaceutical ingredients (API) in sun protection products may threaten the aquatic species, hence recent studies and innovative lab techniques are

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shifting towards plant-based sunscreen products as safer, effective, and eco-friendly alternatives. Discovering new sources of plant extracts with sunscreen properties that could serve to formulate creams or oils with sun-protective properties and finding their best conditions of storage to conserve their properties becomes of primary importance. By investigating the incubation period in various temperature conditions and solvents of the sun protection factor of *S. raeseri* this study aims to contribute to related cosmetic industries by highlighting the potential use of mountain tea as a natural ingredient.

As mentioned in several studies, such as Dutra *et al*. [8] or Zarkogianni and Nikolaidis [9], the Mansur spectrophotometric method is a widely accepted approach for determining the Sun Protection Factor (SPF) in vitro. In this study, the Mansur equation was used to analyze SPF values of the extracts of the endemic Albanian mountain tea, *Sideritis raeseri Boiss & Heldr (S. raeseri*), a plant known for its bioactive properties, in various solvents and at different incubation temperatures over several days.

The effectiveness of sun-protective products is measured by their SPF, which indicates the amount of UV energy required to cause a minimal erythema dose (MED) on protected skin versus unprotected skin.

$$SPF = \frac{MED-protected\ skin}{MED-unprotected\ skin} \quad (1)$$

While previously studies investigated with different methods the chemical compounds and their degradation in various conditions and solvents of *S. raeseri*, this study provides for the first time the influence on the stability of SPF of this plant in two different solvents, water and water-ethanol. This is particularly relevant for cream and oil formulations seeking to minimize synthetic UV filters due to concerns over allergenicity and toxicity, as proved by Stiefel and Schwack [10], as well as the environment pollution.

2. MATERIALS AND METHODS

2.1. The Plant

The wild Albanian "mountain tea", *Sideritis raeseri Boiss & Heldr*, belongs to the *Lamiaceae* family and was studied in this paper for its potential sunscreen properties under different solvents, time incubation, and temperatures with the Mansur "in vitro" method. This plant, typically found at elevations above 600 meters, is a mostly perennial herb that flourishes in calcareous, well-drained, slightly alkaline soils. It prefers rocky slopes with ample sunlight exposure, experiencing dry summers and mild winters.

The tea, made from an aqueous solution of its aerial parts (flowers, stems, and leaves), is widely used for its anti-inflammatory, analgesic, gastroprotective, and antimicrobial benefits, and serves as a dietary supplement for anaemia.

2.2. Plant Preparation

A quantity of 1g dried *S. raeseri* was chopped and precisely weighed using an analytical balance. The weighed sample was placed in an empty, heat-resistant glass beaker, and 50 mL of deionized water was added once it had begun to boil. Then the mixture was allowed to steep for 5 minutes, during which the chromophores began to release their characteristic colour. The resulting solution was cooled at room temperature and subsequently filtered using filter paper with a pore size of 1.2 μm and a nominal thickness of 260 μm. The filtered extract was transferred to a glass Erlenmeyer flask and stored at 3 °C in a refrigerator for 24 hours before analysis. After 24 hours, the solution was centrifuged at 4000 rpm for 8 minutes.

The resulting supernatant was then diluted at a 1:4 ratio with distilled water (e.g., 20 mL supernatant to 80 mL water, or 1 part supernatant to 4 parts 100% ethanol, corresponding to a 20% v/v dilution). The final concentration of the extract used for spectrophotometric measurements is, for both solutions, 4 mg/mL or 4% w/v. Both were prepared for spectrophotometric analysis. The prepared samples were stored at two different temperatures: 11 °C and 29-30 °C.

2.3. Spectrophotometry Measurement

Absorbance measurements were taken from 290 to 320 nm at 5 nm intervals(step) using a UV-VIS spectrophotometer (NADA 756s) with a 10 mm quartz cuvette, using distilled water and water-ethanol mixture as a blank. A blanking procedure for both types of solvents was executed before

starting the measurements. Water and water-ethanol mixtures as solvents do not interfere with UV absorption in tea-aqueous solutions, as they primarily absorb below the 290-320 nm interval. Each Optical Density (O.D) reading was repeated three times, and the SPF values were calculated with the Mansur formula. Measurements were performed daily over a total period of 6 days at two temperatures and with different solvents, including water and a water-ethanol mixture.

2.4. Mansur Formula

Several studies, as previously described [8-9], used the Mansur equation to calculate SPF by measuring the absorbance (from 290 to 320 nm with a 5 nm step) of diluted plant extracts. This "in vitro" method offers a reliable approach to assess the sun-protective properties of tea-aqueous solutions without requiring direct testing on humans against UVA and UVB. The SPF is calculated using the following equation (2):

$$SPF = CF\ x\ \sum_{290}^{320} EE(\lambda) x\ I(\lambda) x\ Abs(\lambda) \quad (2)$$

where *EE* is the erythemal effect spectrum, *I* is the solar intensity spectrum, $(EE(\lambda) x\ I(\lambda)$ is a constant, describing the relationship between the erythemal efficiency spectrum (EE) and the solar simulator intensity spectrum (I) at wavelengths from 290 to 320 nm, *Abs* is the absorbance of sunscreen products, and *CF* is the correction factor (=10).

2.5. Water and Ethanol Spectroscopic Properties as Solvents

Abou-Dahech *et al.* [11] emphasized the crucial role of solvents in the performance of UV filters. Both water and water-ethanol mixtures are solvents with non-aromatic, saturated molecules; their lowest-energy electronic transitions are σ→σ* or n→σ*, which require high energy, which is why the cutoff wavelength is set before 290 nm and doesn't interfere with the tea solutions' absorbance. Mason *et al*. [12], Shalatonin [13], and Saad *et al*. [14] suggested that water absorbance peaks in UVC at ~186 nm and a water-ethanol mixture of approximately 20% v/v absorb near 214 nm wavelength, and a second lower peak appears at ~280 nm [13]. Therefore, both solvents absorb UV light far before the interval of 290 - 320 nm.

2.6. Tea-aqueous Solutions and Their Spectroscopic Properties

Hodaj-Çeliku *et al*. [15] studied phenolic compounds, such as Carvacrol and Thymol, which have been found in high percentages (respectively 36.7% and 0.5% of total essential oils) in tea-aqueous solutions of *S. raeseri*, as well as essential oils including oxygenated monoterpene, oxygenated sesquiterpene, and sesquiterpene. While Carvacrol and Thymol (phenolic monoterpenes) do absorb in the interval 290-320 nm with a shoulder that falls gradually, oxygenated monoterpene, sesquiterpene do not affect absorption in this interval [16-17]. Carvacrol and Thymol both absorb UV light primarily because their chemical structure includes an aromatic ring and a functional hydroxyl group (-OH), and they contain a benzene ring with delocalized π electrons that absorb energy, with the possibility of undergoing π→π* electronic transitions. Also, Żyżelewicz *et al*. [18] in previous studies have reported that *S. raeseri* contains other aromatic photochemical compounds like flavonoids (Aspigenin, Luteolin, and their derivatives), which all absorb in the above-mentioned UV range due to their aromatic structure. All these compounds in the above-mentioned articles that are identified with spectrometric and HPLC-based methods create plant-origin filters against the UV radiation.

As proven by Romanucci *et al*. [19], compounds like Flavonoids (aglycones and glycosides) and Oxygenated monoterpene, sesquiterpenein, play an important role in scavenging free radicals and reducing oxidative stress by mitigating the damage caused by UV radiation. Espín *et al*. [20] studied that the antioxidant capacity has been positively correlated with phenolic content, and these compounds exert their antioxidant effects through several mechanisms. They stabilize free radicals generation from UV radiation by enhancing the photoprotective properties of the plant extract, besides improving sunscreen filters.

3. RESULTS AND DISCUSSION

This study investigated the effects of temperature and incubation period on the shelf-life stability of the SPF of *S. raeseri* in aqueous and water–ethanol mixed solutions as a possibility for use as plant-origin UV filters in sunscreen formulations.

Couteau *et al*. [21] found that the effect of the presence of ethanol on the efficacy of the filters and their photostability varies on the molecule considered. In Europe, Regulation (EC) No. 1223/2009 [22] currently authorizes 27 organic filters and two mineral filters (Titanium dioxide and Zinc oxide). The topical use of ethanol remains a subject of debate; however, Pendlington *et al.* [23] and Kramer *et al.* [24] have reported that ethanol is, *per se*, safe for topical use.

Tables 1, and 2 summarize triplicate O.D. measurements at seven wavelengths and the calculated SPF values in room temperature and refrigerated conditions, incubation periods, and water as solvent. The Standard Deviation (SD) has been calculated based on the Mean SPF values. The low variability of SD, ranging from 0.036 to 0.23 SPF units, and the Relative Standard Deviation (RSD) of less than 3%, indicates good repeatability and reliability of spectrophotometric measurements. Salazar-Orbea *et al*. [25] found that total polyphenols were more stable at 4 °C than at 24 °C, while Pavlovic *et al*. [26] proved that flavonoid content, due to their gradual degradation, decreases by 23% at 35 °C, and degradation was faster at higher temperatures than in refrigerated conditions, while compounds like phenolic monoterpenes are much more stable. They do evaporate in room conditions, but much less in refrigerated conditions. According to Soliman *et al*. [27], phenolic compounds (Carvacrol and Thymol in our study) were highly stable under various stress conditions, including oxidation, hydrolysis, and thermal decomposition. In water as a solvent, the drop of SPF in room conditions is related to evaporation and degradation processes, while the refrigerated conditions serve as a preservative by slowing these processes.

In Figures 1 and 2, SPF drops with respect to incubation period in both room and refrigerated conditions. The slope value of room temperature (-1.3511) versus that of refrigerated conditions (-0.8262) shows that both solutions are degrading (SPF decrease), but at room temperature, it occurs 1.63 times faster than in refrigerated conditions. The ongoing degradation at refrigerated conditions indicates that water alone is not the only problem,

Table 1. O.D. replicate measurements and SPF values for *S. raeseri* at 29 - 31 °C in water as solvent (n = 3).

Day	Rep\λ [nm]	290	295	300	305	310	315	320	SPF	Mean SPF	± SD
Day1	R1	2.88	2.826	2.749	2.653	2.561	2.483	2.427	26.619	26.426	0.2
	R2	2.84	2.786	2.709	2.613	2.521	2.443	2.387	26.23		
	R3	2.86	2.806	2.729	2.633	2.541	2.463	2.407	26.43		
Day2	R1	2.723	2.56	2.393	2.245	2.133	2.041	1.953	22.78	22.57	0.21
	R2	2.683	2.52	2.353	2.205	2.093	2.001	1.913	22.37		
	R3	2.703	2.54	2.373	2.225	2.113	2.021	1.933	22.57		
Day3	R1	2.582	2.449	2.308	2.178	2.089	2.019	1.951	22.08	21.9	0.18
	R2	2.542	2.409	2.268	2.138	2.049	1.979	1.911	21.72		
	R3	2.562	2.429	2.288	2.158	2.069	1.999	1.931	21.9		
Day4	R1	2.47	2.433	2.284	2.159	2.07	2.002	1.941	21.86	21.68	0.18
	R2	2.43	2.393	2.244	2.119	2.03	1.962	1.901	21.51		
	R3	2.45	2.413	2.264	2.139	2.05	1.982	1.921	21.68		
Day5	R1	2.474	2.341	2.208	2.095	2.013	1.972	1.903	21.22	21.04	0.18
	R2	2.434	2.301	2.168	2.055	1.973	1.932	1.863	20.86		
	R3	2.454	2.321	2.188	2.075	1.993	1.952	1.883	21.04		
Day6	R1	2.149	2.005	1.895	1.787	1.712	1.656	1.61	18.12	17.93	0.19
	R2	2.109	1.965	1.855	1.747	1.672	1.616	1.57	17.74		
	R3	2.129	1.985	1.875	1.767	1.692	1.636	1.59	17.93		

Table 2. O.D. replicate measurements and SPF values for *S. raeseri* at 11 °C in water solvent (n = 3).

Day	Rep\λ [nm]	290	295	300	305	310	315	320	SPF	Mean SPF	± SD
Day1	R1	2.88	2.826	2.749	2.653	2.561	2.483	2.427	26.619	26.426	0.2
	R2	2.84	2.786	2.709	2.613	2.521	2.443	2.387	26.23		
	R3	2.86	2.806	2.729	2.633	2.541	2.463	2.407	26.43		
Day2	R1	2.879	2.788	2.669	2.535	2.439	2.37	2.307	25.64	25.44	0.21
	R2	2.839	2.748	2.629	2.495	2.399	2.33	2.267	25.23		
	R3	2.859	2.768	2.649	2.515	2.419	2.35	2.287	25.44		
Day3	R1	2.867	2.776	2.65	2.516	2.416	2.331	2.278	25.41	25.22	0.19
	R2	2.827	2.736	2.61	2.476	2.376	2.291	2.238	25.03		
	R3	2.847	2.756	2.63	2.496	2.396	2.311	2.258	25.22		
Day4	R1	2.785	2.694	2.555	2.406	2.278	2.174	2.088	24.29	24.09	0.21
	R2	2.745	2.654	2.515	2.366	2.238	2.134	2.048	23.88		
	R3	2.765	2.674	2.535	2.386	2.258	2.154	2.068	24.09		
Day5	R1	2.613	2.525	2.404	2.273	2.17	2.087	2.016	22.96	22.77	0.19
	R2	2.573	2.485	2.364	2.233	2.13	2.047	1.976	22.58		
	R3	2.593	2.505	2.384	2.253	2.15	2.067	1.996	22.77		
Day6	R1	2.556	2.48	2.369	2.247	2.146	2.063	1.993	22.657	22.469	0.19
	R2	2.516	2.44	2.329	2.207	2.106	2.023	1.953	22.28		
	R3	2.536	2.46	2.349	2.227	2.126	2.043	1.973	22.47		

regardless of the low temperature, confirming that lower temperatures slow photochemical and oxidative degradation of phenolic constituents but do not stop it [27].

Tables 3 and 4 show the O.D. values in triplicate measurements and the mean SPF for the water-ethanol mixture at room and refrigerated temperatures. The SD calculated values range from 0.036 to 0.22 SPF units, with an RSD >2 % indicating good precision and repeatability of the spectrophotometric measurements.

At room temperature, the SPF decline is sharp and linear (Figure 3) with a negative slope, which confirms high volatility of Carvacrol and Thymol as well as oxidative degradation of flavonoids that affect the calculation of SPF with the Mansur formula [8]. At refrigerated conditions, the drop of SPF values for 6 consecutive days was 9% (Figure 5) and the slope for refrigerated condition -0.4666, both suggesting the role of low temperature in slowing the volatilization activity and oxidative degradation. This difference in the drop of SPF values between the two conditions could be attributed to the presence of ethanol in the mixture as a stabilizer, as reported by Plaskova and Mlcek [28]. As both water and ethanol–water mixture absorbs below 280nm, the observed differences arise from chemical stability, not from spectral overlap [11-12].

Figures 3 and 4 represent the SPF with respect to the incubation period in both conditions (room and refrigerated conditions). The differences in slope values in water-ethanol mixture at room temperature and refrigerated conditions are, respectively, -1.6795 and -0.4666 SPF units, which indicates a degradation is nearly 3.6 times slower in the refrigerated mixture.

The regression analysis in both Figures 3 and 4, based on R^2 value, reveals a stronger linear correlation for refrigerated conditions (R^2 = 0.8978) rather than room conditions (R^2 = 0.8112), indicating a more reliable prediction of degradation in the refrigerated conditions. At 29-30 °C, the degradation could be multifactorial, including evaporation, oxidative degradation of flavonoids, etc.

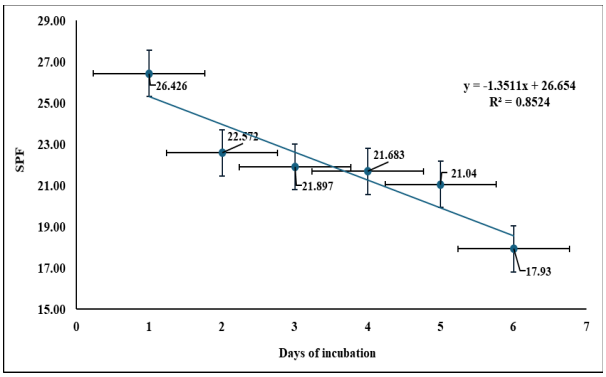

Fig. 1. SPF with respect to the incubation period at room temperature (water).

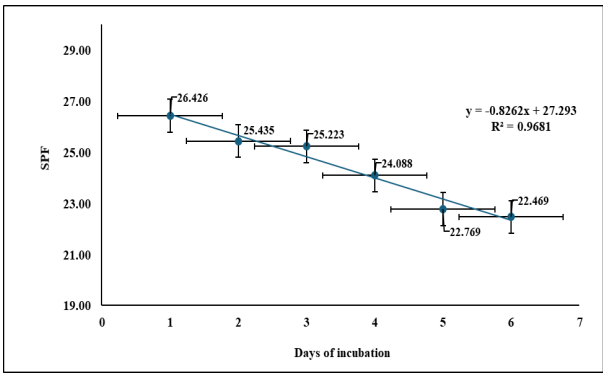

Fig. 2. SPF with respect to the incubation period at refrigerated conditions (water).

Table 3. O.D. replicate measurements and SPF values for *S. raeseri* at 29 - 31 °C in water–ethanol solvent (n = 3).

Day	Rep\λ [nm]	290	295	300	305	310	315	320	SPF	Mean SPF	± SD
Day 1	R1	2.365	2.42	2.4	2.35	2.293	2.24	2.211	23.341	23.281	0.21
	R2	2.325	2.38	2.36	2.31	2.253	2.2	2.171	23.211		
	R3	2.345	2.4	2.38	2.33	2.273	2.22	2.191	23.291		
Day 2	R1	2.17	2.06	1.936	1.838	1.773	1.731	1.525	18.48	18.42	0.056
	R2	2.122	2.012	1.892	1.796	1.734	1.694	1.495	18.37		
	R3	2.146	2.035	1.914	1.817	1.755	1.715	1.510	18.41		
Day 3	R1	1.861	1.742	1.652	1.571	1.524	1.492	1.468	15.79	15.736	0.051
	R2	1.802	1.699	1.603	1.53	1.483	1.454	1.43	15.69		
	R3	1.832	1.721	1.627	1.55	1.504	1.474	1.449	15.73		
Day 4	R1	1.951	1.781	1.645	1.542	1.484	1.451	1.424	15.61	15.547	0.055
	R2	1.902	1.731	1.598	1.501	1.444	1.413	1.387	15.61		
	R3	1.926	1.756	1.621	1.521	1.464	1.431	1.405	15.6		
Day 5	R1	1.821	1.718	1.603	1.536	1.487	1.459	1.431	15.42	15.379	0.041
	R2	1.777	1.672	1.563	1.499	1.451	1.423	1.395	15.348		
	R3	1.799	1.695	1.583	1.518	1.469	1.441	1.413	15.37		
Day 6	R1	1.481	1.438	1.391	1.344	1.326	1.307	1.283	13.42	13.387	0.036
	R2	1.437	1.398	1.347	1.303	1.285	1.267	1.241	13.35		
	R3	1.459	1.418	1.369	1.323	1.305	1.287	1.262	13.39		

Figure 5 shows that the SPF drop related to the first day is 43 % (from 23.28 to 13.39) at room temperature, and 9 % under refrigeration conditions (from 23.28 to 21.20), with the SPF value lower in the water-ethanol mixture. The difference between the first measurement of SPF in both conditions is about 11 % could be considered as normal or may be related to the addition of ethanol in the plant water extraction, creating a less polar solvent with a potential to alter the absorption band through a solvatochromatic effect, as reported by Shirali *et al*. [29].

Water-ethanol solvent at room temperature initially facilitates, by virtue of its being less polar, the solubility of phenolic compounds, but it seems to reduce the SPF value, likely due to its volatility and the increased oxidation of sensitive aromatic compounds. Their gradual photodegradation and oxidation account for the observed decline in SPF

Table 4. O.D. replicate measurements and SPF values for *S. raeseri* at 11 °C in water–ethanol solvent (n = 3).

Day	Rep\λ [nm]	290	295	300	305	310	315	320	SPF	Mean SPF	± SD
Day1	R1	2.365	2.42	2.4	2.35	2.293	2.24	2.211	23.341	23.281	0.21
	R2	2.325	2.38	2.36	2.31	2.253	2.2	2.171	23.211		
	R3	2.345	2.4	2.38	2.33	2.273	2.22	2.191	23.291		
Day2	R1	2.59	2.512	2.424	2.318	2.24	2.183	2.152	23.41	23.2	0.22
	R2	2.55	2.472	2.384	2.278	2.2	2.143	2.112	22.98		
	R3	2.57	2.492	2.404	2.298	2.22	2.163	2.132	23.2		
Day3	R1	2.57	2.483	2.384	2.283	2.203	2.144	2.117	23.05	22.83	0.22
	R2	2.53	2.443	2.344	2.243	2.163	2.104	2.077	22.61		
	R3	2.55	2.463	2.364	2.263	2.183	2.124	2.097	22.83		
Day4	R1	2.533	2.457	2.361	2.256	2.173	2.11	2.074	22.77	22.56	0.21
	R2	2.493	2.417	2.321	2.216	2.133	2.07	2.034	22.35		
	R3	2.513	2.437	2.341	2.236	2.153	2.09	2.054	22.56		
Day5	R1	2.609	2.457	2.361	2.256	2.173	2.11	2.074	21.54	21.31	0.23
	R2	2.569	2.417	2.321	2.216	2.133	2.07	2.034	21.09		
	R3	2.589	2.437	2.341	2.236	2.153	2.09	2.054	21.31		
Day6	R1	2.208	2.23	2.191	2.133	2.079	2.049	2.043	21.41	21.19	0.21
	R2	2.168	2.19	2.151	2.093	2.039	2.009	2.003	20.99		
	R3	2.188	2.21	2.171	2.113	2.059	2.029	2.023	21.18		

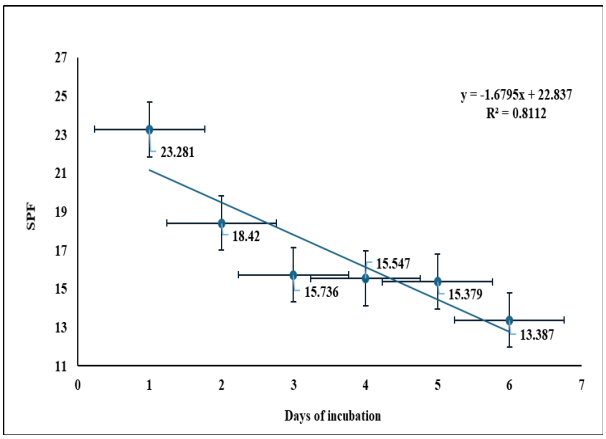

Fig. 3. SPF with respect to the incubation period at room temperature (water-ethanol).

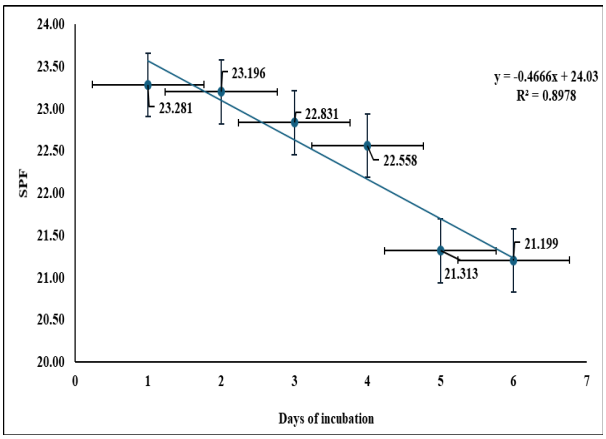

Fig. 4. SPF with respect to the incubation period at refrigerated conditions (water-ethanol).

during prolonged storage, particularly at higher temperatures or in ethanol–water mixtures, as reported by Galmarini *et al.* [30].

The statistical analysis of Table 5, using the Pearson correlation, revealed strong evidence of a true inverse linear relationship between SPF and its incubation time (days). The strongest correlation was observed in the water refrigerated extract (p = 1; r = -0.984), suggesting a highly linear, predictable decrease. On the other side, the statistical analysis shows r = -0.901 for room conditions in water-ethanol mixture, a slightly reduced value which may indicate that this mixture alters the degradation rate. The statistical analysis of Table 6 shows a strong relationship between the type of solvent and SPF values. The r values of 0.952 and 0.968 proved that the two solvents behave almost the same over time, regardless of initial differences in the magnitude of SPF values.

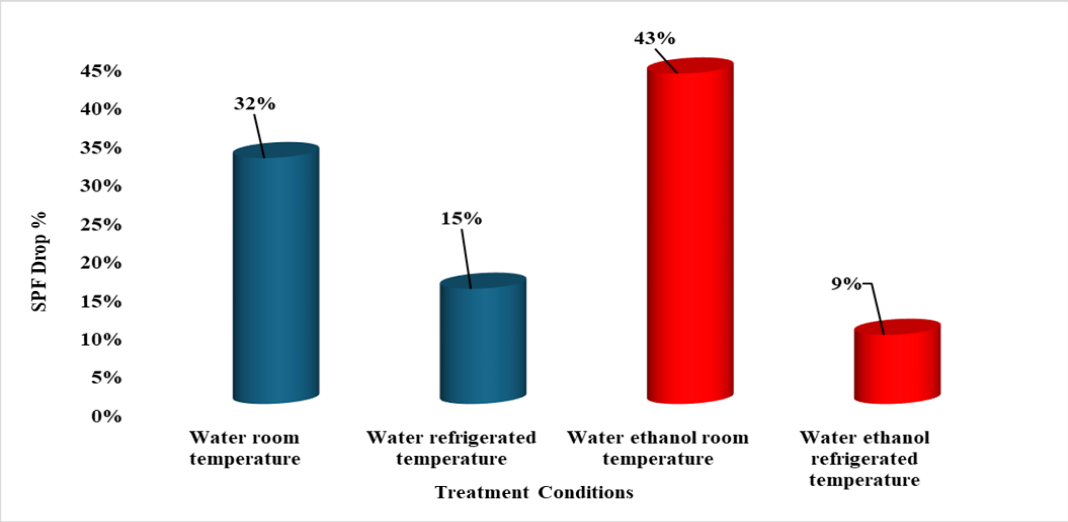

Fig. 5. SPF drop for 6 days for water (blue) vs. water-ethanol (red) at room temperature and refrigerated conditions.

Table 5. Pearson correlation coefficients (r) and their corresponding p-values for SPF vs. Days.

Condition	r (Pearson)	p-value	Significance
W. Room Temp.	−0.923	0.009	($p < 0.01$)
W. Refrigerated	−0.984	0.001	($p < 0.001$)
W.E. Room Temp.	−0.901	0.014	($p < 0.05$)
W.E. Refrigerated	−0.948	0.004	($p < 0.01$)

W. = Water; W.E. = Water-Ethanol mixture

Table 6. Pearson correlation results comparing SPF values between the two solvent types (water vs. water–ethanol).

Condition	r (Pearson)	p-value	Significance
Room Temp.	0.952	0.004	($p < 0.01$)
Refrigerated	0.968	0.002	($p < 0.01$)

4. CONCLUSIONS

This study demonstrated and confirmed that temperature, incubation period, and solvent used are important factors in how well *S. raeseri* maintains its SPF value over time. Specifically, the refrigerated conditions extend the extract shelf-life, likely because colder solvent prevents the rapid photodegradation of key phenolic compounds. The analysis of results also confirmed that the choice of solvent and incubation conditions (temperature) significantly affects the photoprotective potential of these herbal extracts. *S. raeseri* extracts exhibit greater photochemical stability and longer shelf-life at refrigerated conditions with higher SPF values for water solutions. The degradation was slower in a water-ethanol solvent. As an alternative to synthetic UV filters, the use of *S.raeseri* extracts could be recommended for the development of plant-based sun protective cosmetic formulations. This is particularly relevant for formulations seeking to minimize synthetic UV filters, given concerns about allergenicity and environmental pollution. Further studies are needed to evaluate the effect of pH and the half-life of the degradation of SPF in various solvents.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding this publication.

7. ETHICAL STATEMENT

Not applicable. In this study, neither humans nor animals were involved, and no clinical trials were conducted.

8. FUNDING

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9. AUTHORSHIP CONTRIBUTION

Emil Xhuvani conceived, designed, drafted, and analysed the results. Kejda Kristo, Matilda Mema and Irdisa Hima performed the experimental measurement and data collection. All authors agreed to publish the final manuscript.

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Research Article

In Vitro Multiplication and Acclimatization of Banana (*Musa* spp.) Plantlets

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Abstract: Banana (*Musa* spp.) is a horticulturally important fruit crop of economic significance, particularly in tropical and subtropical regions of the world. Vegetative propagation of banana via suckers is hindered by a low multiplication rate and high susceptibility to diseases. The current study optimized efficient protocols for large-scale *in vitro* multiplication and acclimatization of two commercially valued banana cultivars (William and Grand Naine) initially propagated from shoot tip explants. Clusters of shoot buds (1-2 cm) were multiplied on the Murashige and Skoog (MS) medium consisted of different concentrations (0.5, 1.0, 2.0, and 3.0 mg l^{-1}) of benzyl adenine (BA). Medium comprised of different concentrations of activated charcoal (0.5, 1.0, 1.5, and 3.0 mg l^{-1}) was used for healthy shoot and root development. Acclimatization of the plantlets (40 cm) in the greenhouse was carried out on different soils, i.e., peatmoss, river sand, clay soil, hill sand, and desert sand. Results revealed that significantly highest shoot bud multiplication (Avg. 21.2), and plantlet formation was obtained in cv. Grand Naine on the MS medium consisted of 3.0 mg l^{-1} BA. Significantly highest leaf number (4 leaves), leaf length (10.7 cm), root number (12 roots), and root length (13 cm) were noted on the MS medium consisted of 3.0 mg l^{-1} activated charcoal, 0.1 mg l^{-1} naphthalene acetic acid (NAA). Plantlets (15 cm) developed healthy shoot and roots during *in vitro* growth were shifted in the greenhouse for acclimatization and further growth. Significantly higher survival rate (96%) of the acclimatized plantlets in the greenhouse and healthy growth was noted on the soil medium consisted of river sand in cv. Grand Naine after 1M (month). Protocols optimized for *in vitro* and *ex vitro* growth of the two elite banana cultivars (William and Grand Naine) will benefit to the farmers for large-scale cultivation in the area and across the world.

Keywords: Acclimatization, *Ex vitro* Growth, Greenhouse, *In vitro* Plantlets, Shoot Tip Explants.

1. INTRODUCTION

Banana (*Musa* spp.) is one of the important fruit crops belongs to the family Musaceae is the crossbreed of *Musa balbisiana* and *Musa acuminata* [1-3]. Over 1000 banana cultivars and varieties are cultivated across the world [4]. Banana word originated from the Arabic word "banan" meaning the finger [5]. Banana plant is herb, perennial, monocot, and is cultivated in tropical regions of the world in more than 130 countries [6]. Banana is a rich source of vitamin A, C, and B6, as well as proteins, carbohydrates, and minerals including calcium, magnesium, manganese, and potassium [7, 8]. Banana is propagated commercially via tissue culture provides mass propagation, rejuvenation of older varieties, disease elimination, conserving genetic resources, and management of biotic and abiotic stresses [9-11]. Tissue culture-derived plants yield high quality true-to-type fruits, and remain free from pests and diseases [12]. *In vitro* propagation of banana varieties has been tested in many countries as an alternative propagation method to naturally occurring suckers [13-15]. For many years, banana was traditionally propagated through suckers and diploid seeds' germination [16]. However, such efforts remained limited due to diseases, genetic variability and lack of

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uniformity [17]. Recently, different treatments of plant growth regulators (PGRs) were tested for large-scale multiplication of banana plantlets [18]. Banana is cultivated worldwide, and is among the important cash crops being a rich source of important nutrients [19]. Micropropagation at large scale always remains problematic due to the high cost of inputs required, microbial contamination, and lack of protocols to be used on various banana varieties [20]. It was demonstrated that the banana was effectively propagated *in vitro* using shoot tip explants obtained from healthy suckers [21]. Recent studies also reported the micropropagation protocols of banana through shoot tip explants, *in vitro* multiplication and acclimatization [22]. Corms and suckers are used as conventional propagating materials for fruit production [23, 24]. Suckers can be the source of transfer of diseases and pests, and also have low multiplication rate has rendered the interest of farmers to propagate the banana via tissue culture [25, 26]. Plant tissue culture is an efficient and reliable method of propagating the rare and endangered plants on commercial scale in a short time [27].

Micropropagation plays significant role for getting disease-free plants of the fruit crops for economic growth [28]. *In vitro* propagation through shoot tip explants may overcome disease problems because of the aseptic conditions followed during culture process [29]. Banana fruits have high nutritional value, and is low priced fruit making it familiar in developing countries [26]. Micropropagation of banana involves several *in vitro* and *ex vitro* growth stages such as, sterilization of explants, initiation, shoot multiplication, rooting and acclimatization in the greenhouse [16]. Cell division, cell elongation and differentiation can be induced by manipulation of plant growth regulators in the media [30]. Preferred and widely used explants of banana for tissue culture are shoot tips obtained from suckers [16]. Shoot tip explants of banana remain free from the viruses and other pathogens due to occurrence of small vacuoles inside the cells and high nucleocytoplasmic ratio which restricts the pathogen invasion [31]. Several banana varieties previously were micropropagated via tissue culture through shoot tip explants [32]. Sivakumar and Visalakshi [33] conducted study on *in vitro* propagation of banana cv. Poovan using shoot tip explants. Singh *et al.* [26] described the effect of different plant growth regulators on *in vitro* propagation of banana cultivar Grand Naine using shoot tip explants. Several studies described successful *in vitro* regeneration protocols, shoot multiplication, rooting and acclimatization of banana plantlets using shoot tip explants [34-41].

Khairpur district of Pakistan is suitable area for banana cultivation with appropriate soil and climate; therefore, the current study is aimed to optimize protocols for *in vitro* multiplication and further growth of plants in the greenhouse, and in the open field. The present study is expected to establish efficient protocols for shoot bud multiplication initially obtained from immature shoot tip explants, shoot elongation and rooting, and acclimatization of the micropropagated plantlets on different soil types for two commercially valued banana cultivars William and Grand Naine.

2. MATERIALS AND METHODS

2.1. Plant Material

Clusters of shoot buds (initially obtained from shoot tip explants) were cultured on the media consisted of different concentrations of PGRs. Small plantlets (7-8 cm) were isolated from the clusters during each subculture (1-2 months interval) and cultured in jars for further growth and rooting for 1-2 subcultures.

2.2. Media Preparation

Little shoot bud clusters of banana cultivars were cultured on the Murashige and Skoog (MS) media consisted of different concentrations of benzyl adenine (BA), i.e., 0.5, 1.0, 2.0 and 3.0 mg l^{-1} used for shoot bud multiplication and plantlets' development (4-8 cm in length) for a single subculture. Long shoots were detached easily, whereas the remaining buds were recultured on the same medium for multiplication and plantlet formation. Plantlets (7-8 cm in length) were grown on the MS media consisted of different concentrations of activated charcoal, i.e., 0.5, 1.0, 1.5 and 3.0 g l^{-1} in addition to 0.1 mg l^{-1} naphthalene acetic acid (NAA) for the healthy root and shoot development for 1-2 months for a single subculture.

2.3. Culture Conditions

Clusters of shoot buds during multiplication and plantlet formation stage were kept in growth room

under temperature 24 °C ± 2 (16 hours photoperiod), whereas, the plantlets during rooting stage were kept under temperature 27 °C (16 hours photoperiod) for the better growth of root and shoot.

2.4. Acclimatization

Plantlets (15 cm long) were acclimatized in the greenhouse on different soil mixtures obtained from local sources, i.e., river sand, desert sand, peatmoss, clay soil and hill sand. Plantlets were shifted to greenhouse in closed vessels, followed by careful washing with 2 g l^{-1} systemic fungicide (Carbendazim) for 1-2 minutes. Later the plantlets were transplanted on different soil types and covered completely with polyethylene plastic sheet for a week. Plantlets in the greenhouse were ventilated regularly after one week until complete removal of the plastic sheet after four weeks. Fertilizer treatment was started after two months of acclimatization in the greenhouse. Spray of fungicide (Copper Oxychloride) was carried out after every15 days to keep the plants intact from any fungal infection during the growth in the greenhouse.

2.5. Data Analysis

Data were based on three replicates from each cultivar on different treatments used throughout all *in vitro* and *ex vitro* growth stages, and each replicate was based on 20 randomly selected plantlets/ cultures (*in vitro*/ *ex vitro*). Data were subjected to two-way analysis of variance (ANOVA) followed by LSD (≤ 0.05) using the software (IBM SPSS Statistics version 30.0) as per the method of Steel and Torrie [42].

3. RESULTS AND DISCUSSION

3.1. Effect of Different Treatments of BA on Shoot Bud Multiplication and Plantlet Formation

Data in Table 1 show that significantly highest shoot bud multiplication (Avg. 21.2 buds) was noted in cv. Grand Naine followed by cv. William (Avg. 18.5 buds) after one month on the medium consisted of 3.0 mg l^{-1} BA. Medium consisted of 2.0 mg l^{-1} BA induced avg. 15.7 small shoots in cv. Grand Naine and avg. 13.3 small shoots in cv. William after one month (Figure 1(a)). Further, decrease in the BA concentration, i.e., 1.0, 0.5 mg l^{-1}, reduced significantly the average shoot formation in one month in both banana cultivars. Results of the two-way ANOVA showed significant differences, revealed that treatment effect was highly significant ($p < 0.001$) than cultivar ($p < 0.041$), and combined effect of cultivar and treatment ($p < 0.006$). Nevertheless, the high cultivar-treatment interaction shows that the reaction of each cultivar differed according to the level of PGR, with Grand Naine being relatively more responsive to all treatments. The findings clearly indicate that the growth response measured in both banana varieties, William and Grand Naine in the presence of higher concentration of PGRs was greatly increased. The same trend was recorded between 0.5 and 3.0 mg l^{-1} showing that the effect of concentration of PGRs on *in vitro* shoot buds multiplication was strong. Both cultivars had little response at the lowest concentrations (0.5 mg l^{-1}). Nonetheless, at 1.0 and 2.0 mg l^{-1}, a considerable change was noticed, indicating that moderate levels of PGRs are best to induce cell division and multiplication

Table 1. Effect of different concentrations of benzyl adenine (BA) on shoot bud multiplication of banana cultivars (William and Grand Naine).

PGRs (mg l^{-1})	William	Grand Naine
0.5	4.11 ± 0.57d	6.21 ± 0.78d
1.0	8.21 ± 0.11c	9.82 ± 0.45c
2.0	13.3 ± 0.31b	15.7 ± 0.61b
3.0	18.5 ± 1.11a	21.2 ± 0.77a
LSD (0.05%)	0.002	0.001
Variability		
Cultivar	0.041	
Treatment	0.001	
Cultivar × Treatment	0.006	

Mean values in columns with standard error denoted with different letters show significant levels at $p \leq 0.05$.

of the shoot. Optimum response was observed at 3.0 mg l^{-1} at which point Grand Naine performed better than William suggesting cultivar variation in responsiveness. The observed differences are further proven by statistical grouping (a-d) which shows that all levels of treatments were significantly different at ($p < 0.05$), in this case, the results are reliable. In general, the findings indicate that the concentration of 3.0 mg l^{-1} PGR results in the optimum growth in both cultivars, especially in Grand Naine. These results are in line with earlier researches that have cited improved shoot proliferation in banana with higher levels of cytokinin probably because of higher meristematic activity and nutrient mobilization. Current results report shoot multiplication agrees with Shukla *et al.* [8] who obtained shoot proliferation on MS medium consisted of 5 mg l^{-1} benzyl adenine for *Musa acuminata* var. hazari, *Musa paradisica* var. sanikol, and *Musa paradisica* var. kashkol; whereas, shoot proliferation of *Musa acuminata* var. hondakol was achieved on MS medium consisted of 3 mg l^{-1} BA. Generally, in several banana species, adenine-based cytokinins were used for *in vitro* propagation [43-47]. Al-amin *et al*. [48] tested BAP and NAA for *in vitro* regeneration and shoot multiplication of banana. Several studies observed positive effect of benzyl aminopurine (BAP) on shoot initiation and multiplication in different banana cultivars [49-54]. Khalil *et al*. [55] obtained multiple shoots on MS medium with 5 mg l^{-1} BAP, while MS medium with 1 mg l^{-1} NAA, 0.2 mg l^{-1} BAP produced longest shoots after 45 days. Wong [29] reported that increase in BAP concentration resulted in promoting number of shoots per explant. Backiyarani *et al.* [56] analyzed the effect of BA (1, 2, 3, 4, 5, and 6 ppm) on shoot induction, observed that 6 ppm of BA had a significantly positive effect on shoot formation. Another study conducted on the effects of different BA concentrations on different banana cultivars were noted as reproducible [57]. Nguyen *et al.* [58] conducted study on the effects of various levels of BA (0, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 ppm) on shoot multiplication in cv. Grand Naine observed the positive effects of BA. Previous studies described that BA is one of the synthetic cytokinins which assumed tolerance in different developmental forms in the plants promoting the growth [59, 60]. Generally, high frequency shoot bud multiplication of banana cultivars was related to appropriate BA concentrations added in the medium. Shoot bud cultures responded well on the

Fig. 1. (a) Cluster of little shoots of banana, (b) Banana plantlets with well-established leaves and roots ready for shifting in the greenhouse, (c) Shifting of 15 cm long plantlets of banana in the greenhouse, (d) Six months old plants of banana in the greenhouse, (e) 40 cm long healthy banana plant in the just prior to cultivate in the open field, and (f) 40 cm long plant shifted in the open field for further growth.

media supplemented with higher treatments of BA indicate high requirement of cytokinins, i.e., BA or BAP as used in banana tissue culture by several workers. On the contrary, there is risk of somaclonal variations generally when cultures are treated with higher doses of synthetic PGRs during *in vitro* growth. Hence, the attention should be paid to find out the PGRs treatments promoting suitable *in vitro* growth and to avoid any somaclonal variations by applying moderate levels of PGRs throughout all *in vitro* growth stages.

3.2. Effect of Different Treatments of Activated Charcoal on Plantlets' Growth and Rooting

Data in Table 2 indicate that significantly highest leaf number (4 leaves), leaf length (10.7 cm), root number (12 roots) and root length (13 cm) were recorded on the MS medium consisted of 3 g l^{-1} activated charcoal, 0.1 mg l^{-1} NAA in banana cv. Grand Naine (Figure 1(b)). MS medium consisted of 1.5 g l^{-1} activated charcoal, 0.1 mg l^{-1} NAA significantly reduced the leaf number (4 leaves), leaf length (9 cm), root number (7 roots) and root length (10.6 cm) in cv. Grand Naine and in cv. William. Further reduction in all growth parameters was recorded in both cultivars with further reduction in activated charcoal, i.e., 1.0 and 0.5 g l^{-1} with 0.1 mg l^{-1} NAA, however, significantly lowest values were recorded on the medium comprised of 0.5 g l^{-1} activated charcoal after one month. Results of two-way ANOVA showed overall significant effect of cultivar ($p < 0.003$), treatment ($p < 0.002$) and combined effect of cultivar and treatment ($p < 0.005$). The current findings showed that activated charcoal with 0.1 mg l^{-1} NAA is a good option in promoting shoot growth and rooting in banana plantlets. Leaf length, root number, and root length were found to increase steadily with the increase in AC concentration and the highest performance was found to be at 3.0 g l^{-1} in the both cultivars. Although there was minimal difference in the number of leaves, and a significant enhancement in the growth and rooting factors, especially the root number and root length which showed that AC favors rhizogenesis and enhances general plant vigor during *in vitro* growth. This increased performance at the high levels of AC can be credited to its capability to absorb the inhibitor compounds thus stimulating the effectiveness of NAA in the root induction. The existence of genotype differences between William and Grand Naine further underscores cultivar-specific physiological reactions with Grand Naine being more sensitive, especially in root proliferation and elongation. This could be explained by hormonal variations, uptake rate, or metabolic sensitivity to exogenous auxin and absorption via AC. The higher AC concentration (3.0 g l^{-1}) indicates that an ideal ratio between the uptake of inhibitory compounds and the maintenance of auxin concentration is important in terms of *in vitro* shoot development and rooting. Such a combination of treatments can lead to physiological efficiency resulting in the production of vigorous plantlets with enhanced acclimatization potential, which is essential in large-scale micropropagation systems. Several studies [61, 62] utilized indole butyric acid (IBA)

Table 2. Effect of different concentrations of Activated Charcoal + 0.1 mg l^{-1} NAA on plantlet formation and rooting in banana cultivars (William and Grand Naine).

AC (g l^{-1})	William				Grand Naine			
	LN	LL (cm)	RN	RL (cm)	LN	LL (cm)	RN	RL (cm)
0.5	3.0 b	7.3d	3.2d	5.2d	3.0b	6.3d	4.0d	7.1d
1.0	4.0a	7.5c	4.2c	5.8c	3.0b	6.5c	5.0c	7.3c
1.5	4.0a	8.2b	6.0b	10.2b	4.0a	9.0b	7.0b	10.6b
3.0	4.0a	10.2a	9.0a	12.0a	4.0a	10.7a	12.0a	13.0a
LSD (0.05%)	0.06	0.002	0.001	0.001	0.042	0.001	0.001	0.002
Variability								
Cultivar		0.003						
Treatment		0.002						
C × T		0.005						

LN = leaf number, LL = leaf length, RN = root number, RL = root length, C = cultivar, T = treatment. Mean values in columns denoted with different letters show significant levels at $p \leq 0.05$.

Table 3. Effect of different soil types on the survival percentage of *in vitro* grown banana plantlets of cvs. William and Grand Naine during acclimatization in the greenhouse.

Soil medium	Survival %			
	William		Grand Naine	
	1M	6M	1M	6M
River sand	95 ± 1.2a	94 ± 1.1a	96 ± 0.8a	94 ± 0.7a
Desert sand	87 ± 1.0b	84 ± 0.6b	89 ± 0.7b	85 ± 0.5b
Peatmoss	80 ± 0.7c	77 ± 1.2c	83 ± 0.5c	78 ± 0.7c
Clay soil	67 ± 0.5d	60 ± 1.3d	65 ± 1.2d	59 ± 1.1d
Hill sand	62 ± 1.0e	59 ± 0.7e	60 ± 1.1e	57 ± 1.3e
LSD (0.05%)	0.000	0.001	0.000	0.002
Variability				
Cultivar		0.005		
Treatment		0.001		
C × T		0.004		

Mean values in columns with standard error denoted with different letters show significant levels at $p \leq 0.05$.

for rooting in *in vitro* grown plantlets of banana. However, in this study different concentrations of IBA were tested in addition to NAA for rooting. Deo *et al*. [43] observed that NAA was more effective than indole acetic acid (IAA) for *in vitro* rooting in banana plantlets. Banerjee and De Langhe [63] used IBA for root induction in different banana cultivars. Kelta *et al*. [54] observed highest rooting on the MS medium consisted of activated charcoal 200 mg l^{-1}, IBA 1.0 mg l^{-1}. Selvakumar and Parasurama [41] reported that half strength MS medium containing activated charcoal 200 mg l^{-1} and 20 mg l^{-1} adenine sulfate supported rooting in banana cultivars Grand Naine and Elakkia. Deo *et al*. [43] observed healthy rooting in *in vitro* grown banana plantlets using NAA. Activated charcoal has been exploited extensively due to its positive effects on the *in vitro* shoot growth and rooting [64]. Activated charcoal previously was used in the medium to enhance the *in vitro* shoot multiplication and rooting [65-67]. AC also absorbs polyphenols affecting the root growth [68-70]. Similarly, current study observed positive effects of activated charcoal on growth of the plantlets of banana cultured *in vitro*. In addition to activated charcoal, NAA also supported the growth of the roots used in combination with activated charcoal. NAA is widely utilized PGR for *in vitro* rooting in wide variety of plant species. Besides developing dark green colour of the leaves of banana plantlets, the AC also decreases the necrosis in the banana leaves cultured on the medium with activated charcoal indicated normal photosynthesis and chlorophyll development in the plantlets during *in vitro* growth. Banana exhibits rapid *in vitro* growth, therefore subculture number during *in vitro* growth can be reduced, and rapid shifting in the greenhouse avoid genetic variations.

3.3. Effect of Different Soil Types on Survival Percentage of Plants in the Greenhouse after 1M and 6M

Data presented in Table 3 show that significantly highest survival percentage of the acclimatized plantlets in the greenhouse was noted in cv. Grand Naine transplanted on the river sand after 1M (96%) and 6M (94%). Results showed 4% mortality after 1M and further 2% mortality after 6M (total 6% mortality) was recorded in cv. Grand Naine. Better survival percentage of the plants in the greenhouse was also obtained on the desert soil after 1M (89%) and 6M (85%), however, total 15% mortality was recorded in cv. Grand Naine. Further, survival percentage of the plants in greenhouse was recorded on the different soil types after 1M and 6M, i.e., peatmoss (83% and 78% respectively in cv. Grand Naine), clay soil (67% and 60% respectively in cv. William) and hill sand (62% and 59% in cv. William). Results of the two-way ANOVA showed significant differences, with treatment effect ($p < 0.001$) more significant than the cultivar effect ($p < 0.005$), and combined effect of cultivar and

treatment ($p < 0.004$). The cultivar-treatment interaction also suggests that cultivar response is different between soils, therefore it is important to maximize the substrate composition for a particular genotype. The findings clearly indicate that the type of soil played a significant role on the survival percentages of *in vitro* raised banana plantlets of cultivars (William and Grand Naine) over time 1M and 6M in greenhouse. Of all the treatments, plants on the river sand had the highest survival rates of 94-96%, and was statistically superior at 1M and 6M. This performance is due to its proper drainage, aeration which are important characteristics of the soils in successful acclimatization of the tissue cultured plantlets that have under developed root systems and are very sensitive to waterlogging. Plants on the desert sand on the other hand had moderate survival percentage (84-89%), although much lower than the plants were growing on the river sand. This may be because of low capacity of water retention and absence of necessary nutrients hindering the growth of plants in the long term after transplanting. Peatmoss resulted in intermediate survival (87-83%) even though it provides good moisture holding capacity, excess supply of water, and a reduced aeration, may have negatively affected root respiration during acclimatization. The lowest percentages of survival were found in clay soil (59-67%) and hill sand (57-62%). The low nutrient levels and irregular changes in moisture caused by poor aeration, compaction and waterlogging and the hill sand may have failed to allow the growth of the banana plants. Decrease in the survival percentage of treatments between 1M and 6M, demonstrated that the acclimatization stress in the long-term and the environmental variability continue to affect the plantlet establishment regardless of the time period since the initial transplanting. Vasane and Kothari [71] observed that soil, press mud cake, vermicompost (1:1:1) and soil, press mud cake, vermicompost (2:1:1) gave better results of plant growth and survival percentage of *in vitro*-derived banana cv. Grand Naine. Parkhe *et al.* [72] recommended several combinations, i.e., peat soil, farmyard manure (FYM), sand, vermicompost, sand and cocopeat alone and in combinations for banana cv. Grand Naine. Chamling and Bhowmick [73] recommended equal proportions of soil, compost, coir pith and sand for hardening the banana plantlets in the greenhouse. Medhane *et al.* [74] used 0.1% (w/v) Bavistin (Carbendazim) for 2-3 minutes for treating the *in vitro* grown plantlets of banana before transfer on soil; the soil was composed of autoclaved black soil, vermicompost and cocopeat (1:1:1). Similarly, in this study washing of whole the plantlets were carried out with fungicide solution (3 g l^{-1} Carbendazim) for 2-3 minutes before shifting on different soil types in the greenhouse for restricting any fungal infection during acclimatization stage.

Wijerathna and Kumarihami [75] obtained maximum survival rate of banana plants on river silt and desert sand (1:1 and 2:1) during acclimatization of banana plantlets in the greenhouse. Sivakumar and Visalakshi [33] transferred the rooted banana plantlets in plastic pots containing garden soil, farmyard manure and sand (2:1:1). Soil mixtures (soil, sand, manure in the ratio of 3:2:1, FYM, vermicompost, neem cake) used in greenhouse for hardening of tissue-cultured banana plantlets [76]. In contrast to previous studies described different soil mixtures for different banana varieties, current study is also an additional approach for getting maximum survival rate of the *in vitro* grown plantlets of the elite banana cultivars William and Grand Naine. Banana plantlets were carefully taken out of culture vessels and washed in a fungicide solution before to culture on different soils in the greenhouse (Figure 1(c)). Plants of both banana cultivars exhibited healthy growth in the greenhouse (Figure 1(d)). Healthy plants of banana (40 cm) were selected for transfer in the open field (Figure 1(e)). Plants were transferred in the open field showed 100% survival rate due to well-developed roots and shoot obtained during acclimatization stage in the greenhouse (Figure 1(f)). Fertilizer (NPK based on the ratio of 1:1:1) 3-4 grams per plant was applied when plants started to grow in the greenhouse. In addition, spray of 3 g l^{-1} fungicide (Copper Oxychloride) was carried out on the growing banana plants to restrict any fungal infection. Banana is rapidly growing plant; hence, elite and rare disease-free banana cultivars can be propagated via tissue culture on large scale in a shortest time and space.

4. CONCLUSIONS

The findings reinforced the critical role of the medium composition towards the successful micropropagation. The research will help improve the tissue culture protocols and will form a scientifically sound foundation upon which to

scale up propagation systems in horticultural crops like banana. PGRs had significant contribution in all the *in vitro* growth stages. Activated charcoal with NAA was better to achieve healthy plantlets to be able to shift in the greenhouse for successful acclimatization. Banana plantlets exhibited rapid multiplication and plantlet formation, and rooting during *in vitro* growth. Plantlets successfully acclimatized in the greenhouse on different soil types showed better survival and growth with higher survival rate on river sand. Further, study is required to evaluate the plants in the field and to get fruiting in *in vitro* grown plants, and to study genetic stability of the plants obtained via tissue culture. Moreover, the studies should also be conducted to combine this approach with other growth regulators, and how it can be used under *ex vitro* conditions to further improve commercial relevance. Protocols devised in this study for two exotic banana cultivars will significantly improve to multiply the plants of the same and other banana cultivars on large scale cultivation in the area and across the world.

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6. CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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8. AUTHORSHIP CONTRIBUTION

N.S. wrote the manuscript and analyzed the data, A.A. A-S. helped in experimentation and conceptualization, M.A.J. helped in editing and data analysis, A.A.M. helped in editing and data analysis, Z.A.J. helped in experimentation and editing, and G.S.M. helped in experimentation and methodology.

9. DECLARATION

Authors declare that: (i) the results are original, (ii) the same material is neither published nor under consideration for publication elsewhere, (iii) approval of all authors has been obtained, and (iv) in case the article is accepted for publication, its copyright will be assigned to the Pakistan Academy of Sciences.

10. ETHICAL STATEMENT

This work does not include any studies involving human or animal subjects.

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Research Article

Antibacterial and Antibiofilm Activity of Pullulanase from *Paenibacillus macerans* Against Urinary Catheter-Associated Pathogens

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Abstract: Urinary tract infections and urinary catheters are frequently related. Clinicians in underdeveloped nations often encounter urinary tract infections (UTIs), which are among the most prevalent bacterial illnesses. Selecting the appropriate empirical treatment for a patient may be aided by the results of area-specific surveillance studies that seek to identify the bacteria causing UTIs and their patterns of resistance. Pullulinase, an enzyme that hydrolyzes pullulin into maltotriose, panose, and maltooligosaccharides, is capable of inhibiting bacterial colonization and biofilm formation on urinary tract catheters. The goal of this work is to detect *Paenibacillus macerans* isolated from rhizosphere soil as pullulanase producer and to use the purified pullulanase as prebiotic, antibacterial, and antibiofilm agent. Using of modified pullulin-agar for screening pullulanase producers. Purification of pullulanase by DEAE-Cellulose A-50 column followed by sephadex G-150 column. Isolation of urinary catheters bacteria and using of pullulanase and detection biofilm formation on the catheters then applied of pullulanase as antibacterial and antibiofilm agent against urinary catheters bacteria in addition to enhance the growth of lactic acid bacteria. Eleven isolates of *Paenibacillus macerans* (41%) had showed an ability to produce pullulanase with different levels. Serial steps were used Pullulanase purification which included 70% ammonium sulfate saturation, DEAE-Cellulose A-50 ion exchange chromatography and sephadex G-100 gel filtration chromatography with a recovery yield of 22.9%, 8.11 fold of purification and specific activity 15.90 U/mg. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were the most predominant isolates in the urinary catheters. The purified pullulanase has a potential prebiotic characteristic to enhance lactic acid bacteria growth while allegedly impeding the development of pathogens and inhibiting their biofilm formation. Prebiotic properties are typically intended not only to reduce pathogen growth and biofilm formation, but to promote the growth of desirable and beneficial bacteria like lactic acid bacteria. In conclusion, pullulanase can be added to animal feed to increase digestibility and gastrointestinal health. It can also be used to cover catheters to assist avoid infections and bacterial colonization.

Keywords: Pullulanase, Urinary Catheters, Prebiotic, Biofilm Inhibition, *Paenibacillus Macerans,* Enzyme Purification, Lactic Acid Bacteria.

1. INTRODUCTION

Paenibacillus macerans is considered as a facultative anaerobe and a member in paenibacillaceae family [1] that is found in different environments like soil and plants and can perform nitrogen fixation and fermentation, besides their presence in blood cultures of infants with infection [2]. A linear polymer of maltotriose, which is also known as pullulan, and linked with α-saccharoyl linkages, is utilized in various industries [3]. Pullulanases (extra cellular hydrolases that breaks-1, glycosidic linkages in pullulan, starch, and oligosaccharins) are examples of an extracellular debranching enzymes which debride these oligosaccharides from a subtype of glycans that is derived from pullulan [4, 5]. Pullulanase, a significant debranching enzyme widely used for α-1,6 glucosidic hydrolysis in starch, amylopectin, pullulan, and related oligosaccharides, is an industrial enzyme candidate [4]. Due to its special actions on the α-1,6 connections in pullulan a linear α-glucan

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consisting essentially of maltotriosyl units linked by 1,6-α-bonds is preferred for microbial pullulanase in industry. Pullulanases are also hydrolysed in pullulan and amylopectin by the α-1,6 glucoside bond. Different microorganisms like *Klebsiella planticola*, *B. acidopullulyticus*, *B. deramificans*, *B. cereus* and *Geobacillus stearothermophilus* produce pullulanases [6].

Catheters for the urinary system are silicone or latex-filled tubes. Biofilms can easily form on the inside or outside of inserted catheters. The longer the catheter is left in place without changing, the more likely it is that microorganisms will create catheter biofilms [7]. The process of bacteria growing and irreversibly attaching to a surface to create biofilms involves the production of extracellular polymers that help in matrix development and adhesion [8, 9]. In hospital and community care settings, urethral catheters are the most frequently used medical devices. However, their usage puts patients at risk for infection and creates an environment that is perfect for the growth of bacterial biofilms [10, 11]. Individuals receiving long-term urethral catheterization are more susceptible to infections related to biofilms; urease-producing species' crystalline biofilm production frequently results in catheter occlusion and other severe clinical consequences [12]. In critical care units, urinary tract infections are mostly caused by bacterial biofilms that grow on urine catheters. Because of the development of these biofilms, cytobacteriological analysis of patient urine is frequently deceptive. Determining the type of bacteria living on the catheter surface and characterizing these biofilms are therefore crucial [13]. The aim of this research was to investigate pullulanase production by a new strain of *Paenibacillus macerans* isolated from rhizosphere soil, purification of pullulanase and prebiotic, antibacterial, and antibiofilm characteristics are being investigated.

2. MATERIALS AND METHODS

2.1. Detection of Producers of Pullulanase

Samples of soils of rhizosphere at different sites in Mustansiryiah University Garden, Baghdad, Iraq were collected at 27 locations. For ten minutes, one gram of each sample was suspended in ten milliliters of sterile distilled water and forcefully shaken. The surface of the modified pullulin-agar according to [4] that consisted of (g/l) soluble pullulan [10] was then distributed with 0.1 of the suspension fluid, NaCl (2), $MgSO_4.7H_2O$ (0.1), K_2HPO_4 (0.17) and $KH_2PO_4.7H_2O$ (0.12) and Agar (0.15), pH 7.0 [14]. Till the colonies appeared, the plates were then incubated at 30 °C. Pullulanase producers were considered colonies around which a clear hollow zone was observed.

2.2. Biological Diagnosis

Morphological features were observed in the increasing bacterial isolates and identified by Berge's Systemic Bacteriology Manual and identified, for example, size, colour, shape, and colony outline as well as gauze and biochemical tests [15]. By using Vitek 2 with Vitek GPI cards this diagnosis has been confirmed.

2.3. Submerged Fermentation for Pullulanase Production

A modified pullulan agar media has been inoculated on the selected isolate but not agar. The incubation took place for 48 hrs at 30 °C. At 8000 rpm to 10 mins, the cultivation was centrifuged and the supernatant used for crude enzymes.

2.4. Pullulanase Activity

The measurement of reduced pullulan release of sugar tested the activity a mixture of reaction (3 ml) of 0.5 ml pullulan and 2 ml sodium phosphate buffer (0.5 ml) raw enzyme sources (0.1 M, pH 6.5). 0.5 ml $CaCl_2$ (0.02 % w/v) was added to reaction mixture. The reaction was stopped by the ice bath, after incubation was carried out at 40 °C for twenty minutes, then 1 mL of 3-5-dinitrosalicyclic acid reagent was added and the sugar released by enzyme hydrolysis was decreased, followed by incubation in boiling bath for five minutes, measuring enzyme activity at 540 nm. after incubation at 20 mins [16]. The amount of the enzyme, that released one mole of sugar reduction as glucose per minute under standard conditions, is defined as one unit of pullulanase.

2.5. Estimate of Protein Determination

The concentration of protein was determined by the Bradford [17] method and the standard use of bovine serum albumin.

2.6. Purification of Pullulanase

Culture supernatant obtained after incubation in the pullulan broth medium (8,000 g, 30-minute, 4 °C) and subjected to ammonium sulphate prescription with 20-80% saturation, through centrifugation (8,000 g, 30-minute, 4 °C). The sample has been removed overnight at 4 °C (2000 rpm) and the precipitate has been collected at 20 mM phosphate-buffered saline (PBS) with a pH of 7.2, and then dialyzed overnight in a centrifugation buffer at pH 7.0. The dialyzed protein was loaded on a column (2cm x 25cm) of the DEAE-Cellulose A-50 and eluted with same buffer at a flow rate of 0.1-0.4 m NaCl. In the sephadex G-150 column (2 x 70 cm), which was previously balanced with that buffer, the fractions with highest activity of the pullulanase were collected. For further studies after protein concentrations of 280 nm and Pullulanase activity was measured.

2.7. Isolation Urinary Catheters Bacteria

Urinary catheters that hospitalized patients had been using for longer than a week were meticulously removed in an aseptic manner. Each catheter was carefully packed in a sterile glass bottle before being sent straight to the lab for examination. These urinary catheter discs were sliced, and the discs were cultured on Blood agar and MacConkey. Macroscopical and microscopic analysis of cultures carried out in compliance with guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [18]. To authenticate the isolates, the Vitek-2 system was utilized.

2.8. Quantitative Detection of Biofilm Formation by Urinary Catheters Bacteria

The method of quantitative microtiter plates, as detailed by Castilla-Sedano *et al*. [19], was employed. First, 3 mL of brain heart infusion broth was used as the inoculation medium for each uropathogenic bacterial colony that was recovered from a new agar plate. The culture was incubated for 18 to 24 hours at 37 °C with agitation (150 rpm). Following the incubation period, 20µL of the bacterial inoculum was combined with 180µL of brain heart infusion broth and injected into separate sterile, disposable, 96-well polystyrene tissue culture microplates with a U-bottom and an untreated surface. As a negative control, brain heart infusion broth devoid of cells was used to confirm the sterility of the media. For 48 hours, the TCP was incubated at 37 °C. After the incubation period, the contents of each well were carefully removed, and the free-floating planktonic bacterial cells were removed by air-drying them after four washes in 200 µL of sterile phosphate-buffered saline (PBS) with a pH of 7.2. After carefully rinsing the wells three times with deionized water, the wells were stained for 15 minutes with 1% crystal violet. The dye was then dissolved in 96% ethanol for another 15 minutes. An automated micro-ELISA reader was used to measure the optical density (OD) at 590 nm. for every isolation, three separate trials were run. By using the OD values of adhering bacterial cells, biofilm production was classified as negative, weak, or strong, in accordance with the guidelines given by Stepanović *et al.* [20]; the experiment was performed in triplicate.

2.9. Enhancement of Lactic Acid Bacteria by Pullulanase

The effect of pullulanase on the growth of two *Lactobacillus reuteri* isolates, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus sp*., was tested. These bacterial isolates were cultivated 24-hour at 37 °C in 50 ml MRS broth. 1% of inoculums adjusted to 0.5 absorption in 600 nm were subsequently moved to 5 ml of MRS broth with 1% purified pullulanase or without then for 4 hours was incubated at 37 °C. The cell number of MRS agar plating and cultivated overnight at 37 °C was determined following 4 hours of incubation. The experiment was performed in triplicate. Enhanced (percent) activity was identified as [21]:

$$\textit{Enhancement Activity}\ (\%) = \frac{SB-CB}{CB} \times 100$$

where SB is the quantity of MRS colonies with purified pullulanase (cfu/ml) and CB, there are no purified pullulanase (cfu/ml) cells within MRS.

2.10. Effect of Pullulanase on Pathogenic Bacteria Growth

In aerobial conditions 50 ml of the nutrient broth medium was grown in patenous bacterial isolates by shaking at 150 rpm for 24 hours at 37 °C. 5 ml of nutritional broth medium, 5 ml of nutrient broth plus 1% of purified pullulanase, and 1% of the inoculum adjusted to absorb at 600 nm were combined, and

the mixture was shaken at 150 rpm for 4 hours at 37 °C to generate the desired result. The number of cells was determined by nutrient agar tapering and incubation at 37 °C overnight. The experiment was performed in triplicate. Activity of inhibition (%) has been determined as [21]:

$$Inhibition\ Activity\ (\%) = \frac{CB-SB}{CB} \times 100$$

where SB is cell volume in nutrients pullulanase (cfu/ml) and CB is quantity of cells in nutrient pullulanase (cfu/ml) without purification in nutrient pumps.

2.11. Effect of Pullulanase on Biofilm Formation by Pathogenic Bacteria

In a microtiter plate approach, 125 µl of pure pullulanase in 20 mM phosphate-buffered saline (PBS) with a pH of 7.2 was combined with 125 µl of a chosen bacterial culture to measure pullulanase activity against biofilm formation. Following a 24-hours and 48-hours incubation period at 37 °C, the biofilm experiment was conducted as previously indicated. As previously said, the biofilm activity was duplicated. The percentage of biofilm inhibition was computed as follows [22]:

$$Percentage\ of\ biofilm\ inhibition\ (\%) = \frac{OD\ treatment}{OD\ control} \times 100$$

3. RESULTS

3.1. Detection of Producers of Pullulanase

Following pullulan agar plate incubation, the bacterial isolates from soil and agricultural wastes exhibited a clear hollow zone surrounding their colonies, indicating that they were pullulanase producers. These 11(41%) isolates were identified as *Paenibacillus macerans*, based on microscopic and biochemical tests. The pullulan hydrolysis zone ratio of 0.9 to 3.3 (Figure 1) and *Paenibacillus maceramic* SR8, isolated from rhizospheric soil, showed the highest level of pullulanase production.

3.2. Purification of Pullulanase

In *Paenibacillus macerans* SR8 cultivation broth, pullulanase was obtained as an extracellular enzyme. The pullulanase purification results are summarized in Table 1. Precipitation was performed using ammonium sulphate at 70 percent saturation, precipitation of protein from the cell-free surnatant in the specific activity reaching a level of 2.795 U/mg. To extract this salt from the sample, use ammonium sulfate precipitation followed by dialysis. First, the sample was loaded to a column of A-50 DEAE-Cellulose. The elution of the 0.1 - 0.5 M gradient NaCl led to the appearance of three protein peaks and the activity of pullulanase was at the second protein peak (Figure 2). Pullulanase was purified in this step 2.98 times with 26.3% yield. The fractions collected were loaded to G-100 column of sephadex. The active fractions revealed a protein maximum of 22.9 % with a purification fold of 8.11 and a specific activity of 15.90 U/mg, which included the activity of pullulanase in the second peak, as shown in Figure 3.

3.3. Distribution of Gram-Negative Bacterial Species Isolated from Urinary Catheters

Nineteen Gram negative bacterial isolates were found among the twenty-one urine catheter discs that were taken from eleven urinary catheters. These isolates included seven isolates of *A. baumannii,* four isolates of *E. coli,* six isolates of *P. aeruginosa*, and two isolates of *E. aerogenus*.

3.4. Quantitative Detection of Biofilm Formation by Urinary Catheters Bacteria

All bacterial isolates showed signs of being able to produce biofilm at different intensities, according to the results of the TCP technique. Of these isolates, 53%, 21%, and 29% formed strong, moderate, and weak biofilms, respectively (Table 2).

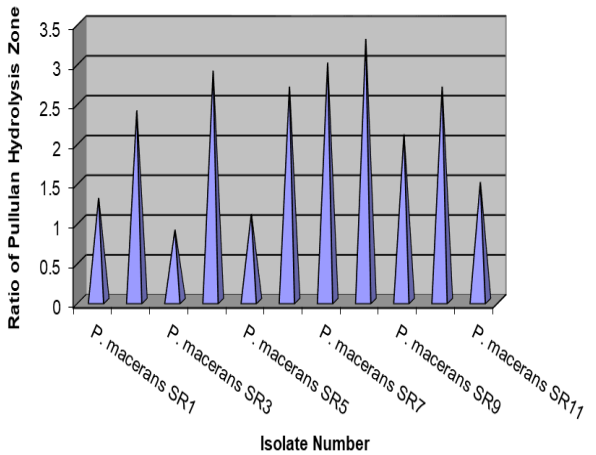

Fig. 1. *Paenibacillus macerans* isolates of rhizosphere soils in pullulan hydrolysis zones.

Table 1. Steps of pullulanase purification from *Paenibacillus macerans* SR_8.

Purification Step	Size (ml)	Pullulanase Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/ mg)	Total Activity	Purification Fold	Yield (%)
Crude extract	150	65.8	33.6	1.96	9870	1	100
$(NH_4)_2SO_4$ precipitation	70	84.7	30.3	2.795	5929	1.43	60.0
DEAE-Cellulose A-50	23	112.8	19.9	5.668	2594.4	2.98	26.3
Sephadex G-100	19	119.3	7.5	15.90	2266.7	8.11	22.9

Table 2. Biofilm formation by different negative bacilli isolated from urinary catheters.

Biofilm Formation Level	*E. coli*	*A. baumannii*	*P. aeruginosa*	*E. aerogenus*	Biofilm Formation (%)
Weak	1	2	2	-	29
Moderate	1	1	1	1	21
Strong	2	4	3	1	53
Total	4	7	6	2	

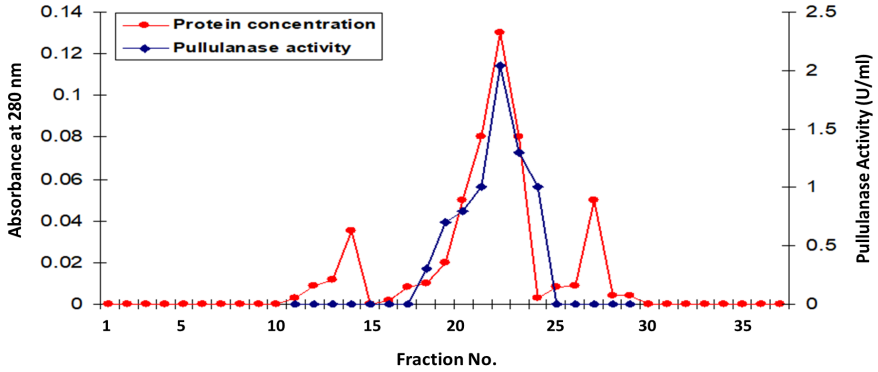

Fig. 2. DEAE-Cellulose A-50 column as ion exchanger was used to purify pullulanase from *Paenibacillus macerans* SR8.

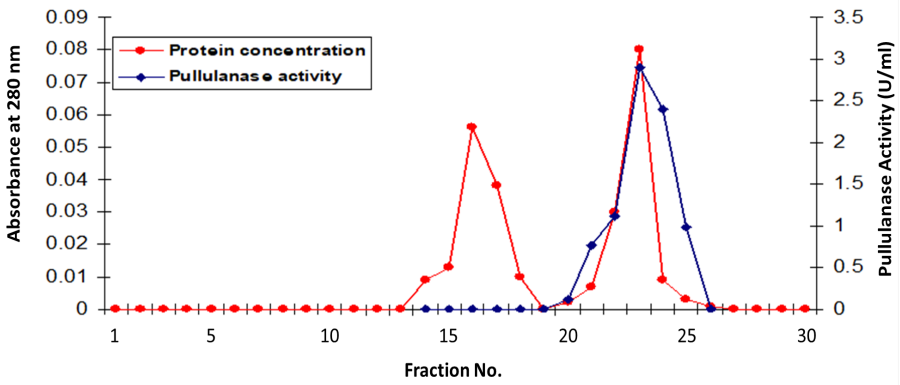

Fig. 3. Gel filtration on a sephadex G-100 column was used to purify pullulase from *Paenibacillus macerans* SR8.

3.5. Enhancement of Lactic Acid Bacteria by Pullulanase

Lactic acid bacteria, including *Lactobacillus reuteri*, *Lactobacillus plantarum, Leuconostoc mesenteroides,* and *Pediococcus* sp., were used to assess the prebiotic qualities of pure pullulanase. Pullulanase stimulated isolates of *Lactobacillus reuteri* isolates with 82–85% followed by *Leuconostoc mesenteroides* with 75-80% while little stimulation for *Pediococcus* sp. isolates with 45-50% as reported in Figure 4.

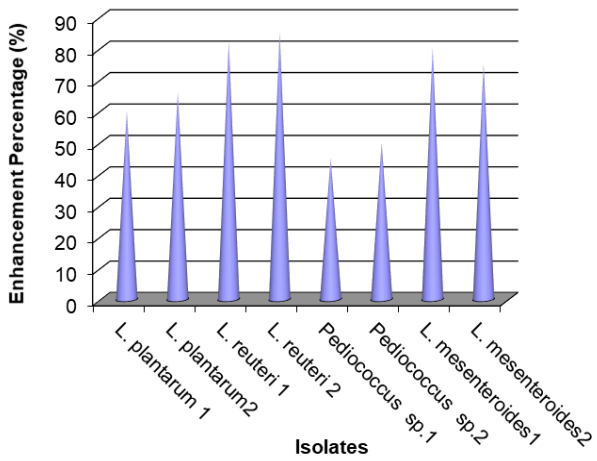

Fig. 4. Purified pullulanase from *Paenibacillus macerans* SR8 enhances lactic acid bacteria.

3.6. Effect of Pullulanase on Pathogenic Bacterial Growth

The pathogenic bacteria such as *A. baumannii*, *E. coli*, *P. aeruginosa* and *E. aerogenus* when combined with purified pullulanase led to inhibition of the bacterial growth with an inhibition percentages ranged between 72 to 83% for *P. aeruginosa* followed by 62 to 68% for *E. coli.* In contrast, lower inhibition rate appeared with *A. baumannii* at 23-44% as shown in Figure 5.

These findings suggest that pullulanase has the potential prebiotic property of enhancing lactic acid bacteria growth while inhibiting pathogenic bacteria growth. Prebiotic properties, in general, do not only inhibit pathogen growth but additionally promote the development of advantageous microorganisms like *Lactobacillus.* This is the first study to look at the purification of pullulanase from *Paenibacillus macerans* and its prebiotic properties.

3.7. Effect of Pullulanase on Biofilm Formation by Pathogenic Bacteria

Using the strongest producers, pullulanase activity against uropathogeinic bacteria's biofilm formation on urinary catheters was examined. It was shown that pullulanase that had been isolated from *Paenibacillus macerans* had antibiofilm action against many genera. Figure 6 shows that the percentage of biofilm inhibition was 44–76% after 24 hours and rose to 58–84% after 48 hours. *A. baumannii* isolates had the highest percentage of inhibition, followed by *P. aeruginosa* isolates with 68–75%, and *E. aerogenus* isolates with 58%.

4. DISCUSSION

Differences in hydrolytic zone diameter in the isolates of *Bacillus* lead to differences in pullulanase genetic expression [3]. In previous reports, pullulanases were mainly extracellular enzymes produced by various kinds of bacteria, especially by *Bacillus* spp. [3, 4]. According to Muslim *et al*. [6], *Paenibacillus macerans*, isolated from agricultural waste, was capable of producing pullulanase using salmon plants as a screening medium. An ammonium sulfate was the most common used salt,

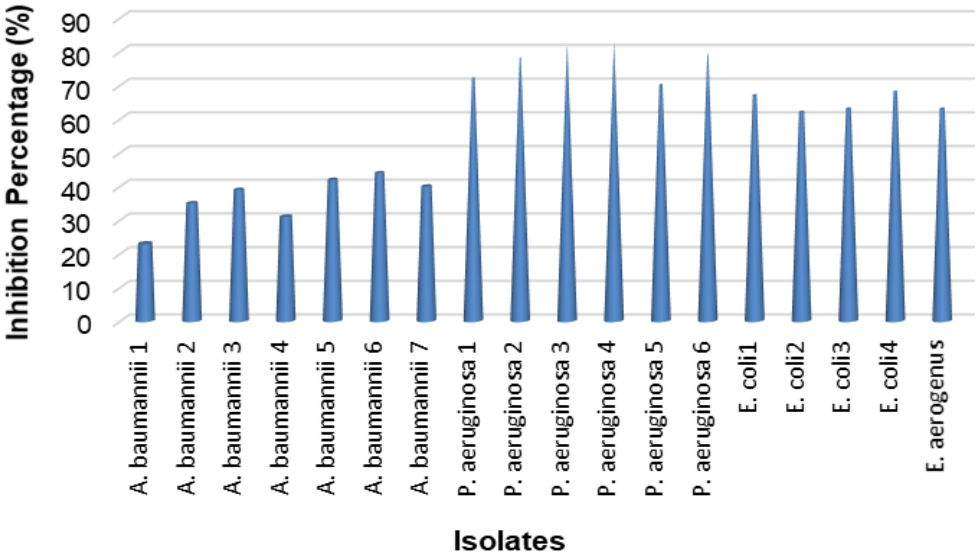

Fig. 5. An inhibition of urinary catheters pathogenic bacteria by purified pullulanase from *Paenibacillus macerans* SR_8.

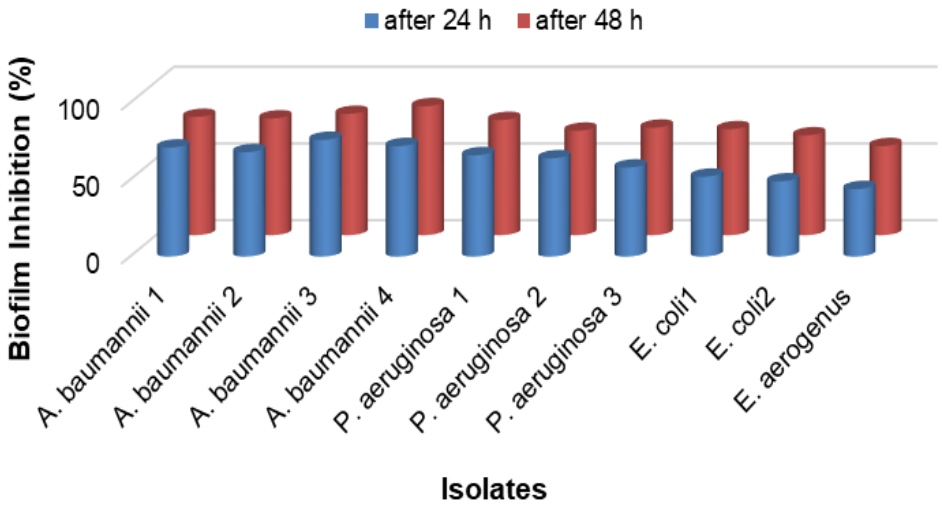

Fig. 6. Inhibition biofilm formation by urinary catheters pathogenic bacteria by purified pullulanase from *Paenibacillus macerans* SR_8.

which supports centrifugation separation because of its low solution density in comparison to other salts, high solubility, lack of buffer capacity, and low cost [23]. Extra-cellular pullulanase Type I has been cleansed with CM Sepharose FF ion exchange chromatography, followed by Sephadex G-150 gel filtration chromatography as the last purification step, folding from 14,367 with the final purification step of *Lactococcus lactis*, type I, by using ammonium-sulfate fractionation and dialysis (rather than ultrafiltration) [24]. A 5-step purification method was used to purify a 150-kDa monomeric pullulanase: (1) an original enzyme was purified 50.0 times for the ultimate specific activity of 3.0 U/mg, (2) Ammonium sulphate salts, (3) dialysis and anion exchange, (4) permeation of the gel, and (5) hydrophobial chromatography. Urinary tract pathogens are commonly isolated and counted using CHROM agar Orientation media, a chromogenic culture medium. It has several benefits, including improved gram-negative bacilli differentiation and easier detection and presumed identification of gram-negative bacilli [25]. It has been demonstrated in a number of earlier investigations that urinary tract pathogens, such as gram-negative bacilli, enterococci, and staphylococci, may be effectively isolated and differentiated using this medium. The majority of strains linked with *A. baumannii, Klebsiella ornithinolytica*, and *Pseudomonas aeruginosa* are responsible for catheter-related urinary tract infections [26]. Urinary catheter biofilm growth and biomineralization can result in serious side effects like infection and blockage. *Proteus mirabilis* and *Pseudomonas aeruginosa*, two uropathogens, were used to assess biofilm development and biomineralization in vitro on catheters retrieved from hospitalized patients [27]. Recurrent, complex UTIs are frequently caused by biofilm-forming bacteria, which are typically linked to MDR bacteria [28]. The creation of novel treatments depends on our ability to comprehend the pathophysiology and variables connected to biofilm formation [29]. The isolated *E. coli* were exposed to the biofilm-forming medium, 125 (62.5%) of the isolates formed biofilms on Congo Red Agar [30]. Because biofilm-forming bacteria have a strong polymeric matrix that prevents antibiotic penetration, they often show better resistance than planktonic cells [31]. Prebiotics are indigestible dietary additives that benefit the host by encouraging the colon's few resident microorganisms to grow and/or function. Pullulan is a non-digestible carbohydrate that is fermented by the microbiota and can change the composition of the intestinal microbiota. It is a -1,6 connected polymer of maltotriose subunits [32]. The natural microflora of the infants'colon was selectively affected by pullulan and its derivatives. While pullulan had no impact on the growth of *Bifidobacterium* and *Lactobacillus*, it did increase their acidifying activity, which was likely the cause of the decrease in the number of *E. coli* bacteria [33]. As a result, animal feed containing distilled pullulanase may be used to increase digestibility and promote gastrointestinal health. In the future, more investigation will be done to ascertain the kind of synbiotic impact that might arise from combining it with a suitable probiotic.

5. CONCLUSIONS

Certain isolates of Gram-negative bacteria, including *A. baumannii, E. coli, P. aeruginosa*, and *E. aerogenus*, were obtained from urinary catheters. Pullulanase may have a prebiotic property that promotes the growth of lactic acid bacteria while ostensibly hindering the growth of pathogens and preventing the creation of their biofilms. Consequently, pullulanase can be added to animal feed to increase digestibility and gastrointestinal health. It can also be used to cover catheters to assist in preventing bacterial colonization, infections, and the care of patients with biofilm-associated infections.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare that there is no competing interest.

8. AUTHORS' CONTRIBUTIONS

Sahira Nsayef Muslim: Conceptualization, Methodology, Supervision. Nehad A. Taher: Formal analysis, Investigation. Wafaa Hassan Muslem: Data curation, Writing - original draft (Corresponding Author). Saba Riad Khudhaier: Validation, Writing - review and editing.

9. FUNDING

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10. ETHICAL STATEMENT

This study does not involve human participants or animal subjects. Therefore, ethical approval was not required.

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Supplementary Data

Phytochemical Diversity and Pharmacological Perspectives of *Vitex leucoxylon* L.f.: A Comprehensive Review

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Table S1. Phytochemical profile of *Vitex leucoxylon* L.f. with compounds and activities.

Class/ Sub-Sub-class	Compound	Plant Part	Molecular formula	Melting point 0C	Molecular weight g/mol	Solvent	Quantification	Biological activity	References
Sugar compound									
Anhydrosugar	1,6-Anhydro-β-D-glucopyranose (levoglucosan)	Leaves	$C_6H_{10}O_5$	170–172	162.14	Ethanol	GC-MS	Preservative	[28]
Monosaccharide (aldoses)	d-Mannose	Leaves	$C_6H_{12}O_6$	133-140	180.15	Ethanol	GC-MS	Anticancer, Antiviral	[43]
Disaccharide	D-Glucose, 6-O-D-galactopyranosyl	Leaves	$C_{12}H_{22}O_{11}$	182	342.3	Ethanol	GC-MS	Preservative	[28]
Oligosaccharide	Maltotriose hydrate	Leaves	$C_{18}H_{32}O_{16}$	132-135	504.43	Methanol	FTIR	Precursor for prebiotics	[44]
Oligosaccharide	Maltopentose hydrate	Leaves	$C_{30}H_{54}O_7$	168	526.74	Methanol	FTIR	Precursor for prebiotics , Inhibition of interleukin-2	[46]
Cyclic oligosaccharide	Gamma-cyclodextrin hydrate	Leaves	$C_{48}H_{80}O_{40}$	267	1344.08	Methanol	FTIR	Enhancement of drug solubility and bioavailability	[46]
Disaccharide	(+)-β-D Lactose β	Leaves	$C_{12}H_{22}O_{11}$	224	342.3	Methanol	FTIR	_	14
Oligosaccharide	Dextrin	Leaves	$C_{18}H_{32}O_{16}$	53-54	504.4	Methanol	FTIR	_	14
Aromatic alcohol glycoside	Benzyl α-D-glucoside	Leaves	$C_{13}H_{18}O_6$	95–103	270.28	Ethanol	GC-MS	_	[28]
Fatty Acids									
Saturated fatty acid	n-Hexadecanoic acid	Leaves	$C_{16}H_{32}O_2$	61–63	256.43	Ethanol	GC-MS	Antioxidant, Anti-inflammatory	[28]
Saturated fatty acid	Octanoic Acid	Leaves	$C_8H_{16}O_2$	239	144.21	Ethanol	GC-MS	Anti-Inflammatory, Perfumery pesticide	[28,47,48]
Polyunsaturated fatty acid	9,12,15-Octadecatrienoic acid	Leaves	$C_{18}H_{32}O_2$	_	292.46	Ethanol	GC-MS	Antihistaminic, Anticancer, Anti-inflammatory, Hepatoprotective	[28]
Saturated fatty acid	Tetradecanoic acid	Bark	$C_{14}H_{28}O_2$	52-58	228.37	Ethanol	GC-MS	Anti-viral, Antibacterial, Antioxidant	[28]
Polyunsaturated fatty acid	9,12-octadecadienoic acid	Bark	$C_{18}H_{32}O_2$	−5 °C	280.45	Ethanol	GC-MS	Anti-inflammatory, Antioxidant	[28]
Monounsaturated fatty acid	Oleic acid	Bark	$C_{18}H_{34}O_2$	13.4	282.46	Ethanol	GC-MS	Antifungal	[28]

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Saturated fatty acid	Octadecanoic acid	Bark	$C_{18}H_{36}O_2$	69.4	284.48	Ethanol	GC-MS	Antioxidant, Antiviral, Antimicrobial	[28]
Saturated fatty acid	n-Decanoic acid	Bark	$C_{10}H_{20}O_2$	31.6	172.26	Dichloromethane	GC-MS	Antibacterial, Anticancer	[28]
Saturated fatty acid	Dodecanoic acid	Bark	$C_{12}H_{24}O_2$	44-46	200.32	Dichloromethane	GC-MS	Antibacterial, Antioxidant	[28]
Saturated fatty acid	Propionic acid	Leaves	$C_3H_6O_2$	-20.5	74.08	Methanol	FTIR	Antioxidant , Anticancer, Antidiabetic	[14]
Unsaturated fatty acid	Hexadecenoic acid	Bark	$C_{16}H_{32}O_2$	60-62	256.43	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Hepatoprotective	[27]
Saturated fatty acid	Tetracosanoic acid	Bark	$C_{24}H_{48}O_2$	75-80	368.64	Chloroform + Methanol	NMR Spectroscopy	Antimicrobial, Hepatoprotective	[27]
Esters									
Aromatic ester	Benzoic acid, 3-hydroxy-, 1-methylethyl ester	Leaves	$C_{10}H_{12}O_3$	28–30	180.2	Ethanol	GC-MS	Antimicrobial, Anti-Inflammatory	[28]
Dimethyl ester	Dimethyl terephthalate	Leaves	$C_{10}H_{10}O_4$	142	194.18	Methanol	GC-MS	Production of plastics, Polyester fibres	[35]
Fatty acid ester	Hexadecanoic acid, methyl ester	Leaves	$C_{17}H_{34}O_2$	29–35	270.45	Ethanol	GC-MS	Antioxidant, Hypocholesterolaemia, Nematicide	[28]
Ethyl esters of fatty acids	Hexanoic acid, ethyl ester	Leaves	$C_8H_{16}O_2$	-67	144.21	Ethanol	GC-MS	Antimicrobial	[28]
Nitro fatty acid ester	4,9-Decadienoic acid, 2-nitro-, ethyl ester	Leaves	$C_{12}H_{19}NO_4$	_	168.24	Ethanol	GC-MS	Antimicrobial	[28]
Acetylenic fatty acid ester	10, 13-Octadecadiynoic acid, methyl ester	Leaves	$C_{19}H_{30}O_2$	_	290.45	Ethanol	GC-MS	Antimicrobial	[28]
Acetylenic fatty acid ester	4-Decynoic acid, methyl ester	Leaves	$C_{11}H_{20}O_2$	Below 25	184.27	Ethanol	GC-MS	Insecticidal, Antimicrobial	[28]
Polyunsaturated fatty acid	6,9,12,15-Docosatetraenoic acid, methyl ester tetramethyltetradeca-4,8-dienoic acid, methyl ester	Leaves	$C_{23}H_{38}O_2$	_	346.56	Ethanol	GC-MS	Anti-cholesterol	[28]
Methyl branched-chain fatty acid ester	Heptanoic acid, 2-methyl-, methyl ester	Seeds	$C_9H_{18}O_2$	15	158	Ethanol	GC-MS	_	[28]
Sorbitan fatty acid ester	Sorbitan monolaurate	Leaves	$C_{18}H_{34}O_6$	54-98	346.46	Methanol	FTIR	_	[14]
_	Methylparaben	Leaves	$C_8H_8O_3$	125–126	152.15	Ethanol	GC-MS	Preservative	[28]
Terpenes									
Sesquiterpene	Caryophyllene	Leaves	$C_{15}H_{24}$	Below 25	204.35	Ethanol	GC-MS	Anticancer, Analgesic, Sedative, Fungicide	[28,50]
Sesquiterpene	Azulene, 1,4-dimethyl-7-(1-methylethyl)	Leaves	$C_{15}H_8$	29-30	198.3	Ethanol	GC-MS	Antiallergic, Antidiabetic	[28]
Triterpene	Squalene	Seeds	$C_{30}H_{50}$	-75	410.73	Ethanol	GC-MS	Anti-Inflammatory, Immune-Enhancing Properties, Detoxifier	[28,51,52]
Terpenoids									
Phytosterol	β-sitosterol	Leaves	$C_{29}H_{50}O$	136-142	414.71	Methanol	GC-MS	Anticancer, Antidiabetic	[53,54,55]
Pentacyclic triterpenoid	Betulinic acid	Leaves	$C_{38}H_{40}O_3$	294-296	456.7	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Hepatoprotective	[27]
Triterpenoid	Corosolic acid	Leaves	$C_{30}H_{48}O_4$	148-150	472.71	Ethanol	IR spectroscopy	Antidiabetic, Anti-inflammatory	[28,56]
Triterpenoid	3β-hydroxy-olean-5, 12-dien-28- oic acid	Bark	$C_{30}H_{46}O_3$	198	454.69	Petroleum ether + ethanol + dichloromethane	1H NMR	_	[28]
Triterpenoid	3β-acetoxyolean-12-en-27- oic acid	Bark	$C_{32}H_{50}O_4$	197	498.74	Petroleum ether + ethanol + dichloromethane	1H NMR	_	[28]
Diterpene alcohol	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Leaves	$C_{20}H_{40}O$	38–39	296.53	Ethanol	GC-MS	Antimicrobial, Anti-Inflammatory	[28]
Sesquiterpene lactone	2(3H)-Benzofuranone, hexahydro-4,4,7α-trimethyl-	Leaves	$C_{11}H_{18}O_2$	78-80	282.26	Ethanol	GC-MS	Antimicrobial, Hepatoprotective	[28,57]
Acyclic diterpene alcohol	Phytol	Leaves	$C_{20}H_{40}O$	25	296.53	Ethanol	GC-MS	Anti-Inflammatory, Diuretic, Cytotoxic, Antioxidant	[28,58]
Monoterpene epoxide	Ascaridole epoxide	Leaves	$C_{10}H_{16}O_2$	_	168.24	Ethanol	GC-MS	Antimicrobial, Antioxidant, Anthelmintic	[28]
Sesquiterpene	Ledol	Leaves	$C_5H_{10}O_3$	104-105	118.13	Ethanol	GC-MS	Antifungal, Expectorant properties	[28]
Diterpene	7α-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	Leaves	$C_{15}H_{26}O$	_	222.37	Ethanol	GC-MS	Anti-Inflammatory, Asthma	[28]
Sesquiterpene	Aromadendrene oxide	Leaves	$C_{15}H_{24}O$	_	220.35	Ethanol	GC-MS	Anticancer, Sedative	[28]
Monoterpene	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	Leaves	$C_{13}H_{20}O$	_	192.3	Ethanol	GC-MS	Antifungal	[28]
Tocopherol	Vitamin E	Leaves	$C_{29}H_{50}O_2$	2.5- 3.5	430.7	Ethanol	GC-MS	Antiaging, Antidiabetic Anti-inflammatory	[28]
Iridoids									
Iridoid glycoside	Agnuside	Leaves	$C_{22}H_{26}O_{11}$	146	466.43	Methanol	GC-MS	Antioxidant, Cardioprotective, Anticancer	[57,59,60]
Iridoid glycoside	Aucubin	Leaves	$C_{15}H_{22}O_9$	181	346.33	Methanol	GC-MS	Antioxidant, Anti-Aging, Anti-Fibrosis, Anticancer, Hepatoprotection, Neuroprotection	[57,61,62]
Monoterpene	Iridiod A	Leaves	_	_	_	Methanol	GC-MS	Anticancer	[31, 57]
Monoterpene	Iridiod B	Leaves	_	_	_	Methanol	GC-MS	Anticancer	[31, 57]

Flavone									
Flavone	5,6,7,4' -tetrahydroxy flavone	Bark	$C_{15}H_{10}O_5$	218-221	270.24	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
O-methylated flavone	5-hydroxy-7, 4' dimethoxy flavone	Leaves	$C_{171}H_4O_5$	168-172	298.08	Petroleum ether + ethanol + dichloromethane	NMR Spectroscopy	-	[28]
Flavone	5- hydroxy-3,6,7,3',4' pentamethoxyflavone	Leaves	$C_{20}H_{20}O_8$	120-121	388.37	Petroleum ether + ethanol + dichloromethane	1H NMR	-	[28]
Flavonoids									
Flavone C-glucoside	2'-O-caffeoylorieutin	Leaves	$C_{30}H_{26}O_{14}$	250-251	523.14	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
Flavonoid glycoside	Vitexin	Bark	$C_{21}H_{20}O_{10}$	264-265	432.38	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
Trihydroxyflavone	Kaemferol 3-O- β-D glucopyranoside	Leaves	$C_{20}H_{18}O_{11}$	178-180	434.35	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
Flavone C-glycoside	Isovitexin	Bark	$C_{21}H_{20}O_{10}$	246-247	432.38	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
O-methylated flavone	5,7 dihydroxy- 6,4' dimethoxy flavanone	Leaves	$C_{17}H_{16}O_6$	179	314.29	Petroleum ether + ethanol + dichloromethane	1H NMR	-	[28]
O-methylated flavanone	5,3'-dihydroxy—7,8,4'-trimethoxy flavanone	Leaves	$C_{18}H_{18}O_7$	_	346.33	Petroleum ether + ethanol + dichloromethane	1H NMR	-	[28]
O-methylated flavanone	7,8 dimethyl herbacetin 3-rhamnoside	Leaves	$C_{23}H_{24}O_{11}$	189	476.43	Petroleum ether + ethanol + dichloromethane	1H NMR	Antioxidant	[28]
Phenolics									
Aromatic aldehyde	Cinnamaldehyde	Leaves	C_9H_8O	7-8	132	Ethanol	GC-MS	Anticancer, Hypoglycemic	[28]
_	Benzenepropanoic acid, α-(hydroxyamino)	Leaves	$C_9H_9NO_3$	_	179	Ethanol	GC-MS	Antimicrobial	[28]
Di-phenol	Hydroquinone	Leaves	$C_6H_6O_2$	172–174	110.11	Ethanol	GC-MS	Antimicrobial	[28]
Hydroxybenzoic acid	Benzoic acid, 4-hydroxy-	Leaves	$C_7H_6O_3$	216–217	138	Ethanol	GC-MS	Antimicrobial, Anticancer, Hypoglycemic	[28]
Phenolic glycoside	6-O-caffeoylarbutin	Leaves	$C_{21}H_{22}O_{10}$	224-225	434.4	Ethanol	IR spectroscopy	Antidiabetic, Anti-inflammatory , Antioxidant	[71]
Hydroxybenzoic acid	3-hydroxy-Benzoic acid	Leaves	$C_5H_{10}O_3$	200	118.13	Ethanol	GC-MS	Antialgal, Antimutagenic, Antiestrogenic, Anti-platelet aggregating, Nematocidal, Antiviral, Antioxidant	[28,63]
Benzopyrone	Coumarin	Leaves	$C_9H_6O_2$	68-73	146.14	Methanol	FTIR	Antimicrobial, Anticoagulant, Anticancer, Anti-ulcers	[14,64,65]
Volatile organic compounds									
Unsaturated aliphatic alcohol	2,4-Pentadien-1-ol, 3-propyl-, (2Z)	Leaves	$C_{11}H_{12}O$	_	160.22	Ethanol	GC-MS	Antioxidant , Anti-inflammatory	[28]
Unsaturated aliphatic alcohol	10-Undecen-1-ol	Seeds	$C_{11}H_{22}O$	-3	170.29	Ethanol	GC-MS	Antimicrobial	[28]
Unsaturated aliphatic alcohol	3-Buten-2-ol	Seeds	C_4H_8O	-100	72	Ethanol	GC-MS	Antimicrobial	[28]
Aliphatic aldehyde	Tridecanal	Leaves	$C_{13}H_{26}O$	14	198.35	Methanol	FTIR	Antimicrobial, Antifungal, Cytotoxicity	[14]
Aliphatic aldehyde	Dodecyl aldehyde	Leaves	$C_{12}H_{24}O$	12	184.32	Methanol	FTIR	_	[14,48]
Aliphatic aldehyde	Nonyl Aldehyde	Leaves	$C_9H_{18}O$	-18	142.24	Methanol	FTIR	Antidiarrheal, Antifungal	[14,48,68,69]
Acyclic diene	1,5-Heptadiene, 2,6-dimethyl	Leaves	C_9H_{16}	–70 °	124.22	Ethanol	GC-MS	Antimicrobial, Antioxidant	[28,92]
Aliphatic ketone	3- nonanone	Leaves	$C_9H_{18}O$	-8	142.24	Methanol	GC-MS	_	[14]
Others									
Polyol	Glycerin	Leaves	$C_3H_8O_3$	17.8	92	Ethanol	GC-MS	Preservative, Antimicrobial	[28]
Aliphatic diol	1,14-Tetradecanediol	Seeds	$C_{14}H_{30}O_2$	85-90	230.39	Ethanol	GC-MS	Antimicrobial	[28]
Furanoid aldehyde	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Leaves	$C_6H_6O_3$	28-34	126	Ethanol	GC-MS	Preservative, Antimicrobial	[28]
Aliphatic amide	Butyramide	Leaves	C_4H_9NO	114-116	87.12	Methanol	FTIR	_	[14, 48]
Aromatic ether	Benzene, (ethenyloxy)-	Leaves	C_8H_8O	-50	120	Ethanol	GC-MS	_	[28]
Long-chain aliphatic ether	Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	Leaves	$C_{20}H_{40}O_2$	_	312.53	Ethanol	GC-MS	Antimicrobial	[28]
Alkyl glyceryl ether	2-(9-octadecenyloxy)ethanol (Z)	Leaves	$C_{20}H_{40}O_2$	_	312.53	Ethanol	GC-MS	Luciferase, Antioxidant	[28]
_	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Leaves	$C_6H_8O_4$	75-78	144	Ethanol	GC-MS	Antimicrobial	[28]
Acetal	Butane, 1,1-diethoxy-3-methyl	Leaves	$C_9H_{20}O_2$	167-168	160.25	Ethanol	GC-MS	Antidiabetic Antidermatitic, Antileukemic, Anticancer	[28]
Furan derivative	Furan, 2-propyl	Leaves	$C_7H_{10}O$	–55 °C	110.16	Ethanol	GC-MS	_	[28]
Cardiac glycoside	Lanatoside A	Leaves	$C_{49}H_{76}O_{19}$	245-248	1017.1	Methanol	FTIR	_	[14]
Imidazole	2-Methyl-5,5-diphenyl-4-(methylthio)imidazole	Seeds	$C_{17}H_{16}N_2S$	_	280.39	Ethanol	GC-MS	Antimicrobial	[28]

Alkane	Tetradecane	Bark	$C_{14}H_{30}$	5.9	198.39	Dichloromethane	GC-MS	Antibacterial, Antifungal	[28]
Aromatic hydrocarbon	Benzene, 1-(1,5-dimethylhexyl)-4-methyl	Bark	$C_{15}H_{24}$	_	204.36	Dichloromethane	GC-MS	_	[28]
Alkane	Hexadecane	Bark	$C_{16}H_{34}$	18.18	226.44	Dichloromethane	GC-MS	Antimicrobial, Antioxidant	[28]
Alkane	Nonadecane	Bark	$C_{19}H_{40}$	32	280.54	Dichloromethane	GC-MS	Antimicrobial, Antioxidant	[28]
Alkane	Heptacosane	Bark	$C_{27}H_{56}$	59	380.74	Dichloromethane	GC-MS	Overcoming P-Glycoprotein (P-Gp)-Mediated Drug Resistance,	[28,70]
Alkane	Octacosane	Bark	$C_{28}H_{58}$	60-64	394.77	Dichloromethane	GC-MS	Antimicrobial, Antifungal	[28]
Aromatic hydrocarbon	1,2,4-Trimethylbenzene	Bark	C_9H_{12}	-43.78	120.19	Petroleum ether	GC-MS	Antagonist Inhibition Of Daf-12 Receptor	[28]
Alkane	Heneicosane	Bark	$C_{21}H_{44}$	40.5	296.58	Petroleum ether	GC-MS	Antimicrobial, Pesticidal	[28]
Alkane	Tricontane	Leaves	$C_{30}H_{62}$	63-67	422.83	Methanol	FTIR	Anticancer	[31]
Alkyl hydroxylamine	Hydroxylamine, O-decyl	Bark	$C_{10}H_{23}NO$	34	173.3	Dichloromethane	GC-MS	_	[28]
Fatty alcohol	Heptadecanol	Bark	$C_{17}H_{36}O$	55-57	256.47	Chloroform + Methanol	NMR Spectroscopy	Anti-inflammatory, Antimicrobial, Hepatoprotective	[27]
Monoacylglycerol	1-Monolinoleoylglycerol trimethylsilyl ether	Leaves	$C_{27}H_{54}O_4Si_2$	_	498.89	Ethanol	GC-MS	_	[28]
Aliphatic nitriles	Hexanenitrile	Seeds	$C_6H_{11}N$	-80	97.16	Ethanol	GC-MS	Antimicrobial	[28]
Pyrimidine nucleobase	Thymine	Leaves	$C_5H_6N_2O_2$	316-317	126	Ethanol	GC-MS	_	[28]
Isothiocyanate	Neopentyl isothiocyanate	Leaves	$C_6H_{11}NS$	_	129.226	Ethanol	GC-MS	Antimicrobial	[28]
Sulfone	2,3-Dihydrothiophene 1,1-dioxide	Leaves	$C_4H_6O_2S$	48.5	118.15	Ethanol	GC-MS	Antiviral	[28]
Sulfone	Phenyl sulfone	Leaves	$C_{12}H_{10}O_{25}$	123-129	218.27	Methanol	FTIR	Anti-HIV,	[14, 48]
Sulfinic acid	Benzenesulfinic acid	Leaves	$C_6H_6O_2S$	83-84	142.18	Methanol	FTIR	_	[14, 48]
_	Propane, 1-(ethenylthio)-	Leaves	$C_5H_{10}S$	–113 °C	102	Ethanol	GC-MS	Antimicrobial	[28]
Dialkyl phthalate	Di-n-octylphthalate	Bark	$C_{24}H_{38}O_4$	-25	390.56	Petroleum ether	GC-MS	Anticancer	[28]
Dialkyl phthalate	Dibutyl phthalate	Seeds	$C_{16}H_{22}O_4$	_	278.34	Ethanol	GC-MS	Antimicrobial, Antifouling	[28]
Dialkyl phthalate	Didodecyl phthalate	Seeds	$C_{32}H_{54}O_4$	21-23	502.78	Ethanol	GC-MS	Antimicrobial, Antifouling	[28]
Dialkyl phthalate	1,2-Benzenedicarboxylic acid, diisooctyl ester	Leaves	$C_{24}H_{38}O_4$	_	390.56	Ethanol	GC-MS	Antimicrobial, Antifouling	[28]
Dialkyl phthalate	1,2-Benzenedicarboxylic acid, butyl8-methylnonyl ester	Bark	$C_8H_{16}O_2$	_	144.21	Ethanol	GC-MS	_	[28]
Bile acid derivative	Ethyl iso-allocholate	Leaves	$C_{26}H_{44}O_5$	160	436.63	Ethanol	GC-MS	Antidiabetic, Antioxidant	[28,71]
Monoterpene	Cyclohexene, 3-methyl-6-(1-methylethylidene)-	Seeds	$C_{10}H_{16}$	_	136.24	Ethanol	GC-MS	_	[28]

Table S2. Pharmacological activities of *Vitex leucoxylon L.f.*

Plant Part	Pharmacological activity	Extract	Dose route of administration	Standard Used	Control/Assay	Method	Induced chemical	Model/Microbial strain	Result	Reference
Leaves	Antiosteoporotic	90 % Ethanol	500 mg/kg	Calcium & Vitamin D_3	-	*in vivo*	Chronic alcohol abuse (20 % ethanol/saline)	Wistar albino rats (Female)	Serum Ca^{2+}, phosphorus ↑; ALP, urinary Ca^{2+}, phosphorus, creatinine ↓.	[8]
Leaves	Hepatoprotective	Alcoholic extract	500 mg/kg	-	-	*in vivo*	CCl_4	BALB/C mice	At 500 mg/kg, alcoholic extract improved ALP, bilirubin, SGOT, and SGPT, showing hepatoprotective effect.	[12]
Bark	Anti-inflammatory	Ethanol	500 mg/kg	-	-	*in vivo*	Carrageenan (1% w/v, 0.1 ml)	Wistar albino rats	43.82% inhibition	[15]
Bark	Anti-inflammatory	Aqueous	500 mg/kg	-	-	*in vivo*	Carrageenan (1% w/v, 0.1 ml)	Wistar albino rats	46.06% inhibition	
Bark	Anti-inflammatory	-	-	Indomethacin, 10 mg/kg	-	*in vivo*	Carrageenan (1% w/v, 0.1 ml)	Wistar albino rats	50.60% inhibition	
Leaves	Analgesic	-	-	-	Distilled water	*in vivo*	Phenylbutazone (100 mg/kg)	Wistar albino rats	21.08 ± 3.96 writhes that reduced writhing	[16]
Leaves	Analgesic	Cold aqueous Infusion	100 mg/kg	-	-	*in vivo*	-	Wistar albino rats	22.00 ± 5.31 writhes	
Leaves	Analgesic	-	200 mg/kg	-	-	*in vivo*	-	Wistar albino rats	19.60 ± 4.30 writhes	
Leaves	Analgesic	Ethanol	200 mg/kg	-	-	*in vivo*	-	Wistar albino rats	24.00 ± 6.91 writhes	
Leaves	Analgesic	-	400 mg/kg	-	-	*in vivo*	-	Wistar albino rats	36.40 ± 4.57 writhes	
Leaves	Anti-inflammatory	Ethanol	100–400 mg/kg	Phenylbutazone, 100 mg/kg	-	*in vivo*	Carrageenan paw edema (acute inflammation)	Wistar albino rats	0.42 ± 0.08 ml (Significant)	
Leaves	Anti-inflammatory	Ethanol	100–400 mg/kg	Phenylbutazone, 100 mg/kg	-	*in vivo*	Cotton pellet granuloma (chronic inflammation)	Swiss albino rats	47.32 ± 4.17 mg/100 g (non-significant)	
Bark	Anti-inflammatory	Methanol	100 mg/kg	-	Vehicle (0.5% CMC)	*in vivo*	50 µL FCA injected in left hind paw on day 14.	Swiss albino rats	VL-89/185A at 100 mg/kg produced **36.92% inhibition of paw edema**.	[18]

Bark	Anti-inflammatory	Methanol	250 mg/kg	-	Vehicle (0.5% CMC)	*in vivo*	50 µL FCA injected in left hind paw on day 14.	Swiss albino rats	VL-89/185A at 250 mg/kg showed **46.41% inhibition of paw edema**.	[18]
Bark	Anti-inflammatory	-	10 mg/kg	Prednisolone	Vehicle (0.5% CMC)	*in vivo*	50 µL FCA injected in left hind paw on day 14.	Swiss albino rats	Prednisolone at 10 mg/kg achieved **59.74% inhibition of paw edema**.	
Leaves (AgNP synthesis)	Antibacterial	Aqueous	5 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 6mm, *Escherichia coli* 10mm, *Pseudomonas aeruginosa* 8mm, *Klebsiella pneumoniae* 5mm and *Salmonella typhi* 10mm	[21]
-	-	-	10 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 8, *Escherichia coli* 14, *Pseudomonas aeruginosa* 10, *Klebsiella pneumoniae* 8, *Salmonella typhi*16	
-	-	-	15 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 10mm, *Escherichia coli* 20mm, *Pseudomonas aeruginosa* 16mm, *Klebsiella pneumoniae* 12mm and *Salmonella typhi* 20mm	
Leaves (CuNPs synthesis)	Antibacterial	Aqueous	5 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 10mm, *Escherichia coli* 12mm, *Pseudomonas aeruginosa* 10mm, *Klebsiella pneumoniae* 8mm and *Salmonella typhi* 12mm	
-	-	-	10 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 12mm, *Escherichia coli* 18mm, *Pseudomonas aeruginosa* 18mm, *Klebsiella pneumoniae* 12mm and *Salmonella typhi* 18mm	
-	-	-	15 µg/ml	Ofloxin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Staphylococcus aureus* 18mm, *Escherichia coli* 22mm, *Pseudomonas aeruginosa* 20mm, *Klebsiella pneumoniae* 18mm and *Salmonella typhi* 22mm	
Leaves (AgNP synthesis)	Antifungal	Aqueous	5 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 8mm, *Candida tropicalis* 6mm and *Aspergillus niger* 6mm	
-	-	-	10 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 12mm, *Candida tropicalis* 8mm and *Aspergillus niger* 10mm	
-	-	-	15 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 16mm, *Candida tropicalis* 10mm, *Aspergillus fumigatus* 4mm and *Aspergillus niger* 14mm	
Leaves (CuNPs synthesis)	Antifungal	Aqueous	5 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 8mm, *Candida tropicalis* 6mm and *Aspergillus niger* 6mm	
-	-	-	10 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 12mm, *Candida tropicalis* 8mm and *Aspergillus niger* 10mm	
-	-	-	15 µg/ml	Ketokonazole (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Candida albicans* 16mm, *Candida tropicalis* 10mm, *Aspergillus fumigatus* 4mm and *Aspergillus niger* 14mm	
Leaves	Antioxidant	Ethanol	10 µg/ml	-	DPPH radical scavenging	*in vitro*	-	-	IC_{50} - 0.084	
Bark	Antioxidant	Ethanol	10 µg/ml	-	DPPH radical scavenging	*in vitro*	-	-	IC_{50} - 0.171.	
Seed	Antioxidant	Ethanol	10 µg/ml	-	DPPH radical scavenging	*in vitro*	-	-	IC_{50} - 0.758.	
Leaves	Antioxidant	Ethanol	10 µg/ml	-	Superoxide Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.317.	
Bark	Antioxidant	Ethanol	10 µg/ml	-	Superoxide Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.480	
Seed	Antioxidant	Ethanol	10 µg/ml	-	Superoxide Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.591	
Leaves	Antioxidant	Ethanol	10 µg/ml	-	Hydroxyl Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.130	
Bark	Antioxidant	Ethanol	10 µg/ml	-	Hydroxyl Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.166	
Seed	Antioxidant	Ethanol	10 µg/ml	-	Hydroxyl Radical Scavenging Activity	*in vitro*	-	-	IC_{50} - 0.268	
Leaves	Antioxidant	Ethanol	10 µg/ml	-	Lipid Peroxidation Assay	*in vitro*	-	-	IC_{50} - 0.209.	
Bark	Antioxidant	Ethanol	10 µg/ml	-	Lipid Peroxidation Assay	*in vitro*	-	-	IC_{50} - 0.200	
Seed	Antioxidant	Ethanol	10 µg/ml	-	Lipid Peroxidation Assay	*in vitro*	-	-	IC_{50} - 0.213	

Leaves	Hepatoprotective	Chloroform & Methanolic	50 mg/kg to 200 mg/kg	Silymarin	-	*in vivo*	CCl_4	Wistar albino rats (Male)	Liver function (GOT, GPT, ALP, bilirubin) showed hepatoprotection in all extracts except bark methanol.	
Leaves	Antibacterial	Chloroform	250 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 10 mm, *Bacillus cereus* 10 mm, *Bacillus pumilis* 10 mm, *Escherichia coli* 10 mm and *Proteus vulgaris* 9 mm	
	Antibacterial	-	500 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 12 mm, *Bacillus cereus* 13 mm, *Bacillus pumilis* 11 mm, *Streptococcus aureus* 9 mm, *Escherichia coli* 13 mm, *Proteus vulgaris* 12 mm and *Pseudomonas aeruginosa* 9 mm	
	Antibacterial	Methanol	250 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 11 mm, *Bacillus cereus* 25 mm, *Bacillus pumilis* 19 mm, *Streptococcus aureus* 16 mm, *Escherichia coli* 22 mm, *Proteus vulgaris* 20 mm, *Pseudomonas aeruginosa* 19 mm and*Streptomyces marienensis* 22 mm	
	Antibacterial	-	500 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 12 mm, *Bacillus cereus* 28 mm, *Bacillus pumilis* 22 mm, *Streptococcus aureus* 18 mm, *Escherichia coli*25 mm, *Proteus vulgaris* 27 mm, *Pseudomonas aeruginosa* 23 mm and *Streptomyces marienensis* 26 mm	[27]
Stem bark	Antibacterial	Chloroform	250 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 10 mm, *Streptococcus aureus* 10 mm, *Escherichia coli* 9 mm, *Proteus vulgaris* 11 mm, *Pseudomonas aeruginosa* 10 mm and *Streptomyces marienensis* 10 mm	
	Antibacterial	-	500 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 12 mm, *Bacillus cereus* 10 mm, *Bacillus pumilis* 10 mm, *Streptococcus aureus* 13 mm, *Escherichia coli*12 mm, *Proteus vulgaris* 14 mm, *Pseudomonas aeruginosa* 12 mm and *Streptomyces marienensis* 12 mm	
	Antibacterial	Methanol	250 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 16 mm, *Bacillus cereus* 23 mm, *Bacillus pumilis* 14 mm, *Streptococcus aureus* 14 mm, *Escherichia coli*22 mm, *Proteus vulgaris* 18 mm, *Pseudomonas aeruginosa* 14 mm and *Streptomyces marienensis* 18 mm	
	Antibacterial	-	500 µg/disc	Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Bacillus subtilis* 19 mm, *Bacillus cereus* 28 mm, *Bacillus pumilis* 22 mm, *Streptococcus aureus* 18 mm. *Escherichia coli*24 mm. *Proteus vulgaris* 20 mm, *Pseudomonas aeruginosa* 18 mm and*Streptomyces marienensis* 20 mm	
Leaves	Antifungal	Chloroform	250 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 18 mm, *Rhizopus oryzae* 12 mm and*Saccharomyces cerevisiae* 30 mm	
	Antifungal	-	500 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 18 mm, *Rhizopus oryzae* 14 mm and *Saccharomyces cerevisiae* 35 mm	
	Antifungal	Methanol	250 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 22 mm, *Rhizopus oryzae* 19 mm and *Saccharomyces cerevisiae* 20 mm	
	Antifungal	-	500 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 22 mm, *Rhizopus oryzae* 22 mm and *Saccharomyces cerevisiae* 20 mm	
Stem bark	Antifungal	Chloroform	250 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 14 mm, *Rhizopus oryzae* 10 mm and *Saccharomyces cerevisiae* 22 mm	
	Antifungal	-	500 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Aspergillus niger* 20 mm, *Rhizopus oryzae* 12 mm and*Saccharomyces cerevisiae* 27 mm	
	Antifungal	Methanol	250 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Rhizopus oryzae* 10 mm and *Saccharomyces cerevisiae* 18 mm	
	Antifungal	-	500 µg/disc	Nystatin Chloramphenicol (30 µg/cup)	-	*in vitro*	-	Cylinder plate assay	*Rhizopus oryzae* 16 mm and *Saccharomyces cerevisiae* 20 mm	
Leaves	Antibacterial	Ethanol	2000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*Escherichia coli* 14.5 mm, *Klebsiella pneumoniae* 11mm, *Enterobacter aerogenes* 15mm, *Vibrio cholerae* 18mm, *Salmonella paratyphi* 16mm, *Staphylococcus aureus* 11.5 and *Streptococcus faecalis* 12	
-	-	-	1000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 9mm, *K. pneumoniae* 8mm, *E. aerogenes* 10mm, *V. cholerae* 14mm, *S. paratyphi* 10mm, *S. aureus* 10mm and *S. faecalis* 9mm	[28]
-	-	-	500 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*V. cholerae* 11mm and *S. paratyphi* 8mm	
-	-	-	250 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	No activity	
-	-	Methanol	2000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 11mm, *K. pneumoniae* 9mm, *E. aerogenes* 12mm, *V. cholerae* 15mm, *S. paratyphi* 13mm, *S. aureus* 12mm and *S. faecalis* 11mm	
-	-	-	1000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 9mm, *E. aerogenes* 10mm, *V. cholerae* 12mm, *S. paratyphi* 10mm, *S. aureus* 10mm and *S. faecalis* 9mm	
-	-	-	500 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*V. cholerae* 10mm and *S. paratyphi* 9mm	
-	-	-	250 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	No activity	
-	-	Petroleum ether	2000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 10mm, *K. pneumoniae* 12mm, *E. aerogenes* 10mm, *V. cholerae* 11mm, *S. paratyphi* 12mm, *S. aureus* 11mm and *S. faecalis* 10mm	

-	-	Dichloromethane	2000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 9mm, *K. pneumoniae* 11mm, *E. aerogenes* 10mm, *V. cholerae* 12mm, *S. paratyphi* 11mm, *S. aureus* 10mm and *S. faecalis* 9mm	[28]
-	-	Aqueous	2000 µg/disc	Gentamycin (10 µg/disc)/Kanamycin (10 µg/disc)	-	*in vitro*	-	Disc diffusion method	*E. coli* 10mm, *K. pneumoniae* 12mm, *E. aerogenes* 10mm, *V. cholerae* 13mm, *S. paratyphi* 12mm, *S. aureus* 11mm and *S. faecalis* 10mm	
Bark	Antioxidant	Ethyl acetate	100 µg/ml	-	DPPH radical scavenging	*in vitro*	-	-	The ethyl acetate bark extract of *Vitex leucoxylon* L.f. showed 88.52% DPPH scavenging at 100 µg/ml, close to BHT (91.45%).	[30]
Bark	Antioxidant	Ethyl acetate	100 µg/ml	-	Nitric oxide scavenging	*in vitro*	-	-	The ethyl acetate bark extract of *Vitex leucoxylon* L.f. showed 74.00% nitric oxide scavenging at 100 µg/ml, compared to BHT (82.24%).	
Bark	Antioxidant	Ethyl acetate	100 µg/ml	-	Superoxide Radical Scavenging Activity	*in vitro*	-	-	The ethyl acetate extract of *Vitex leucoxylon* L.f. bark demonstrated 74.22% superoxide scavenging activity at 100 µg/ml, against BHT (81.76%).	
Bark	Antioxidant	Ethyl acetate	100 µg/ml	-	Hydroxyl radical scavenging	*in vitro*	-	-	The ethyl acetate bark extract of *Vitex leucoxylon* L.f. showed 79.04% hydroxyl scavenging at 100 µg/ml, above BHT (73.03%).	
Leaves	Hepatoprotective	Ethanol	500 mg/kg	5-Fluorouracil (20mg/kg/day)	Normal saline (0.9 %)	*in vivo*	Diethylnitrosamine 200mg/kg	Wistar albino rats	Hepatoprotective	[31]
Leaves	Anti-inflammatory	Hydroalcoholic	50 µg/ml	-	-	*In vitro*	0.36% NaOH-induced hypotonic haemolysis.	Human red blood cell membrane stabilization	96.63 % inhibition	[34]
Leaves	Anti-inflammatory	Hydroalcoholic	100 µg/ml	-	-	*in vitro*	-	Human red blood cell membrane stabilization	90.97 % inhibition	
Leaves	Anti-inflammatory	Hydroalcoholic	1000 µg/ml	-	-	*In vitro*	-	Human red blood cell membrane stabilization	84.16 % inhibition	
Leaves	Anti-inflammatory	Ethanol	50 µg/ml	-	-	*in vitro*	-	Human red blood cell membrane stabilization	99.03 % inhibition	
Leaves	Anti-inflammatory	Ethanol	100 µg/ml	-	-	*in vitro*	-	Human red blood cell membrane stabilization	90.02 % inhibition	
Leaves	Anti-inflammatory	Ethanol	1000 µg/ml	-	-	*in vitro*	-	Human red blood cell membrane stabilization	84.43 % inhibition	
Leaves	Anti-inflammatory	-	-	Prednisolone - 50 µg/mL	-	*in vitro*	-	Human red blood cell membrane stabilization	99.03 % inhibition	
Leaves	Anti-inflammatory	-	-	Prednisolone- 100 µg/mL	-	*in vitro*	-	Human red blood cell membrane stabilization	97.23 % inhibition	
Leaves	Anti-inflammatory	-	-	Prednisolone-500 µg/mL	-	*in vitro*	-	Human red blood cell membrane stabilization	98.85 % inhibition	
Bark	Antipyretic	Ethyl acetate extract	500 mg/kg	Aspirin (300 mg/kg)	-	*in vivo*	Brewer's yeast (20% suspension)	Wistar albino rats	Ethyl acetate extract (500 mg/kg) lowered rectal temperature in pyretic rats at 2–4 h.	[35]
Leaves	Anticancer	Ethanol	250 mg/kg	5-Fluorouracil (20 mg/kg)	DAL untreated mice	*in vivo*	-	Mice	Ethanol extract showed stronger antitumor activity.	
Aerial parts	Anticancer	Aqueous	-	-	-	*in vitro*	-	Mice	A549 lung cancer cells, IC_{50} was 315.57 µg/mL and Against NCI-H460 cells, IC_{50} was 560.48 µg/mL.	
Leaves	Anthelmintic	Chloroform, Ethyl acetate, Methanol, Ethanol and Aqueous	50 to 250 mg/20 ml	Piperazine citrate (100 µg/ml)	-	*in vivo*	-	*Pheretima posthuma*	Methanol extract showed well result at 250 mg/20 ml.	[38]
Leaves	CNS Depressant	Ethanol	50mg/1 ml & 100 mg/1 ml	Chlorpromazine (25 mg)	5 % gum acacia 1ml/kg	*in vivo*	Hypothermia	Wistar albino rats	At 250 and 500 mg/kg, it significantly reduced locomotor activity, peaking at 1 h, though less than the standard drug.	[39]
Leaves	Insecticidal	Powder itself	0.5gm/100 gm to 2.5 gm/100 gm	-	-	*in vivo*	-	*Corcyra cephalonica*	At 0.5 g/100 g, fecundity was 210.57 with 86.56% survival, dropping to 178.38 and 70.54% at 2.5 g/100 g.	[40]

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2. A. Fayyaz, N. Ali, Z.A. Umar, H. Asghar, M. Waqas, R. Ahmed, R. Ali, and M.A. Baig. CF-LIBS based elemental analysis of Saussurea simpsoniana medicinal plant: a study on roots, seeds, and leaves. *Analytical Sciences* 40(3): 413-427 (2024). DOI: 10.1007/s44211-023-00480-9

3. W. Bialek and S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100: 258-263 (2008). https://doi.org/10.1103/PhysRevLett.100.258101

b. Books

4. W.R. Luellen (Ed.). Fine-Tuning Your Writing. *Wise Owl Publishing Company, Madison, WI, USA* (2001).

5. U. Alon and D.N. Wegner (Eds.). An Introduction to Systems Biology: Design Principles of Biological Circuits. *Chapman & Hall/CRC, Boca Raton, FL, USA* (2006).

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7. S. Brown and L.A. Boxer. Functions of Europhiles. In: Hematology, (4^{th} ed). W.J. Williams, E. Butler, and M.A. Litchman (Eds.). *McGraw Hill, New York, USA* pp. 103-110 (1991).

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10. L.M. Highland and P. Bobrowsky. The landslide handbook–A guide to understanding landslides. Circular 1325. *US Geological Survey, Reston, Virginia* (2008).

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