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Research Article

Assessment of the antimicrobial potential of the selected phytochemically riched medicinal plants against the antibiotic resistant pathogenic bacterial strains

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Abstract

Tribal communities of Tanawal have been known for the formulations of herbal remedies and their supply to pharmaceutical industries for more than 50 years in KPK, Pakistan. This study aims to report on the ethnomedicinal uses and evaluation of the medicinal efficacy of eight selected medicinal plants of Tanawal region, KPK. These plants were selected due to their popularity and frequency of utilization among communities in the study area. Anti-microbial activities of three different kinds of plant extracts (ethanol, methanol, n-hexane) were evaluated against four strains of bacteria *Bacillus subtilis* (ATCC 6058), *Pseudomonas aeruginosa* (ATCC 7222) (gram-positive) and *Escherichia coli* (ATCC 25928), *Staphylococcus aureus* (ATCC 6537) (gram-negative) bacteria. An agar disc diffusion assay was performed to investigate the anti-bacterial activity. Results exhibited significant control of all extracts over bacterial growth but the results of ethanolic extracts were most prominent. Moreover, the choice of plant has a statistically significant influence on the zone of inhibition in the case of gram-positive strains but less effect for the gram-negative strains. Further, analysis revealed a significant effect of extract concentration on the zone of inhibition for both gram-positive and negative bacterial strains. Some concentrations are even better than the reference drugs i.e. chloramphenicol and streptomycin.

Keywords: antibacterial activity; ANOVA; bacterial strains; ethnomedicine; taxa

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Introduction

Herbs were first used in the formulation of medicines and the pharmacological treatment of diseases (Teng *et al.,* 2016). Many antibiotics used today to treat bacterial and other infections were initially extracted from natural sources, such as ethnomedicinal plants (Lewis *et al.,* 2024). Plant-based antimicrobials are an enormously underutilized source of drugs with tremendous therapeutic potential. One of the first pharmacological treatments used by healers was the use of herbs, and even today, 25% of all conventional pharmaceuticals are still made from plants in the old-fashioned way (Akbar, 2020).

Drug resistance has steadily increased as a result of widespread antibiotic usage, which is frequently therapeutically necessary. The majority of the world's population in low-income societies is severely affected by this phenomenon (Fongang *et al.,* 2023). Since resistance mechanisms have been found and documented for all known antimicrobials now used in clinical settings, antibiotic-resistant bacterial populations pose a growing threat to both animal and human health (Tadesse *et al.,* 2012; Naher *et al*., 2023).

In the globally wide-reaching, precisely in the unindustrialized countries, infectious bacterial diseases from different species of *Bacillus, Staphylococcus, Escherichia,* and *Pseudomonas* are the primary agents to cause severe contagions and the major cause of human deaths (Anju *et al.,* 2023). Today, it is estimated that more than two-thirds of the world's population rely on plant-derived drugs; For instance, Quinine (Cinchona) and berberine (Berberis), morphine (Nicotiana), vincamine (Catharanthus) are the antibiotics acquired from plant life which are exceedingly operative counter to microscopic organisms (*Staphylococcus aureus, Escherichia coli*) (Alamgir and Alamgir, 2018; Bremner, 2021) some 7000 medicinal compounds used in the Western pharmacopeia are derived from plant (Coe and Anderson, 1996).

In the Tanawal region of KPK, Pakistan, a number of medicinal have been reported for their ethnomedicinal value (Bibi *et al.,* 2022). Many of these plants are quite famous in the study area and utilized for the cure of a broad range of disorders like skin infections, gastrointestinal disorders, urinary tract, and respiratory infections etc. Bibi *et al.* (2022) studies reported that *Silybum marianum* L.*, Xanthium strumarium* L.*, Mallotus philippensis* (Lam.) Müll.Arg., *Vitex negundo* L., *Indigofera heterantha* Wall. Ex Brandis, *Rumex hastatus* D.Don, *Datura innoxia* Mill., and *Tribulus terrestris* L. have comparatively higher use values that reflect their popularity in the studied area. *Tribulus* is considered to increase general body debility and strengthening of the body while the dried herb of *Rumex hastatus* is boiled in water and then taken orally considered very useful in internal lesions, ulcers, and stomachaches while *Mallotus philippensis* has been considered useful to cure dysentery. A powdered form of *Vitex negundo* according to the local elders and healers has been employed in intestinal dysentery. *Indigofera* and *Xanthium* were associated with skin ailments, a piece of information provided by the local traditional health practicians to cure skin scabies and smallpox in children (Bibi *et al.,* 2022).

However, limited experimental evidence is available regarding the antibacterial activity of these medicinally significant plants. Hence, the main aim of the current study includes the utilization of statistical analysis such as ANOVA to describe the findings more accurately and the assessment of *in vitro* antibacterial profile of selected ethno-medicinal plants (*Silybum marianum* L., *Xanthium strumarium* L., *Mallotus philippensis* (Lam.) Müll.Arg., *Vitex negundo* L., *Indigofera heterantha* Wall. ex Brandis, *Rumex hastatus* D.Don, *Datura innoxia* Mill., *Tribulus terrestris* L) based on data gathered from local inhabitants to isolate and create novel broad range antibiotic compounds.

Materials and Methods

Sampling area and its climatic condition

Eight plant species including *Silybum marianum* L., *Xanthium strumarium* L., *Mallotus philippensis* (Lam.) Müll.Arg., *Vitex negundo* L., *Indigofera heterantha* Wall. Ex Brandis, *Rumex hastatus* D.Don, *Datura innoxia* Mill., *Tribulus terrestris* L. were selected based on their ethno-medicinal properties reported in published ethnobotanical data of Tanawal area KPK, Pakistan (Bibi *et al.,* 2022).

The Tanawal region is located 74 kilometres north of the capital Islamabad in the province of Khyber Pakhtunkhwa in Pakistan, at an elevation of 1374 meters above the sea. Its inhabitants are predominantly agriculturists. The valley is located between the latitude of 34.36 (34° 21' 30 N) and longitude of 73.07 (73° 4' 0 E), with an average elevation of 1374 meters above the sea (Figure 1).

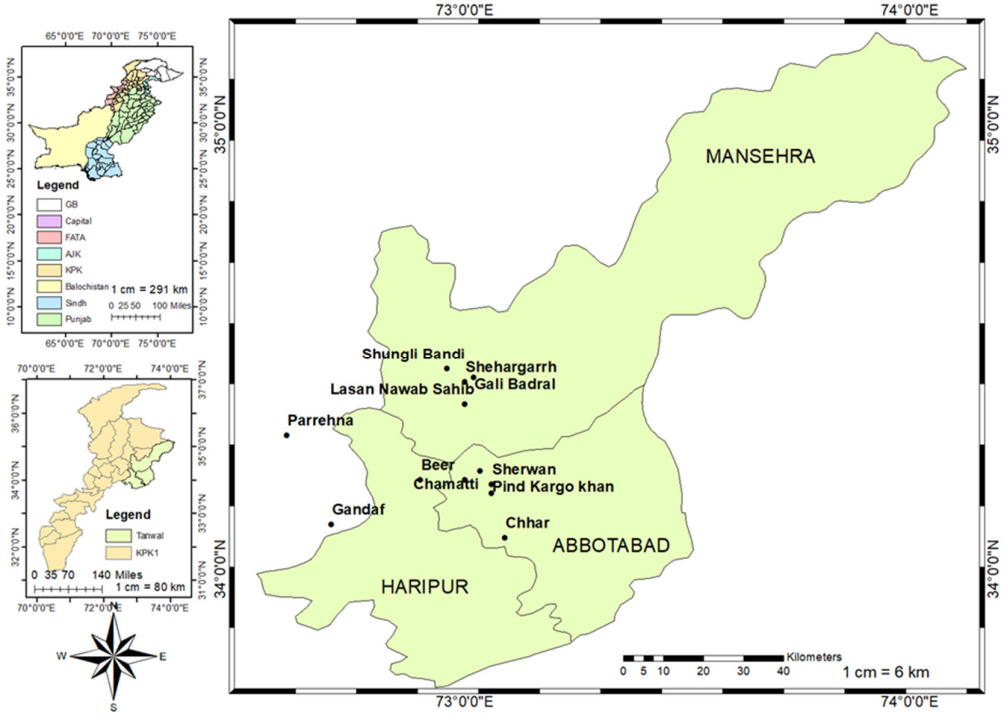

Figure 1. Index map of sampling sites from Tanawal, KPK

The climate of the area is a moist temperate type and the region has two rainy seasons: January-February and June-July respectively. The elevated areas are mountainous where snowfall occurs in winter. During the data collection, botanical descriptions such as root, stem, leaf, seasonal availability, habit, habitat, and climatic zones have been recorded. Samples were identified with the help of Flora of Pakistan (http://www.efloras.org) (Nazish *et al.,* 2019; Nazish and Althobaiti, 2022). The collected plant material was shade-dried for 3-4 days and then ground finely (Figure 2). The fine powdered material was kept in air-tight zipper bags and stored at 4 °C for the preparation of crude extracts in many solvents of diverse polarity i.e. methanol, ethanol, and n-hexane.

Figure 2. A. *Datura innoxia* Mill**. B.** *Indigofera heterantha* Wall. ex Brandis **C.** *Mallotus philippensis* (Lam.) Müll.Arg. **D.** *Rumex hastatus* D.Don **E.** *Silybum marianum* L **F.** *Tribulus terrestris* L. **G.** *Xanthium strumarium* L. **H.** *Vitex negundo* L.

Preparation of the plant extracts

The 20 grams of each plant powder was soaked in a flask in 200 ml of the solvent (methanol, ethanol, and n-hexane) for 3 days with occasional shaking to facilitate extraction. After 3 days the mixture was filtered through Whatman No.1 filter papers in a Buchner funnel using a suction pump. The same amount of solvent was added to the leftover residue of filtration and the whole process was repeated for a further 3 days. This cycle was repeated for 18 days to get the final extracts. Later the extracts were concentrated to dryness with the help of a rotary evaporator (Brinkmann rotavapor, Model # R) at 40 °C and under reduced pressure. Concentrated extracts were stored at 4 °C in the refrigerator (Serdar *et al*., 2015; Nahir *et al.,* 2023).

Further dilution was prepared by dissolving dry extracts (16.5 mg) in 10 ml of DMSO. This stock solution of 16.5 mg/10 ml was further diluted to 13.5 mg/10 ml, and 11.5 mg/10 ml concentrations of the extract. Solutions of a standard antibiotic 2 mg/ml of each of chloramphenicol and streptomycin were also prepared. The standard antibiotics and pure DMSO solutions were used for positive and negative controls respectively.

Antibacterial activity

The agar disc diffusion assay was performed to investigate anti-bacterial activity (Krishnan *et al*., 2019). Four strains of bacteria *Bacillus subtilis* (ATCC 6058), *Pseudomonas aeruginosa* (ATCC 7222), gram-positive, and *Escherichia coli* (ATCC 25928), *Staphylococcus aureus* (ATCC 6537) gram-negative bacteria were used.

Preparation of bacterial cultures

Nutrient broth medium (MERCK) was used to grow bacteria for inocula preparation. It was composed of peptone (5 gm/L) and meat extract (3 gm/L) while nutrient agar medium (MERCK) was composed of peptone (5 g/L), meat extract (3 gm/L), and agar-agar (12 gm/L). Nutrient broth medium was prepared by dissolving 0.8 g/100 ml of nutrient broth in distilled water while nutrient agar medium was prepared by dissolving 2 g of nutrient agar in 100 ml of distilled water (pH 7.0) and was autoclaved (Bakhtiyari *et al.,* 2022).

McFarland 0.5 $BaSO_4$ turbidity standard

McFarland 0.5 $BaSO_4$ was used to compare the turbidity of bacterial culture. The standard was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 N H_2SO_4. Barium sulfate turbidity standard (4 to 6 ml) was taken in a screw-capped test tube and was utilized to compare the turbidity (Khormali *et al.,* 2023).

Assay procedure (Agar well method)

A nutrient agar medium was prepared by suspending nutrient agar (MERCK) 2 g in 100 ml distilled water (pH 7.0) and autoclaved. It was allowed to cool up to 45 °C. Then it was seeded with 1 ml of prepared inocula to have 106 CFU per ml. Petri plates (14 cm) were prepared by pouring 75 ml of seeded nutrient agar and allowed to solidify. Wells were made in the agar plate with sterile cork borer (8 mm). Using micropipette, 100 µl of test solutions (plant extract) was poured in respective well. For positive control solutions of a standard antibiotic 2 mg/ml of each of chloramphenicol and streptomycin and for negative control (DMSO) was poured to each Petri plate. Finally, the Petri plates were incubated at 37 °C for 24 h. Then clear (inhibition) zones were detected around each hole. DMSO alone (0.1 ml) was used as a control under the same condition for each sample, and, by subtracting the diameter of inhibition zone resulting with DMSO from that obtained in each case, antibacterial activities were calculated as a mean of 3 replicates. Diameter of the clear zones, showing no bacterial growth, around each well was measured with the help of vernier caliper. Triplicate plates were prepared for each sample.

Statistical analysis

Two-way ANOVA was performed on Microsoft Excel-2021 (XL Miner Analysis Tool Pak).

Results

Here, eight ethnomedicinal plants of a Tanawal region, KPK, Pakistan used for different remedies by local communities were tested against gram-positive (*Bacillus subtilis*, *Pseudomonas aeruginosa*) and gram-negative (*Escherichia coli, Staphylococcus aureus*) bacterial cultures to investigate their antibacterial potential. Botanical descriptions such as root, stem, leaf, seasonal availability, habit, habitat, and climatic zones are represented in Table 1. In addition, the ethnomedicinal importance of studied plants was cross-verified with the available ethnobotanical or ethnomedicinal literature from Pakistan and worldwide (Table 2). This data showed that these plants have diverse ethnomedicinal importance in different cultures. The phytochemical literature also exhibited the presence of antibacterial compounds such as alkaloids, flavonoids, terpenes, phenolics, and volatile oils.

The values of zones of inhibition represented the degree of antimicrobial effect of plant extracts on all four bacterial strains (Figures 3-5). All three extracts (methanol, ethanol, and n-hexane) were effective against bacterial cultures, but the ethanolic extracts were found to be more prominent extract for antibacterial activity. The results of antibacterial activity were presented according to the tested concentrations of extracts.

Table 1. Morphological characteristics of the studied taxa

Family	Scientific Name	Common name	Habitat	Habit	Life span	Level of threats in Pakistan	Status of species	Roots	Stem	Leaves	Fruits	Flower	Season
Asteraceae	*Silybum marianum* (L.) Gaertn.,	Oontkatara	Fertile soils and sheep camps	Herb	Biennial	Low	Tropical-Subtropical	Taproot	Glabrous or slightly woolly	Leaves have shiny, green upper surfaces and are noticeably variegated with white markings.	Plume is made up of fine bristles	Purple thistle flower heads develop at the apex of the stems.	April to July
Asteraceae	*Xanthium strumarium* L.	Choteaghokroo	Roadsides and along riverbanks	Herb	Annual	Low	Tropical	Taproot	Short, stout, hairy slightly ribbed	Broad alternate triangular-ovate/ suborbicular Light-bright green while irregular lobes having long petiole	Obovoid, enclosed in the hardened involucre, with two hooked beaks and hooked bristles. fruits have hooked bristles and two strong hooked beaks	Monoecious and are pollinated by insects. Flower heads are in terminal and axillary racemes and are white or green.	July- October

Euphorbiaceae	*Mallotus philippensis* (Lam.) MüllArg.	Kameela	Forest clearings	Tree	Perennial	Low	Tropical-Subtropical	Taproot	Slender branched	Alternate and simple, or leathery, ovate to lanceolate, cuneate to rounded. acute or acuminate, hairy and reddish glandular beneath, and reddish-brown in color	depressed-globose; 3-lobed stellate; puberulous; with abundant orange or reddish glandular granules; 3-seeded	Male flowers in terminal to axillary, solitary or fascicled paniculate spikes, flower with numerous stamens, small; female flowers spikes or slender racemes, stellate hairy, 3 papillose stigmas	April- july
Lamiaceae	*Vitex negundo* L.	Marwaan	*Open Foresrs*	Herb		Low	Tropical	Taproot	Aromatic, deciduous shrub	tri- or penta-foliate leaves on quadrangular branches	small, ovoid or obovoid, four seeded drupes, black when ripe	Bluish-purple coloured the flowers are small, lanceolate, in panicles up to 30 cm long	July to October
Papiolanaceae	*Indigofera heterantha* Wall. ex Brandis.	Kainthee	Grassy Field	Herb		Low		Branched tap root	Erect, sub-erect, Branclets, argente-canisent	Pinnnate, short petioled, leaflets small, opposite, 17-25, lanceolate-oblong, sub coraceous, covered with white bristles above, glaucous and thinly argenteo, canisent below	Small pod sub cylindrical, glabrous	Racemes pedicillate, 12-20-flowered; calyx companulate; corolla purpurlish red, canisent externally	June through October
Polygonaceae	*Rumex hastatus* D.Don	Khatimbal	Cultivated beds	Herb	annual, biennial and perennial	Low	Subtropical	Tap root	Vertical, climbing to horizontal, branched, not void or sulcate.	Petioles of the same length as the blade; blade hastate, panicles terminal with erect divergent	Valves pinkish, orbicular or kidney shaped, membranous, nearly pellucid, with small rounded projection at base, base deeply heart shaped, edge nearly intact	Polygamous. Male flowers: tepals nearly identical. Female flowers: outer tepals elliptic	March-April
Solanaceae	*Datura innoxia* Mill.	Tatulaa	Unculti vated Fields	Herb	annual	High	Tropical-Subtropical	Branched taproot	Slender and tend to become hollow with age	Coarse with whole margin and are gray-velvety turning dark green	Brown hard spherical-shaped capsules, densely covered in slender spines less than 10mm long	White, solitary, large funnel shaped and up to 200 mm in length	April- September
Zygophyllaceae	*Tribulus terrestris* L.	Bhakrra	Dry loose and Sandy Soil.			Low	Temperate	Slender, fibrou, cylindrical, frequently branched	About 2 meters long,	Inverse and brief approximately 1.25 cm in length.	Rough projections and are wooded around 1 cm in breadth which possesses spikes about 6 mm long	Yellow petal flowers and thorny fruits.	September - March

Table 2. Ethnomedicinal utilization and phytochemistry of studied taxa having anti-microbial potential

Plant species	GIT	Sexual Disorders	Immunity booster	Respiratory Disorders	Urinary Tract Infections	Dermal Disorders	Secondary metabolites reported in the literature
Silybum marianum (L.) Gaertn.	+ (Abdel-Latif *et al.,* 2023)	+ (Devi *et al.,* 2019)	+ (Abdel-Latif *et al.,* 2023)	+ (Wang *et al.,* 2020)	+ (Abed *et al.,* 2018)	+ (Fan *et al.,* 2019)	Flavonoids, tannins, coumarins, volatile oil, terpenoids (Eldalawy *et al.,* 2021)
Xanthium strumarium L.	+ (Usman *et al.,* 2021)	+ (Kozuharova *et al.,* 2019)	+ (Lin *et al.,* 2014)	+ (Linh *et al.,* 2021)	+ (Buha and Acharya, 2020)	+ (Buha and Acharya, 2020)	Alkaloids, flavonoids, terpenes, phenolics (Abdulkhaleq *et al.,* 2022; Ezghayer *et al.,* 2024)
Mallotus philippensis (Lam.) Müll.Arg.	+ (Kumar *et al.,* 2020)	+ (Mishra *et al.,* 2015)	+ (Gill *et al.,* 2018)	+ (Dharajiya *et al.,* 2015)	+ (Rizvi *et al.,* 2024)	+ (Saikia *et al.,* 2006)	Coumarin, diterpenoids, flavonoids, Isocoumarins, phenols, steroids Triterpenoids (Bilal *et al.,* 2022)
Vitex negundo L.	+ (Dave *et al.,* 2018)	+ (Jan *et al.,* 2017)	+ (Gerometta *et al.,* 2020)	+ (Dzoyem *et al.,* 2014)	+ (Benil *et al.,* 2023)	+ (Kayani *et al.,* 2015)	Casticin, Chrysophanol D, fructose, Iso-orientin, *p*-hydroxybenzoic acid (Bharti *et al.,* 2023)
Indigofera heterantha Wall. ex Brandis.	+ (Dzoyem *et al.,* 2014; Benil *et al.,* 2023)	+ (Kayani *et al.,* 2015; Benil *et al.,* 2023)	+ (Zeb *et al.,* 2021)	+ (Benil *et al.,* 2023)	+ (Benil *et al.,* 2023)	+ (Verma *et al.,* 2020)	Alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins, triterpenoids (Ullah *et al.,* 2022)
Rumex hastatus D.Don	+ (Najafabadi *et al.,* 2020)	+ (Najafabadi *et al.,* 2020)	+ (Soliman *et al.,* 2019)	+ (Najafabadi *et al.,* 2020)	+ (Soni *et al.,* 2012)	+ (Najafabadi *et al.,* 2020)	Alkaloids, terpenes, phenolics, flavonoids (Andleeb *et al.,* 2018)

Datura innoxia Mill.	+ (Mahmood *et al.*, 2011)	+ (Ayuba *et al.*, 2012; Deshpande *et al.*, 2023)	+ (Chouhan *et al.*, 2024)	+ (Mahmood *et al.*, 2011)	+ (Sharma *et al.*, 2021)	+ (Naseri and Khazaei, 2019)	Alkaloids, amino acids, carbohydrates, cardiac glycosides, flavonoids, phenolic compounds, tannins (Sharma *et al.*, 2021)
Tribulus terrestris L.	+ (Kheirollahi *et al.*, 2019)	+ (Kheirollahi *et al.*, 2019)	+ (Al-Bayati and Al-Mola, 2008)	+ (Abbas *et al.*, 2024)	+ (Al-Fatimi, 2019)	+ (Al-Fatimi *et al.*, 2007)	Alkaloids, cinnamic acid amides flavonoids, lignanamides saponins, steroids (Ştefănescu *et al.*, 2020)

Zone of inhibition

At 11.5 mg/ml

At the concentration of 11.5 mg/ml for *Bacillus subtilis,* a maximum value of the Zone of inhibition was recorded for *T. terrestris* (8.5mm) in methanolic and ethanolic extract while minimum values were observed for *I. heterantha* (5.5 mm) in n-hexane (Figure 3A). For *Pseudomonas aeruginosa,* a maximum value (9 mm) was noticed for *X. stumarium* in methanolic extract, on the other hand, methanolic extracts of *S. marianum*, *T. terrestris,* and *X. stumarium* were recorded as minimum (Figure 3B). For *Staphylococcus aureus*, the maximum value of (8 mm) was attained for *V. negundo* and *I. heterantha* in methanolic extract while *M. phillipensis* proved to be more effective in n-hexane extract on the contrary *I. heterantha* showed minimum average values (Figure 3C). In *Escherichia coli* cultures *M. phillipensis* showed the highest average values while *S. marianum* in ethanolic extract exhibited the lowest zone of inhibition (Figure 4D).

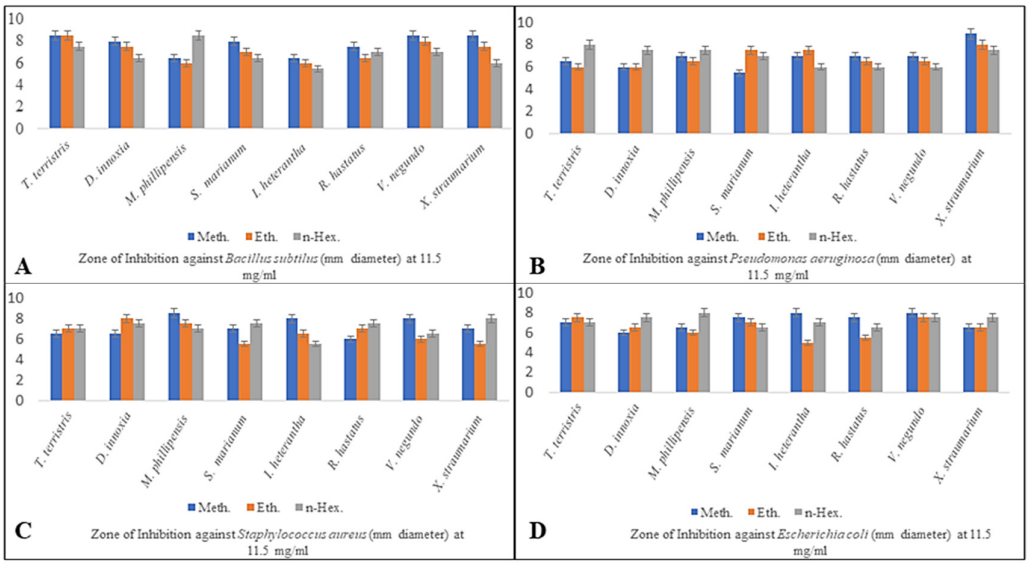

Figure 3. Zone of Inhibition of plants against at 11.5 mg/ml against bacterial strain **A.** *Bacillus subtilis* **B.** *Pseudomonas aeruginosa* **C.** *Staphylococcus aureus* **D.** *Escherichia coli*

At 13.5 mg/ml

At the concentration of 13.5 mg/ml of *Bacillus subtilis,* the highest inhibition (11 mm) was recorded for *T. terrestris* in methanolic extract while ethanolic extract of *M. phillipensis* showed the minimum inhibitory effect of (7 mm) (Figure 4A). Against *Pseudomonas aeruginosa,* the highest value (12 mm) was recorded for *V. negundo and X. stumarium* in methanolic extract while the methanolic extract of *S. marianum* showed minimum effect (7 mm) (Figure 4B). Against *Staphylococcus aureus* ethanolic extract of *X. stumarium* showed the highest inhibition (12 mm) while *D. innoxia* showed minimum inhibition (7.5 mm) in ethanolic extract

(Figure 4C). *Escherichia coli* had a maximum inhibition of (11.5 mm) in n-hexane extracts of *V. negundo* and *M. phillipensis* while the methanolic extract of *D. innoxia* showed minimum inhibition (7 mm) in methanolic extract (Figure 4D).

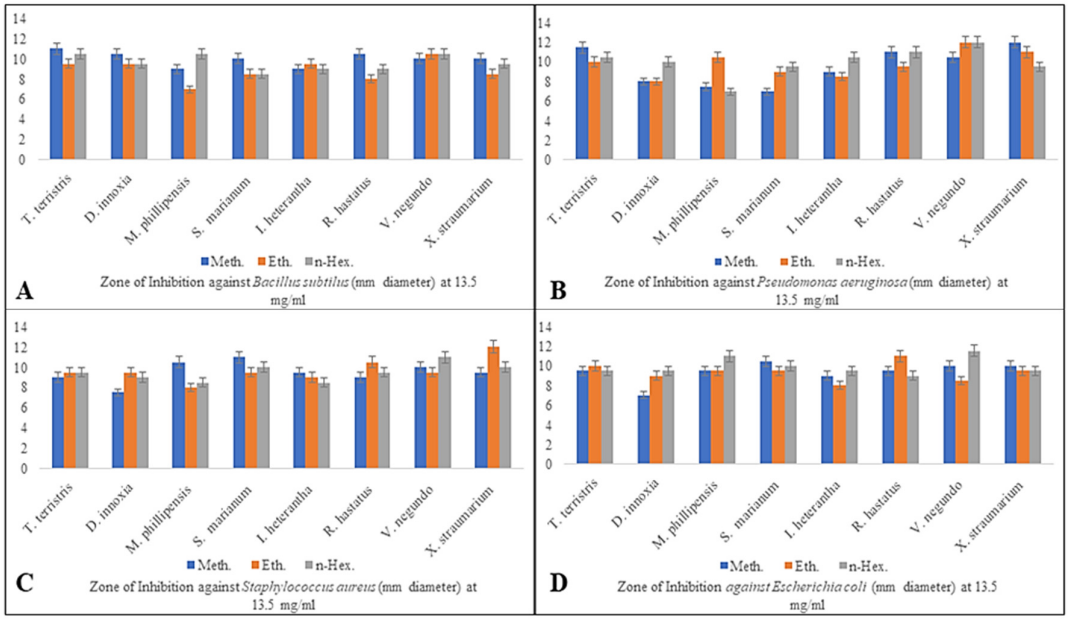

Figure 4. Zone of Inhibition of plants against at 13.5 mg/ml against bacterial strain **A.** *Bacillus subtilis* **B.** *Pseudomonas aeruginosa* **C.** *Staphylococcus aureus* **D.** *Escherichia coli*

At 16.5 mg/ml

At the concentration of 16.5 mg/ml against *Bacillus subtilis,* chloramphenicol showed a maximum value of 11.75 mm in *D. innoxia* while a minimum value (6.75 mm) in n-hexane extract of *R. hastatus* (Figure 5A). For *Pseudomonas aeruginosa* maximum values were recorded in chloramphenicol for *D. innoxia* and *V. negundo* (11.5 mm) (Figure 5B) For the *Staphylococcus aureus, D. innoxia* exhibited maximum value in streptomycin while *S. Marianum* had least value in n-hexane (Figure 5C). Lastly, *Escherichia coli* and *D. innoxia* had maximum value in streptomycin and *R. hastatus* exhibited minimum values (Figure 5D). *Vitex negundo* L., *Silybum marianum* (L.) Gaertn., and *Xanthium strumarium* L. exhibited moderate bactericidal activity against all four bacterial strains (Table 3).

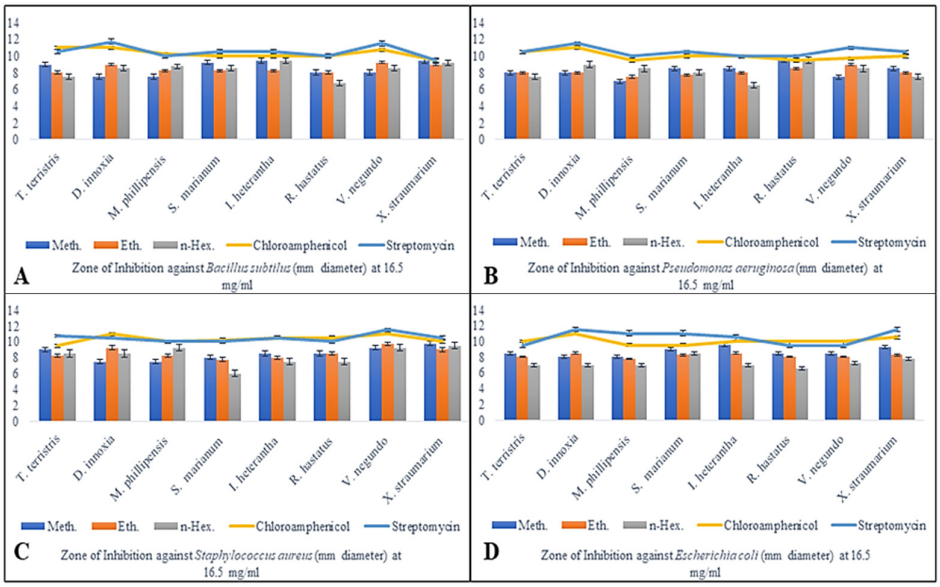

Figure 5. Zone of Inhibition of plants against at 16.5 mg/ml against bacterial strain **A.** *Bacillus subtilis* **B.** *Pseudomonas aeruginosa* **C.** *Staphylococcus aureus* **D.** *Escherichia coli*

Table 3. Plant species with the zone of inhibition against gram-positive and gram-negative bacteria

Plant Species	Extract	Zone of inhibition in (mm diameter) at 11.5 mg/ml				Zone of inhibition in (mm diameter) at 13.5 mg/ml				Zone of inhibition in (mm diameter) at 16.5 mg/ml			
		G+VE		G-VE		G+VE		G-VE		G+VE		G-VE	
		Bacillus subtilis	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus aureus*
Tribulus terrestris L.	Meth.	8.5	6.5	7	6.5	11	11.5	9.5	9	9	8	8.5	9
	Eth.	8.5	6	7.5	7	9.5	10	10	9.5	8	8	8	8.25
	n-Hex.	7.5	8	7	7	10.5	10.5	9.5	9.5	7.5	7.5	7	8.5
	Chloraamphenicol	-	-	-	-	-	-	-	-	11	10.5	10	9.5
	Streptomycin	-	-	-	-	-	-	-	-	10.5	10.5	9.5	10.75
Datura innoxia Mill.	Meth.	8	6	6	6.5	10.5	8	7	7.5	7.5	8	8	7.5
	Eth.	7.5	6	6.5	8	9.5	8	9	9.5	9	8	8.5	9.25
	n-Hex.	6.5	7.5	7.5	7.5	9.5	10	9.5	9	8.5	9	7	8.5
	Chloraamphenicol	-	-	-	-	-	-	-	-	11	11	11	11
	Streptomycin	-	-	-	-	-	-	-	-	11.75	11.5	11.5	10.5
Mallotus philippensis (Lam.) Müll.Arg. .	Meth.	6.5	7	6.5	8.5	9	7.5	9.5	10.5	7.5	7	8	7.5
	Eth.	6	6.5	6	7.5	7	10.5	9.5	8	8.25	7.5	7.75	8.25
	n-Hex.	8.5	7.5	8	7	10.5	7	11	8.5	8.75	8.5	7	9.25
	Chloraamphenicol	-	-	-	-	-	-	-	-	10.25	9.5	9.5	10
	Streptomycin	-	-	-	-	-	-	-	-	10	10	11	10
Silybum marianum (L.) Gaertn.	Meth.	8	5.5	7.5	7	10	7	10.5	11	9.25	8.5	9	8
	Eth.	7	7.5	7	5.5	8.5	9	9.5	9.5	8.25	7.75	8.25	7.75

	n-Hex.	6.5	7	6.5	7.5	8.5	9.5	10	10	8.5	8	8.5	6
	Chloraamphenicol	-	-	-	-	-	-	-	0	10	10	9.5	10.25
	Streptomycin	-	-	-	-	-	-	-	0	10.5	10.5	11	10
Indigofera heterantha Wall. ex Brandis	Meth.	6.5	7	8	8	9	9	9	9.5	9.5	8.5	9.5	8.5
	Eth.	6	7.5	5	6.5	9.5	8.5	8	9	8.25	8	8.5	8
	n-Hex.	5.5	6	7	5.5	9	10.5	9.5	8.5	9.5	6.5	7	7.5
	Chloraamphenicol	-	-	-	-	-	-	-	0	10	10	10	10.5
	Streptomycin	-	-	-	-	-	-	-	0	10.5	10	10.5	10.5
Rumex hastatus D.Don	Meth.	7.5	7	7.5	6	10.5	11	9.5	9	8	9.5	8.5	8.5
	Eth.	6.5	6.5	5.5	7	8	9.5	11	10.5	8	8.5	8	8.5
	n-Hex.	7	6	6.5	7.5	9	11	9	9.5	6.75	9.5	6.5	7.5
	Chloraamphenicol	-	-	-	-	-	-	-	0	10	9.5	10	10.5
	Streptomycin	-	-	-	-	-	-	-	0	10	10	9.5	10
Vitex negundo L.	Meth.	8.5	7	8	8	10	10.5	10	10	8	7.5	8.5	9.25
	Eth.	8	6.5	7.5	6	10.5	12	8.5	9.5	9.25	9	8	9.75
	n-Hex.	7	6	7.5	6.5	10.5	12	11.5	11	8.5	8.5	7.25	9.25
	Chloraamphenicol	-	-	-	-	-	-	-	0	10.75	9.75	10	11
	Streptomycin	-	-	-	-	-	-	-	0	11.5	11	9.5	11.5
Xanthium strumarium L.	Meth.	8.5	9	6.5	7	10	12	10	9.5	9.5	8.5	9.25	9.75
	Eth.	7.5	8	6.5	5.5	8.5	11	9.5	12	9	8	8.25	9
	n-Hex.	6	7.5	7.5	8	9.5	9.5	9.5	10	9.25	7.5	7.75	9.5
	Chloraamphenicol	-	-	-	-	-	-	-	0	9.5	10	10.5	10
	Streptomycin	-	-	-	-	-	-	-	-	9.5	10.5	11.5	10.5

Statistical interpretation

Gram +ve Bacteria

The analysis indicates that the choice of plant has a significant main effect on the zone of inhibition against *Bacillus subtilis* (F(df) = 0.804964238, p-value = 0.908139076) and *Pseudomonas aeruginosa* (F(df) = 0.818227874, p-value = 0.890497693) (Figure 6). This suggests that different plants exhibit varying levels of effectiveness in inhibiting the growth of bacteria. The analysis further reveals a significant main effect of concentration on the zone of inhibition for *Bacillus subtilis* (F(df) = 93.12799582, p-value = 0) and *Pseudomonas aeruginosa* (F(df) = 89.49969374, p-value = 0). This indicates that different concentrations of the leaf extract significantly impact the inhibitory effects against *Bacillus subtilis* and *Pseudomonas aeruginosa*. It implies that certain concentrations are more effective in inhibiting bacterial growth than others. Overall, the results suggest that the plant species and concentration influence the zone of inhibition against both bacteria. This indicates that certain plants and specific concentrations of the leaf extract possess antimicrobial properties that are effective against both bacteria (Table 4).

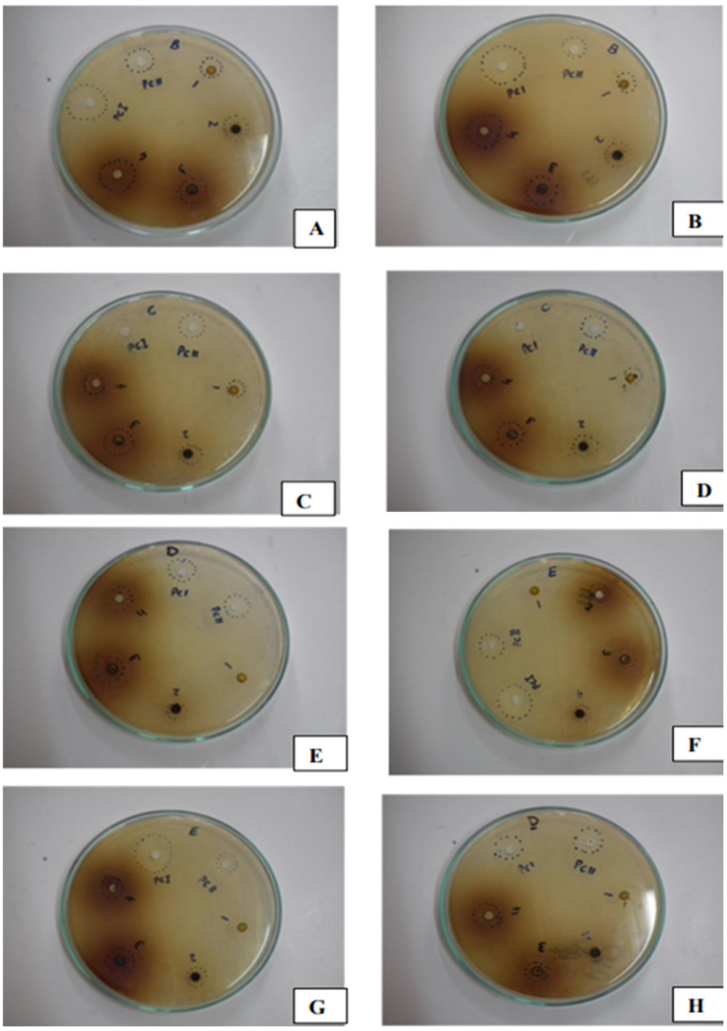

Figure 6. (A) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Escherichia coli*, 1= *Silybum marianum,* 2= *Datura innoxia* 3= *Indigofera heterantha*, 4=*Mallotus philippensis*, PC1=Streptomycin, PCII= Chloramphenicol; (B) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Escherichia coli* 1= *Rumex hastatus*, 2= *Xanthium strumarium*, 3= *Vitex negundo*, 4= *Tribulus terrestris*, PC1=Streptomycin, PCII= Chloramphenicol; (C) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Staphylococcus aureus,* 1= *Silybum marianum,* 2= *Datura innoxia* 3= *Indigofera heterantha*, 4=*Mallotus philippensis*, PC1=Streptomycin, PCII= Chloramphenicol; (D) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Staphylococcus aureus* 1= *Rumex hastatus*, 2= *Xanthium strumarium*, 3= *Vitex negundo*, 4= *Tribulus terrestris*, PC1=Streptomycin, PCII= Chloramphenicol; (E) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Bacillus subtilis*, 1= *Silybum marianum,* 2= *Datura innoxia* 3= *Indigofera heterantha*, 4=*Mallotus philippensis*, PC1=Streptomycin, PCII= Chloramphenicol; (F) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Bacillus subtilis* 1= *Rumex hastatus*, 2= *Xanthium strumarium*, 3= *Vitex negundo*, 4= *Tribulus terrestris*, PC1=Streptomycin, PCII= Chloramphenicol; (G) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Pseudomonas aeruginosa*, 1= *Silybum marianum,* 2= *Datura innoxia* 3= *Indigofera heterantha*, 4=*Mallotus philippensis*, PC1=Streptomycin, PCII= Chloramphenicol; (H) Zone of inhibition of ethanolic plant extracts at 16.5 mg/ml against *Pseudomonas aeruginosa* 1= *Rumex hastatus*, 2= *Xanthium strumarium*, 3= *Vitex negundo*, 4= *Tribulus terrestris*, PC1=Streptomycin, PCII= Chloramphenicol

Gram –ve bacteria

The analysis reveals that the choice of the plant does not have a significant main effect on the zone of inhibition against *Staphylococcus aureus* (F(df) = 0.816407495, p-value = 0.893038038) and *Escherichia coli* (F(df) = 0.80577938, p-value = 0.90711274). This suggests that the different plants tested do not exhibit substantial variations in their effectiveness in inhibiting the growth of Staphylococcus bacteria. Moreover, the significant main effect of concentration on the zone of inhibition *Staphylococcus aureus* (F(df) = 92.28680135, p-value = 0) *Escherichia coli* (F(df) = 89.4272261, p-value = 0). This implies that different concentrations of the leaf extract significantly impact the inhibitory effects against these gram-negative strains. Certain concentrations are more effective in inhibiting bacterial growth than others.

Overall, the findings confirm that the choice of the plant does not have a statistically significant influence on the zone of inhibition against both gram-negative bacteria (Figure 7). However, the choice of concentration of the leaf extract does have a significant impact (Table 4).

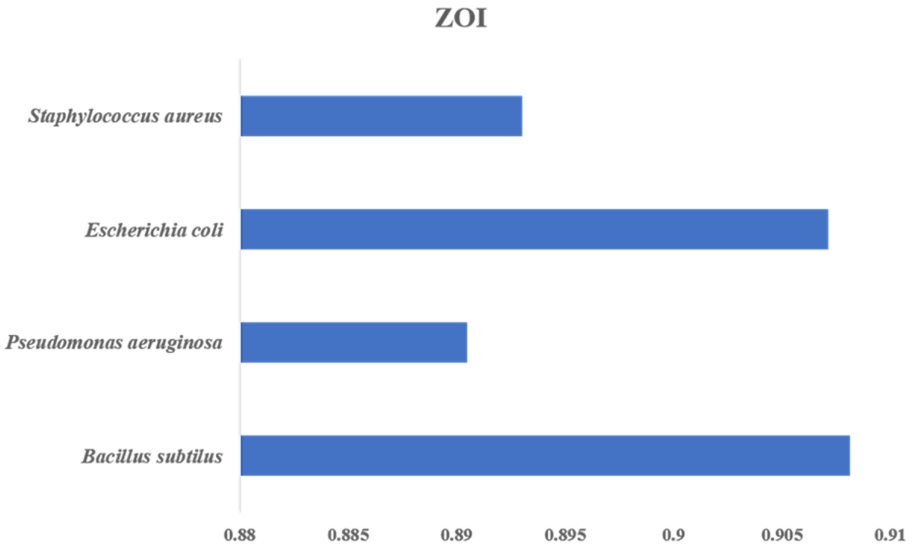

Figure 7. Statistical differences among the Zone of inhibitions of different plant extracts for different testing bacteria using ANOVA

Table 4. Statistical interpretation of results through two-way ANOVA

Zone of inhibition													
Bacillus subtilis							***Pseudomonas aeruginosa***						
Source of Variation	SS	df	MS	F	P-value	F crit	Rows	670.7076	119	5.636199	0.818228	0.890498	1.29097
Rows	659.3306	119	5.540593	0.804964	0.908139	1.29097	Columns	1233.001	2	616.5007	89.49969	0	3.033758
Columns	1282.006	2	641.0028	93.128	0	3.033758	Error	1639.415	238	6.888299			
Error	1638.161	238	6.88303				Total	3543.124	359				
Total	3579.497	359					Rows	670.7076	119	5.636199	0.818228	0.890498	1.29097
Escherichia coli							***Staphylococcus aureus***						
Source of Variation	SS	df	MS	F	P-value	F crit	Source of Variation	SS	df	MS	F	P-value	F crit
Rows	642.0243	119	5.395162	0.805779	0.907113	1.29097	Rows	665.9151	119	5.595925	0.816407	0.893038	1.29097
Columns	1197.535	2	598.7674	89.42723	0	3.033758	Columns	1265.128	2	632.5641	92.2868	0	3.033758
Error	1593.549	238	6.695582				Error	1631.33	238	6.854329			
Total	3433.108	359		0	3.033758		Total	3562.373	359				

Discussion

The emphasis has been drawn on the medicinal aspects and antibacterial potential of ethnobotanical plants. Various researchers in different parts of the world have contributed to explore the ethnomedicinal flora and antimicrobial capabilities of important plants (Ali *et al.,* 1999; Aziz *et al.,* 2016; Ali and Qaiser, 2009; Anwar *et al.,* 2009; Atta *et al.,* 2023; Beura and Raul, 2024; Aboukhalaf *et al.,* 2024). *Indigofera heterantha* locally called kainthee is an affiliate of the Leguminosae family found in the Himalayan region of Pakistan (Tanawal Valley) exhibited remarkable antibacterial activity against all the selected pathogenic bacteria. Rahman *et al.* (2011) conducted a study on the antibacterial activities of some wild medicinal plants collected from the western Mediterranean coast, Egypt, and found them as natural alternatives for infectious disease treatment. They showed results similar to our findings. The antibacterial activity of the methanol extracts of the aerial parts of the *Datura innoxia* showed a significant amount of bactericidal activity contradictory to the results of Eftekhar *et al.* (2005) that showed little or no antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. *Vitex negundo* L. (Verbenaceae) is a small, sweet-smelling herb scattered throughout the Tanawal region. It was one of the extensively used plants with high medicinal importance in the study area. Major constituents such as alkaloids, flavonoids, tannins, and essential oils play a significant role in antibacterial activity by disrupting bacterial cell membranes and inhibiting bacterial growth (Abbas *et al.,* 2024). The entire plant is medicinally significant and possesses anti-allergic, insecticidal, antibacterial, antifungal, and mosquito-repellent activities (Akbar, 2020). In the present study, it showed its maximum activity against *Bacillus subtilis* and *Pseudomonas areoginosa*. Betulinic acid, ursolic acid, and b sitosterol are some of its active constituents isolated from its leaves (Ladda and Magdum, 2018), while Silybin (formerly called silymarin) hauls out from the fruits of *Silybum marianum* (Compositae), current findings about this plant confirms the reported components of the plant (Lee *et al.,* 2003; Křen and Valentová, 2022). *Rumex hastatus* is a diuretic, refrigerant, and freshening agent and the roots of *Rumex hastatus* are purgative (Radyowijati *et al.,* 2003). The antibacterial activity of *R. hastatus* was established and its methanolic extract of roots showed noteworthy concentration-dependent antibacterial activity. Identification of natural antibacterial agents from various sources can act effectively against disease-causing foodborne bacteria which is one of the major concerns throughout the world (Patra *et al.,* 2015). The occurrence of saponins, flavonoids, alkaloids, lignanamides, and cinnamic acid amides has been reported in *T. terrestris* (Tadesse *et al.,* 2012) that are solely the agents behind its significant ability to inhibit bacterial growth. *Mallotus philippensis* contains a large portion of hydroxy-acetophenone along other natural composites that influence the antibacterial activity (Cheenpracha *et al.,* 2019).

Conclusions

These plants could be considered a low-priced environmental kindly and justifiable source of bio bactericides. Also, the experiment provides some scientific rationalization for the manipulation of extracts to cure infectious diseases. The lower activity might advocate the nonexistence or inadequacy of bio-energetic components in an extract. However, it is essential that crude extracts such as these requisites can be further purified through fractionation to isolate and ascertain the compounds accountable for antibacterial activity. We also conclude that the studied area was first sightseen in history and has a diverse ethnomedicinal important flora that could be explored in number of ways.

Authors' Contributions

Writing the original article: T.A. and A.M.; reviewing and editing: W.H., M.A.Z., M.N., B.A., M.N., M.U.H., A.H. and E.F.A; funding acquisition: A.H. and E.F.A. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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