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Analyzing the antimicrobial efficacy of the economically important tree *Knema linifolia (Roxb.) Warb*

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ABSTRACT

Knema linifolia is widely used for fuel wood, fodder and healthcare purposes. This plant treats various diseases in different parts of India, including Assam, Meghalaya, Alipurduar and Darjeeling districts of West Bengal. This study was carried out to determine the bactericidal properties of various parts of *K. linifolia* aqueous extract. The aqueous extract of the leaves, bark, stem and plant sap were tested against *Escherichia coli* (gram-negative bacteria) & *Staphylococcus aureus* (gram-positive bacteria). Among the tested extracts, both the leaf and bark extracts were found to have high bactericidal potential and can kill more than 60% of both bacterial strains with a concentration of 300µg/mL through an agar diffusion test. The MIC (Minimum Inhibitory Concentration) values for the leaf and bark extracts were recorded at ≤1000µg/mL & ≤500µg/mL, respectively. It has also been found that both the bark and leaf extracts contain high tannins, which might be essential for the antibacterial properties of *Knema* sp. There is currently a lack of proper documentation on using *K. linifolia*, which makes it challenging to conduct clinical or commercial research to support new uses in modern phototherapy. This study aims to fill this gap and provide significant information that could lead to changes in modern medicine.

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1 Introduction

Medicinal or herbal plants have always been considered an important source of antimicrobial drugs in various countries worldwide. Over the last few decades (almost 30 years), there has been a significant increase in the use of highly effective herbal ingredients for treating multiple diseases. It has been reported that about eighty percent of the global population started depending on phytomedicinal sources to support their basic healthcare needs (Chipinga et al. 2018). The reasons could be (i) people use these herbal medications as complementary therapy because healthcare costs have significantly increased in the last few decades and (ii) the use of herbal remedies or natural antidotes is a cultural trend due to the ineffectiveness or resistance issues with the conventional pharmaceuticals (Ndamba et al. 1994). A wide range of diseases has shown minimal response to pharmaceutical antidotes, and the plausible solution could be herbal supplements (Chipinga et al. 2018).

Herbal supplements are effectively utilized in treating the following conditions, such as anti-inflammation healing skin infections, to cure splenic problems & complications during pregnancy in different parts of India and have been popular in local cultures over many decades. Multiple reports have shown that natural compounds are highly effective as an alternative antimicrobial component, combating drug resistance against commercially available antimicrobials (Yang et al. 2022). In such a wide range of medicinal plants, *K. linifolia* is a valuable species, containing high amounts of bioactive compounds, and therefore, it becomes the area of research in this study (Steenkamp et al. 2004). Only a handful of literature has been explored to introduce this plant species briefly. *K. linifolia* (Knema tree) falls under the family Myristicaceae and is a deciduous plant mainly found within the tropical region of Assam and West Bengal (Barstow and Timberlake 2018). Different members of this genus show various benefits, especially as medicinal plants to treat various diseases. The diseases that the use of this plant can treat are chronic fever, jaundice, splenic problems, high inflammations, problems related to breathing trouble and compromised taste bud responses (Alen et al. 2000; Wiart et al. 2004; Dhawan 2012; Salleh and Ahmad 2017; Supriya and Sreekanth 2021; Saising et al. 2022). The tree has extensive medicinal properties, widely known among the locals of Assam and northern parts of West Bengal to treat various infections and rare illnesses. However, few research works have been published on the diversified use of the medicinal properties of *K. linifolia* and the other species of the genus *Knema*. Also, no or little work has been carried out on screening, assessment, and isolation of phytoconstituents and biological evaluation of the stem, leaf or bark extracts of *K. linifolia* for antimicrobial, anti-cancer, and anti-protozoan activities. Only two reports have been published so far, which discussed the presence of various phytochemicals like tannins, terpenes, flavonoids, steroids, etc., in some other species of the genus *Knema* (Salleh and Ahmad 2017; Supriya and Sreekanth 2021**).** The absence of proper knowledge and documentation of the genus *Knema* and over-exploitation by the local people marked many species of the genus *Knema*, including *Knema linifolia*, as "decreasing" species in the IUCN red list of threatened species (Sheeja et al. 2013). Only a few *K. linifolia* trees have been reported alive in India, mainly in Assam, Meghalaya, Alipurduar & Darjeeling districts of West Bengal (Barstow and Timberlake 2018). To search for effective alternative therapy modalities, it is important to understand the adequacy and helpfulness of *K. linifolia*, which can be utilized to treat different bacterial and other contagious diseases commercially. Hence, this research was carried out to discover the microbicidal properties of *K. linifolia* plant extracts, which could add valuable facts to the existing information pool in developing new safe antimicrobial substances from natural products. Therefore, this study has been carried out to evaluate the antimicrobial efficacy of *K. linifolia* bark, leaf, stem and plant sap samples crude extracts against two bacterial strains, *E. coli* and *S. aureus*. Along with this, the phytochemical content present in these different parts of *K. linifolia* has also been measured using standard biochemical tests.

2 Materials and Methods

2.1 Plant Sample Collection & Extraction

The Samples (Leaf, Bark, Sap, Steam) of *K. linifolia* (Roxb.) Warb were collected from the Alipurduar district of West Bengal, India. The Botanical Survey of India (BSI), Kolkata, identified the species. The collected samples (leaf, bark, sap, and stem) were brought to the laboratory, cleaned thoroughly with a brush to remove dust and debris, then washed in running tap water (pH:7), followed by drying in a hot air oven (37ºC) stored into tightly closed containers till extraction at room temperature. For extraction preparation, 200 ml of sterile water and 20g of each plant material (including bark, leaves, stems, and sap-derived compounds) were macerated in a mortar and pestle and left at room temperature for 48 hours with occasional shaking. This was followed by the filtration of the mixture through a Whatman channel paper no. 1 (7.0 cm). The same procedure has been repeated five times using water as the solvent to increase the yield. Then, the precipitates were kept at 50°C until completely dried into powder form. The dried materials of the leaf, bark, stem and sap extracts were finally held at 4°C for further experimental usage (Kamanula et al. 2017). The final concentration used in this study was 100 µg/ml.

2.2 Microorganisms and Microbial culture media

In the form of bacterial agents, *Escherichia coli* (*E. coli*; ATCC 35218) and *Staphylococcus aureus* (*S. aureus*; ATCC 25923) were used for the experiments. Mueller Hinton agar (Sigma) media was prepared as per company guidelines under aseptic conditions.

2.3 Antimicrobial Activity: Cup-plate Method

The antibacterial efficacies of different natural compounds have been regularly tested through the cup-plate method, using small holes as the reservoir of the experimental natural substances (Brantner et al. 1994). The method followed according to (Zaika 1988). In brief, 50 ml of Mueller Hinton agar has been used to prepare a standard Petri plate. After leaving the medium to settle down in the plate for 1 hour, holes/cups with a diameter of 7mm were made in the petri plates. 40 µl of the sterilized molten medium was used to seal the bottom of each cup. The cups were then filled by adding three different concentrations (100, 200, 300 µl) of the *K. linifolia* bark, leaf, stem & plant sap extract, while the sterilized distilled water was used as a negative control (1.0 mg/ml; data not shown). 100 µg/ml of ampicillin was added as positive control to one of the cups. These plates were then kept at 37°C for the next 24 hours. The experiment had been replicated thrice. The zone of inhibition was measured in diameter (mm) for *E. coli* and *S. aureus* with a vernier calliper (Das et al. 2012).

2.4 Minimal inhibitory concentration (MIC) of Bark and Leaf Aqueous Extract

Minimal inhibitory concentration (MIC) tests were considered as measuring boundaries to find the impacts of antimicrobial compounds. MIC was done in a flat-bottomed 96 well microtitre plate against *E. coli* and *S. aureus* for 24 hours at 35-37°C as described by (Paul Bhattacharya et al. 2020). Each concentration of the aqueous extract from leaf, bark, stem, and plant sap was used, and the experiment was performed in triplicate. The final concentration of *K. linifolia* bark and stem extracts ranged from 3.9-1000 µg/mL. Additional negative and positive controls were used for the experiment.

2.5 Quantitative Estimation of Phytochemicals

Phytochemicals are quantified from the leaf and bark-dried powder of *K. linifolia.* Subjective examinations were conducted for tannins, terpenoids, saponins, phenolic compounds and flavonoids. Standard chemicals used are tannic acid (SRL 92101), Linalool (Sigma-L2602), Diosgenin (TCI-D1474), Gallic acid (Sigma-G7384) & Quercetin (Sigma-Q4951) for the isolation and identification of tannins, terpenoids, saponins, phenolic compounds and flavonoids (Mandal et al. 2013; Mujeeb et al. 2014). To determine the total amount of tannins, 5g of *K. linifolia* leaf and bark powder was dissolved in 20 mL of 50% methanol and stirred for 1hr in the presence of heat. After filtration, the filtrate was mixed with 20mL of distilled water, 10ml of 17% sodium carbonate and 2.5mL of Folin-Denis reagent and left stranded for 20 minutes. The resulting bluish-green mixture was analyzed at 760nm wavelength, and the tannin content was calculated by comparing it with the respective standard.

Similarly, 5g of leaf and bark dried powder (wi) were dissolved in 9 mL of ethanol and kept for 24 hours before filtration to determine the total terpenoid amount. The filtrate was further extracted using 10mL of pet ether. The extracts were kept for complete evaporation and complete drying (wf), and the total yield was calculated by using the formula (wi-wf/wi×100) (Malik et al. 2017).

To determine the total amount of saponins, 5g of finely powdered leaf and bark were dissolved in 100 mL of Isobutyl alcohol and stirred for 5 hours before adding 20 mL of 40% saturated magnesium carbonate solution. After filtration, 2 mL of 5% $FeCl_3$ solution and 50 mL of distilled water were supplemented to the filtrate and kept for 30 minutes until the colourless solution turned blood red. The resulting blood-red solution was measured at 380nm, and the saponin content was calculated by comparing it with the respective standard.

For determining the total amount of phenolic compounds, 5g of the leaf and bark-dried powder were boiled in the presence of 50 mL of ether, kept for 15 minutes, and further distributed in a ratio of 2:1 (distilled water: extract). The resulting solution was further supplemented with 2 mL of NH_4OH, followed by adding 5 mL of pentanol and incubated for 30 minutes at room temperature. The absorbance was recorded at 505 nm, and the total phenol content was calculated by comparing it with the respective standard.

5 g of finely powdered leaf and bark were boiled in 2M HCl for 30 minutes to determine the flavonoid amount. After filtration, an equal volume of ethyl acetate was added dropwise to the filtrate. The weight of precipitated flavonoids was determined and reported as mg/g.

2.6 Statistical Analysis

All the statistical analyses were done using a Paired two-tailed Student's t-test. Each experiment was repeated thrice, and data was presented as mean ± standard deviation (SD) (Bhaumik et al. 2009).

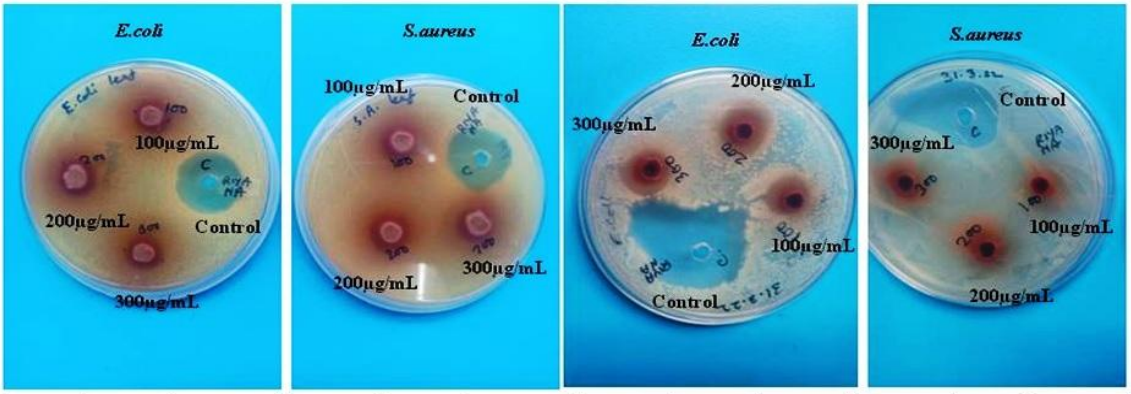
3 Results

Freshly collected plant parts, viz, leaves, fruits, and seeds of the species *Knema linifolia (Roxb.)* Warbs were collected from the Jaldapara National Park, Alipurduar, West Bengal, during the summer. The Herbarium leaves, fruit and seed samples (Figure 1) were submitted at the National Herbarium of India, Indian Botanical Garden of Calcutta and Unique Species ID and certificate provided by BSI office, Kolkata (Specimen No.:RB-01 Specimen Name: *Knema linifolia (Roxb.)Warb*).

3.1 Antibacterial Effect of Different Parts Aqueous Extract of *Knema linifolia*

The inhibitory activities of the aqueous leaf, bark, stem and sap extracts of *K. linifolia* were evaluated against gram-positive bacteria

Figure 1 *K. linifolia* (*Roxb.) Warb* A) Tree, B) Leaf, C) Leaf twig and mature fruits, D) Plant Sap ,E) Bark, F) Stem. Pictures of different parts of *Knema linifolia (Roxb.) Warb* taken at the Jaldapara national park, West Bengal (26.6960° N, 89.2855° E) on 25th April, 2022.



	ZOI (mm)	%inhibition
Control	2.3±0.43	100
100µg/ml	1.2±0.11	52.17
200µg/ml	1.3±0.24	56.52
300µg/ml	1.4±0.24	60.86

	ZOI (mm)	%inhibition
Control	2.2±0.36	100
100µg/ml	1.3±0.21	59
200µg/ml	1.3±0.18	59
300µg/ml	1.4±0.2	63.63

	ZOI (mm)	%inhibition
Control	2.7±0.17	100
100µg/ml	1.6±0.26	59.25
200µg/ml	1.7±0.05	62.96
300µg/ml	1.8±0.1	66.66

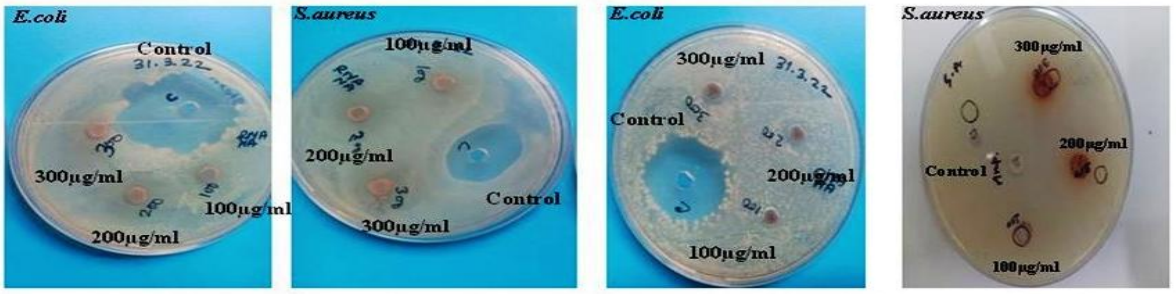
	ZOI (mm)	%inhibition
Control	2.6±0.33	100
100µg/ml	0.8±0.17	30.76
200µg/ml	1.7±0.09	65.38
300µg/ml	1.8±0.15	69.23

Leaf Extract

Bark Extract

A B C D

Figure 2 Antimicrobial activity of leaf & bark extracts of *K. linifolia*. Zone of inhibition (mm) produced by three different concentrations (100µg/mL, 200µg/mL & 300µg/mL) of leaf extract against *E.coli* (A) and *S.aureus* (B); Zone of inhibition (mm) produced by three different concentrations (100µg/mL, 200µg/mL & 300µg/mL) of bark extract against *E.coli* (C) and *S.aureus* (D). Ampicillin has been used as control (100µg/mL). All the experiments repeated thrice (n=3) and data shown are mean±SD.



E.coli	ZOI (mm)	%inhibition
Control	2.3±0.60	100
100µg/ml	1±0.10	43.47
200µg/ml	1.1±0.16	47.82
300µg/ml	1.13±0.19	49.13

S. aureus	ZOI (mm)	%inhibition
Control	2.2±0.59	100
100µg/ml	1±0.10	45.45
200µg/ml	1.05±0.1	47.72
300µg/ml	1.07±0.8	48.63

Stem

E.coli	ZOI (mm)	%inhibition
Control	2.3±0.58	100
100µg/ml	1±0.1	43.47
200µg/ml	1.1±0.11	47.82
300µg/ml	1.12±0.2	48.69

S.aureus	ZOI (mm)	%inhibition
Control	2.2±0.57	100
100µg/ml	0.8±0.1	36.36
200µg/ml	1.±0.1	45.45
300µg/ml	1.1±0.1	50

Plant sap

A B C D

Figure 3 Antimicrobial activity of stem & plant sap extracts of *K. linifolia*. Zone of inhibition (mm) produced by three different concentrations (100µg/mL, 200µg/mL & 300µg/mL) of stem extract against *E.coli* (A) and *S.aureus* (B); Zone of inhibition (mm) produced by three different concentrations (100µg/mL, 200µg/mL & 300µg/mL) of plant sap extract against *E.coli* (C) and *S.aureus* (D). Ampicillin has been used as control (100µg/mL). All the experiments repeated thrice (n=3) and data shown are mean±SD.

Table 1 Minimum Inhibitory Concentrations of *K. linifolia* leaf and bark extract *S. aureus* and *E.coli*

Microorganism		Turbidity in broth (µg/ml)								
		3.9	7.8	15.6	31.2	62.5	125	250	500	1000
E. coli	Leaf Extract	+	+	+	+	+	+	+	-	-
	Bark Extract	+	+	+	+	+	+	-	-	-
S. aureus	Leaf Extract	+	+	+	+	+	+	+	+	-
	Bark Extract	+	+	+	+	+	+	-	-	-

S. aureus and gram-negative bacteria *E. coli*. Results presented in Figures 2 & 3 revealed the efficacy of leaf, bark, stem, and plant sap extracts. Among the tested various extracts, 300 µg/mL of both the leaf and bark extracts were shown to have significant antibacterial efficacy as both can kill more than 60% of both *S. aureus* and *E. coli*, compared with the ampicillin control (Figure 3). The stem and plant sap extracts failed to show considerable bactericidal properties (≤50%; Figure 3). As the leaf and bark crude extracts were more potent than the stem and plant sap crude extracts, all the further experiments were performed using only the leaf and bark crude extracts.

3.2 Minimal inhibitory concentration of *K. linifolia* leaf and Bark extracts

The growth inhibitory potential of leaf and bark extracts of *K. linifolia* against both *S. aureus* & *E. coli* were determined through MIC values (Table 1). To decide the MIC value, the concentrations of the leaf and bark extracts used were 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 & 1000 µg/mL. Among these, the MIC for the leaf extract was 500µg/mL and 1000 µg/mL for *E. coli* and *S. aureus*, respectively. In the case of bark extract, the MIC value was recorded at 250µg/mL for *E. coli* and *S. aureus* (Table 1). The MIC values for leaf and bark extracts were ≤1000µg/mL & ≤500µg/mL for *E. coli* and *S. aureus*, respectively. These results show that bark crude extract has more potential to kill both the *E. coli* and *S. aureus* strains.

3.3 Quantitative phytochemical analysis of *Knema linifolia* aqueous extracts

The phytochemical studies of leaf and bark crude extracts of *K. linifolia* were carried out in this study to find out the presence of different phytocompounds, namely, tannins, terpenoids, saponins,

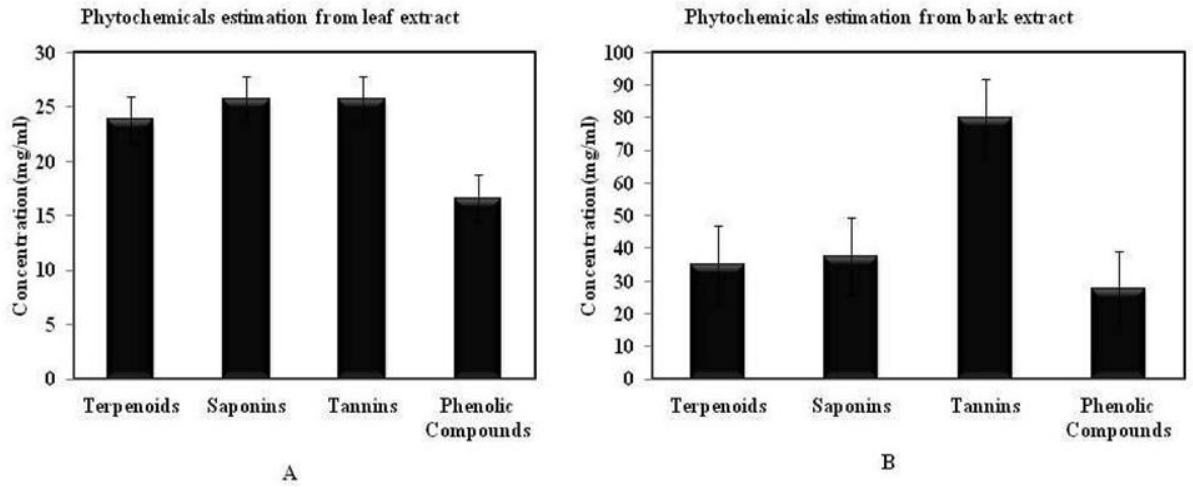


Figure 4 Quantitative estimation of phytochemicals from leaf and bark *K. linifolia* (A) Quantitative analysis of tannins, terpenoids, saponins & phenolic compounds in the leaf extract; (B) Quantitative analysis of tannins, terpenoids, saponins & phenolic compounds in the bark extract. All the experiments repeated thrice (n=3) and data shown are mean±SD.

Table 2 Qualitative analysis of phytochemical from bark and leaf extract of, sap and stem of *K. linifolia*

Components	Bark	Leaf
Flavonoids	-	+
Tannins	+++	+++
Saponins	++	++
Anthraquinones	-	-
Alkaloids	-	-
Anthocyanins	-	-
Terpenes/Terpenoids	-	++
Phenolic Compounds	+	+

flavonoids and phenolics by the standard techniques (Mujeeb et al. 2014). The presence of considerable terpenoids, tannins, phenolic compounds and saponins in the bark and leaf extracts of *K. linifolia* has been confirmed when compared against the respective standards (Figure 4; Table 2). No or negligible quantity of flavonoids was present in both the extracts (data not shown). The concentration of terpenoids, saponins, and phenolics was almost equivalent (23-38 µg/g) in leaf and bark extracts. But, the amount of tannin varies greatly in bark compared to the concentrations of other phytocompounds. The bark extract of *K. linifolia* contained more than 80µg/g of tannin (Figure 4).

4 Discussion

India is a country with great ethno-medicinal diversity. With the growing cost of medicines, reoccurrence of drug-resistant strains and side effects of medicine, it is important to discover alternate phytomedicinal sources that might be proven as a solution to combat this situation (Roy Chowdhury et al. 2022). *K. linifolia* is a plant of high ethnomedicinal values among the local tribes of the eastern and northeastern parts of India (Supriya and Sreekanth 2021). However, lack of proper documentation leads to the confinement of this knowledge only to the local peoples, leading to less preservation and plantation of this plant and the probable extinction of this plant species shortly (Barstow and Timberlake 2018). In this research, we are trying to outline the antimicrobial potency of the aqueous extract of different parts (leaf, bark, stem, and plant sap) of *K. linifolia*. Also this study is, however, the first-ever documentation to quantify the phytochemicals present in the bark and leaf of *K. linifolia* to the best of our knowledge.

The taxonomical analysis was done through morphological characterization by the BSI office, Kolkata, based on the herbarium sheet, fruit and seed samples collected from Jaldapara National Park, Alipurduar district, west Bengal (Figure 1). This study is of notable significance as it showed that *K. linifolia* plant aqueous extract can kill gram-negative and gram-positive bacteria

(Figure 2, Table 1). The sequential antimicrobial study used aqueous leaf, bark, stem and plant sap extracts against *E. coli* and *S. aureus* strains. A cup plate assay with crude extracts from various plant organs showed that leaf and bark crude extracts are more potent in inhibiting the gram-negative and gram-positive strains. The inhibition percentages for leaf extracts are 60.86 and 63.63 against *E. coli* and *S. aureus*, respectively. Bark extract shows more potential by killing 66.66% of *E. coli* and 69.23% of *S. aureus* with the same concentration (300µg/mL) as leaf extract (Figure 2). This data has also been supported by the MIC values for the aqueous bark extract, i.e. 500µg/mL & 250µg/mL for *S. aureus* and *E. coli*, respectively (Table 1).

To find out the probable reason behind the antimicrobial properties of the leaf and bark crude extracts, the phytochemical analysis for certain natural compounds, such as tannins, terpenoids, saponins, flavonoids and phenolic compounds, were assessed against their respective standard compounds. Flavonoids were present in nominal quantity in leaf and bark (data not shown). Terpenoids, saponins and phenolic compounds are almost equivalent in leaf and bark extracts (ranging from 23µg/mL to 38µg/mL; Figure 4 and Table 2). In contrast, bark extract contains an elevated amount of tannins (80µg/mL), which could probably be the rationale for the high microbicidal potential of crude extract of *K. linifolia* bark against both the *E. coli* and *S. aureus* strains (Figure 4 & Table 2). The medicinal properties of tannins have been well documented in various literature (Chung et al. 1988; Kaczmarek 2020). The *K. linifolia* plant sap is also dark red or maroon coloured, resembling that of the blood, which might be due to the very high concentration of tannins present in the plant (Roy Chowdhury et al. 2022), as evident from Table 2.

Conclusion and Future Prospects

In conclusion, it can be summarized that the bark and leaf aqueous extracts of *K. linifolia* showed impressive antimicrobial potency against gram-negative and gram-positive bacteria, possibly due to the high concentration of tannins in the plant. Based on the data, it is evident that *K. linifolia* could be used as a potential alternative phytomedicinal source, but more studies need to be done in this aspect. Further, the antimicrobial, anti-fungal and anti-protozoan efficacy of different fractions of *K. linifolia* leaf and bark extract could be explored soon.

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Conflict of interest

No conflict of interest has been declared by any of the authors.

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