Qualitative Phytochemical Analysis, *In Vitro* Antioxidant Activities and Antimicrobial Assessment of Methanolic Leaf, Root and Stem Extracts of *Rhizophora apiculata*

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Abstract

For the purpose of treating many illnesses, traditional medicine is based on mangrove plants that are derived from a number of secondary metabolites. The *Rhizophora* species of mangroves are known to be widespread on tropical waterways. Uncontrollable use of broad-spectrum antibiotics has been associated with a rise in the frequency of multidrug resistant bacterial strains and an increased incidence of bacteria that are less susceptible to antibiotic treatment. A large number of opportunities for discovering plant species which could release secondary metabolites, have antimicrobial properties or provide other therapeutic applications should be provided by investigating the richness of mangroves. With the evaluation of *in vitro* antioxidant and antimicrobial properties against some human pathogenic bacteria, the main objective of this study was to investigate the bioactive components present in the mangrove species *Rhizophora apiculata*.The phytochemistry investigation was carried out in accordance with the standard methods of science. Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique, free radical scavenging activity was examined *in vitro*. In order to provide antimicrobial activity, the method of agar well diffusion was used. The present study indicates that mangroves *Rhizophora apiculata* may be an optimal source of biological compounds which have been shown to neutralize pathogenic organisms in humans.

Keywords: Mangrove, Phytochemicals, Antioxidant, Antimicrobial, Pathogenic, DPPH

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INTRODUCTION:

Infectious diseases continue to pose a significant threat to global health [1,2,3]. The use of numerous antimicrobial drugs has led to multidrug resistance, necessitating the search for alternative antimicrobial agents from natural resources [4,5]. Plants are a well-known source of active ingredients that prevent the growth of many bacteria[6]. India is one of the 17 mega-biodiverse countries, ranking eighth in terms of species diversity. It features a wide variety of plant genetics, many of which are used medicinally. In India, the demand for medicinal herbs for use in folk medicine has increased over the years.

The diverse collection of salt-tolerant plants known as mangroves are found in tropical and subtropical intertidal areas in estuaries. There are around 70 different mangrove species worldwide [7,8]. Indian mangroves cover an area of 36,000 square kilometers and include 65 species from 39 different genera [9]. Where freshwater and saltwater systems converge, these plants must thrive in a demanding environment. At the boundary between sea and land, they can adapt morphologically and withstand extreme salinity conditions and strong temperature gradients [10].These plants are equipped with special and diverse classes of phytochemicals to survive in these demanding environments. The presence of bioactive phytochemicals and secondary metabolites in mangrove plants including phenols, flavonoids, tannins, alkaloids, saponins and others could explain why they are used to cure various diseases [11,12,13]. Various species of mangrove plants are used in traditional medicine and pharmacology [14,15,16]. Several authors have suggested mangrove plants as potential sources of natural antioxidant and antimicrobial compounds that could be used in drugs to treat cancer, bacterial and fungal infections [17,18,19]. Therefore, in order to predict the biological effects of these plants, it is necessary to identify the phytochemical elements of their bioactive components[20].A mangrove plant from the *Rhizophoraceae* family called *Rhizophora apiculata* is widely used in traditional

medicine to treat bacterial diseases [21,22]. *R. apiculata* has been shown to block human immunodeficiency virus (HIV) in previous studies [23], and its extracts have also been reported to promote the growth and survival rates of clownfish infected with *Vibrio alginolyticus.*

Amino acids, lipids, proteins and DNA can be damaged by oxidation due to the high concentration of free radicals and other reactive species created by aerobic respiration in the body. Numerous chronic and degenerative diseases such as atherosclerosis, heart disease, aging, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others are associated with oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [24,25,26]. Antioxidants are the most efficient means of eliminating the free radicals responsible for oxidative stress. Free radicals are molecules that antioxidants can stabilize or deactivate before they cause cell damage. By preventing and correcting damage caused by ROS and RNS, promoting their scavenging of free radicals and suppressing oxidative diseases, both exogenous and endogenous antioxidants act as efficient free radical scavengers[27]. Commercially available synthetic antioxidants used to treat disorders associated with oxidative stress include butylated hydroxytoluene, butylated hydroxyanisole, and tertiary butylhydroquinone [28].Therefore, natural sources of antioxidants such as plant derivatives are more popular. There is a strong trend towards using natural antioxidant sources such as plant derivatives as many Indian medicinal herbs are used to treat diseases caused by oxidative stress [29]. Secondary metabolites from plants, mainly phenols and flavonoids, can reduce the production of ROS and treat chronic diseases caused by oxidative stress [30]. Many people in Asia and Africa use the important medicinal mangrove plant *Rhizophora apiculata* in traditional medicine. *R. apiculata* has long been used in traditional medicine for its biological properties, which include antiviral, antibacterial, and antifungal properties [15].

Phytochemicals have been divided into two groups: primary components (proteins, amino acids, chlorophyll, and common sugars) and secondary components (phenolic compounds, tannins, saponins, essential oils, alkaloids, flavonoids, terpenoids, and other components). Researchers are now focused on studying the effects of plant phytochemicals on human and animal health. Phytocomponents are bioactive substances naturally found in plants that act as a unit with fiber and nutrients to ward off disease and stress [21, 22]. Studies suggest that the raw extracts of various mangrove species have antibacterial and antioxidant effects as mangrove plants are a rich source of secondary metabolites. Therefore, this work is an attempt to analyze the phytochemical constituents to investigate the antioxidant and antibacterial abilities of the crude extract from leaves, roots and stems of *R. apiculata* against both gram-positive and gram-negative human pathogenic bacteria [1].

MATERIALS AND METHODS

Description of Study Sites: Sites for the study include the Pichavaram mangrove forest, a coastal region in Tamil Nadu, India, where many different species of mangroves, including *R. apiculata*, thrive. The Pichavaram mangrove forest in Tamil Nadu is an important ecosystem that contributes to the protection and development of the local coastal areas.

Plant Taxonomy: The identification of the mangrove species *R. apiculata* was successfully performed at the Plant Taxonomy Laboratory, Department of Botany, Annamalai University, Tamil Nadu.

Collection of Plant Materials: The fresh leaves, roots and stems of *R. apiculata* were collected in a pollution-free zone of Pichavaram coast in February 2022, identified by an experienced plant taxonomist (Dr. D. Kumarasamy, Professor, Department of Botany, Annamalai University).

Pretreatment of plant materials: Plant materials were collected, divided into sections, thoroughly cleaned under running water and dried in the shade at room temperature for over two months as a pretreatment. In order to produce a powder form (roughly pulverized) from the plant samples, the dried plant components were further homogenized.

Preparation of crude plant extracts: Using methanol as the organic solvent, hot crude extracts of R. apiculata were prepared in the following proportions: 1:3 (w/v) for the leaves and 1:5 (w/v) for the stem and Root. For Soxhlet extraction, 100 g of coarsely ground leaf, stem, and root samples were combined with 300 mL of solvent for the leaves and 500 mL for the stems and roots. All residual solvents contained in the extract were evaporated in a rotary evaporator at 45°C to concentrate the resulting extracts. All extracts were stored in a refrigerator at 4°C until used for analysis. The phytochemicals and the antioxidant and antibacterial activity were then analysed qualitatively.

Percentage of yield: The percentage of yield was calculated using the formula:

$$\text{Percentage (\%) yield} = \frac{\text{WCE}}{\text{WSP}} \times 100$$

Where WCE is the weight of crude extract and,

WSP is the weight of the dried sample

Phytochemical Analysis:

Saponins, flavonoids, terpenoids, alkaloids and phenols were among the phytocomponents studied in leaf, root and stem extracts of *R. apiculata*.

Test for Saponins: A 0.5 g sample of crude extract was placed in a test tube with 3 ml of distilled hot water to check for the presence of saponins. The mixture was then vigorously shaken for one minute. Foaming indicates the presence of saponins.

Test for Flavonoids: A dilute ammonia solution was placed in a test tube with 0.5 g of the extract and 10 ml of distilled water to confirm the presence of flavonoids. After filtering, 1mL H_2SO_4 conc. was added. A yellow color indicates the presence of flavonoid compounds.

Test for Terpenoids: A sample weighing 0.8 g and 10 ml of methanol was placed in a test tube. After the test tube was shaken well, 5 ml of the plant extract sample was filtered out. In the following step, 2 ml of chloroform was incorporated into the selected plant extract sample. The development of a reddish-brown coloration is a sign that terpenoids are present in plants.

Test for Alkaloids: Alkaloids were checked by combining 0.2 g of the sample with 3 ml of hexane solution in a test tube and shaking vigorously before filtering. Then 5 ml of 2% HCl was added, stirred and filtered into a test tube. Picric acid was added drop by drop. Alkaloid is indicated by yellow precipitate.

Test for Phenols: In a test tube, add 4-5 drops of FeCl3 solution to 2 milliliters of raw plant extract. If, after the addition of FeCl3, a bluish-black hue forms or the extract turns dark green, then phenol is present.

Determination of antioxidant activity

2,2-Diphenyl-1-Picrylhydrazyl radical scavenging assay (DPPH): The DPPH free radical scavenging assay is a well-established assay and is widely used to assess the free radical scavenging activity of natural and synthetic antioxidant compounds using spectroscopy. This test is based on the reduction of DPPH in methanol in the presence of hydrogen donating antioxidant components of plant extracts due to the formation of the non-radical form of DPPH. The color change is monitored at 517 nm. Briefly, a 1 mL aliquot of a 0.3 mM DPPH ethanol solution was added to 2.5 mL of various concentrations of *R. apiculata* leaf, root and stem methanolic extracts (20, 40, 60, 80, 100 µg/mL) and standard ascorbic acid (~30 µg/ml) incubate at room temperature in the dark; After 20 minutes, the absorbance was measured at 517 nm. Ethanol was

used as a blank. DPPH solution (1 ml, 0.3 mM) plus ethanol (2.5 ml) serves as a negative control. All tests were performed in triplicate (n=3) and mean values were calculated. Half-maximal inhibitory concentration (IC_{50}) values were also calculated. Lower absorbance of the reaction mixtures indicates higher free radical scavenging activity.

$$\text{DPPH radical scavenging}(\%) = \frac{(A_{sample} - A_{sample\ blank})}{A_{control}} \times 100$$

Where A_{sample} is the absorbance of the test samples with DPPH solution,

$A_{sample\ blank}$ is the absorbance of the test sample only, and

$A_{control}$ is the absorbance of the DPPH control

Antimicrobial test

Test germs: The gram-positive bacteria *Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA) and the gram-negative bacteria *Klebsiella pneumoniae* and *Salmonella typhi* were evaluated. The clinical isolates of these bacteria were obtained from the Department of Microbiology, Rajah Muthiah Medical College, Annamalai University.

Preparation of a standardized inoculum: All bacterial strains were aerobically cultured for 24 hours at 37 degrees Celsius in Muller-Hinton agar (M-H Agar), sub-cultured every month and stored at 4 degrees Celsius for further use.

Antibacterial Activity: Antibacterial activity was evaluated using the agar well diffusion method. All extracts were first brought to room temperature and then used for bioassay using the agar-well diffusion method. Petri dishes were sterilized, then 20 ml of Müller-Hinton agar was poured in to check the bacterial susceptibility test. These plates were held flat and the agar allowed to solidify. After solidification, 5 indentations were made using a sterilized 7 mm diameter cork borer. The crude extracts with different concentrations, corresponding to 100, 50

and 25 mg/ml, were filled into the wells with a micropipette to check the antibacterial activity. Ciprofloxacin and Vancomycin were used as positive controls (30 mg/ml) for gram-negative and gram-positive bacteria, respectively, and DMSO was used as negative control. The plates were incubated at 37°C for 24 hours, then the zone of inhibition for each extract of the test plates was measured on each organism. All tests were run in triplicate and each positive result was retested to ensure its effectiveness. Zone sizes were measured in millimetres (mm) and bacterial activity expressed as mean inhibition.

Statistical Analysis:

Results were expressed as the mean standard deviation in triplicate for chemical analysis and as four standard deviations for antibacterial activity. The statistical analysis was carried out using one-sided analysis of variance (ANOVA) and subsequent Duncans comparison. The correlation between phytochemical composition and free radical scavenging activity was performed using Pearson's analysis.

RESULTS AND DISCUSSION

Phytochemical Analysis:

The main components of the secondary metabolites were sought in the crude leaf, root and stem extract using a qualitative analysis based on precipitation and staining reactions by specific reagents. In short, saponin was determined by the formation of a stable foam. The appearance of the dark green extract after the addition of ferric chloride indicated the presence of a phenolic composition. Flavonoid was indicated by the reaction of metal and the appearance of a yellow color. Terpenoid and alkaloid were tested using Salkowski's test and Dragendroff's solution, respectively. The red-brown ring formed at the junction of two layers and indicates the presence of terpenoids. The appearance of orange-yellow precipitates confirmed the presence of alkaloids. The results of this screening for phytochemicals are shown in Table 1. All parts of

these plants have identified the presence of phytochemicals in different ways. However, high levels of secondary metabolites phenol and flavonoid were observed in all three parts of *R. apiculata* tested.

Table 1. Phytochemical Screening Performed on Methanolic Crude Extracts of *Rhizophora apiculata*Root, Stem and Leaf.

Phytoconstituents	Verification Methods	Precipitationand Colors	Leaf Extract	Root Extract	Stem Extract
Saponin	Frothing Test	Stable foam	+	-	+++
Flavonoid	Reaction of Metal	Yellow color	++	+	+
Terpenoid	Salkowski's Test	Reddish-brown ring	+	-	-
Alkaloid	Dragendroff's Test	Orange-yellow precipitate	-	++	++
Phenol	Ferric Chloride Test	Dark green or Bluish-black extract	++	+++	+

+++: highly abundant; ++: moderately abundant; +: present; -: absent.

Antioxidant Activity:

Free radical scavenging activity was measured in vitro using the DPPH method for each of the three extracts. The data are listed in Table 2-4; Fig. 1-3. The antioxidant activity of methanolic extracts of *R. apiculata* root, leaf and stem was carried out at different doses of 20, 40, 60, 80 and 100 µg/ml. The percentage of free radical scavenging of DPPH was determined by

spectroscopic analysis at 517 nm of quenched DPPH after reaction with samples. The IC_{50} radical scavenging efficiency of *R. apiculata* extracts ranged from 12.40% to 14.16%. All *R. apiculata* extracts showed a very high level of scavenging activity.

Table 2:Calculation of % Radical Scavenging and IC_{50} from DPPH Assay (Root)

Concentration (µg/ml)	Control	Sample (Root)	%RSA	IC_{50}
20	1.943	0.812	58.2	12.40
40	1.982	0.678	65.792	12.80
60	1.966	0.512	73.95	13.19
80	1.958	0.456	76.71	13.58
100	1.966	0.257	86.93	13.97

Table 3: Calculation of % Radical Scavenging and IC_{50} from DPPH Assay (Leaf)

Concentration (µg/ml)	Control	Sample(Leaf)	%RSA	$IC5_0$
20	1.982	0.984	50.35	12.40
40	1.982	0.768	61.25	12.80
60	1.982	0.549	72.3	13.19
80	1.982	0.294	85.17	13.58
100	1.982	0.428	78.4	13.97

Table 4: Calculation of % Radical Scavenging and IC_{50} from DPPH Assay (Stem)

Concentration (µg/ml)	Control	Sample (Stem)	%RSA	IC_{50}
20	1.943	1.052	45.85	12.56
40	1.982	0.986	50.25	12.96
60	1.966	0.874	55.55	13.36
80	1.958	0.456	76.71	13.76
100	1.966	0.643	67.29	14.17

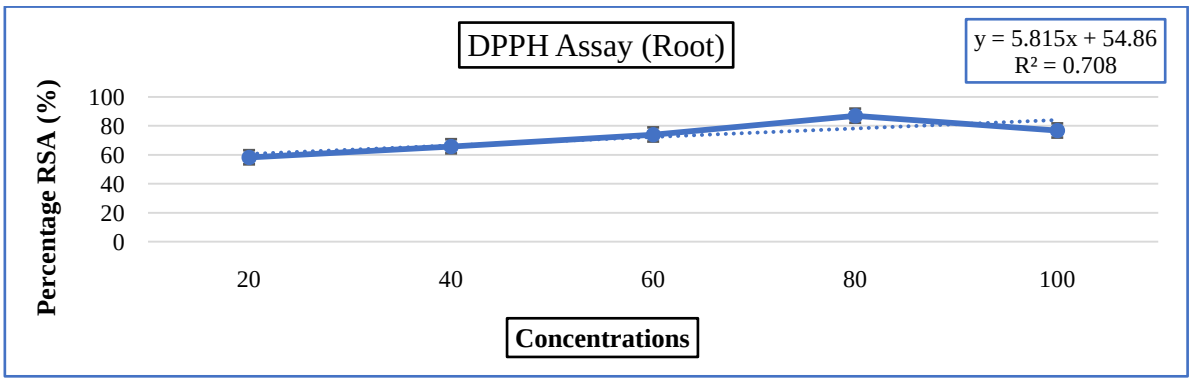


Fig. 1: **Graph showing Antioxidant activities of *Rhizophora apiculata* methanolic root extract**

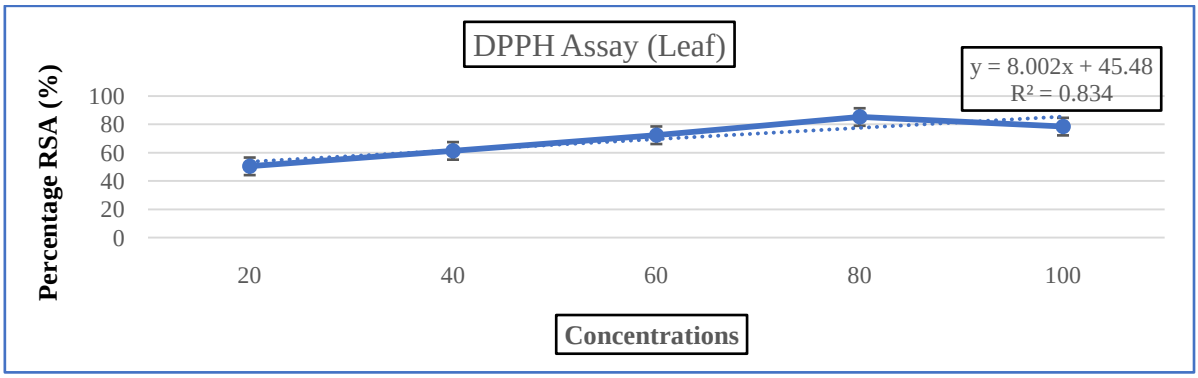


Fig. 2: Graph showing Antioxidant activities of *Rhizophora apiculata* methanolic leaf extract.

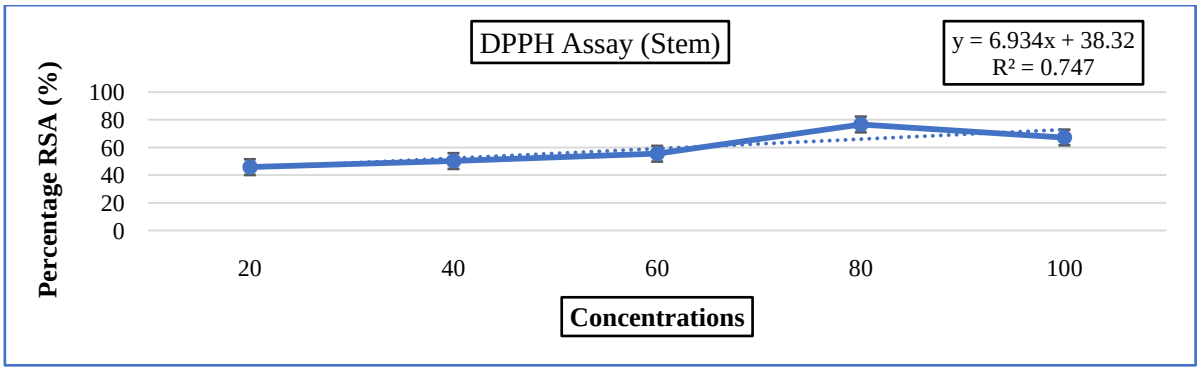


Fig. 3: Graph showing Antioxidant activities of *Rhizophora apiculata* methanolic stem extract.

Antimicrobial test:

The antibacterial test was performed by the agar well diffusion method and the zone of inhibition was measured in triplicate and expressed as the mean standard deviation of the zone of inhibition

(in mm) as shown in Tables 5 and 6; Fig. 4 and 5. The extracts of different concentrations of 25, 50 and 100 µg/ml were tested against gram-positive bacteria (*Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA) and gram-negative bacteria (*Klebsiella pneumoniae* and *Salmonella typhi*). The zone of inhibition of leaf extracts against gram-positive bacteria ranged from 8.25 to 27.87 and 10.25 to 21.5 for gram-negative bacteria, while the zone of inhibition of root extracts ranged from 7.5 to 13.62 for gram-positive bacteria and 6.12 to was 10 for gram-negative bacteria. The results also showed that these extracts act in a dose-dependent manner, as the enlargement of the zone was observed with the increase in concentration.

Table 5: Inhibitory action of *Rhizophora apiculata* methanolic leaf extracts against the following pathogenic bacteria.

Pathogenic bacteria	Experimental units	Inhibition zone (mm) ± Standard deviation
Klebsiella pneumoniae	T_1	12.75 ± 0.41
	T_2	20.5 ± 1.66
	T_3	21.5 ± 1.66
	T_4	26.37 ± 1.22
	T_0	0 ± 0.00
Salmonella typhi	T_1	10.25 ± 0.41
	T_2	13.62 ± 1.22
	T_3	14.87 ± 1.56
	T_4	14.87 ± 1.56
	T_0	0 ± 0.00
Enterococcus faecalis	T_1	8.25 ± 0.41
	T_2	9.75 ± 0.41
	T_3	10 ± 1.16
	T_4	12.87 ± 0.72
	T_0	0 ± 0.00
Methicillin-resistant *Staphylococcus aureus* (MRSA)	T_1	25.12 ± 0.72
	T_2	27.87 ± 0.72
	T_3	27.87 ± 0.72
	T_4	29.75 ± 0.41
	T_0	0 ± 0.00

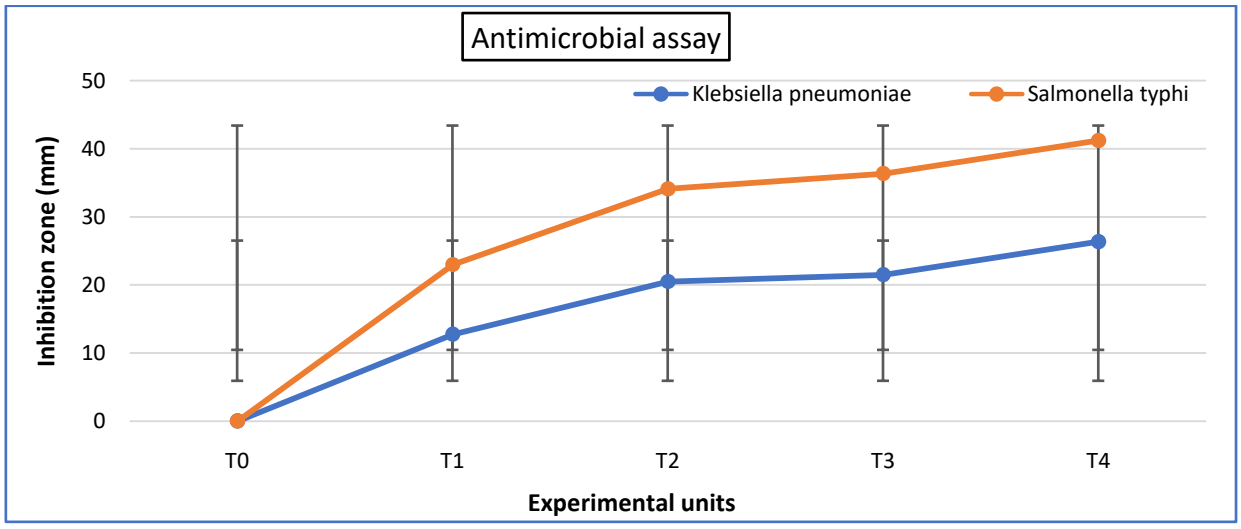
T_1: 25%, T_2: 50%, T_3: 100%, T_4: Positive control, T_0: negative control.

Table 6: Inhibitory action of *Rhizophora apiculata* methanolic root extracts against the

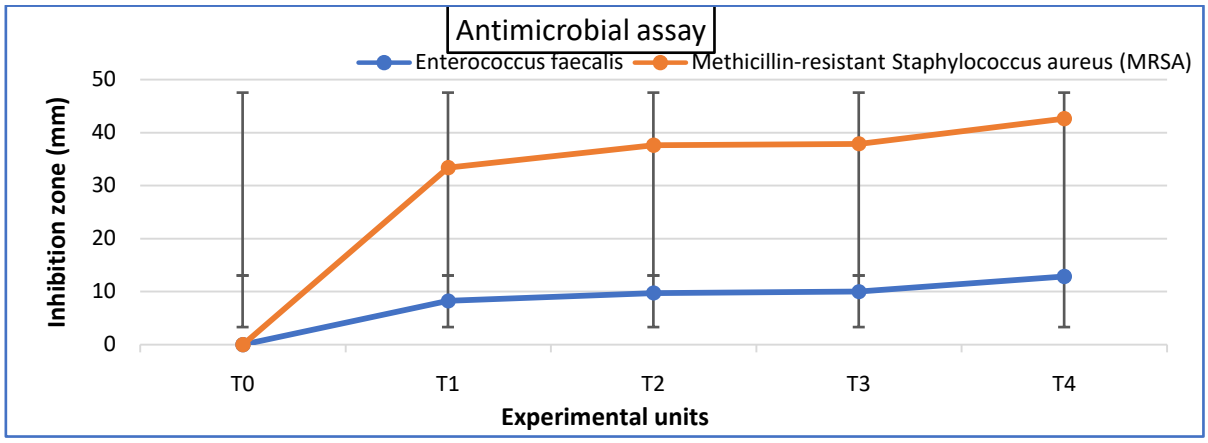
following pathogenic bacteria.

Pathogenic bacteria	Experimental units	Inhibition zone (mm) ± Standard deviation
Klebsiella pneumoniae	T_1	7.5 ± 1.66
	T_2	10.37 ± 1.22
	T_3	11.62 ± 1.22
	T_4	15.12 ± 0.72
	T_0	0 ± 0.00
Salmonella typhi	T_1	10.25 ± 0.41
	T_2	12.87 ± 1.56
	T_3	13.62 ± 1.22
	T_4	14.87 ± 1.56
	T_0	0 ± 0.00
Enterococcus faecalis	T_1	8.25 ± 0.41
	T_2	9.75 ± 0.41
	T_3	10 ± 1.16
	T_4	15 ± 1.16
	T_0	0 ± 0.00
Methicillin-resistant *Staphylococcus aureus* (MRSA)	T_1	6.12 ± 0.72
	T_2	6.25 ± 0.41
	T_3	9.5 ± 1.66
	T_4	10.75 ± 0.41
	T_0	0 ± 0.00

T_1: 25%, T_2: 50%, T_3: 100%, T_4: Positive control, T_0: negative control.

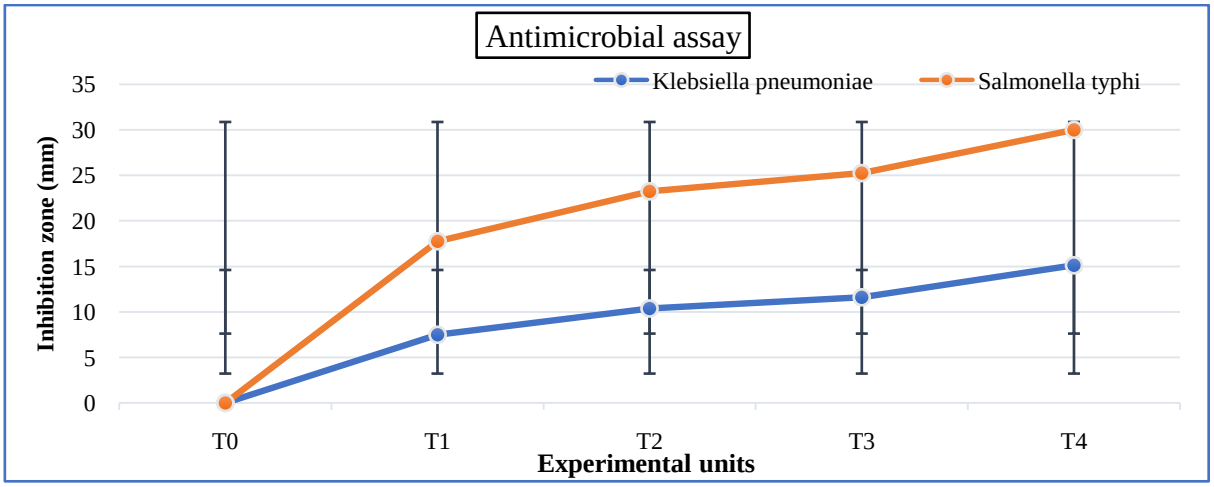


(a) Gram -ve Bacteria

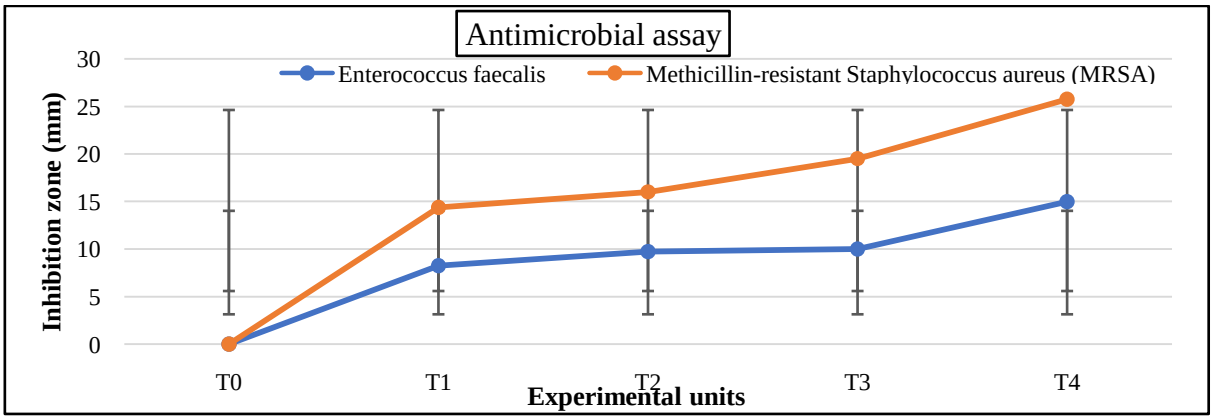


(b) Gram +ve Bacteria

Fig. 4: Graph showing anti-bacterial activities of *R. apiculata* methanolic leaf extract and standard antibiotic. T_0-Negative control, T_1-25% Crude extract, T_2-50% Crude extract, T_3-100% Crude extract and T_4- Positive control (Antibiotic-30µg/ml).



(a) Gram -ve Bacteria



(b) Gram +ve Bacteria

Fig. 5: Graph showing anti-bacterial activities of *R. apiculata* methanolic root extract and standard antibiotic. T_0-Negative control, T_1-25% Crude extract, T_2-50% Crude extract, T_3-100% Crude extract and T_4- Positive control (Antibiotic-30µg/ml).

Conclusion:

The overall analysis indicates that a successful attempt to characterize the phytochemical and antimicrobial activity of mangrove *R. apiculata* was undertaken. It shows that antimicrobial compounds are valuable, ecologically safe and economically viable. The results of this study also demonstrate that *R. apiculata* exhibits potent DPPH radical scavenging activity in methanol extracts. The activity is consistent with the presence of phytochemicals in this plant. These phytochemical properties can be explored for the development of natural antioxidants. Therefore, further studies focus on isolating bioactive compounds and identifying pure components in each extract, which could be used as lead molecules in the development of new drugs from mangroves in the field of medicinal plants.

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