Quantification of secondary metabolites and antimicrobial efficacy in leaves of *Bruguiera gymnorrhiza* – A true mangrove at west coast of Kerala

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Abstract

To evaluate and analyze the active compounds and quantification of secondary metabolites such as total phenol content, flavonoid content and H_2O_2 content in the leaves of *Bruguiera gymnorrhiza* and carry out the antimicrobial activity of the large leafed mangrove so as to determine its medicinal properties as an efficient drug.

The ethanol leaf extract was analyzed using GC-MS method. Quantification of secondary metabolites like total phenol, flavonoid, and H_2O_2 content was carried out on both hexane and ethanol extracts. The antimicrobial activity was carried out on the ethanol leaf extract against standard Ciprofloxacin.

The ethanol leaf extract of the sample showed more compromising results than that of the hexane extract. The flavonoid content and the phenol content are said to be more in the ethanol extract than that of the hexane extract. The H_2O_2 content also came out to be more in the ethanol extract. The Gas Chromatography Mass Spectrometer of the ethanolic extract of the leaf sample revealed about 25 compounds with Formamide, n, n-dimethyl showing the high peak area. The antimicrobial activity of the leaf sample of the ethanolic extract showed more activity with the bacteria *Streptococcus pyogenes*.

The present study revealed that *Bruguiera gymnorrhiza* can be used as a potential drug and further studies are required on it.

Keywords: Mangrove, *Bruguiera*, Secondary metabolites, Antimicrobial, GC-MS

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1. Introduction

Mangroves are the halophytic plant communities that consist of mainly shrubs or small trees growing along the coastal areas or the brackish water. Mangroves have a well-developed salt filtration system and a complex root system to tolerate the salt-water interaction and wave action. Our main focus of study is on the *Bruguiera gymnorrhiza* (L.) Lamk. Of the family Rhizophoraceae a true mangrove of the mangrove family. The plant is extensively studied for its good medicinal activity. The root extract of the plant is said to have high resistance against both gram negative and gram positive bacteria (S. Acharya et al. 2020).This is a large leafed mangrove widely spread across the pacific mainly from Southeast Asia to the Ryukyu Islands of southern Japan into Micronesia and Polynesia and southward to subtropical Australia. The presence of active constituents of the plant is said to vary from place to place, certain factors like temperature, humidity, soil, rainfall is said to affect the makeup of a plant. The west coast is said to have a more divergent form of life forms which is an ideal location for collection. In India, the screening of secondary metabolites of this plant is mainly made from the material collected from the region of the Bay of Bengal, works from the western part of the country hasn't gone much and there is a habitual loss of these species of plants. The *B. gymnorrhiza* is said to be a more shade tolerant mangrove species and is also showing increased basal area composition within the intact forests dominated by *R .apiculata* (Putz and Chang 1986).The leaves are said to be opposite, simple, elliptical, dark green, and coriaceous (leathery), aggregated at the tips of apical shoots in clusters of about 12 leaves. The leaf bladesare elliptic tending to oblong. Stipules are green or yellowish. This large-leafed mangrove is viviparous, meaning that the species produce seeds that germinate on the parent plant. The dispersal unit, a viviparous seedling, is called a hypocotyl. An apparent fruit stage can't be found in this plant, instead a hypocotyl emerges singly from an attached calyx that are located from the third to fifth nodes below the apical shoot. Expended calyces are found to remain attached even after mature propagules fall from parent trees (James and Norman 2006).The bark of the plant is said to be rich in tannin content and can be used for bio treating for tanning leather. The medicinal uses of fruits encompasses of the treatment of shingles and eye diseases (Rudjiman 1992) and (Othman 1998).In the present study, we have analyzed the active compounds, quantification of the secondary metabolites, and antimicrobial activity in leaves of *B. gymnorrhiza*. The work is also aimed at creating an efficient drug for certain bacterial disease which is currently under process and also can be entailed on the importance of ethno-pharmaceutical medicine which can be effectual against human pathogens and also as an effective compound against the corona virus which is claiming thousands of life worldwide.

2. Materials and Methods

The leaves of *B. gymnorrhiza* were collected from Malipuram of Cochin islands, Kerala, India. The shade dried leaf sample of*B. Gymnorrhiza* was powdered to 25g successively extracted using 250mL of ethanol and hexane by using the Soxhlet extractor for 8-10 hours (Gafner et al. 1998). The extract was filtered through the Whatmann No.1 filter paper to remove all the undissolved matter, including cellular materials and other constituents that are insoluble in the extraction solvent.

2.1. Total Flavonoid Content

The 50 µL of crude extract (1 mg/ml ethanol) were made up to 1mL with methanol, mixed with 4mL of distilled water and then 0.3ml of 5% $NaNO_2$ solution; 0.3ml of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2mL of 1mol/L NaOH solution were added, and the final volume of the mixture was brought to 10mL with double-distilled water. The mixture was allowed to stand for 15 min, and the absorbance was measured at 510nm (Patel et al. 2010), (Satish Kumar et al. 2008) and (Patel et al. 2012).

2.2. Total Phenol content

The Folin-Ciocalteu test was performed by referring to the standard method (Velioglu et al. 1998),with some modifications. The crude sample was prepared by liquefying 10 mg of the extract in 10 ml of the solvent to yield a concentration of 1 mg/ml. About 100 µL of the extract (1 mg/ml) was combined and mixed with 0.75 ml of the Folin-Ciocalteu reagent in the test tube. The liquid mixture was allowed to stand for 5 minutes at a room temperature. The mixture was then added about 0.75 ml of sodium carbonate (Na_2CO_3), and the test tube was shaken gently to mix them. After 90 minutes, the observance of the mixture was measured using the UV-Vis spectrophotometer at 725nm.

2.3. Hydrogen Peroxide Content (H_2O_2)

The 100µl of plant extract with 5mL of 0.1% (W/V) Tri-Chloroacetic Acid (TCA). This homogenate was then centrifuged at 12000× g for 15 minutes. To the 0.5 ml of the supernatant 0.5ml of potassium phosphate buffer (p^H 7.0) and 1mL of KI were added. After vortexing the mixture its absorbance was read at 390nm (Velikova et al. 2000).

2.4. GC-MS Analysis

GC-MS analysis of the ethanol extract of *B.gymnorrhiza*leaf was performed using Shimadzu Japan gas chromatography QP2010 plus with a fused gas chromatography (GC) column coated with polymethylsilicon (0.25mm x 50m).

2.5. Antimicrobial Activity

The test organisms used were clinical isolates *viz, Salmonella paratyphi, Staphylococcus aures* and *Streptococcus pyogenes.* The human fungal pathogens like *Candida albicans* and *Aspergillus fumicatus,* were obtained from the Dept. Of Microbiology, Bharathiar University, Coimbatore, India. The antibacterial samples were tested by the well diffusion method. Different concentration of the extracts (100 µg/ml) was prepared by reconstituting with methanol. The test microorganisms were seeded into the respective medium by spread plate method 10 µl (10 cells/ml) with the 24h cultures of bacteria growth in nutrient broth. After solidification the filter paper wells (5 mm in diameter) impregnated with the extracts were placed on test organism-seeded plates. Chloramphenicol (10 µg) used as standard for antibacterial test. The antibacterial assay plates were incubated at 37°C for 24 hrs. The diameters of the inhibition zones were measured in mm (Bauer et al. 1996). The antifungal activity was tested by the well diffusion method. The potato dextrose agar plates were inoculated with each fungal culture (10 days old) by point inoculation. The filter paper wells (5 mm in diameter) impregnated with 100 µg concentrations of the synthesized silver nanoparticles were placed on test organism-seeded plates. Chloramphenicol (10 µg well 1) used as positive control. The activity was determined after 72 hours of incubation at 28°C. The diameters of the inhibition zones were measured in mm(Taylor et al. 1996).

3. STASTICAL ANALYSIS

Obtained results were recorded from triplicate observations and articulated as mean $\pm$ SD. Microsoft Excel 2013 were used for the statistical evaluation of the data.

4. Results

4.1. Total phenol and flavonoid content

The ethanolic leaf extract of *B. gymnorrhiza* has revealed by spectrophotometricaly and it showed significant results of total phenolic contents and hexane extract showed the lower value of phenolic content when compared to ethonolic extract.

The total flavonoid content in *B. gymnorrhiza* showed more significant in leaf ethonolic extract when compared to hexane extract. (Fig 1&2), the results showed that total phenol and flavonoid contents are higher in ethonolic extract when compared to hexane extract.

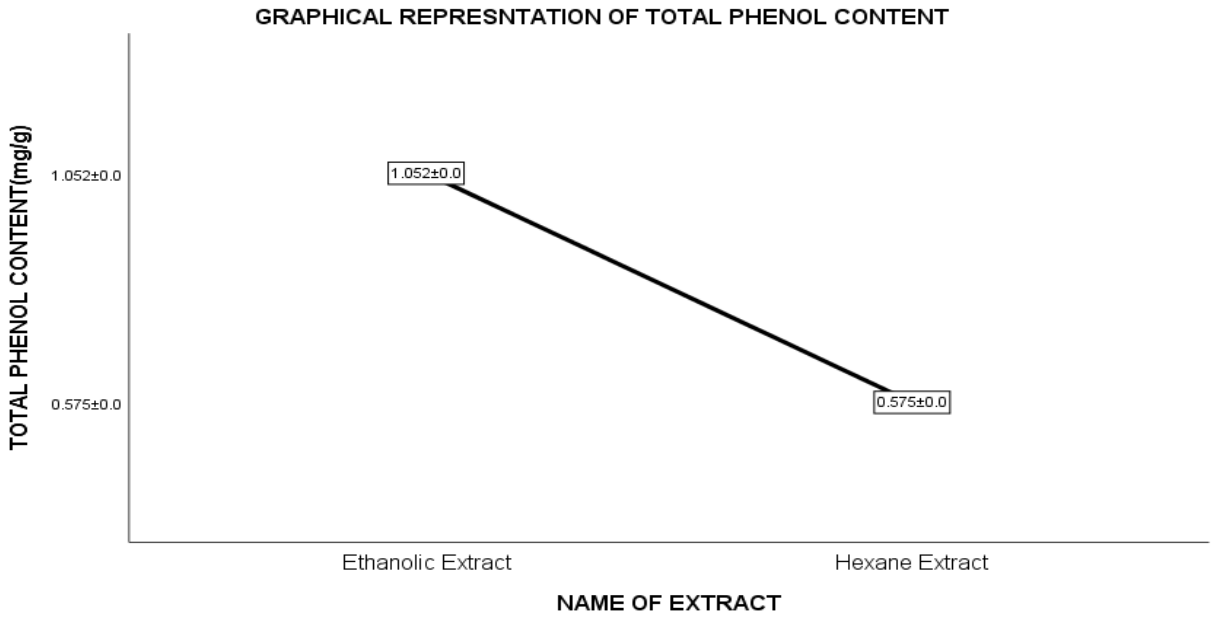


Fig.1: Total phenol content in leaves of *B. gymnorrhiza*

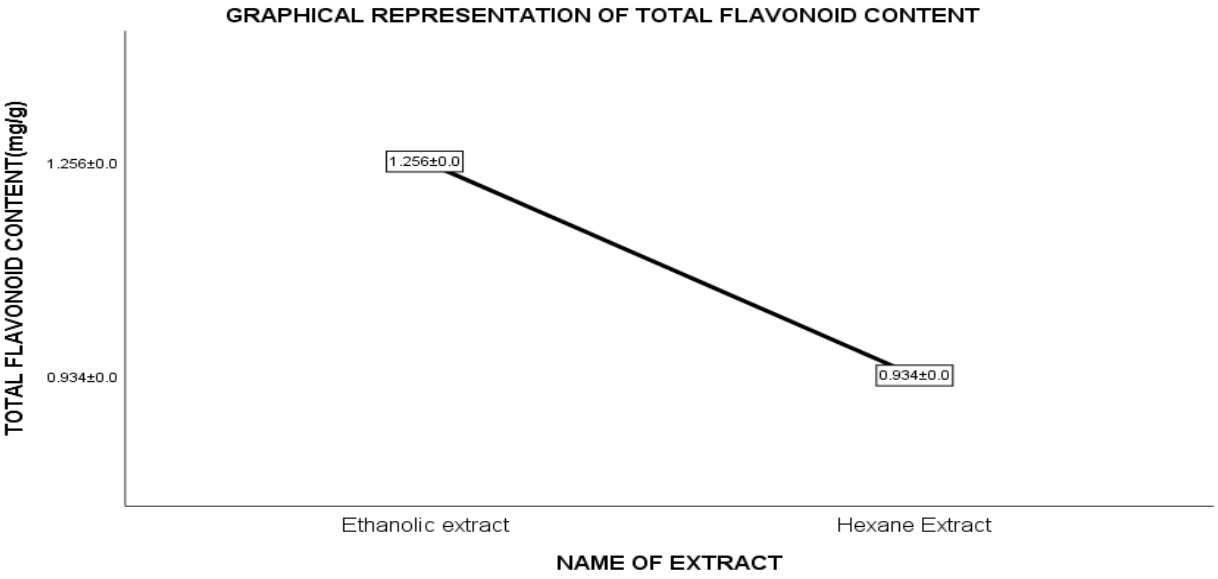


Fig.2: Total flavonoid contentin leaves of *B. gymnorrhiza*

4.2. Hydrogen peroxide(H2O2) Content

The H_2O_2content in the leaf extract of *B. gymnorrhiza* found to be more in the ethanol extract and it was lower in hexane extract when compared to ethanolic extract. (Fig-3).

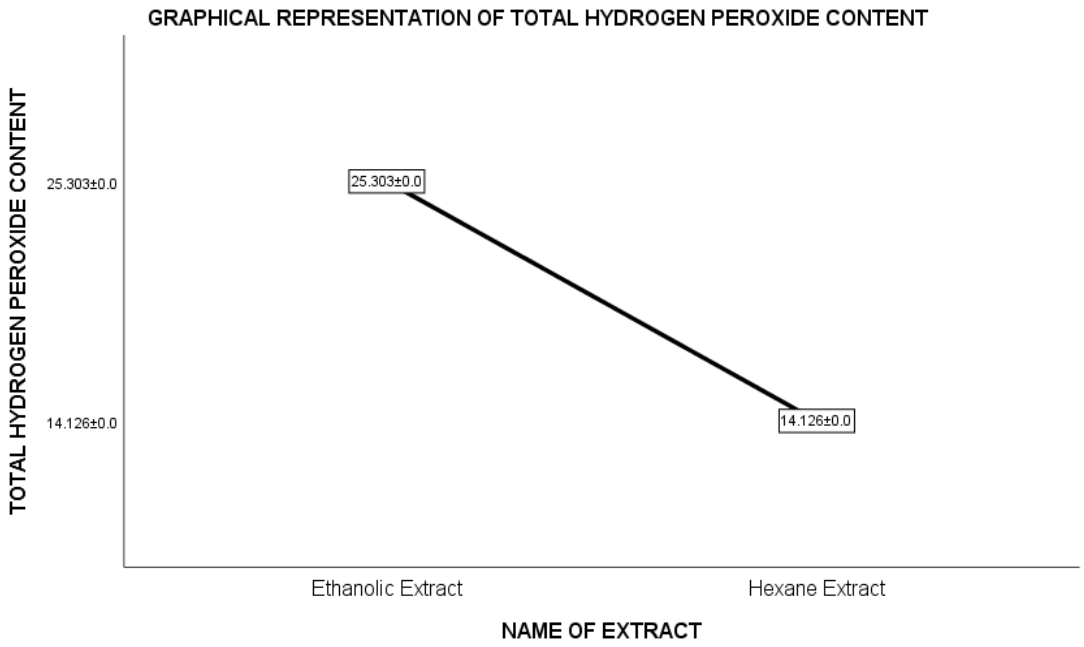
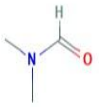
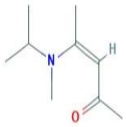
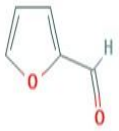
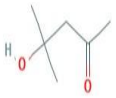
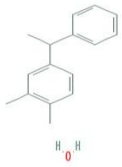
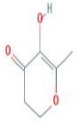


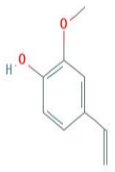
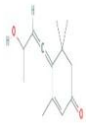
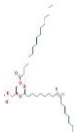
Fig.3: Hydrogen peroxide (H_2O_2) content in leaves of *B. gymnorrhiza*

4.3. GC-MS Analysis

GC-MS analysis was done on the ethanolic extract of the leaf sample as the sample being more polar than hexane. The leaf extract of the plant *B. gymnorrhiza* (Ethanol extract) were analyzed by GC-MS. The presence of components was confirmed by comparing the mass spectra of analyzing components with standard mass spectra of NIST and Willey library. In the GC-MS analysis of *B. gymnorrhiza*, 25 compounds were confirmed in the ethanol extract of a Cochin leaf sample. The active principles with their retention time (Rt), molecular formula (MP), molecular weight (MW), peak area (%), structure, medicinal uses, respectively, were presented in the table of the GC-MS given below (Table 1).

Table 1: GC-MS Analysis in leaf ethanolic extract of *B. gymnorrhiza*

S.No	Retention time	Name of the chemical compound	Molecular formula	Molecular weight (g/mol)	Peak Area %	Structure of the chemical compound	Properties
1	2.550	Formamide, N,N-dimethyl-	C_3H_7NO	73.09 g/mol	8.50		Fatal to life or health.
2	2.604	Carbonic acid, diethyl ester	$C_5H_{10}O_3$	118.13g/mol	3.82		Used as flavoring agents.
3	2.793	3-penten-2-one, 4methyl	$C_9H_{17}NO$	155.24g/mol	9.38		Insect repellent
4	3.050	2-Furancarboxaldehyde	$C_5H_4O_2$	96.08g/mol	1.52		Irritant or hazardous
5	3.234	2-hydroxy-2-methyl-4pentanone	$C_6H_{12}O_2$	116.16g/mol	3.05		Flavoring agents
6	4.216	Benzene, 1,2-dimethyl	$C_{16}H_{18}$	210.31g/mol	4.81		Toxic to enviornment
7	6.026	2-propanol, 1,1,1-Trichloro-2-methyl	$C_4H_9Cl_3O_2$	195.47g/mol	0.77		Injurious to humans
8	7.456	7-tridecanone	$C_{13}H_{26}O$	198.34g/mol	2.80		Used for fragnance
9	8.719	2,3-dihydro-5-hydroxy6-methyl	$C_6H_8O_3$	128.13g/mol	2.00		Injurious to humans
10	9.264	Disulfide, methyl propyl	$C_4H_{10}S_2$	122.3g/mol	11.16		Used as food additive
11	12.695	2-Furancarboxaldehyde, 5-(hydroxymethyl)	$C_6H_6O_3$	126.11g/mol	2.66		Food additive
12	13.135	3,4,5,6-Tetramethyloctane	$C_{12}H_{26}$	170.33g/mol	0.28		Used a biomarker

13	15.477	2-methoxy-4-Vinylphenol	$C_9H_{10}O_2$	150.17g/mol	0.28		Flavoring agent
14	16.884	9-oxa-Bicyclo[3.3.1]nona-3,6dien-2-one	$C_8H_8O_2$	136.15g/mol	1.15		Can be used as biomarker
15	18.345	3-hexadecene, (z)-	$C_{16}H_{32}$	224.42g/mol	0.73		Used as dyes
16	18.627	Hexadecane	$C_{16}H_{34}$	226.44g/mol	0.68		Health hazard
17	23.261	9-octadecene, (e)-	$C_{18}H_{36}$	252.5g/mol	1.04		Health hazard and used for lipid maps
18	23.698	2-cyclohexen-1-one, 4(3-hydroxy-1-buten)	$C_{13}H_{18}O_2$	206.28g/mol	1.07		Used for cosmetic manufacture
19	27.662	Phosphonic acid, dioctadecyle	$C_{42}H_{82}NO_8P$	760.1g/mol	1.05		Biomarker
20	28.612	3,7,11,15-tetramethyl2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5g/mol	3.12		Flavoring agent or Adjuvant
21	30.817	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42g/mol	2.31		Used as additives and cosmetic agents
22	40.337	Hexacosane	$C_{26}H_{54}$	366.7g/mol	0.40		May be used as pesticide
23	41.846	Eicosane, 2-methyl	$C_{21}H_{44}$	296.6g/mol	0.70		Used in manufacture of paraffin wax
24	43.303	Heptacosane	$C_{27}H_{56}$	380.7g/mol	1.34		Production of detergents
25	49.133	2H-1-benzopyran-6-ol, 3,4-dihydro-2,5,7,8	$C_{35}H_{53}NO_3$	535.8g/mol	1.21		Used as emulsions

4.4. Antimicrobial Activity

The ethanolic extract of *B. gymnorrhiza* leaves showed the highest antimicrobial activity against each 3 bacterial strains such as *Staphylococcus aureus, Salmonella paratyphi,* and *Streptococcus pyogenes* and two fungal strains of *Candida albicans* and *Aspergillus fumicatus* depicts the results. The gram positive bacteria *Streptococcus pyogenes* showed highest inhibition in 100% concentration extract and the gram negative bacteria *Salmonella paratyphi* showed inhibition in 50% concentration. The antifungal activity of leaves of *B. gymnorrhiza* against two fungal spp. Namely *Candida albicans* and *Aspergillus fumicatus* shows a maximum inhibition zone was observed in high concentration of 100%. The minimum inhibition was observed in low concentration 50% of *Aspergillus fumicatus.* (Table 2 & 3).

Table 2: Antibacterial activity in leaf ethonolic extracts of *B. gymnorrhiza*

S.No	Pathogenic bacteria	Zone of inhibition (mm)		
		Ethonolic extract concentrations		Standard (Ciprofloxacin)
		50%	100%	
1.	*Staphylococcus aureus*	4.1+0.1	6.1+0.1	08+0.1
2.	*Salmonella paratyphi*	3.1+0.1	6.0+0.1	09+0.1
3.	*Streptococcus pyogenes*	3.2+0.1	7.1+0.0	11+0.1

(All data are expressed in MEAN±SD)The experiment was conducted in triplicates (n=3)

Table 3: Antifungal activity in leaf ethonolic extracts of *B. gymnorrhiza*
(All data are expressed in MEAN±SD).The experiment was conducted in triplicates (n=3)

S.No	Pathogenic fungus	Zone of inhibition (mm)		
		Ethonolic extract concentrations		Standard (Ciprofloxacin)
		50%	100%	
1.	*Candida albicans*	2.1±0.1	5.0±0.0	07±0.1
2.	*Aspergillus fumicatus*	2.0±0.0	5.1±0.1	07±0.1

5. Discussion

B. gymnorrhiza belongs to coastal forests, part of the plant constituents may be polar in nature. The present study on the leaves of *B. gymnorrhiza* confirmed that the extraction solvent in the preparation of samples has a significant role in the phytochemical and antimicrobial activities. It was also reported (Haq et al. 2011) that the ethanolic leaf extract of *B. gymnorrhiza* contain phenolic content about 189. 4±0. 6 which is very higher than that obtained in the sample from the west coast of Kerala i.e. 1.052±0.0. The variation can be observed for the difference in geographical area and the amount of solute concentration in the soil. The concentration of phenolic compounds and flavonoid content is said to be more in the root extracts of *R. apiculata* than in *A. illicifolius* (Asha et al. 2012), this was distinguished based on the plant material we have chosen, leaves of *B. gymnorrhiza* also showed a considerable amount of the secondary metabolite content. These phenolics and flavonoids possess many useful properties including antiallergic, anti-inflammatory, antiviral, antioxidant, estrogenic, enzyme inhibition, vascular and cytotoxic antitumor activity. Other than the leaves of *B. gymnorrhiza,* it was reported that, the presence of phytochemical compounds like phenols and flavonoids in the extract of *Avicenna marina* and *R. stylosa* leaves (Fokuia et al. 2014). The antibacterial activity of the ethanolic extract of *B. gymnorrhiza* leaves showed more inhibition against *Streptococcus pyogenes* compared with other organisms like *Salmonella paratyphii, Staphylococcus aureus*. The antifungal activity was also conducted on the same ethanolic extract of *B. gymnorrhiza* leaves and the inhibition were founded in *Aspergillus fumicatus*. As reported earlier the ethanol extract of the leaves showed more antimicrobial activity compared to other solvents like methanol and chloroform extract, the chloroform extracts of leaves didn't show any inhibition against the growth of pathogenic bacteria as reported in previous work (Haq et al. 2011). The whole plant of *R. mucronata* inhibited the growth of all bacteria strains. The bark extract gave the highest activity in both tested bacteria with inhibition zone value of 8.8 mm and 8.6 mm against *S. aureus* and *E. coli,* respectively. *R. mucronata* inhibited *S. aureus* than *E. coli* due to the substances of each part (Rahmi et al. 2012), which clearly shows the family *Rhizophoraceae* as good anti-bacterial, anti-molluscidal, and anti-feedant agents.

Acknowledgement The authors are grateful to acknowledge the PG & Research department of Botany, PSG college of Arts & Science, Coimbatore for providing the necessary facilities during the study.

Conflict of interest statement: We declare no conflict of interest.

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