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Antifouling Activity of Extracts from Mangroves against Biofouling Bacteria Isolated from Boats in Royapuram, Chennai, India

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ABSTRACT

Keywords

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Biofouling is a process of attachment and growth of micro- and macro-foulers on submerged natural or manmade marine structures causing huge economic and environmental losses worldwide. Marine organisms are rich source of structurally novel and biologically active metabolites and can be one of the best replacement options as natural antifoulants against toxic heavy-metal-based paints. Mangroves possess a diverse array of bioactive compounds; hence, an attempt was done to evaluate its antifouling (AF) potential. In this study, a biofoulant sample was collected from boats, and culturable marine bacteria were isolated on Zobell's marine agar medium. The 16SrRNA sequencing of five isolates was done and confirmed to be *Vibrio parahaemolyticus*, *Vibrio nereis*, *Micrococcus luteus*, *Exiguobacterium profundum* and *Salinicococcus shispanicus*. *In vitro* screening for AF potential of methanolic extract of two mangroves (leaves, bark and root), viz. *Bruguiera cylindrica* and *Rhizophora apiculata* collected from Pichavaram against biofouling bacteria was done by the agar diffusion method. The results indicated that mangroves have potent AF components and can be used as a natural source of antifoulant.

Introduction

Biofouling involves colonization of structures that are either living or artificial mainly in the aquatic environment by micro- (bacteria, diatoms, spores of macroalgae and protozoans) and macro-foulers (barnacles, bryozoans, algae and tubeworms). Biofilm formation by bacteria is the key step during marine fouling which serves as a focus for the attachment and growth of invertebrates, sessile plants and animals (Camps *et al.*, 2011).

Mature biofouling communities are complex, highly dynamic ecosystems, which once established are extremely difficult to eradicate creating extensive problems in marine technology such as roughness on the ships' hulls, reducing efficiency, speed and maneuverability (Manilal *et al.*, 2010). The problem is so severe worldwide that the expenditure incurred on antifouling (AF) measures alone is approximately US\$ 6.5 billion a year (Bhadury *et al.*, 2004).

AF includes both defensive biological processes used by marine organisms to limit epibiosis and methods used to protect submerged structures such as ships' hulls (Yebra *et al.*, 2004), aquaculture equipment (Braithwaite and McEvoy 2004) and optical devices (Patil *et al.*, 2007). One of the most promising alternatives to tributyltin or heavy-metal-based paints is the development of naturally occurring AF compounds from marine organisms. To date, a variety of Natural Product Antifoulants (NPAs) have been isolated from different marine organisms, including marine bacteria, algae, sponges, corals, seaweeds, seagrasses and mangrove plants (Deepa *et al.*, 2014; Qian *et al.*, 2010; Raveendran *et al.*, 2009). A wide variety of laboratory AF assays have been developed using different test organisms including bacteria, diatoms, algae, barnacle, mussels and other species, which are major contributors to fouling (Briand 2009).

Mangroves are widespread in tropical and subtropical regions, growing in the saline intertidal zones of sheltered coastlines and contain biologically active antimicrobial compounds (Abeyasinghe *et al.*, 2006; Ravikumar *et al.*, 2011). Several species of mangroves have been reported to contain secondary metabolites like alkaloids, phenolics, steroids and terpenoids, which are of toxicological, pharmacological and ecological importance (Bandaranayake 2002). But, there is scant information on the isolation of antifouling compounds from mangrove species. Therefore, an attempt was made to evaluate the AF potential of two mangrove species, viz. *Bruguiera cylindrica* and *Rhizophora apiculata* against the biofouling bacteria.

Materials and Methods

Collection of mangroves

Two species of mangroves, viz. *Bruguiera cylindrica* and *Rhizophora apiculata*, were

collected and identified from Pichavaram (11°26'N:73°48'E), India. The samples were washed with distilled water to remove salt, dirt and other associated debris. Clean samples were then dried under shade for one week at room temperature. The leaves, bark and root of the mangroves were separated and ground.

Extraction of crude bioactives

The leaves, bark and root of the mangroves were extracted in methanol at 1:3 ratio for 72 h and evaporated to dryness using a rotary evaporator at 40°C. The residues were preserved at 4°C for further analysis.

Isolation of biofouling bacteria

Biofouling samples were collected from boats in Royapuram (13°7'31.98" N, 80°17'51.52" E), Chennai, India. The biofilms were scraped off from five different locations and placed in a sterile container; an additional 100 ml of sterilized seawater was added and placed in an icebox. The sample was subjected to vigorous vortexing for 5 min and serially diluted using sterilized seawater. A volume of 100 µl of the diluents was spread on sterile Zobell's Marine Agar 2216 (Himedia, Mumbai). The plates were incubated at room temperature (27°C–30°C) for 5 days, and isolated colonies showing different morphological characteristics were identified using minimum biochemical tests (Das *et al.*, 2007) and confirmed by 16SrRNA sequencing. The purified isolates were then cultured on Zobell's Marine Slant and stored at 4°C.

16S rRNA sequencing

Genomic DNA was isolated using an InstaGene™ Matrix Genomic DNA isolation kit and amplified by using PCR primers 27F (5'AGAGTTTGATC MTG

GCTCAG-3') and 1492 (5'TACGGYT ACC TTGTTACGACTT-3'). The sequencing was done using 518F (5'-CCAGCAGCCGC GGTAATACG-3') and 800R (5'-TACCAG GGTATCTAATCC-3') primers. Sequence reactions were performed using ABF prism BIG Dye™ Termination cycle sequencing kits with AmpliTaq^R DNA polymerase (FS enzyme) Applied Biosystem. The 16S rRNA sequence was blast using NCBI Blast similarity search tool.

AF bioassay

The antimicrofouling activity of different concentrations (100 µg/ml and 200µg/ml) of leaf extracts against five test organisms was done by the agar diffusion method (Nathan *et al.*, 1978). A volume of 100 µg/ml of methanolic extract of bark and root of mangroves was also assayed similarly. All the assays were done in triplicate. After incubation, the plates were observed for the zone of inhibition around the wells, and the zone size was measured in millimetres.

Antimicrobial efficacy was graded based on the zone diameter as high activity (> 15 mm), moderately active (10–14 mm), trace activity (5–9 mm) and no activity (<4 mm) (Rios *et al.*, 1988).

Minimum inhibitory concentration assay

Determination of MIC value for methanolic extract that showed high antibacterial activity was determined by broth dilution method (Ravi *et al.*, 2011). Various dilutions of (3.25, 6, 12, 24.5, 50, 100, 500, and 1000 µg/ml) of leaf extracts was prepared with DMSO and mixed with 50 µl of 24 h old bacterial inoculum.

After incubation, turbidity were measured and the lowest concentration of the extract inhibiting the growth of test bacteria was observed and tabulated.

The identification of functional groups in crude mangrove parts was also determined through FTIR (Shimadzu).

Results and Discussion

Marine bacteria from boats in the form of biofilms or microfouling were isolated and cultured, and five morphologically distinct isolates were identified as *Vibrio parahemolyticus* BF-1, *Vibrio nereis* BF-2, *Salinicoccus hispanicus* BF-3, *Micrococcus luteus* BF-4 and *Exiguobacterium profundum* BF-5 using 16S rRNA sequencing. The efficacy of the crude methanolic extracts of *Bruguiera cylindrica* and *Rhizophora apiculata* was evaluated against these five biofouling bacteria (Tables 2 and 3). The effectiveness of the active compounds present in the plant extract showed growth inhibition as clear areas surrounding the well.

Methanolic leaf extract of *Rhizophora apiculata* at 100 µg/ml concentration exhibited a wide spectrum of activity, with maximum zone of inhibition against *Vibrio parahemolyticus* BF-1 (16 mm) and minimum against *Vibrio nereis* BF-2 (Figure 1). *Bruguiera cylindrica* showed maximum activity against *Exiguobacterium profundum* BF-5 (17mm) and minimum against *Vibrio nereis* BF-2 (12mm) as shown in Figure 2. At 200 µg/ml concentration the maximum zone of inhibition for *Rhizophora apiculata* was 24 mm against *Vibrio parahemolyticus* BF-1 and *Exiguobacterium profundum* BF-5 and 25 mm for *Bruguiera cylindrica* against *Micrococcus luteus* BF-4. Antimicrobial activity of both the mangroves was less against *Vibrio nereis* BF-2 and *Salinicoccus hispanicus* BF-3.

Crude leaf extracts of *Bruguiera cylindrica* and *Rhizophora apiculata* showed higher AF activity than bark and root extract

irrespective of concentration. The bark of *Bruguiera cylindrica* showed better AF activity against all bacteria compared to *Rhizophora apiculata* with maximum against *Vibrio parahemolyticus* BF-1 and *Micrococcus luteus* BF-4 as shown in Figure 3. The root extract of both the mangroves showed almost similar inhibition results against the biofouling bacteria (Figure 4). The difference in the antimicrobial effect of mangroves could be due to the quantity of antimicrobial components present in each form. The observation can be rationalized in terms of the polarity of the compounds being extracted by the solvent, intrinsic bioactivity and ability to diffuse in the media used for the assay (Nair *et al.*, 2007).

The extract of *Rhizophora apiculata* showed MIC value of 50 µg/ml against *Vibrio parahemolyticus* BF-1, *Exiguobacterium profundum* BF-5 and 100 µg/ml against *Vibrio nereis* BF-2, *Salinicoccus hispanicus* BF-3 and *Micrococcus luteus* BF-4.

Likewise, *Bruguiera cylindrica* exhibited MIC value of 50 µg/ml against *Micrococcus luteus* BF-4 and *Exiguobacterium profundum* BF-5 followed by MIC value of 100 µg/ml against the remaining bacteria (Table 4). Overall, the mangrove extracts of *Bruguiera cylindrica* and *Rhizophora apiculata* showed better antibacterial activity against the strains *Vibrio parahemolyticus* BF-1, *Exiguobacterium profundum* BF-5 and *Salinicoccus hispanicus* BF-3.

Fourier Transform Infrared Spectroscopy (FTIR) analysis revealed the presence of bonds such as O–H stretch, H-bond (alcohols, phenols), C–H stretch (alkanes), C–O stretch (alcohols, carboxylic acids, esters and ethers), C–Br (alkyl halides) in all the extracts as shown in Table 4 (Figures 5–10). The inhibitory activity is mainly correlated with these active functional groups.

Table.1 Details of BLAST analysis, percentage of similarity and NCBI accession numbers of biofouling bacteria isolated from boats

S. No.	Assigned code	Sequence length	Similarity %	BLAST results	NCBI's accession number
1.	BF-1	1486	99	<i>Vibrio parahemolyticus</i>	KR137715
2.	BF-2	1473	99	<i>Vibrio nereis</i>	KR137716
3.	BF-3	1507	99	<i>Salinicoccus hispanicus</i>	KR137717
4.	BF-4	1467	99	<i>Micrococcus luteus</i>	KR137718
5.	BF-5	1496	99	<i>Exiguobacterium profundum</i>	KR137719

Table.2 Antibacterial activity of leaf extract (zone of inhibition in mm).

S. No	Name of the organism	<i>Rhizophora apiculata</i>		<i>Bruguiera cylindrica</i>	
		100 µg/ml	200 µg/ml	100 µg/ml	200 µg/ml
1	<i>Vibrio parahemolyticus</i> BF-1	16	24	13	23
2	<i>Vibrio nereis</i> BF-2	12	20	12	21
3	<i>Salinicoccus hispanicus</i> BF-3	15	20	13	22
4	<i>Micrococcus luteus</i> BF-4	14	21	14	25
5	<i>Exiguobacterium profundum</i> BF-5	15	24	17	24

Table.3 Antibacterial activity of bark and root extracts (zone of inhibition in mm).

S. No	Name of the organism	<i>Rhizophora apiculata</i>		<i>Bruguiera cylindrica</i>	
		Bark	Root	Bark	Root
1	<i>Vibrio parahemolyticus</i> BF-1	12	12	12	12
2	<i>Vibrio nereis</i> BF-2	11	10	11	10
3	<i>Salinicoccus hispanicus</i> BF-3	12	11	12	11
4	<i>Micrococcus luteus</i> BF-4	11	11	11	11
5	<i>Exiguobacterium profundum</i> BF-5	12	10	12	10

Table.4 MIC of methanolic leaf extracts

S.No	Name of the organism	<i>Rhizophora apiculata</i> µg/ml	<i>Bruguiera cylindrica</i> µg/ml
1.	<i>Vibrio parahemolyticus</i> BF-1	50	100
2.	<i>Vibrio nereis</i> BF-2	100	100
3.	<i>Salinicoccus hispanicus</i> BF-3	100	100
4.	<i>Micrococcus luteus</i> BF-4	100	50
5.	<i>Exiguobacterium profundum</i> BF-5	50	50

Fig.1 Antibacterial activity of leaf extract of *Rhizophora apiculata*

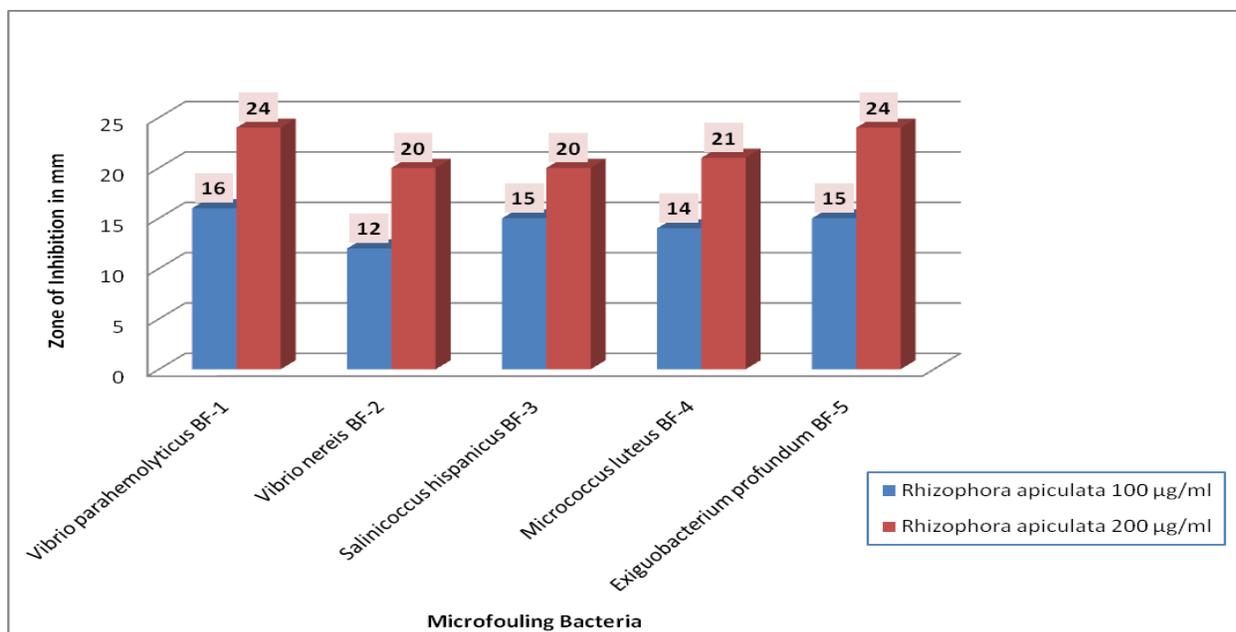


Table.5 Identification of functional groups through FTIR analysis

Name of the species	Frequency (cm ⁻¹)	Bond	Functional group
<i>Rhizophora apiculata</i>			
Leaves	3408 (b,m)	O–H stretch, H-bonded	Alcohols, phenols
	2954 (s)	C–H stretch	Alkanes
	1734 (s)	C=O stretch	Aliphatic esters, aldehydes
	1647 (m)	N–H bend	1°amines
	1058 (s)	C–O stretch	Alcohols, carboxylic acids, esters and ethers
	876 (s)	C–H “opp”	Aromatics
	653 (s)	C–Br stretch	Alkyl halides
Bark	3422 (s,b)	O–H stretch, H-bonded	Alcohols, phenols
	2925 (m)	C–H stretch	Alkanes
	1739 (s)	C=O stretch	Aliphatic esters, aldehydes
	1628 (m)	N–H bend	1°amines
	1043 (m)	C–O stretch	Alcohols, carboxylic acids, esters and ethers
	775 (m)	C–Cl stretch	Alkyl halides
Root	3395 (b)	O–H stretch, H-bonded	Alcohols, phenols
	2925 (s)	C–H stretch	Alkanes
	1736 (m)	C=O stretch	Aliphatic esters, aldehydes
	1634 (s)	N-H bend	1°amines
	1044 (s)	C-O stretch	Alcohols, carboxylic acids, esters and ethers
<i>Bruguiera cylindrical</i>			
Leaves	3416 (b)	O-H stretch, H-bonded	Alcohols, Phenols
	2925 (m)	C-H stretch	Alkanes
	1736 (s)	C=O stretch	Aliphatic esters, aldehydes
	1623 (m)	N-H bend	1°amines
	1324 (m)	C-N stretch	Aromatic amines
	1038 (m)	C-O stretch	Alcohols, carboxylic acids, esters and ethers
	660 (m):	C=C-H:C-H bend	Alkynes
Bark	3422 (s,b)	O-H stretch, H-bonded	Alcohols, Phenols
	2925 (m,n)	C-H stretch	Alkanes
	1739 (s,n)	C=O stretch	Aliphatic esters, aldehydes
	1628 (m,sh)	N-H bend	1°amines
	1043 (m,b)	C-O stretch	Alcohols, carboxylic acids, esters and ethers

	775 (s,n)	C-Cl stretch	Alkyl halides
Root	3418 (m,b)	O-H stretch, H-bonded	Alcohols, Phenols
	2926 (m,n)	C-H stretch	Alkanes
	1738 (s,n)	C=O stretch	Aliphatic esters, aldehydes
	1623 (s,n)	N-H bend	1°amines
	1036 (s,b)	C-O stretch	Alcohols, carboxylic acids, esters and ethers

Fig.2 Antibacterial activity of leaf extract of *Bruguiera cylindrica*

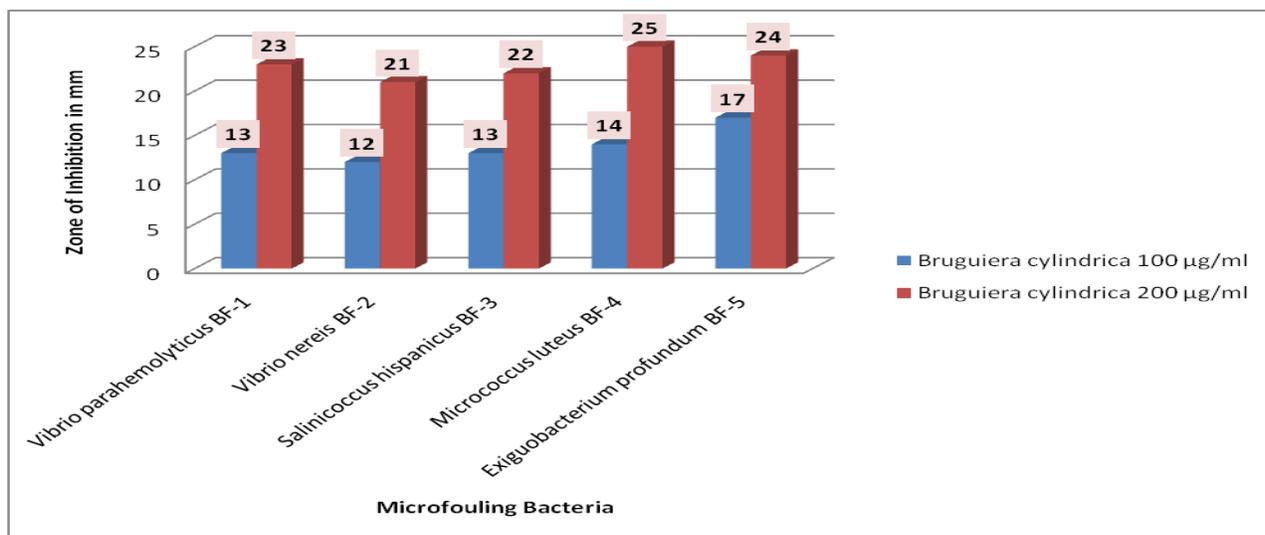


Fig.3 Antibacterial activity of bark extract

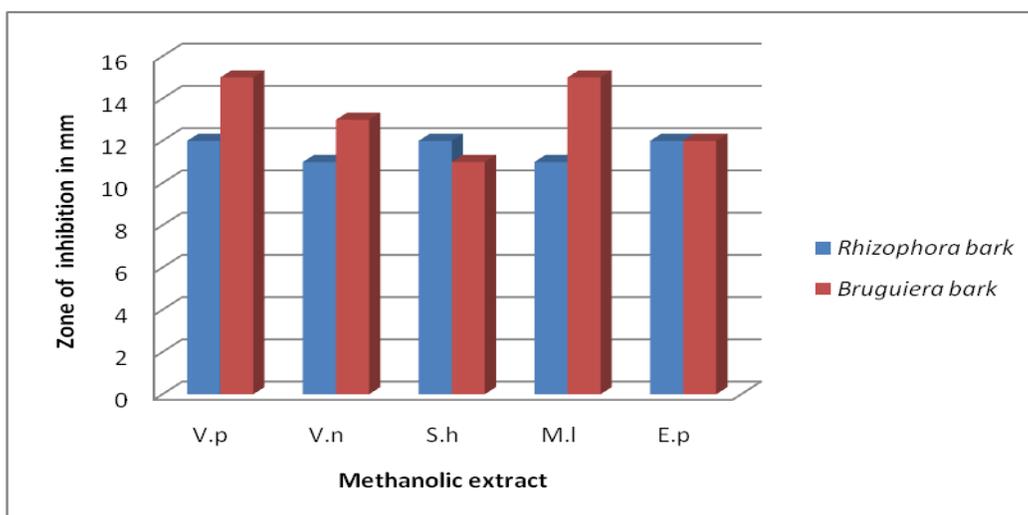


Fig.4 Antibacterial activity of root extract

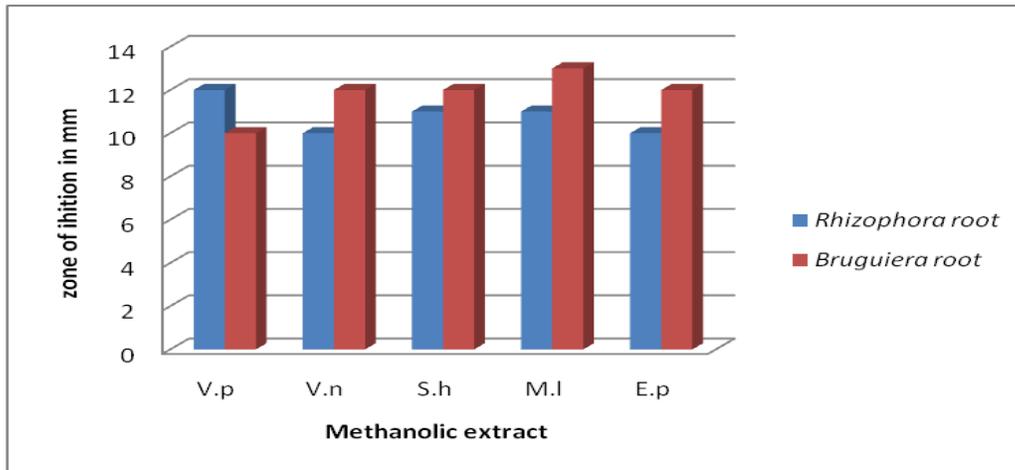


Fig.5 FTIR spectrum of *B. cylindrica* leaves

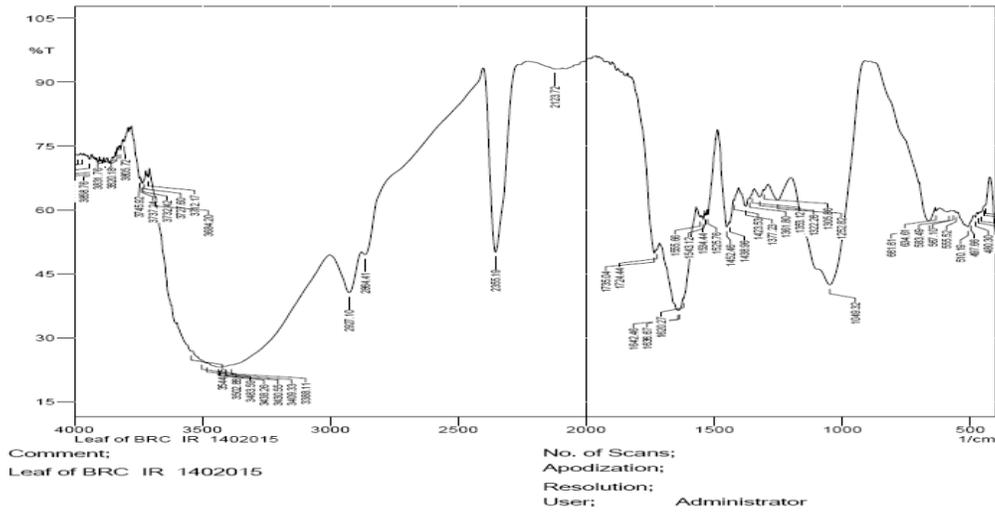


Fig.6 FTIR spectrum of *R. apiculata* leaves

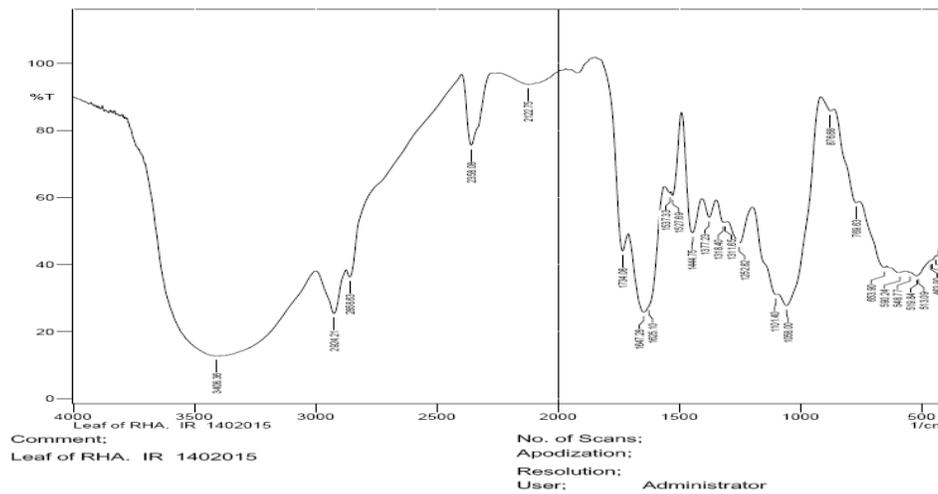


Fig.7 FTIR spectrum of *B. cylindrica* bark

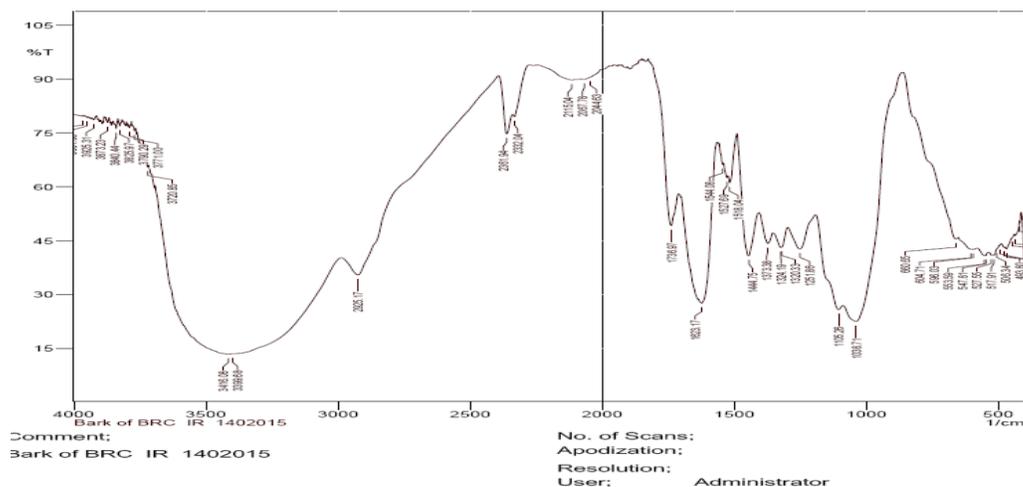


Fig.8 FTIR spectrum of *R. apiculata* bark

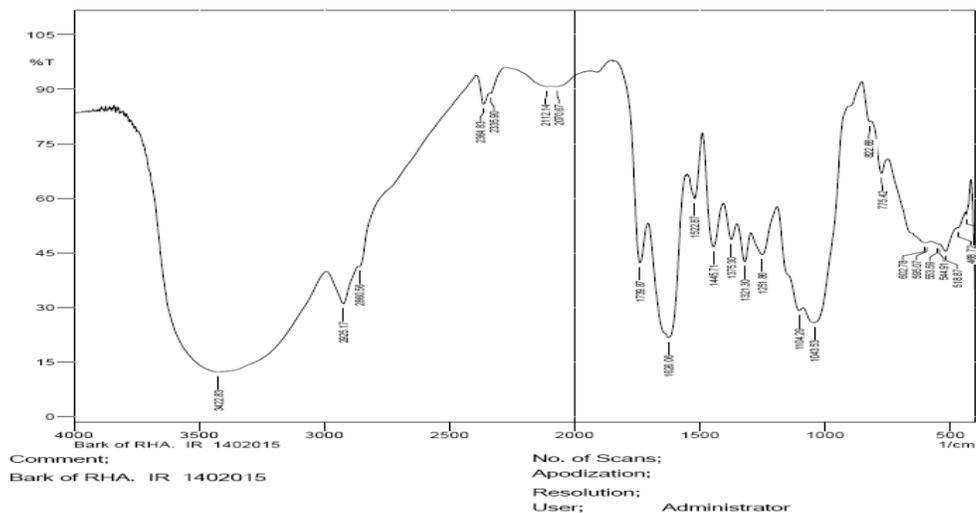


Fig.9 FTIR spectrum of *B. cylindrica* root

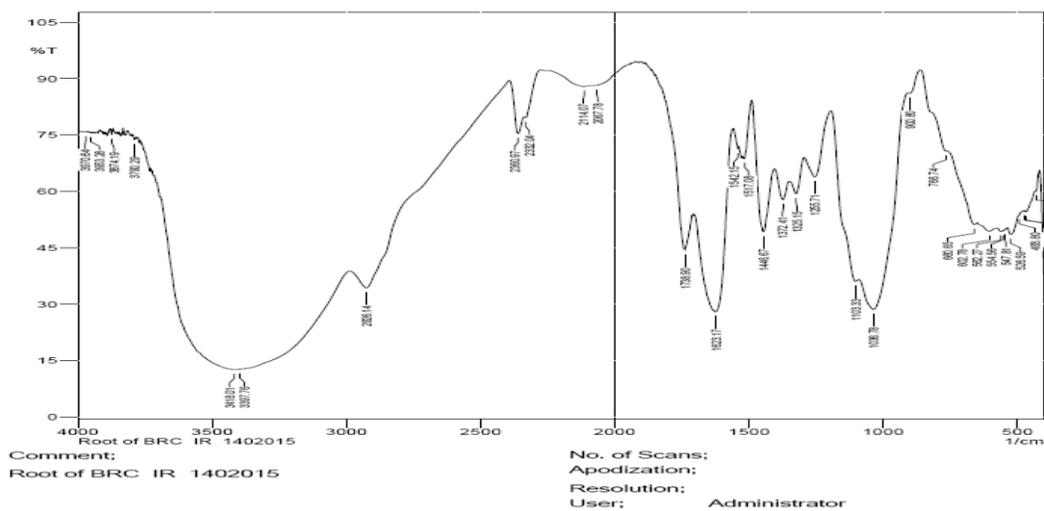
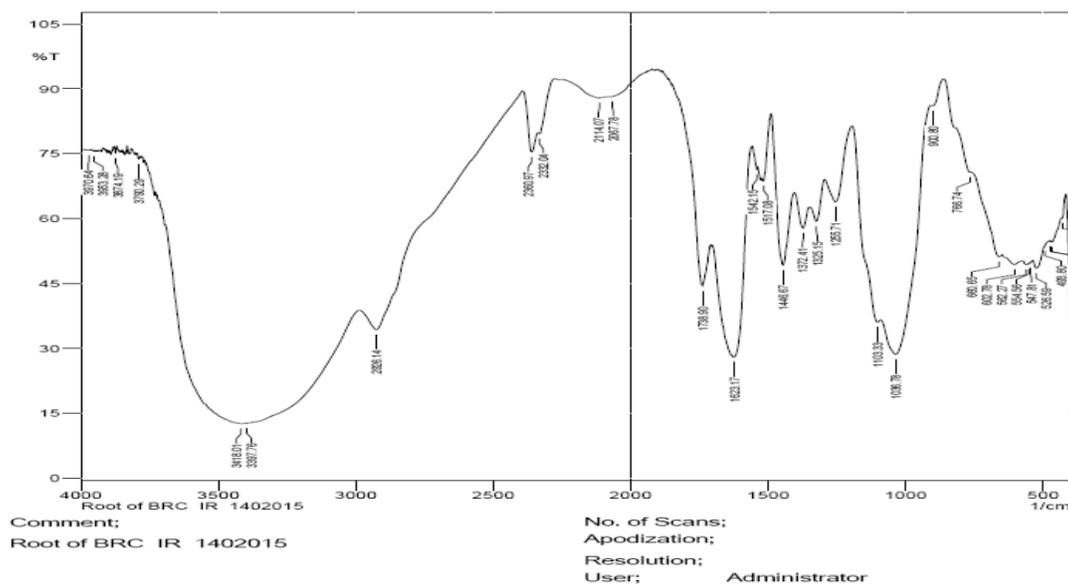


Fig.10 FTIR spectrum of *R. apiculata* root

AF agents derived from natural products may be less environmentally harmful than the current toxic chemicals, having less activity against non-target species. The extracts of mangroves were reported to have AF properties (Chen *et al.*, 2008). The present study also clearly showed that extracts from mangroves possess antimicrobial property against tested fouling bacteria. The effectiveness of the active compounds present in the plant extract showed growth inhibition as clear areas surrounding the well.

In vitro screening results showed that both the mangroves possess maximum antimicrobial activity against *Vibrio parahaemolyticus* BF-1 and *Exiguobacterium profundum* BF-5 and least against *Vibrio nereis* BF-2. These preliminary data suggest that extracts of *Rhizophora apiculata* and *Bruguiera cylindrica* may be used to develop an environmentally safe antifoulant to control fouling organisms. The inhibition activity is mainly correlated with the major functional groups like amino, hydroxyl, carbonyl and

aliphatic group. The bonds such as O-H stretch, H-bonded, C-H stretch, C-O stretch, C-Br were present in all extracts and could be mainly involved in inhibition activity. Prabhakaran *et al.*, have reported that mangroves showed higher activity against biofilm-forming bacteria compared to seaweed and seagrass extracts and should be further investigated with various fractions of the extracts.

The extracts of mangroves were reported to have AF properties (Chen *et al.*, 2008). Similar studies done by Deepa *et al.*, observed that *Excoecaria agallocha* and *Kandelia candel* showed promising AF results against fouling bacteria (*V. vulnificus*, *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus*), diatoms (*N. subinflata* and *N. palea*) and barnacle cyprid. Significant AF activity against *P. vulgata* was observed with methanol extract from *A. marina*, followed by extracts of *B. cylindrica* and *A. ilicifolius* (Manilal 2009). Antioxidant activities of bark extract from mangroves, *Bruguiera cylindrica* (L) Blume and *Ceriops decandra per* indicates its potential as

dietary supplements to treat chronic diseases caused by overproduction of free radicals (Krishnamoorthy *et al.*, 2011). Ethanolic extract of *Avicenna marina* had different levels of antibacterial activity against antibiotic-resistant bacteria and eye pathogen (Ravikumar *et al.*, 2011). Antisettlement against *Balanus albicostatus* was reported with *Ceriops tagal* because of diterpene methoxy-ent-8-14-pimarenely-15-one and it showed LC50 of 10 μ /cm² (Chen *et al.*, 2008).

In conclusion, the study of marine organisms for their bioactive potential has gained momentum in recent years and may provide valuable information for fouling control in the marine environment. This is the first and successful attempt on the studies of AF activity of mangroves against biofouling bacteria from boats. This study concludes by stating that bioactive compounds present in tested mangroves possess AF potential and will definitely aid in the development of natural antifoulants in the near future. Further studies are needed to identify the pure compounds, establish exact antibacterial mechanism and formulate it as effective antifoulants.

Nucleotide sequence accession numbers

All the five sequences were submitted to Genbank, the National Centre for Biotechnology Information (NCBI) through Bankit, and the accession numbers were given as: KR137715, KR137716, KR137717, KR137718 and KR137719.

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