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Evaluating the antibacterial and anticandidal potency of mangrove, *Avicennia marina*

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ABSTRACT

Objective: To evaluate the antibiotic activity of mangrove plant, *Avicennia marina* (*A. marina*) against human and shrimp pathogens and to delineate bioactive constituents by gas chromatography-mass spectrometer (GC-MS) profiling.
Methods: The antimicrobial activity of the different polar and non-polar extracts of *A. marina* was inspected by well diffusion technique against 16 bacterial pathogens and two fungal pathogens.
Results: Of the six organic extracts examined, methanolic extract of *A. marina* fairly repressed the growth of all bacterial and fungal pathogenic strains tested. In general, mangrove extract was more active against the bacterial pathogens while against yeasts, the activity was lesser. The antibiotic activity was attributed to the presence of diverse bioactive secondary metabolites. The chemical profiling of the methanolic extract was performed by GC combined with mass spectrometry. The results of GC-MS showed that the main phytoconstituents were benzeneethanol,4-hydroxy- (RT = 12.173), followed by benzaldehyde,3-methyl- (RT = 6.811). Finally, the GC-MS data evinced that the antimicrobial activity of *A. marina* was due to the synergistic effect of all constituents or the activity of major constituents.
Conclusions: Considering the urgent need of novel antibiotics, the present study brings out a new insight on the exploration of mangroves for antibiotic production in future.

1. Introduction

Mangrove floras have historically been extensively explored for their versatile medicative usances[1,2]. The applications include the treatment of rheumatism, small pox, abscess, scabies, sores, boils and ulcers as peer-reviewed by Bandaranayake[3]. Their efficacy may be ascribed to the existence of diverse bioactive principles. It is speculated that mangrove flora biosynthesize the rifest array of secondary metabolites to vye against biotic and abiotic stress factors in their natural habitat. Albeit, scores of natural bioactivities have been discovered, the demand for novel therapeutic compounds is still imperative in concern with newly emerging diseases and multidrug-resistant strains of microorganisms. The marine floral bioactive principles have lower adverse reactions than those currently used synthetic antimicrobial derivatives and can defy the growth of pathogens by diverse mechanisms. Hence, the application of natural bioactivities allows an alternative scope in the management of multidrug-resistant microbial strains[4]. The screening of mangroves with higher biological activities could facilitate the discovery of novel natural products, which is suitable for lead compounds in biopharmaceutical sectors. Thence, more researches pertained to the bioactivity screening of mangrove floras should be accentuated.

For the species of mangrove plants, *Avicennia marina* (*A. marina*) is globally distributed and officinally used to ameliorate a range of physical ailments and foster healing since antiquity[5]. For instance, retrospective studies justified that *A. marina* bears reputed bioactive properties such as anticancer, antiviral and antifungal activities[6-8]. Mangrove plants of *A. marina* are plenteously distributed in the wetlands of South Indian littoral. Howbeit, the efficacy of *A. marina* from the study area is not thoroughly scrutinized. Data obtained from our earlier study articulated that methanolic extract of *A. marina* which is sourced from the mangrove wetland of Ayiramthengu located in Kollam District (8°54' N and 76°38' E) (southwest coast of India) showed anti-*Vibrio*, cytotoxic, antifouling activities[9]. As yet, the antimicrobial activities of *A. marina* from other regions of southwest coast of India have seldom

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been explored. In this background, the present study is initiated to examine the antibiotic activity of *A. marina* and its bioactive constituents.

2. Materials and methods

2.1. Collection and extraction of secondary metabolites from *A. marina*

Field collection of tender foliage of mangrove, *A. marina* was made from the mangrove wetland of Puthenvelikkara located in Eranakulam (10°11'51.7" N and 76°14'34.5" E) expanse (southwest coast of India). The voucher specimen was identified to the genus and specie level by Prof. Ravi N, an eminent mangrove taxonomist in India. Freshly-collected specimens were immediately transported to the laboratory on ice and subjected to organic solvent extraction. Prior to extraction, 10 g of plant leaves were gently rinsed with filtered water to eliminate salt and extraneous contaminants. Once rinsed, samples were excised into thin sections using a sterile scissor and extracted in different solvents of increasing polarity such as chloroform, dichloromethane, ethyl acetate and methanol. The extraction procedure was described in detail previously[2]. The resultant aliquot was filtered using muslin cloth sheets and concentrated to near dryness in a distillation system, also was weighed and placed in the refrigerator at 4 °C until it was used for antibiotic assay.

2.2. Microbial test cultures

Antibiotic activity of the mangrove extracts was examined against 16 strains of human and animal bacterial pathogens and two strains of clinical *Candida* spp. The bacterial and fungal strains used in assay were enlisted in the Table 1. The human and shrimp pathogens with MTCC number were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India while the *Vibrio* isolates were culled from *Penaeus monodon* smote with vibriosis[10]. The clinical bacterial and fungal pathogens were obtained from the clinical laboratory. All the bacterial strains were maintained on nutrient agar slants (Hi-Media) at (37.0 ± 0.1) °C as described elsewhere[1]. Sabouraud dextrose agar slant was used for the routine propagation of *Candida* spp. Fresh overnight bacterial cultures were prepared for experiments by transferring a loop-full of inoculums from stock cultures to test the tubes of nutrient broth sterilized at 121 °C for 20 min.

2.3. Antibacterial assay

The crude mangrove extracts were inspected for antimicrobial activity utilizing the well diffusion assay technique[2]. In short, sterile Mueller-Hinton agar was dispensed into Petri plates and uniformly swabbed with 100 µL of suspension containing 10^8 CFU/mL of appropriate strains of pathogens. The well was made on the seeded surface using a sterile cork borer and 100 µL of appropriate extract of particular concentration (5 mg/mL) was placed inside the wells. Organic solvent used for the extraction was used as positive control against the bacteria. The experimental Petri plates were incubated at 37 °C for 24 h and the diffusion-inhibition area was calculated in mm^2 and compared with controls. The extracts showing an inhibitory area of 20 mm^2 was deemed active. The assays were conducted in triplicate to validate the findings statistically.

Table 1

Panel of pathogens used for antimicrobial assay.

Groups	Organisms	
Human pathogens	Gram-positive bacteria	*S. aureus* (MTCC 96)
		Streptococcus mutans (MTCC 890)
		Micrococcus luteus (MTCC 106)
	Gram-negative bacteria	*Klebsiella pneumoniae* (MTCC 109)
		Shigella flexneri (MTCC 1457)
		V. mimicus (MTCC 4434)
Clinical isolates		*S. aureus*
		Pseudomonas sp.
		Proteus vulgaris
		E. coli
		Klebsiella sp.
Shrimp pathogens		*V. parahaemolyticus* (MTCC 451)
		V. vulnificus (MTCC 1145)
Shrimp *Vibrio* isolates		*V. harveyi* (Vb2)
		V. alginolyticus (Vb3)
		Vibrio fischeri (VA1G)
Candida spp. (clinical)		*C. albicans*
		C. tropicalis

S. aureus: *Staphylococcus aureus*; *V. mimicus*: *Vibrio mimicus*; *E. coli*: *Escherichia coli*; *V. parahaemolyticus*: *Vibrio parahaemolyticus*; *V. vulnificus*: *Vibrio vulnificus*; *V. harveyi*: *Vibrio harveyi*; *V. alginolyticus*: *Vibrio alginolyticus*; *C. albicans*: *Candida albicans*; *C. tropicalis*: *Candida tropicalis*.

2.4. Anticandidal assay

The anticandidal assay was performed according to the methodology described by Manilal *et al*.[11]. The Sabouraud dextrose agar (Hi-Media) was used for anticandidal screening and routine propagation of yeast strains. Cell suspensions containing approximately 10^7 CFU/mL cells of yeasts were prepared and evenly spread onto the surface of the agar plates using a sterile swab sticks. Thereafter, wells were prepared using a sterile cork borer. The resultant wells were carefully filled with 100 µL of mangrove extract. The well with solvent used for extraction was considered as negative control. The assay was performed in triplicates of individual Petri dishes. The diameter of the inhibition halo after 48 h was considered to be indicative of bioactivity and the area of zone of inhibition was calculated.

2.5. Gas chromatography-mass spectroscopic (GC-MS) analysis of *A. marina*

The crude extract was chemically analysed by GC-MS using a Clarus 500 GC from Perkin-Elmer equipped with mass detector turbo mass gold-Perkin Elmer TurboMass 5.2 spectrometer and an elite-5 MS (5% diphenyl/95% dimethylpolysiloxane), 30 × 0.25 mm × 0.25 µm of capillary column was used with helium at a 1 mL/min as a carrier gas. The GC oven temperature was kept at 110 °C for 2 min, programmed to 280 °C at the rate of 5 °C/min and kept constant at 280 °C for 10 min. The split ratio was adjusted to 1:20 and the injection volume was 2 µL. The injection and detector temperature were 250 °C. The GC-MS electron ionization mode was 70 eV. Mass scan range was from *m/z* 45–450 Da. The peaks of the GC were subjected to mass-spectral analysis. The identification of peaks was carried out using National Institute of Standards and Technology, Version 2.0 (2005).

2.6. Data analysis

The results of the antimicrobial activity were expressed as mean ± SD. The mean values were calculated using One-way ANOVA and SPSS for Windows version 20.0 (Statistical Package for Social Services, Chicago, IL, USA).

3. Results

The results from the overall inhibitory pattern of six organic extracts of *A. marina* against fungal and bacterial pathogens were shown in Table 2. The methanolic extract of *A. marina* exerted the highest rank of activity (100%) which curbed the growth of all bacterial and fungal strains. Whilst, the extracts obtained from ethyl acetate, dichloromethane and ethanol showed moderate to lower rank of activity. In contrast, chloroform and poly butylenes succinate extracts of *A. marina* were found to be inactive. Comparing the results of inhibitory properties of all organic extract, it was evinced that the methanolic extract was the best solvent for the maximum extraction of secondary metabolites. Furthermore, no inhibiting effect was shown by positive control solvent, methanol. Therefore, methanol was selected for further studies.

Table 2

Overall inhibitory activity of different solvent extracts of *A. marina* against different panel of test pathogens.

Solvents	Antimicrobial activity (%)				
	Human Pathogens (MTCC)	Clinical isolates	Fungal strains	Shrimp pathogens (MTCC)	*Vibrio* isolates
Chloroform	0.0	0.0	0.0		
Dichloromethane	33.3	20.0	0.0	100.0	66.6
Ethyl acetate	100.0	100.0	50.0	100.0	100.0
Ethanol	66.6	50.0	0.0	50.0	33.3
Methanol	100.0	100.0	100.0	100.0	100.0
Phosphate buffer saline	0.0	0.0	0.0	0.0	0.0

Overall activity was expressed as relative antimicrobial activity of respective solvent extracts against particular group of test pathogens. Zone of inhibition ⩾ 20 mm^2 was considered as active.

3.1. Antibacterial activity of *A. marina* against human bacterial and fungal pathogens

In this study, methanolic extract of *A. marina* demonstrated the highest and broadest spectrum of inhibitory activity against all the tested bacterial pathogens of human. The area of inhibition ranged from (244.95 ± 15.60) mm^2 to (431.75 ± 10.10) mm^2 was recorded against the human type culture strains (Figure 1) and (105.17 ± 9.90) to (246.30 ± 23.60) mm^2 against the clinical pathogens respectively (Figure 2). Of the six type culture pathogens, Gram-positive bacteria was so sensitive, particularly the *S. aureus* which exhibited marked inhibitory value of (431.75 ± 10.10) mm^2. In the case of clinical pathogens, the highest inhibitory value of (246.30 ± 23.60) mm^2 was displayed against *S. aureus*, however the inhibitory value against Gram-negative pathogen, *E. coli* (105.17 ± 9.90) mm^2 was found to be moderate. On the other hand, anticandidal activity of *A. marina* was lower in the range of (56.44 ± 7.50) mm^2 to (58.19 ± 23.40) mm^2 against *C. albicans* and *C. tropicalis* respectively (Figure 2).

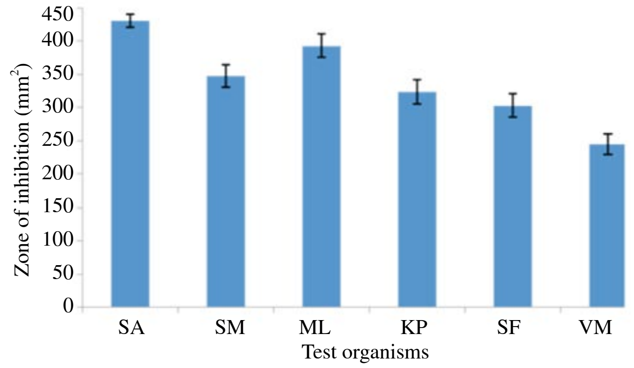
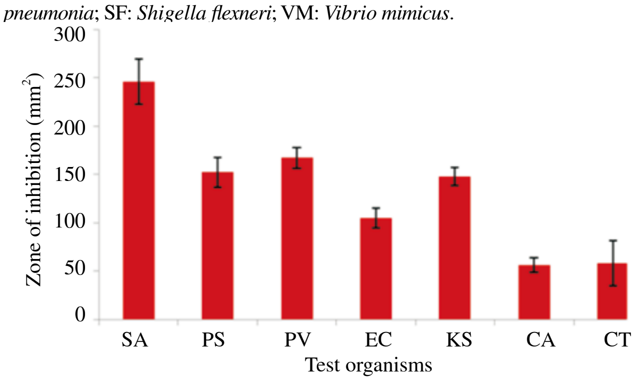


Figure 1. Antibacterial potential of *A. marina* against human type culture strains.

The activities were presented in the inhibition area of the halo. SA: *S. aureus*; SM: *Streptococcus mutans*; ML: *Micrococcus luteus*; KP: *Klebsiella pneumonia*; SF: *Shigella flexneri*; VM: *Vibrio mimicus*.



Figiure 2. Antimicrobial potential of *A. marina* against human clinical strains.

The activities were presented in the inhibition area of the halo. SA: *S. aureus*; PS: *Pseudomonas* sp.; PV: *Proteus vulgaris*; EC: *E. coli*; KS: *Klebsiella* sp.; CA: *C. albicans*; CT: *C. tropicalis*.

3.2. Anti-*Vibrio* activity of *A. marina* against pathogenic type cultures and isolates of shrimp

The anti-*Vibrio* activity of *A. marina* against type culture and isolated *Vibrio* strains were very prominent. The crude extract of *A. marina* produced notable inhibitory values between the ranges of (107.14 ± 15.70) mm^2 and (392.23 ± 11.30) mm^2 (Figure 3). The highest inhibitory values were manifested against *V. vulnificus* [(392.23 ± 11.30) mm^2], followed by *V. alginolyticus* [(369.41 ± 17.40) mm^2]. However, *V. harveyi* (107.14 ± 15.70) mm^2 was found to be least sensitive *Vibrio*.

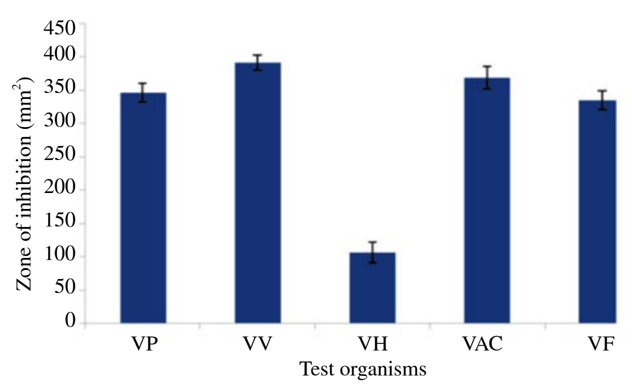


Figure 3. Antibacterial potential of *A. marina* against *Vibrio* pathogens.

The activities were presented in the inhibition area of the halo. VP: *V. parahaemolyticus*; VV: *V. vulnificus*; VH: *V. harveyi*; VAC: *V. alginolyticus*; VF: *Vibrio fischeri*.

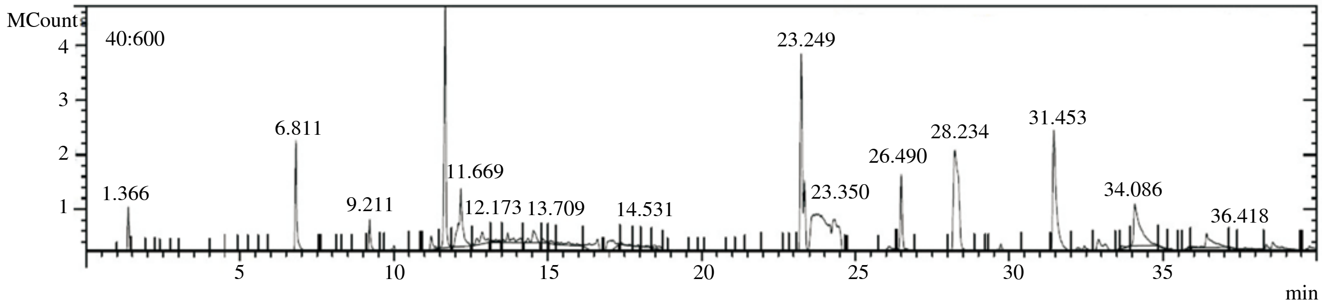


Figure 4. GC-MS chromatogram of the methanolic extract of *A. marina*.

3.3. GC-MS profile of methanolic extract of A. marina

In order to examine the chemical constituents responsible for antimicrobial activity, the crude methanolic extract was subjected to GC-MS analysis. The preliminary GC-MS analysis of crude methanolic extract of *A. marina* on the basis of spectral data revealed the presence of a mixture of volatile compounds. In this study, a total of nine prominent peaks were observed with retention times as presented in Figure 4 and Table 3. The major constituents analysed were benzeneethanol,4-hydroxy- (RT = 12.173), followed by benzaldehyde,3-methyle- (RT = 6.811).

Table 3

The major phytoconstituents identified from the crude extract of *A. marina* by analysis of GC-MS.

No.	RT	Peak name	Height
1	6.811	Benzaldehyde,3-methyle-	934 931
2	9.211	2-Methoxy-4-vinylphenol-	173 086
3	11.669	Cyclobuta(1,2:3,4)dicyclooctene,hexadec	846 689
4	12.173	Benzeneethanol,4-hydroxy-	6 118 893
5	13.709	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)b	39 996
6	14.531	Lycorenan-7-one,9,10dimethoxy-1-methyl-	22 320
7	23.249	1,2-Dicarboxy-3-(4,chlorophenyl)2,3(1H)	553 511
8	23.350	Phenol,2-(1,1-dimethylethyl)-4-(1-methy	202 806
9	26.490	Methyl p-(2-phenyl-1-benzimidazolyl)benz	230 652

RT: Retention time.

4. Discussion

Mangrove plants have long been represented as an accessible source of secondary metabolites with a spectrum of therapeutic and pharmacological potentials[3]. There is a profound attention in studying the antimicrobial potency of mangroves. As a part of our on-going research on the bioactivities of mangroves[4,9,12], this study focuses on the antimicrobial activity of *A. marina* sourced from the South Indian littoral. The study area, southwest coast of India, is recognized as an exclusive habitat for diverse species of mangroves and mangrove associates[1,2,4,9,12]. Approximately, 15 species of mangroves and 33 species of mangrove associates are thus far recorded in the wetlands, intertidal zones and estuarine areas of South Indian littoral and species remain to be documented[13]. However, studies pertained to the in-depth analysis of bioactivity and chemical profiling of mangroves and mangrove associates of the southwest coast of India are still rudimentary and minimal[1,2,12]. Therefore, the purpose of this study was to inspect the antimicrobial activity of *A. marina* sourced from the South Indian littoral. The reason for preferentially collecting *A. marina* was based on the results of our previous bioactivity studies on same specie sourced from another geographical region of South Indian littoral[9]. The preliminary antibiotic screening of *A. marina* revealed that there is a distinct difference between the antibiotic activities against test organisms with regards to the type of solvents used for the extraction. Of the six solvents extract tested, crude extract of *A. marina* obtained from methanol was noteworthy, which subjugated the growth of all test pathogens. The high rank of inhibitory action of the methanolic extract might be due to the presence of higher concentration of the antibiotic constituents. In addition, maximal extent of antibiotic activity was recorded against the bacterial pathogens while minimal range of activity was produced against the yeast. However, antimicrobial activity observed in other solvent extracts was much inferior and therefore excluded in the further studies. As potent inhibitory activity was detected in methanolic extract, it can be inferred that the antibiotic compounds present in the *A. marina* are fairly soluble in methanol than else others. In concordance with our studies, Dhayanithi *et al*. purported that methanolic leaf extract of *A. marina* showed the highest antibacterial activity[14].

In general, it can be opined that the methanolic extract of *A. marina* was the most effective against Gram-positive type culture bacteria whereas the lower rank of activity was manifested to be moderate against Gram-negative ones. Among the Gram-positive bacteria, *S. aureus* was observed to be the most susceptible. The *S. aureus* is a normal flora of humans and one of the preeminent causes of nosocomial bacteraemia. Clinically potent antibiotics to manage multidrug resistant *S. aureus* have not been developed so far. Therefore, results obtained in this study support the possible use of mangrove bioactives as a source of antistaphylococcal agent in future. On the other hand, the most resistant human type culture strain was Gram-negative bacteria, *V. mimicus*. The resistant of Gram-negative bacteria might be due to the variation in cell membrane and permeability.

Antimicrobial resistance of clinical pathogens has been a grave problem worldwide. In this study, the mangrove extract excellently inhibited the growth of all tested clinical pathogens. In the case of clinical strains, extract showed notable inhibitory activity against *S. aureus*. The inhibitory profile of *A. marina* against wide spectrum of human pathogens has been well established for over 3 decades. In addition, previous study proved that other species of *Avicennia* exhibited antibacterial activity against multidrug resistant clinical pathogens[4].

Among the different human fungal pathogens, species of genus *Candida* can cause serious systemic infections which increased substantially over the last decade and they are the most common opportunistic pathogen in patients infected with AIDS virus[15]. In the present study, mangrove extract exhibited minimal degree of activity against the *Candida* sp. when compared with bacterial pathogens. The resistance might be due to the lower concentration of extract used and therefore, much higher concentration of extract is necessary for the strong inhibition. Thus, *A. marina* can be considered as a novel source for extracting new molecules which show antibiotic activity against *Candida* sp. Literature appertained to the anticandidal activity of *A. marina* is scanty[16,17]. This report is the first to describe the possible anticandidal activity of *A. marina* from the South Indian littoral.

Shellfish like shrimps can preferentially accumulate microorganisms such as *V. parahaemolyticus* and *V. vulnificus* which results in a higher incidence of food poisoning acquired through shellfish consumption. Literature survey revealed that there has been relatively few studies that specifically reported the anti-*Vibrio* activity of *A. marina*[9]. In this study, the crude extract successfully inhibited the growth all tested *Vibrio* pathogens. It was further found that anti-*Vibrio* activity of *A. marina* evidenced in this study had also been determined in our previous study[9]. Howbeit, anti-*Vibrio* activity of *A. marina* sourced from the Kollam coast has not shown reproducible results and is found to be currently diminished (data not shown). The overall results of present study evidence a wide antimicrobial spectrum of the methanolic extract of *A. marina,* which emphasizes the possibility of developing novel antibiotics.

The chemical prospecting of *A. marina* dates back to 1961[18]. Hitherto, chemical studies of *A. marina* have revealed the presence of more than 66 chemical components therein[19]. The results of GC-MS profiling revealed that the main phytoconstituents were benzeneethanol, 4-hydroxy- (RT = 12.173), followed by benzaldehyde, 3-methyle- (RT = 6.811). The antibiotic potency displayed by the *A. marina* could be associated with the high percentage of benzeneethanol,4-hydroxy and benzaldehyde,3-methyle which is already envisaged to possess antibiotic activity[20]. There are some recent reports on GC-MS analysis of *A. marina* from the Indian coast[14,21]. In conclusion, results of the present study surmised that mangrove plant, *A. marina* is a rich and novel source of drug leads for antibiotics, warranting further comprehensive studies on chemical elucidation of active constituents as well as the mechanism of antibiosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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