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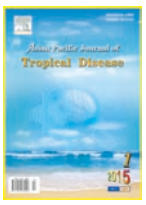

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# An *in vitro* antagonistic efficacy validation of *Rhizophora mucronata*

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PEER REVIEW

**Peer reviewer**

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**Comments**

In the article authors described the result of screening for antibacterial activities of *Rhizophora* and preliminary study regarding the bioactive metabolites. The bioactive metabolites were identified by GC-MS studies. The study is quite relevant and novel in the field of mangrove natural products.
Details on Page 31

ABSTRACT

**Objective:** To assess the *in vitro* antimicrobial efficacy of *Rhizophora mucronata (R. mucronata)* collected from the mangrove wetland of Ayiramthengu (southwest coast of India) against potential human and shrimp pathogens and to analyse the allelochemical constituents by gas chromatography-mass spectrometer (GC-MS) profiling.
**Methods:** Agar diffusion assay was used to investigate the efficacy of *R. mucronata* extracted in different polar and non-polar solvents. The antimicrobial activity was assessed against six type cultures of human and seven type cultures of shrimp pathogens.
**Results:** In the present study, methanol was found to be the best solvent for extracting the antimicrobial principles from *R. mucronata*. The results of the antimicrobial assay inferred that this mangrove could be a potential source of antibiotics for controlling the bacterial pathogens in human and shrimp. Furthermore, phytoconstituents of the crude extract were identified by GC-MS analysis. The GC-MS profile of the crude extract revealed that the main constituent was, Ethanone,1-(2-hydroxy-5-methylphenyl (Rt=9.213), which might have a functional role in the antibiotic activity.
**Conclusions:** Collectively, the overall results implies the mangrove, *R. mucronata* could be utilized as a renewable natural source for the development of novel biotherapeutics to combat human and shrimp pathogens.

KEYWORDS

Mangrove extract, Antimicrobial activity, *Rhizophora*, Human pathogens

## 1. Introduction

The bioscreening of natural products have revved up due to the paucity of safe antimicrobial drugs and the perilous upsurge of new and re-emerging infectious diseases. The antibiotics from natural source are efficacious, biodegradable, less toxic and cost effective and therefore, it could supplant the costly synthetic antibiotic drugs. Biopotentiality of mangrove vegetal makes them as a reservatory for the development of pharmaceuticals, fish and animal feed additives, agrichemicals and natural pigments[1]. The officinal usage of mangrove flora dates back to several centuries ago and still continues[1]. The mangrove preparations used successfully in the treatment of infectious diseases and aliments are envisaged to possess antimicrobial potency.

The mangrove, *Rhizophora mucronata* (*R. mucronata*) Lam., commonly known as red mangrove, typically distributed in East Africa and India through Asia as well as Indonesia to the Western Pacific, wet tropical regions of Australia[2]. It has folkloric curative properties against diabetes, diarrhoea, nausea, haematuria, haemorrhages

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and angina. The pertinent data from literature corroborated that *R. mucronata* posses antiviral[3], antibacterial[4], antidiabetic[5], cytotoxic, analgesic, antidiuretic activity[6], nematocidal[7], antidiarrheal[8], hepatoprotective and antioxidant properties[9]. The diverse biological activities manifested by the *R. mucronata* are due to the presence of various types of steroids, diterpenoids and triterpenoids[10].

Data about the distribution of different species of genus *Rhizophora* from the southwest coast of India (Kerala coast) is already articulated[11]. Similarly, antimicrobial activity of other species of *Rhizophora* from the same collection site has been previously corroborated[12]. Howbeit, antibiotic potential and chemical analysis of *R. mucronata* from the southwest coast of India is not addressed till now. For this reason, the present study is intended to evaluate the antagonistic potency and gas chromatography-mass spectrometer (GC-MS) analysis of mangrove, *R. mucronata* collected from the southwest coast of India.

## 2. Materials and methods

### 2.1. Collection and extraction of bioactives from R. mucronata

Aerial foliage of predominant mangrove, *R. mucronata*, was sourced from the outer fringes of mangrove wetland of Ayiramthengu located in Kollam vicinity (09°12′ N and 76°47′ E). The collected specimen was identified taxonomically by an eminent taxonomist, Prof. Ravi N, Sree Narayana College, Kollam. For the isolation of bioactives, the specimen was exhaustively extracted in different solvents of increasing polarity as described by Manilal and Idhayadhulla[13].

### 2.2. Test microorganisms

The different solvent extracts of *R. mucronata* were evaluated against a battery of human and shrimp pathogenic bacteria (Table 1). The human and shrimp pathogens with MTCC number were obtained from Institute of Microbial Technology, Chandigarh, India. Culture of these bacteria were grown in nutrient broth (Himedia®) at 37 °C and maintained on nutrient agar slants at 4 °C and sub-cultured prior to experimental use.

### 2.3. Antimicrobial assay

Antimicrobial assay described by Manilal and Idhayadhulla[13] was adopted. Briefly, respective pathogens were swabbed in Mueller-Hinton agar plates. In each triplicate of plates, wells were punched using a sterile cork hole borer and were filled with 120 μL of appropriate organic extract. Subsequently, the plates were incubated at (30±2) °C for 24 h. The well with solvent used for dissolution was considered as negative control while chloramphenicol (1 mg/mL) and nalidixic acid (1 mg/mL) was used as the positive control for shrimp and human pathogens. Clear zone of inhibition appeared around wells were considered indicative of antimicrobial activity. The inhibitory activity was measured by calculating the area of clear zone. The antibiogram of pathogen was analyzed for skewness using SPSS 20.0 software.

**Table 1**
Panel of pathogens used for antimicrobial assay.

| Group | Species |
|---|---|
| Human pathogens (MTCC) | *Staphylococcus aureus* (MTCC 96) |
| | *Streptococcus mutans* (MTCC 890) |
| | *Klebsiella pneumonia* (MTCC 109) |
| | *Shigella flexneri* (MTCC 1457) |
| | *Micrococcus luteus* (MTCC106) |
| | *Vibrio mimicus* (MTCC 4434) |
| Shrimp pathogens (MTCC) | *V. alginolyticus* (MTCC 4439) |
| | *V. alcaligenes* (MTCC 4442) |
| | *Vibrio* vulnificus (MTCC 1145) |
| | *V. parahaemolyticus* (MTCC 451) |
| | *V. harveyi* (MTCC 3438) |
| | *Vibrio fischeri* (MTCC 1738) |
| | *A. hydrophila* (MTCC 1739) |

MTCC: Microbial Type Culture Collection.

### 2.4. GC-MS

The crude extract was chemically analysed by GC-MS using a Clarus 500 Perkin-Elmer Gas Chromatograph equipped with mass detector Turbo mass gold-Perkin Elmer Turbomass 5.2 spectrometer and an Elite-5 MS (5% diphenyl/95% dimethyl poly siloxane), 30 mm×0.25 mm×0.25 μm of capillary column was used with helium at a 1 mL/min as a carrier gas. The gas chromatography 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 was 250 °C. The GC-MS electron ionization mode was 70 eV. Mass scan range was from m/z 45-450 amu. The peaks of the gas chromatography were subjected to mass-spectral analysis. Peak identification was carried out using NIST Version 2.0 (2005).

### 2.5. Data analysis

All the results were expressed as mean±SD. Mean values were assessed using One-way analysis of variance using SPSS for Windows version 20.0 (Statistical Package for Social Services, Chicago, IL, USA).

## 3. Results

The evaluation of overall activity of the different organic extracts obtained from *R. mucronata* against both human and shrimp pathogens is shown in Table 2. The extract obtained from methanol was the most active one (100.00%) which subjugated the growth of all tested pathogens. However, the extract obtained from chloroform, ethyl acetate and ethanol was much less effective than the methanolic extract. The hexane and phosphate buffer saline extract showed no activity against the organisms tested. The *in vitro* inhibitory level of methanolic extract of *R. mucronata* were categorised as follows: the inhibitory area more than 250 $mm^2$ was considered high; 100-250 $mm^2$ the antibacterial activity was considered moderate; 20-100 $mm^2$ the antibacterial activity was considered weak; less than 20 $mm^2$ the extracts were considered inactive.

**Table 2**

Overall activity of different solvent extract of *R. mucronata* against different panel of test pathogens.

| Solvents | Antimicrobial activity (%) | |
|---|---|---|
| | Human Pathogens (MTCC) | Shrimp pathogens (MTCC) |
| Hexane | 0.00 | 0.00 |
| Chloroform | 14.28 | 28.57 |
| Ethyl acetate | 66.60 | 85.71 |
| Ethanol | 50.00 | 71.42 |
| Methanol | 100.00 | 100.00 |
| Phosphate buffer saline | 0.00 | 0.00 |

Overall activity was expressed as relative antimicrobial activity of respective solvent extracts against 13 test pathogens. Zone of Inhibition≥20 $mm^2$ was considered as active.

Amongst the two groups of pathogens screened, higher degree of activity was produced against the shrimp pathogens in the range of (88.17±16.90) to (265.78±5.70) $mm^2$ (Figure 1). *Vibrio parahaemolyticus (V. parahaemolyticus)*, *Vibrio alginolyticus (V. alginolyticus)*, *Vibrio harveyi (V. harveyi)* and *Vibrio alcaligenes (V. alcaligenes)* were the most susceptible pathogens to the crude methanolic extract. On contrary, *Aeromonas hydrophila (A. hydrophila)* showed a little resistance and exhibited an area of inhibition (88.17 ±16.90) $mm^2$. Likewise, against the human pathogens tested the zone of inhibition was ranged between (205.23±8.90) to (81.80±9.60) $mm^2$ (Figure 2). The highest area of inhibition of (205.23±8.90) $mm^2$ was manifested against *Staphylococcus aureus (S. aureus)* whereas the least area of inhibition was against *Vibrio mimicus (V. mimicus)* (81.80±9.60) $mm^2$. This results implied that *R. mucronata* contains antibacterial principles that can be detected by chemical analysis.

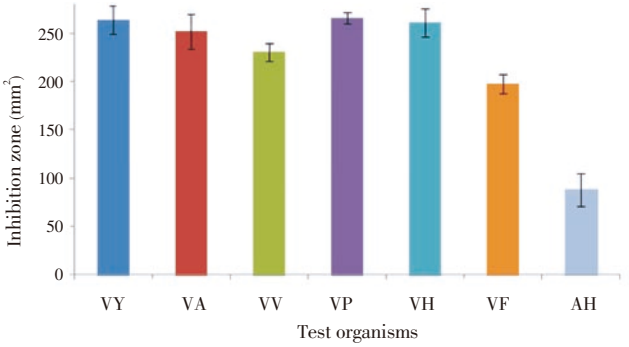


**Figure 1.** Antibacterial potential of methanolic extract of *R. mucronata* against shrimp pathogens.

The activity index was calculated as $mm^2$ area based on the diameter halo displayed. VY: *V. alginolyticus*; VA: *V. alcaligenes*; VV: *V. vulnificus;* VP: *V. parahaemolyticus*; VH: *V. harveyi*; VF: *V. fischeri*; AH: *A. hydrophila*.

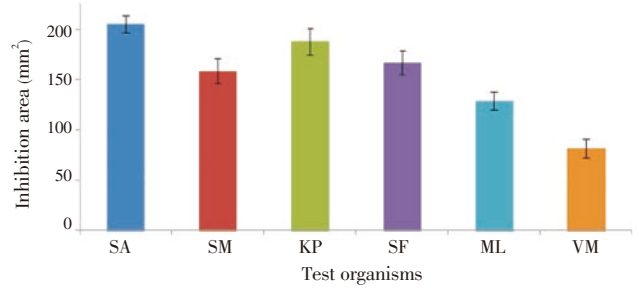


**Figure 2.** Antibacterial potential of methanolic extract of *R. mucronata* against human pathogens.

The activity index was calculated as $mm^2$ area based on the diameter halo displayed. SA: *S. aureus*; SM: *Streptococcus mutans*; KP: *K. pneumonia*; SF: *S. flexneri*; ML: *M. luteus*; VM: *V. mimicus*.

In order to obtain the preliminary data on the bioactive components, methanolic extract of *R. mucronata* was subjected to GC-MS analysis. The crude methanolic extract of *R. mucronata* on the basis of spectral data by GC-MS analysis was found to be a mixture of secondary metabolites. A total of 12 peaks were observed with retention times as presented in Figure 3 and Table 3. The GC-MS analysis of the crude extract evinced that the main phytoconstituent was found to be Ethanone,1-(2-hydroxy-5-methylphenyl) (Rt=9.213) followed by Benzene ethanol,4-hydroxy (Rt=12.164 min).

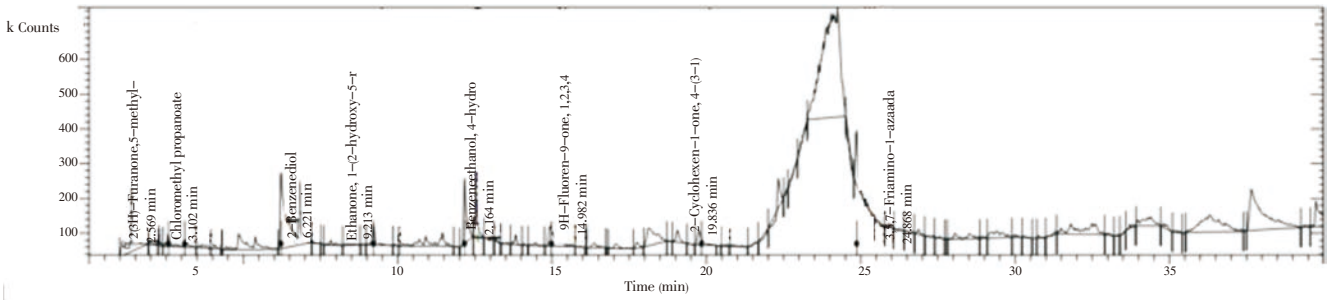


**Figure 3.** GC-MS profile of crude extract of *R. mucronata*.

**Table 3**
Phytoconstituents identified from the crude methanolic extract of *R. mucronata* by GC-MS analysis.

| No. | Rt | Peak name | Height |
|---|---|---|---|
| 1 | 2.569 | 2(3H)-Furanone,5 Methyl- | 11 557 |
| 2 | 3.102 | Chloromethylpropanoate | 18 064 |
| 3 | 4.845 | Ethanone,1-(2-hydroxy-6-methoxyphenyl) | 5 246 |
| 4 | 5.391 | Benzofuran,2-ethenyl- | 11 552 |
| 5 | 6.221 | 1,2-Benzenediol | 106 639 |
| 6 | 6.811 | Benzofuran,2,3-dihydro | 74 441 |
| 7 | 9.213 | Ethanone,1-(2-hydroxy-5-methylphenyl) | 116 048 |
| 8 | 12.164 | Benzene ethanol,4-hydroxy | 108 515 |
| 9 | 12.509 | 2-Hydroxy-4-methyl benzaldehyde | 68 340 |
| 10 | 14.982 | 9H-Fluoren-9-one,1,2,3,4,4a,9a-hexahydr | 5 329 |
| 11 | 19.836 | 2-cyclohexen-1-one,4-(3-hydroxy-1-buten | 4 421 |
| 12 | 24.868 | 3,5,7-Triamino-1-azaadamantane | 51 954 |

Rt: retention times.

## 4. Discussion

Bioactive natural products sourced from marine flora and fauna continue to be used in pharmaceutical preparations either as pure compounds or as extracts[14]. The mangrove vegetal has been used for several purposes including antimicrobial effects since time immemorial[1]. It is a recognized fact that mangrove flora are a potential source of new drugs, with high activity and low toxicity. There is a great variety of compounds that can be extracted and sequestered from mangrove flora. In the present study, *R. mucronata* collected from the southwest coast of India were extracted in different solvents and screened against a panel of human and shrimp pathogens. The methanolic extract of *R. mucronata* showed significant activity against all the tested pathogens. This result envisages that active principles in *R. mucronata* are well soluble in methanol than else others. In contrast to our results, Kusuma *et al.* opined that *n*-hexane and chloroform extract of *R. mucronata* exhibited strong antimicrobial activity[15]. In the present study, notable activity was manifested against shrimp pathogens. There is a dearth of report regarding the antimicrobial activity of *R. mucronata* against shrimp pathogens. The results from the antimicrobial activity against *Vibrio* spp. are in consonances with previous work done against Lobster pathogens[16]. Similarly, our earlier study also demonstrated the antimicrobial activity of another species of *Rhizophora* against *V. harveyi*, *V. vulnificus*, *V. alcaligenes* and *V. alginolyticus*[12]. In the case of human pathogens, activity against *S. aureus* was higher than that of other bacteria tested. In agreement with our results, Gurudeeban *et al.* acknowledged the same pattern of sensitivity of *S. aureus* towards *R. mucronata* sourced from the southeast coast of India[17]. Our studies are in line with other reports on antimicrobial properties of *R. mucronata* against human pathogens using different parts of the plant and extraction processes[18-20]. In addition, Manilal *et al.* reported the antimicrobial activity of *R. apiculata* against multidrug resistant human pathogens[12]. The overall results envisage that *R. mucronata* is a prolific source of antibiotic compounds and could have relevance in the medical and veterinary field.

Plant-derived secondary metabolites are useful in synthesizing novel antimicrobial drugs with new pharmacological efficacy by repeated structural modification. For that, knowledge from preliminary screening is necessary. Probing of chemical constituents from *R. mucronata* has been addressed elsewhere[21-24]. Till now 23 metabolites have been identified from this species[10]. Howbeit, mangrove *R. mucronata* collected from the southwest coast of India is insufficiently studied either chemical or biologically. Due to the possibilities of inhibiting the growth of human and shrimp pathogens with *R. mucronata* extract, GC-MS studies were carried out to determine the bioactive principles. GC-MS analysis on the methanolic extracts of *R. mucronata* evaluated in this study revealed that the main phytoconstituents are Ethanone,1-(2-hydroxy-5-methylphenyl) (Rt=9.213 min) and Benzene ethanol, 4-hydroxy (Rt=12.164 min) and these metabolites can be attributed to the antibiotic activity. In accordance to our results, previous study reported the antibacterial activity of *Terminalia chebula* possessing Ethanone,1-(2-hydroxy-5-methylphenyl[25]. The observed activity of *R. mucronata* could be due to independent or synergistic action of these constituents. The chemotaxonomy of *R. mucronata* was reviewed by Nebula *et al.* with more than 20 diverse metabolites has been noted[10]. The bioactivity and chemical composition of plants can change according to geographical distribution, seasonality and extraction methodology[26]. However, this report is the first to explore the antimicrobial potency of *R. mucronata* from the southwest coast of India. In addition, this study also serves as a base line data for further research on this species.

From these results, it is clearly apparent that *R. mucronata* holds potent bioactive compounds that can efficiently repress the growth of human and animal pathogens. Further bioassay monitored purification will open perspectives on the discovery of bioactives that could be utilized in the field of human and veterinary grade bio-therapeutics.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Comments

### *Background*

The officinal usage of mangrove metabolites to managing diseases in human and animals has been reported since time immemorial. In this background, the authors have justified their research to demonstrate the antimicrobial activity of mangrove, *Rhizophora* sourced from the Indian coast against animal and human pathogens.

### *Research frontiers*

It is well known that the mangroves from different locales possess an ample amount of diverse bioactive metabolites. The article is exclusively a good work in the field of

mangrove bioactives. While considering the significance of natural antibiotics, this study is highly commendable. Antibiotics are being used against human and shrimp pathogens for the last few decades but as it resulted in disease resistance and accumulation of antibiotics. In this context, it has become the need of the hour to find an alternative measure to control the pathogens.

*Related reports*

In prima facia, manuscript is well written and findings are interesting and adds value to the use of plant metabolites for controlling human and shrimp infectious pathogens.

*Innovations & breakthroughs*

The article provides a novel information to the international readers regarding the antimicrobial efficacy of *Rhizophora* against both human and animal pathogens. Even though there are many publications about the antimicrobial activity of *Rhizophora*, preliminary literature survey indicated that there is no publications regarding the antimicrobial activity of *Rhizophora* against both human and animal pathogens.

*Applications*

As per the result described by the authors, mangrove *Rhizophora* could be utilized as a reliable source of antibiotics for the control of human and shrimp pathogens after *in vivo* efficacy validation in future.

*Peer review*

In the article, authors described the result of screening for antibacterial activities of *Rhizophora* and preliminary study regarding the bioactive metabolites. The bioactive metabolites were identified by GC-MS studies. The study is quite relevant and novel in the field of mangrove natural products.

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