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An *in vitro* efficacy validation of mangrove associates

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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 extracts from 12 species of mangrove associates, and an in-detail study of one of the most potent species, namely *C. inophyllum*. The compounds were identified by GC–MS studies. Findings are quite interesting and novel in the field of bio-screening of mangrove associates.

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ABSTRACT

Objective: To investigate the *in vitro* antimicrobial potential of mangrove associates against a battery of human and shrimp pathogenic bacteria and to elucidate its antimicrobial principles.

Methods: In the present study, 12 species of mangrove associates collected from the Kollam (southwest coast of India) vicinity were extracted in different organic solvents of increasing polarity. The resultant extracts obtained from the respective species were examined for the antimicrobial activity against a panel of shrimp and human pathogens by agar diffusion assay.

Results: Of the 12 species evaluated, three species of mangrove associates [*Calophyllum inophyllum* (*C. inophyllum*), *Cerbera odollam* and *Dalbergia candenatensis*] were found to be active. The broadest and highest rank of activity was observed in the crude extract of *C. inophyllum*. Amongst the pathogens tested, shrimp pathogenic *Vibrios* were the most sensitive organisms while human pathogens were found to be a bit resistant. In the present study, ethyl acetate was found to be the best solvent for extracting antimicrobial metabolites. The bioactive principles present in the crude extract of *C. inophyllum* were chemically elucidated by gas chromatography–mass spectrometer. The gas chromatography–mass spectrometer studies revealed the presence of two principal compounds such as 1–Dimethyl(phenyl)silyloxyhexadecane (24.73%) and β–d–Mannofuranoside, O–geranyl (50%) which might play functional role in the chemical defense against microbial invasion.

Conclusions: Based on the overall findings, it could be inferred that the mangrove associate *C. inophyllum* is a promising candidate for the development of plant–based human and veterinary grade antibiotics in future.

KEYWORDS

Mangrove associates, Antimicrobial, Crude extract, Bioactives

1. Introduction

Mangrove associates are considered as a nonexclusive species that are principally distributed in a terrestrial or aquatic–habitat but also occur in the mangrove ecosystem^[1,2]. These flora are non–arborescent,

herbaceous, sub–woody and climber species that growing verdantly in regions bordering the tidal periphery of mangrove habitats worldwide^[3]. Marine vegetation including the mangrove associates have been an astonishing source of antimicrobial agents and have drawn the attention of pharmaceutical fraternities

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in recent decades. Screening of organic extracts from mangroves and other marine vegetation has intensified due to the emergence of multidrug resistant microorganisms^[4]. In fact, mangrove associates contain many bioactives that can defy the growth of virus, bacteria and fungus. For instance, many species of mangrove associates are renowned for their officinal usage to treat wide range of ailments including hepatitis, coughs, diabetes, piles, rheumatism, conjunctivitis and intestinal disorders^[5]. Secondary metabolites so far sequestered from several species of mangrove associates were reported to have antitumor, antileukemic, analgesic, anti-inflammatory and bactericidal activity^[5,6]. These references show that mangrove associates constitute a source of lead compounds for the synthesis of novel biopharmaceuticals.

In India, 86 species of mangrove associates are so far reported^[7]. The study area, Kollam coast (southwest coast of India) is also endowed with diverse varieties of mangrove associates. Preliminary survey evidenced that more than 15 species of mangrove associates were distributed in various wet land regions of Kollam. Bioactivity of true mangrove species from the southwest coast of India is well corroborated^[8,9]. However, there is a dearth of report pertaining to the antimicrobial activity of mangrove associates from the Indian coast. In this background, the present study was initiated to explore the antimicrobial efficacy of different mangrove associates.

2. Materials and methods

2.1. Sampling of vegetation

Twelve plant specimens [*Acanthus ilicifolius* (*A. ilicifolius*), *Acrostichum aureum* (*A. aureum*), *Calamus rotang*, *Calophyllum inophyllum* (*C. inophyllum*), *Cerbera odollam* (*C. odollam*), *Clerodendrum inerme* (*C. inerme*), *Derris scandens* (*D. scandens*), *Derris trifoliata* (*D. trifoliata*), *Dalbergia candenatensis* (*D. candenatensis*), *Ipomoea pes-caprae*, *Morinda citrifolia*, *Premna latifolia* (*P. latifolia*)] used for the screening were collected from the coastal area of Kollam (8°54' N and 76°38' E) and Ayiramthengu (09°12' N and 76°47' E).

The study area, Ayiramthengu wet land is a hotspot of diverse marine floral and faunal assemblages particularly mangroves and mangrove associates. The most common species of mangrove associates are *A. aureum*, *A. ilicifolius*,

C. inerme, *Derris* sp., and *P. latifolia*. Most of these species have not yet explored chemically or biologically. Morphological features of collected samples were analyzed and reconfirmed with the help of Prof. Ravi, Sree Narayana College, Kollam, Kerala, India.

2.2. Preparation of the extracts

The aerial parts of collected plant specimens were washed in sterile water to remove the dirt and associated debris. The cleaned specimens were chopped into small pieces, macerated in different polar and non-polar solvents of increasing polarity (hexane, chloroform, ethyl acetate, methanol and ethanol) using a mortar and pestle at room temperature. The resulted extracts were vortexed and filtered using double folded muslin cloth and the filtrate was centrifuged at 10000 r/min for 10 min (REMI). The residual solvent was allowed to air dry and the gummy residue was collected in air tight plastic vials and stored at 4 °C.

2.3. Test microorganisms

Antimicrobial activity of organic plant extractives were assessed using different microorganisms such as six type cultures [microbial type culture collection (MTCC)] of shrimp *Vibrio* pathogens and five human clinical isolates obtained from Medilab Speciality Laboratories Pvt. Ltd, Kochi, Kerala, S. India (Table 1). All the bacterial strains 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.

Table 1
Panel of pathogens used for the antimicrobial assay.

Group	Species
Human pathogens	<i>Proteus vulgaris</i>
	<i>Pseudomonas</i> sp.
	<i>Staphylococcus aureus</i>
	<i>Escherichia coli</i>
	<i>Klebsiella</i> sp.
Shrimp pathogens (MTCC)	<i>Vibrio alginolyticus</i> (MTCC 4439)
	<i>Vibrio alcaligenes</i> (MTCC 4442)
	<i>Vibrio vulnificus</i> (MTCC 1145)
	<i>Vibrio parahaemolyticus</i> (MTCC 451)
	<i>Vibrio harveyi</i> (MTCC 3438)
	<i>Vibrio fischeri</i> (MTCC 1738)

2.4. Antimicrobial assay

The antibacterial assay was performed as per the

methodology described in our previous study^[4]. Briefly, fresh cultures of respective pathogens (approximate 10^6 CFU/mL) were swabbed over the surface of Mueller–Hinton agar plates. In each triplicate of plates, cylindrical wells were punched using a sterile cork hole borer. Appropriate organic extract (5 mg/mL) were filled up to the brim of each well. Parallel reference standard (1 mg/mL of chloramphenicol and nalidixic acid) and negative control (dissolution solvents) were used to validate the inferences. The plates were incubated at 37 °C for 24 h. After incubation, antimicrobial activities of crude extracts were interpreted by calculating the area of inhibition zone around the well. All samples were tested in triplicate. The antibiogram was statistically analysed for the determination of skewness among the tested organisms using SPSS 20.0 software.

2.5. Determination of the mechanism of antibiosis

The minimal inhibitory concentration (MIC) was determined by the broth dilution method as described by Manilal *et al.* with a little modification^[8]. Highly sensitive bacterial strains including *Vibrio alginolyticus* (*V. alginolyticus*) and *Staphylococcus aureus* (*S. aureus*) were used as test organisms. The determined MIC concentration was plated on growth medium to determine the minimum bactericidal concentration (MBC). When the ratio of MBC/MIC is ≤ 2 , the active fractions were considered as bactericidal otherwise as bacteriostatic. If the ratio is ≥ 16 , the fractions were considered as ineffective.

2.6. Gas chromatographic–mass spectroscopic (GC–MS) analysis

The crude extract of highly active mangrove associate, *C. inophyllum* was subjected to 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 \times 0.25 mm \times 0.25 μ m of capillary column was used with helium at 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 μ L. 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).

2.7. Data analysis

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

3. Results

In the present study, altogether twelve species of mangrove associates sourced from the southwestern coast of India were subjected to extraction process using organic solvents of increasing polarity. Totally, sixty extracts were prepared from twelve species of mangrove associates. The results of the preliminary screening process showed that out of sixty extracts evaluated, crude ethyl acetate extracts of six species showed measurable zone of inhibition against the assay microorganisms (Table 2).

Table 2

Overall activity of different solvent extract of mangrove associates against test organisms.

No	Species of mangrove associates	Overall activity (%)	
		Shrimp Vibrio pathogens (MTCC)	Clinical pathogens
1	<i>A. ilicifolius</i>	20	0
2	<i>A. aureum</i>	0	0
3	<i>Calamus rotang</i>	0	0
4	<i>C. inophyllum</i>	100	100
5	<i>C. odollam</i>	100	60
6	<i>C. inerme</i>	0	0
7	<i>D. scandens</i>	20	0
8	<i>D. trifoliata</i>	20	20
9	<i>D. candenatensis</i>	100	80
10	<i>Ipomoea pes-caprae</i>	0	0
11	<i>Morinda citrifolia</i>	0	0
12	<i>P. latifolia</i>	0	0

Overall activity was expressed as relative antimicrobial activity of respective mangrove associates against 11 tested pathogens. Zone of inhibition ≥ 20 mm² was considered as active.

The formation of inhibitory zone was considered to be an effective indicator of the ability of the mangrove associates to synthesize bioactive metabolites. The extract prepared from methanolic and ethanolic solvent of six species showed slight activity towards the test organisms (data not shown). Under this experimental condition, no conspicuous zone of inhibition was observed for hexane, chloroform

extracts and controls. Among the six mangrove associates, *C. inophyllum*, *C. odollam* and *D. candenatensis* showed the highest activity. Of these, *C. inophyllum* surpasses the activity of other mangrove associates. It efficiently repressed the growth of all tested microorganisms. The crude ethyl acetate extract of *C. inophyllum* produced an inhibitory zone in the range of (127.55±14.50) to (168.14±7.40) mm² for shrimp *Vibrio* pathogens and (83.95±8.10) to (142.44±8.70) mm² for clinical isolates (Figure 1).

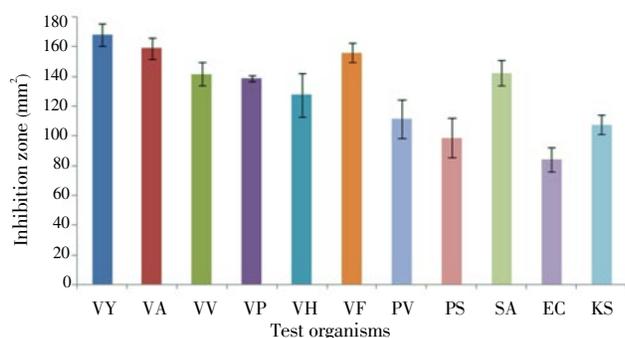


Figure 1. Antimicrobial activity of ethyl acetate extract of *C. inophyllum* against shrimp and clinical pathogens.

The activity index was calculated as mm² area based on the diameter halo displayed. MTCC cultures: VY–*V. alginolyticus*, VA–*Vibrio alcaligenes*, VV–*Vibrio vulnificus*, VP–*Vibrio parahaemolyticus*, VH–*Vibrio harveyi*, VF–*Vibrio fischeri*; Clinical isolates: PV–*Proteus vulgaris*, PS–*Pseudomonas* sp., SA–*S. aureus*, EC–*Escherichia coli*, KS–*Klebsiella* sp.

Inhibitory spectrum was elevated particularly against the shrimp pathogens. Among the shrimp *Vibrios*, *V. alginolyticus* was the most sensitive pathogen which produced an inhibitory zone of (168.14±7.40) mm² whereas in clinical isolates, *S. aureus* was the most susceptible. The MIC was determined against highly sensitive test strains including type culture *V. alginolyticus* and clinical pathogen *S. aureus*. The determined MICs against *V. alginolyticus* and *S. aureus* were 1900 and 2500 µg/mL and the concomitant MBC values were 2100 and 2700 µg/mL. Since the MBC/MIC ratio was less than 2, the active principles can be considered to be a bactericidal agent (Table 3). The second most active mangrove associate was *D. candenatensis* which exhibited an inhibitory zone ranged between (118.22±8.60) to (170.41±4.70) mm² for shrimp pathogens and (86.04±5.20) to (122.06±6.80) mm² for clinical isolates (Figure 2). The third most active mangrove associate *C. odollam* produced an inhibitory zone of (101.5±7.20) to (128.64±5.20) mm² for shrimp *Vibrios* and (65.54±5.40) to (127.99±6.10) mm² for clinical isolates (Figure 3). The other three mangrove associates such as *D. trifoliata*, *D. scandens* and *A. ilicifolius* presented only meager activity. Based on the broadest and highest spectrum of activity, *C. inophyllum* was further chosen for chemical investigation by GC–MS analysis.

Table 3
MIC and MBC of *C. inophyllum*.

Test strain	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
<i>V. alginolyticus</i>	1900	2100	1.10
<i>S. aureus</i>	2500	2700	1.08

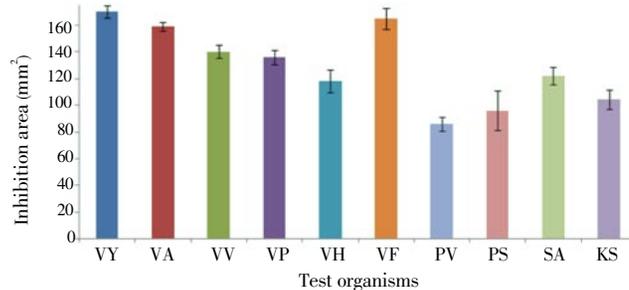


Figure 2. Antimicrobial activity of ethyl acetate extract of *D. candenatensis* against shrimp and clinical pathogens.

The activity index was calculated as mm² area based on the diameter halo displayed. MTCC cultures: VY–*V. alginolyticus*, VA–*Vibrio alcaligenes*, VV–*Vibrio vulnificus*, VP–*Vibrio parahaemolyticus*, VH–*Vibrio harveyi*, VF–*Vibrio fischeri*; Clinical isolates: PV–*Proteus vulgaris*, PS–*Pseudomonas* sp., SA–*S. aureus*, KS–*Klebsiella* sp.

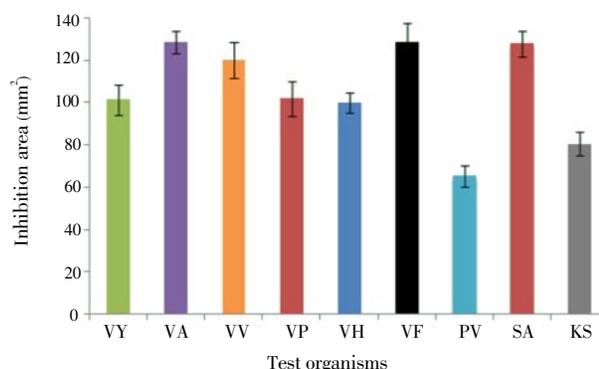


Figure 3. Antimicrobial activity of ethyl acetate extract of *C. odollam* against shrimp and clinical pathogens.

The activity index was calculated as mm² area based on the diameter halo displayed. MTCC cultures: VY–*V. alginolyticus*, VA–*Vibrio alcaligenes*, VV–*Vibrio vulnificus*, VP–*Vibrio parahaemolyticus*, VH–*Vibrio harveyi*, VF–*Vibrio fischeri*; Clinical isolates: PV–*Proteus vulgaris*, SA–*S. aureus*, KS–*Klebsiella* sp.

The bioactive chemical constituents present in the crude extract of *C. inophyllum* were preliminarily elucidated by GC–MS. Totally five peaks were observed (Figure 4). The spectral data revealed a single prominent peak with retention time and molecular weight of 23.96 and 316 respectively (Figure 4).

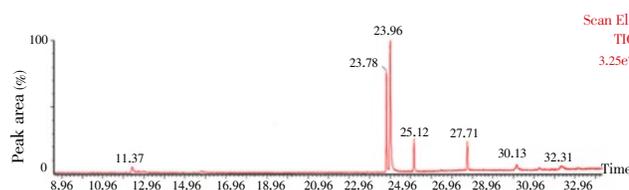


Figure 4. GC–MS profile of the crude extract of *C. inophyllum*.

The MS data matched perfectly with a compound of

molecular formula $C_{16}H_{28}O_6$ which is analogous to β -d-Mannofuranoside, O-geranyl in the National Institute of Standards and Technology library. The retention time and molecular weight of the compounds corresponding to the other peaks are presented in Table 4.

Table 4

Components identified in the crude extract of *C. inophyllum* by GC-MS study.

Rt	Name of the compound	MF	MW	Peak area (%)
11.37	10-Undecen-1-ol	$C_{11}H_{22}O$	170	4.08
23.78	1-Dimethyl(phenyl)silyloxyhexadecane	$C_{24}H_{44}OSi$	376	24.73
23.96	β -d-Mannofuranoside, O-geranyl	$C_{16}H_{28}O_6$	316	50.82
25.12	1-iodo-2-methylundecane	$C_{12}H_{25}I$	296	10.87
27.71	1-Iodo-2-methylnonane	$C_{10}H_{21}I$	268	9.51

Rt: retention time, MF: molecular formula, MW: molecular weight.

4. Discussion

In the recent decades, there is a spurt in the researches pertained to plant based natural products. Natural products sourced from the plants were considered as the basis of first pharmaceutical practice and they continue to play a pivotal role in modern antimicrobial chemotherapy[10]. For instance, 135 drugs currently used for the management of human ailments are derived from plants[11]. Like other flora, mangroves associates also produce a versatile number of metabolites against microbial invasions. These secondary metabolites are potential candidates for possible use in the field of managing wide array of dreadful pathogenic organisms. There is a considerable number of literature pertained to the bio-efficacy studies of true mangroves from the Indian coast. Howbeit, the literatures with regards to antimicrobial activity of mangrove associates are skimpy.

Albeit the mangrove associates are well flourished in the study area, their bioactivities were not explored so far. This is the first report that envisaged the antimicrobial activity of mangrove associates from the southwest coast of India. Among the 12 mangrove associates screened, *C. inophyllum* evinced the broadest and highest spectrum of antimicrobial activity irrespective of their types. The comparative analysis of antimicrobial activity of *C. inophyllum* extracted in different organic solvents showed that activity was significantly higher for ethyl acetate extract followed by ethanol. These results evidenced that the solvents had a key role on the extraction of bioactive principles[8] and also ensures the existence of bioactive compounds in the crude extract of *C. inophyllum*. It showed an excellent activity against the shrimp pathogens, particularly towards the *V. alginolyticus*. The most significant result is that it was of effective activity against *S. aureus*, the most common clinical pathogen associated with nosocomial infections.

As observed in the present study, several species of genus *Calophyllum* showed inhibitory potency against *S. aureus*[12,13]. The antimicrobial activity of *C. inophyllum* sourced from other locales are already acknowledged by many authors[14–16]. According to Ling *et al.*[17], oil extracted from the nut of *C. inophyllum* is used to treat rheumatism, scabies, ringworm and dermatosis. Furthermore, many researchers have reported the anti-HIV and anti-tumor efficacy of *C. inophyllum*[18–20]. Literature survey revealed that GC-MS analysis of *C. inophyllum* sourced from the southwest coast of India has not yet been investigated. The GC-MS analysis of crude ethyl acetate extract of *C. inophyllum* evinced the presence of two major compounds such as 1-Dimethyl(phenyl)silyloxyhexadecane (24.73%) and β -d-Mannofuranoside, O-geranyl (50%) which might have a principal role in the chemical defense against microbial invasion. Data pertaining to the bioactive constituents of *C. inophyllum* from other locales was well documented by many researchers[21,22]. Based on the overall findings, it could be inferred that the mangrove associate, *C. inophyllum* is a promising candidate for the development of plant-based human and veterinary grade antibiotics in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

It is an approved concept that plant based antibiotics has been used to manage many diseases in human and animals since a long time. In this background, the authors have justified their research to demonstrate the antimicrobial activity of mangrove associates.

Research frontiers

In *prima facie* this article is exclusively a novel work in the field of marine floral bioactives. Considering the importance of natural antibiotics, this study is highly commendable. In the recent times, over use and miss use of human and veterinary grade synthetic chemotherapeutics has resulted in the development of many multidrug resistant

pathogens. Hence it is high time to develop an effective biocontrol measurement for the management of these pathogens.

Related reports

About 135 drugs currently used for the management of human ailments are derived from plants. In addition, several species of genus *Calophyllum* showed inhibitory potency against *S. aureus*. The antimicrobial activity of *C. inophyllum* sourced from other locales are already acknowledged by many authors.

Innovations and breakthroughs

The article provides a novel information to the international readers regarding the antimicrobial efficacy of 12 species of mangrove associates. As per the literature survey there is no publications regarding the bioactivity screening of different mangrove associates.

Applications

As per the result narrated by the authors, mangrove associates could be utilized as a source of antibiotics for the control of human and shrimp pathogens.

Peer review

In the article authors described the result of screening for antibacterial activities of extracts from 12 species of mangrove associates, and an in detail study of one of the most potent species, namely *C. inophyllum*. The compounds were identified by GC–MS studies. Findings are quite interesting and novel in the field of bio–screening of mangrove associates.

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