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Antimicrobial potential of marine organisms collected from the southwest coast of India against multiresistant human and shrimp pathogens

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SUMMARY: Diverse marine flora and fauna collected from the southwest coast of India was evaluated for its antimicrobial potential against shrimp *Vibrio* and multiresistant human pathogens. In total, 47 species of various taxa of marine organisms (29 flora and 18 fauna) were screened for antimicrobial activity. The marine flora includes twenty species of seaweeds, two species of mangroves, four species of cyanobacteria and three species of microalgae. The marine fauna comprises three species of porifera, twelve species of molluscans, one species of sea urchin, one of sea cucumber and one of cnidarian. The organic extractives were tested against five type cultures (Microbial Type Culture Collection) of prominent shrimp *Vibrio* pathogens, including *V. parahaemolyticus, V. vulnificus*, *V. harveyi, V. alcaligenes* and *V. alginolyticus,* and five multiresistant clinical pathogens: *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus epidermidis.* Among the marine organisms screened, seaweeds showed a broad spectrum of antibacterial activity. The highly active seaweed *Falkenbergia,* a heteromorphic sporophyte of *Asparagopsis taxiformis* (Delile) Trevisan, was evaluated further to purify the active compounds using different chromatographic systems, including reverse phase HPLC and GC-MS. The analysis revealed that the most abundant metabolites are oleic acid (51.33%) followed by n-hexadecanoic acid (42.87%).

Keywords: antibacterial activity, *Falkenbergia*-phase, sea urchin, cyanobacteria, sea cucumber, microalgae, seaweeds.

RESUMEN: Potencial de los antimicrobianos de los organismos marinos de la costa sureste de la India frente a patógenos multirresistentes del camarón y humanos. – A muestras de flora y fauna marina diversas recogidas de la costa suroeste de la India se les evaluó su potencial antimicrobiano frente a *Vibrios* patógenos de camarón y multirresistentes humanos. En total fueron seleccionadas 47 especies de diferentes taxones de organismos marinos (29 de flora y 18 de fauna) para evaluar la actividad antimicrobiana. La flora marina incluía veinte especies de algas marinas, dos de manglares, cuatro de cianobacterias y tres de microalgas. La fauna marina se componía de tres especies de poríferos, doce de moluscos, una de erizo de mar, una de pepinos de mar y una de cnidario. Los extractos orgánicos se probaron en cinco tipos de cultivos (microbianos, colección de cultivo tipo) de *Vibrio* patógeno de camarón incluyendo *V. parahaemolyticus, V. vulnificus, V. harveyi, V. alcaligenes y V. alginolyticus y* cinco patógenos clínicos multirresistentes como *Staphylococcus aureus*, *Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae y Staphylococcus epidermidis*. Entre los organismos marinos examinados, las algas mostraron un amplio espectro de actividad antibacteriana. La gran actividad del alga *Falkenbergia,* - esporofito heteromórfico de *Asparagopsis taxiformis* (Delile) Trevisan, se evaluó además mediante la purificación de los compuestos activos por cromatografía de fase inversa incluyendo HPLC y GC-MS. El análisis reveló que los metabolitos más abundantes eran el ácido oleico (51.33%), seguido por el ácido n-hexadecanoico (42.87%).

Palabras clave: actividad antibacteriana, *Falkenbergia*-fase, erizo de mar, cyanobacteria, pepino de mar, microalgas, algas marinas.

INTRODUCTION

The marine environment (oceans, seas, coastal backwaters, estuaries, bays and fjords) is considered to be a unique source of earth's biological diversity, as it covers 70.8% of earth's surface and comprises more than 200000 described species of invertebrates and algae (Winston, 1988), which represents only a small percentage of the marine biodiversity (Malakoff, 1997). Due to their particular environment many marine invertebrates such as sponges, jelly fish, sea anemones, bryozoans and corals (Bhakuni and Jain, 1990) exhibit unique physiological and structural characteristics which enable them to survive in extremes of pressure, salinity and temperature. Historically, the ocean represents a virtually idyllic resource for the discovery of novel bioactive metabolites with useful activities (Cragg *et al*., 1997). Marine bioactive compounds, from small to medium molecular weight, are produced for the purpose of greater survivability or fecundity. However, they are not essential for the survival of the host organism. The production of secondary metabolites in marine organisms is known to be highly dependent on various bio-geographic conditions (Hay, 1996). The role and adaptive significance of secondary metabolites has been a topic of debate for a long time (Stone and Williams, 1992). According to Harper *et al*. (2001), secondary metabolites are adaptive and play a key role in the host's defence against pathogens, parasites, predators, competitors and epibiota.

With more intensive studies for natural therapies, marine-derived products are a promising source for the discovery of novel bioactive compounds. Marine natural products first began to be discovered in the late 1970s. Currently a multitude of chemically unique metabolites have been identified from a relatively small part of the biological and chemical diversity of the oceans, many of which are endowed with pharmacodynamic properties (Ireland *et al.,* 1993). With the advent of modern technologies, various marine animals, such as tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans and sea slugs, are being studied for their bioactive compounds (Harvey, 2000). The overall objective of the present study is to evaluate organic extractives of marine flora and fauna for the potential development of antibiotics to control shrimp and human bacterial pathogens.

MATERIALS AND METHODS

Sample collection

Eighteen species of sedentary/slow-moving invertebrate, and twenty nine species of micro and macroflora were collected from the Kollam coast (Thirumullavaram coast) (southwest coast of India) during the lowest tide (08º54'N and 76º38'E). Seaweeds and mangroves were handpicked when their abundance was observed (from November to April 2008). The samples of sponges, molluscans, sea anemones, sea urchins and sea cucumbers were collected during the post monsoon season (July to September 2008) by snorkelling at a depth of 1 to 3 m.

Preparation of extracts from marine algae and mangroves

The collected samples were transported to the laboratory immediately, washed twice with sterile seawater to remove epizoones, epiphytes, sand, calcareous matter and other adhering detritus matters. Samples were blotted in between tissue paper to dry them. The dried samples were weighed, and stored in a sterile ziplap bag at -20ºC until extraction. Voucher specimens were preserved in 5% (v/v) formaldehyde-seawater solution for identification. A known weight (5 gm) of tissue was pounded with methanol at room temperature, vortexed and filtered through double folded muslin cloth, and the filtrate was centrifuged (Eppendorf A G, Germany) at 8679 rpm for 10 min. The supernatant was evaporated to solvent free in a rotary vacuum evaporator (Yamato, Yamato Scientific, Japan). The gummy extract was collected in screw-capped vials and stored at 4ºC. The aliquots were prepared with methanol and tested for their antimicrobial activity against human and shrimp pathogens.

Cyanobacterial biomass production and extraction

The cyanobacteria were scraped off rocks and transported to the laboratory in Scott Duran bottles with 50 ml of sterile seawater. Four cyanobacteria belonging to the orders *Nostocales* and *Chroococcales* were isolated from the Kollam coast and identified by Dr. Panikkar (algologist) from the Department of Botany, Sree Narayana College, Kol-

lam. The cynobacterial biomass was produced in Erlenmeyer flasks incubated at 23±2ºC with shaking at 60 rpm and 12:12 h photoperiod with 2000 lux illumination for 4 weeks. Cells were harvested after 4 weeks by centrifugation at 5000 rpm and extracted as described above.

Microalgal biomass production and extraction

The cultures of identified algal species *Chaetoceros* sp., *Isochrysis galbana* and *Chlorella marina* were grown under constant illumination in f/2 medium and incubated under a 12:12 h photoperiod with 2000 lux illumination at 23±2ºC with shaking at 60 rpm. Microalgae were harvested after approximately a 15-day production period by centrifugation at 5000 rpm and the pellets were washed several times with sterile water. A known biomass (5 gm) was extracted with methanol and the supernatant was subjected to bioassays.

Preparation of extracts from marine fauna

Marine organisms (sponges, sea cucumber, sea urchin, sea anemone and molluscan) were washed individually with running water, chopped, pulverized into small pieces and extracted with methanol as described above.

Fractionation and purification of active compounds from the highly active seaweed *Falkenbergia*-phase of *A. taxiformis*

The methanolic extract of *Falkenbergia* (10 gm) was loaded in a silica gel (60-120 mesh) (Merck, USA) column packed with petroleum ether and eluted with petroleum ether and ethyl acetate (9:1 to 1:9 and 100% ethyl acetate) followed by ethyl acetate and methanol (9:1 to 1:9 and 100% methanol) to yield seven fractions. Individual fractions were collected and tested for antimicrobial activity.

The fraction with antimicrobial properties was further purified by preparative TLC using silica gel G as the stationary phase and 1% methanol in dichloromethane as the mobile phase. After the development of chromatogram, the resolved spots were analyzed by spraying with 50% sulphuric acid for detecting the lipophilic compounds. The TLC resolved spots were scraped out and eluted with methanol and finally centrifuged at 8819 rpm for 5 min. The supernatant with antimicrobial activity was subjected to HPLC (Shimadzu Chromatographic System Kyoto, Japan) at 254 nm absorbance with methanol at a flow rate of 1 ml/min; head pressure at 25 kgf/cm^2. The whole setup was maintained at room temperature (25ºC) and the resultant major peaks were subjected to GC-MS analysis. Phycoconstituents were detected using a Hewlett Packard 5890 Series II gas chromatographic system (Hewlett Packard, Waldbronn, Germany) equipped with HP-5971 mass selective detector (MSD, Hewlett Packard, Palo Alto, CA, USA), and a capillary column (30m × 0.25mm ×0.25mm) was used with helium at a 1 ml min^{-1} as a carrier gas. The GC oven temperature was kept at 110°C for two minutes, programmed to 280°C at the rate of 5°C min^{-1} and kept constant at 280°C for 10 minutes. 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. The mass range was from *m/z* 45 to 450 amu. Peak identification was carried out using NIST Version 2.0 (2005).

Test microorganisms

To characterize the antimicrobial activity, the methanolic extracts were evaluated against a battery of shrimp and multiresistant human pathogenic bacteria. Antimicrobial activity was determined against (i) five species of type cultures (MTCC- Microbial Type Culture Collection) of fish/shrimp *Vibrio* pathogens including *V. harveyi* (MTCC 3438), *V. alginolyticus* (MTCC 4439), *V. vulnificus* (MTCC 1145), *V. parahaemolyticus* (MTCC 451) and *V. alcaligenes* (MTCC 4442), (ii) five multiresistant pathogens obtained from clinical laboratories including the gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis* and the gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The resistant patterns of these human pathogenic isolates were confirmed using selective antibiotics (streptomycin, oxytetracycline, ampicillin and erythromycin) in a preliminary experiment. The isolates were established as multiresistant pathogens and deposited in the Marine Bioprospecting Laboratory, Department of Microbiology, Bharathidasan University.

Antimicrobial assay

The antimicrobial assay was carried out following the methodology of Selvin and Lipton (2004). Briefly, the base layer was prepared with 10 ml

(1.5%, w/v) of Mueller Hinton agar (Himedia, India). Five sterile porcelain beads of 5 mm in diameter were placed on the base layer at 60° angles apart. The overlaid seed layer was prepared by pouring 15 ml of media containing 0.2 ml of prepared inoculum (~ 0.2 OD at 630 nm). The porcelain beads were removed carefully with sterile forceps. The resultant wells were filled with 120 µl of the appropriate algal extract. The well with solvent used for dissolution was considered the negative control. The assay was performed in triplicates of individual Petri dishes. The diameter of the inhibition halo after 24 h of incubation at 37°C was considered to be indicative of bioactivity. The net halo diameter was calculated after subtracting the diameter of the well (5 mm).

Determining the mechanism of antibiosis (bacteriostatic or bactericidal)

The minimal inhibitory concentration (MIC) was determined by the broth dilution method. The 96-well microtiter plates were filled with 100 µl of the seaweed extract prepared in Mueller-Hinton broth (Himedia, India). The microtiter plates were incubated at 37°C for 48 h. In each microtiter plate, one row was set as a control (without column fractions) and another row was used as standard (chloramphenicol and nalidixic acid). After incubation, the OD was read at 610 nm (Spectronic 20 UV-Vis spectrophotometer, Thermo Scientific, Madison, WI, USA). MICs were recorded as the lowest concentration inhibiting visible growth. To measure the minimal bactericidal concentrations (MBC), the MIC cultures were plated on fresh Mueller-Hinton agar with 5% lysed horse blood and incubated for 24 h at 37°C. A reduction of at least 90% of the colonies, compared with the culture of the initial inoculum of the strain, was regarded as evidence of bactericidal activity. When the ratio of MBC/MIC was ≤2 the active fractions were considered to be bactericidal, otherwise they were considered bacteriostatic. If the ratio was ≥16 the fractions were considered to be ineffective.

TABLE 1. – List of marine organisms collected from the Kollam coast.

Organisms	Species
Chlorophyta	
	Enteromorpha compressa (Linn.) Nees
	Cladophora albida (Nees) Kuetzing
	Boodlea composita (Harvey) Brand
	Ulva lactuca Linnaeus
	Bryopsis pennata Lamouroux
	Chaetomorpha brachygona Harvey
	Caulerpa parvula Svedelius
	Ulva rigida Agardh
Phaeophyta	
	Chnoospora bicanaliculata Krishnamurthy and Thomas
	Sargassum cinereum Agardh
	Feldmannia columellaris (Boergesen) Islam
	Dictyota adnata (Zanardini)
	Padina tetrasromatica Hauck
	Lobophora variegata (Lamouroux) Womersley ex Oliveira
Rhodophyta	
	Gelidium pusillum (Stackhouse) Le Jolis
	Gelidium micropterum Kuetzing
	Laurencia sp.
	Grateloupia filicina (Lamouroux) Agardh
	Portieria horemanii (Lyngbye) Silva
	Falkenbergia- phase of *Asparagopsis taxiformis* (Delile) Trevisan
Microalgae	
	Chlorella marina (Butcher)
	*Isochrysis galbana (*Parke)
	Chaetoceros sp.
Mangroves	
	Rhizophora apiculata (Blume)
	Avicinia officinalis (Linnaeus)
Cyanobacteria	
	*Oscillatoria salina (*Biswas)
	Phormidium valderianum (Delp.) Gomont
	Calothrix contarenii (Zanardini) Bornet et Flahault
	Synechococcus sp.
Molluscans	
	Cypraea tigris (Linne)
	Cypraea vitellus (Linne)
	Tibia curta (Sowerby)
	Conus sp.
	Trochus radiatus (Gmelin)
	Haliotis varia (Linne)
	Turbo bruneus (Roding)
	Strombus sp.
	Bulla ampulla (Linne)
	Mytilus edulis (Linnaeus)
	Patella vulgata (Linnaeus)
	Limnaea sp.
Sea urchin	
	Tripneustes gratilla (Linnaeus)
Sea cucumber	
	Bohadschia marmorata (Jaeger)
Sea anemone	
	Anthopleura sp.
Sponges	
	Callyspongia subarmigera Ridley
	Acanthella elongata Dendy
	Haliclona sp.

RESULTS AND DISCUSSION

Study area

The study area, Kollam coast, spreads over 45 km along the southwest coast of India. It has two estuaries, Paravoor and Astamudi, and many incursions of fresh water from agriculture runoff. A ubiquitous laterite and scattered granite boulder capping over the substrate in the intertidal and upper subtidal zones of the coast makes it a highly preferred and desirable habitat for a wide range of organisms. Although this coastal system is extraordinary for its biodiversity and hydrological functions, the flora and fauna of

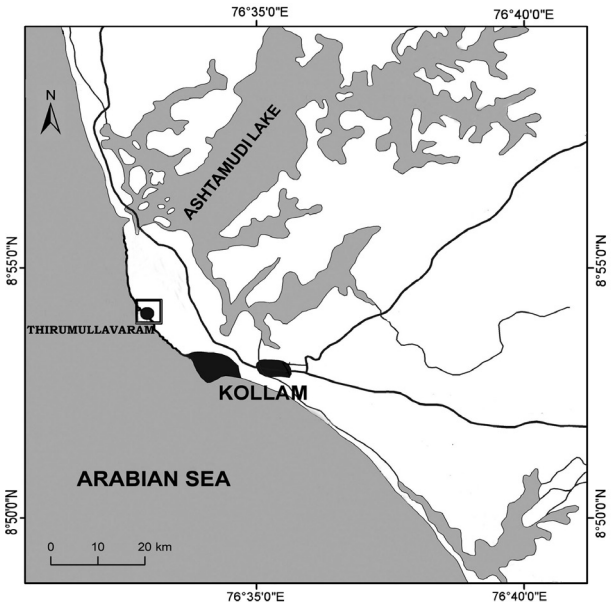

FIG. 1. – Map showing the studied area of the Kollam coast (south-west coast of India)

the area have been studied very little, except for the algal communities. In the present study, 47 species of marine organisms belonging to different flora and fauna were recorded. In spite of the diverse floral and faunal assemblages, the present study focused on the bioactivity of the dominant marine species in order to conserve the low intensity species (Table 1 and Fig. 1).

Overall bioactivity

Of the 47 marine organisms screened, 33 species (9 species of fauna and 24 species of flora) showed antimicrobial activity in different ranges with respect to the phyla, species and test organisms used. Among the active 33 species, the methanolic extract of *Falkenbergia* exhibited the broadest and highest antimicrobial activity (Table 2). The highly active seaweed, the *Falkenbergia*-phase of *A. taxiformis*, was fractionated and purified using different chro-

TABLE 2. – Antimicrobial activity of flora and fauna collected from the Kollam coast.

Marine organisms	Diameter of inhibition zone (mm)									
	Shrimp pathogens*					Multiresistant human pathogens*				
	Vv	Vp	Vh	Vac	Va	Se	Pa	Bs	Kp	Sa
E. compressa	16±1.25	9±2.23	12±2.74	17±2.41	8±3.12	0±00	0±00	0±00	8±1.5	0±00
C. albida	10±2.31	7±2.65	8±1.32	10±2.19	7±3.06	0±00	0±00	0±00	0±00	0±00
B. composita	7±3.21	9±2.05	10±1.65	6±2.46	10±1.45	0±00	0±00	0±00	0±00	0±00
B. pennata	5±1.07	4±1.75	0±00	0±00	0±00	0±00	0±00	0±00	0±00	0±00
C. parvula	3±1.52	0±00	0±00	4±0.56	0±00	0±00	0±00	0±00	0±00	0±00
F. columellaris	0±00	3±1.85	0±00	3±1.46	0±00	0±00	0±00	0±00	0±00	0±00
D. adnata	5±1.42	8±2.03	7±1.37	5±1.76	0±00	0±00	0±00	0±00	0±00	0±00
P. tetrasromatica	12±2.67	11±2.06	0±00	0±00	14±2.96	0±00	0±00	8±2.12	0±00	0±00
L. variegata	24±2.21	9±3.23	9±4.23	14±3.25	8±2.22	8±2.26	9±3.24	9±3.63	0±00	8±2.1
G. pusillum	6±1.65	9±2.61	0±00	0±00	7±2.16	0±00	0±00	0±00	0±00	0±00
G. micropterum	0±00	8±1.08	0±00	7±2.62	0±00	0±00	0±00	0±00	0±00	0±00
Laurencia sp.	23±2.05	20±3.56	14±2.46	18±2.85	22±3.46	11±2.56	8±3.75	0±00	0±00	0±00
G. filicina	08±2.16	9±2.75	11±2.79	0±00	8±2.85	0±00	0±00	0±00	0±00	0±00
P. horemanii	7±1.45	0±00	11±2.52	9±2.12	0±00	0±00	0±00	0±00	0±00	0±00
Falkenbergia-phase	31±2.65	28±3.21	26±2.85	33±2.34	32±1.25	21±2.31	19±1.45	23±1.86	15±2.64	21±3.12
C. marina	0±00	5±2.26	0±00	9±3.24	9±2.35	0±00	0±00	0±00	0±00	0±00
I. galbana	0±00	4±2.74	0±00	8±3.26	8±2.46	0±00	0±00	0±00	0±00	0±00
Chaetoceros sp.	4±1.56	7±1.64	5±2.21	10±2.65	12±1.87	0±00	0±00	0±00	0±00	0±00
R. apiculata	14±2.76	0±00	13±3.95	9±2.64	21±2.85	0±00	6±1.86	6±1.96	7±2.64	7±2.45
A. officinalis	0±00	14±2.65	0±00	0±00	15±1.64	0±00	5±2.62	0±00	0±00	0±00
P. valderianum	8±2.64	9±2.31	8±2.08	14±2.45	0±00	9±2.08	0±00	0±00	7±2.9	0±00
C. contarenii	0±00	0±00	7±3.08	9±4.05	0±00	0±00	0±00	0±00	0±00	0±00
Synechococcus sp.	0±00	9±1.78	9±1.95	9±2.68	13±2.45	0±00	0±00	0±00	0±00	0±00
C. tigris	8±3.23	6±2.45	0±00	5±2.780	0±00	0±00	0±00	0±00	0±00	0±00
T. bruneus	0±00	9±3.21	8±1.46	10±1.06	9±2.65	0±00	0±00	8±2.94	0±00	0±00
T. curta	0±00	7±2.33	0±00	8±00	0±0.0	0±00	0±00	0±00	0±00	0±00
Limnaea sp.	8±4.65	0±00	5±2.68	8±2.54	7±3.45	0±00	0±00	7±1.98	0±00	0±00
T. gratilla	0±00	0±00	0±00	11±2.85	8±2.49	0±00	0±00	0±00	0±00	0±00
B. marmorata	11±2.05	0±00	13±2.97	9±3.45	8±3.62	0±00	0±00	0±00	7±2.86	0±00
Anthopleura sp	0±00	8±2.34	0±00	0±00	11±2.15	0±00	0±00	0±00	0±00	0±00
C. subarmigera	8±2.63	0±00	6±2.12	5±2.63	0±00	0±00	0±00	0±00	0±00	0±00
A. elongata	12±2.87	8±3.14	8±2.61	8±2.14	7±3.21	0±00	0±00	0±00	0±00	0±00
Haliclona sp.	17±2.63	9±2.69	0±00	16±1.64	9±2.07	5±3.05	0±00	8±2.34	0±00	8±3.13

*Shrimp pathogens- *V. parahaemolyticus* (Vp), *V. vulnificus* (Vv), *V. harveyi* (Vh), *V. alcaligenes* (Vac), and *V. alginolyticus* (Va). MDR human pathogens- *Staphylococcus aureus* (Sa), *Pseudomonas aeruginosa* (Pa), *Bacillus subtilis* (Bs), *Klebsiella pneumoniae* (Kp), and *Staphylococcus epidermidis* (Se).

matographic systems including TLC, reverse phase HPLC and GC-MS.

Antibacterial activity of marine flora

Of the 10 bacterial species tested, shrimp pathogens were the most sensitive to the methanolic extract of the *Falkenbergia*-phase of *A. taxiformis* which produced mean zones of inhibition ranging between 26 and 33 mm. Among the shrimp pathogens, *V. alcaligenes*, *V. alginolyticus* and *V. vulnificus* were the most susceptible species with marked inhibition zones (33, 32 and 31 mm respectively) (see Table 4).

The *in vitro* screening of the red alga *Falkenbergia* showed 100% inhibitory activity against all the tested shrimp and human pathogens. Moderate activity (15 to 23 mm) was observed against multiresistant clinical pathogens. Of the five tested species of clinical pathogens, the gram positive bacterium *B. subtilis* was highly susceptible with an inhibition zone of 23 mm. The methanolic extract showed moderate activity against another two gram positive bacteria, *S. epidermidis* (21 mm) and *S. aureus* (21 mm). Among the gram negative bacteria tested, maximum activity was observed against *P. aeruginosa* (19 mm), whereas *K. pneumoniae* (15 mm) appeared to be the less susceptible. Based on the present findings, this marine red alga could be a potential source of antibiotics for controlling clinical and shrimp aquaculture pathogens.

Marine red algal species from the southwest coast of India are recognized for their antimicrobial properties (Selvin and Lipton, 2004). The antimicrobial activity of Bonnemaisoniales taxa has been highlighted previously by several authors (Paul *et al*., 2006; Salvador *et al*., 2007; El-Baroty *et al*., 2007). Nylund *et al.* (2005) reported that the crude organic extract of *Bonnemaisonia hamifera* inhibited the growth of nine bacteria. Later, the same author demonstrated that polyhalogenated 2-heptanone from this alga, strongly inhibits the colonization and growth of numerous marine bacteria (Nylund *et al*., 2008). Another Bonnemaisonialean species, *Asparagopsis armata,* showed remarkable activity against some fish pathogenic bacteria (Bansemir *et al*., 2006). The most characteristic algal bioactive compounds that have exhibited significant anti-HIV activity, according to the various biochemical assays designed for chemotherapeutic strategies, are sulphated galactans extracted from the seaweed, *A. taxiformis* (Haslin *et al.,* 2001).

Lobophora variegata appeared to be the second most potent brown algae among the flora screened for bioactivity. The inhibition was in the range of 8 to 24 mm against nine species of shrimp and clinical pathogens. This species demonstrated antifungal activity against the saprophyte, *Dendeyphella salina* (Puglisi *et al*., 2007).

The mangrove taxa, *R. apiculata* and *A. officinalis* from the southwest coast of India were studied for antimicrobial activity for the first time. The inhibitory activity of mangroves against shrimp pathogens was found to be promising. The methanolic extracts of both mangroves exhibited antimicrobial activity against shrimp and human pathogens. *R. apiculata* showed an inhibition range of 7 to 21 mm against all the tested pathogens. *A. officinalis* showed inhibitions of 15, 14 and 5 mm against two shrimp *Vibrios* and one species of human clinical pathogen respectively. The results of the present study suggest that the mangrove extracts can be used as alternate medicine for shrimp and human diseases. Bioactivity of mangroves has already been reported in many works (Premnathan *et al*., 1996; Abeysinghe and Wanigatunge, 2006). However, though mangroves are potentially active, the exploration of mangroves for pharmaceutical compounds is in a preliminary screening phase (Bandaranayake, 1998).

Table 2 shows the antimicrobial activity of the cyanobacteria analyzed in this study. Isolation of bioactive compounds from marine cyanobacteria has been reported in several publications during the last few decades (Falch, 1996; Papke *et al*., 1997) Recently, hundreds of biomedically relevant compounds from cyanobacteria have been reported. These include various classes of secondary metabolites such as polyketides, amides, alkaloids, lactones, peptides, lipopeptides and compounds of mixed biosynthetic origin (Kleinkauf and Dohren, 1997). *Phormidium valderianum* was found to be the most active cyanobacteria compared to *Calothrix contarenii* and *Synechococcus* sp. The extract from *P. valderianum* was found to be inhibitory against six pathogens such as *V. parahaemolyticus, V. vulnificus, V. harveyi, V. alcaligenes, S. epidermidis* and *K. pneumoniae*. *C. contarenii* and *Synechococcus* sp. were active against *V. harveyi, V. alcaligenes*, *V. parahaemolyticus* and *V. alginolyticus.* However, no antibacterial activity was found in the extract of *Oscillatoria salina.* The antimicrobial activity of cyanobacteria against human pathogens is not very promising. However, Martins *et al*. (2008) noted the

antibiotic activity of the marine *Synechococcus* sp. against gram-positive bacteria.

Chaetoceros sp. showed moderate activity against *V. alginolyticus* (12 mm) and *V. alcaligenes* (10 mm). The results of the present study (Table 2) evidenced that *V. alcaligenes* and *V. alginolyticus* are more susceptible to all microalgae tested, in agreement with the observations of previous researchers who noted vibriocidal activity of microalgae (Naviner *et al*., 1999; Regunathan and Wesley, 2004).

Antimicrobial activity of marine fauna

It is well known that sponges produce diverse secondary metabolites to ward off predators and parasites (Pawlik *et al*., 2002), for competitive exclusion of other sessile life (Becerro *et al*., 1995) and for self protection against infection. The first therapeutic agents from the marine environment were made from the sponge, *Tethya crypta* (Bergmann and Feeney, 1951). So far more than 5300 different natural bioactive products have been discovered (Faulkner, 2000, 2001, 2002). Further, the crude extracts of marine sponges from the Indian coast have a high incidence of antibacterial activity against shrimp/fish *Vibrios* (Selvin and Lipton, 2004a).

In the present study, the methanolic extract of *Haliclona* sp. was active against the bacteria, *V. parahaemolyticus, V. alginolyticus, B. subtilis, S. aureus, S. epidermidis* and especially against the shrimp pathogens, *V. vulnificus* and *V. alcaligenes*. This was followed by *Acanthella elongata* which inhibited *V. alcaligenes, V. vulnificus, V. harveyi, V. alginolyticus* and *V. parahaemolyticus* (Table 2). It was found that clinical pathogens exhibited more resistance to sponge extracts when compared to shrimp *Vibrios.* The results of the present study suggest that sponge secondary metabolites could be used as an antibiotic substance to manage shrimp and human bacterial pathogens.

Among the 12 molluscans screened, the extracts of *Turbo bruneus*, *Tibia curta Cypraea tigris* and *Limnaea* sp. showed a moderate inhibitory effect against shrimp pathogens (Table 2). The highest inhibition values recorded were produced by *T. bruneus* against *V. alcaligenes* (10 mm), *V. parahaemolyticus* (9 mm) *V. harveyi* (8 mm) and *V. alginolyticus* (9 mm). Similar activity was reported for this taxon against marine biofilm-forming bacteria (Ramasamy and Murugan, 2005). In addition, only *T. bruneus* and *C. tigris* achieved an inhibition zone against the multiresistant human pathogen, *B. subtilis* (8 mm). The antimicrobial activity of molluscan reported here agrees with the results of previous studies (Haug *et al*., 2004; Mercado *et al*., 2005).

The methanolic extract of the sea cucumber, *Bohadshia mormorata* showed its highest *in vitro* antibacterial activity against the shrimp pathogens, *V. harveyi* (13 mm) and *V. vulnificus* (11 mm), whereas its lowest activity was observed against the pathogens, *V. alcaligenes* (9 mm) and *V. alginolyticus* (8 mm) (Table 2). Only *K. pneumoniae* (7 mm) was sensitive to the methanolic extract of the sea cucumber. It was earlier reported by Ridzwan *et al*. (1995), that methanolic extracts of *Bohadschia argus* has no inhibitory activity against bacteria, whereas the phosphate buffered saline extract has inhibitory activity. As noted by Villasin and Pomory (2000), the extract from the body wall of the sea cucumber *Parastichopus parvimensis* had antibacterial properties.

The sea urchin *Tripneustes gratilla* showed antibacterial activity against *V. alcaligenes* (11 mm) and *V. alginolyticus* 8 mm. Our results are in agreement with the earlier antibacterial activity of marine echinoderm, *Paracentrotus lividus* against *V. alginolyticus* (Stabili *et al*., 1996). Further, it has been reported that different body parts of the sea urchin, *Strongylocentrotus droebachiensis* possess antibacterial activity (Haug *et al*., 2002).

The Cnidaria, *Anthopleura* sp. showed weak activity against the shrimp and human pathogens tested. It inhibits the growth of *V. alginolyticus* (11 mm) and *V. parahaemolyticus* (8 mm) (Table 2). Similar observations were reported from the benthic sea anemone, *Stichodactyla haddoni* collected from the Indian coast against the fish pathogen, *Aeromonas hydrophila* (Williams *et al*., 2007).

Fractionation and purification of active seaweed *Falkenbergia*-phase of *A. taxiformis*

The fraction that exhibited antimicrobial activity was purified using preparative TLC to obtain a single spot with an *Rf* value of 0.641. The active TLC resolved spot was again purified with reverse phase HPLC. The HPLC profile showed two peaks with retention times (min) of 2.69 and 3.00 respectively at a wavelength of 254 nm (Fig. 2). The eluted HPLC peak that retained antimicrobial activity (data not shown) was chosen for GC-MS analysis.

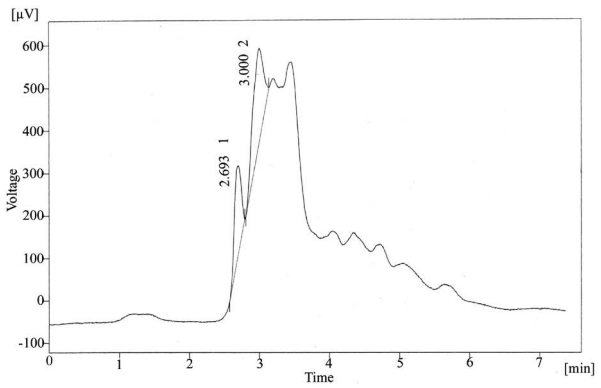

FIG. 2. – HPLC profile of active TLC fraction of *Falkenbergia*-phase of *A. taxiformis.*

Spectrum of the active fraction

The GC-MS chromatogram of *Falkenbergia*-phase of the A. taxiformis active fraction is shown in Figure 3 and the relative percentage of identified compounds is summarized in Table 3. The main phycoconstituents of the active fraction were oleic acid (51.33%) and n-hexadecanoic acid (42%) followed by dodecyl acrylate (1.47%), nitroacetonitrite (1.02), 2-nonen-1-ol, (E) (1.02%) and N-decanoic acid (0.85%), among others (Table 3).

In recent years, algal fatty acids have been emerging as prominent bioactive lead compounds. *Falkenbergia*-phase has been recognized for its many biological activities (Salvador *et al*., 2007). Our results are in accordance with the earlier report of Khotimchenko and Vaskovsky (1990) who recognized the C_{16}, C_{18}, C_{20} fatty acids to be dominant in red algae. Previous studies have already demonstrated the occurrence of the antibacterial property of oleic acid in some plant species (Dilika *et al*., 2002) and its antimycobacterial property in red algae (Saravanakumar *et al*., 2008). In agreement with our results Arun kumar *et al*. (2001) isolated fatty acids from the green algae, *Enteromorpha flexuosa* and showed its inhibition against the plant pathogen, *Xanthomonas oryzae*. As stated by Kanias *et al*. (1992), the antibiotic property of algal species could be confined to the presence of a mixture of organic acids such as oleic, capric, lauric, linoleic, myristic, palmitic and stearic acids.

Mechanism of antibiosis

The MBC/MIC ratios were determined to identify whether the active principles were bactericidal or bacteriostatic compounds. The results of MIC and MBC of the active principles from *Falkenbergia* are presented in Table 4. Since the MBC/MIC ratios obtained were less than 1, the active principles

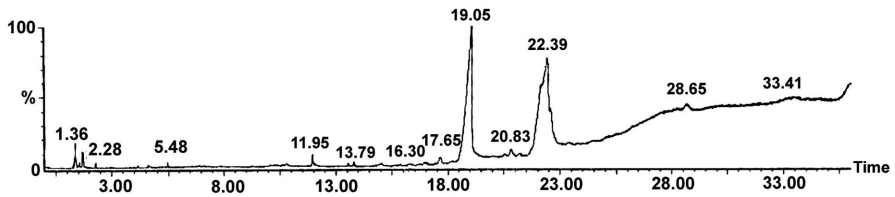

FIG. 3. – GC-MS chromatogram of the active fraction of *Falkenbergia*-phase of *A. taxiformis*

TABLE 3. – Compounds identified from the active fraction of *Falkenbergia*-phase of *A. taxiformis* using GC-MS.

Sl. No	Retention time	Name of the compound	Molecular formula	Molecular weight	Peak Area%
1	1.36	Nitroacetonitrite	$C_2H_2N_2O_2$	86	1.02
2	1.68	Ethanamine,2-(methlthio)-	C_3H_9NS	91	0.39
3	2.28	Pentane,3-methyl-	C_6H_{14}	86	0.17
4	4.16	1-Hexane,2,5-dimethyl-	C_8H_{16}	112	0.11
5	4.60	3-Pentanol, 3-ethyl	$C_7H_{16}O$	116	0.17
6	5.48	1-Hexene, 4-Methyl-	C_7H_{14}	98	0.17
7	6.91	1- Heptyn-4-ol	$C_7H_{12}O$	112	0.11
8	9.25	(2S, 3S)-(-)-3-Propyloxiranemethanol	$C_6H_{12}O_2$	116	0.11
9	10.22	1- Cyclopentyl-2,2-dimethyl-1-propanol	$C_{10}H_{20}O$	156	0.11
10	11.95	N-Decanoic acid	$C_{10}H_{20}O_2$	172	0.85
11	13.54	Oxirane,(butoxemethyl)	$C_7H_{14}O_2$	130	0.17
12	17.64	2-Nonen-1-ol, (E)-	$C_9H_{18}O$	142	1.02
13	19.05	N-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	42.87
14	20.83	Dodecyl acrylate	$C_{15}H_{28}O_2$	240	1.47
15	22.39	Oleic acid	$C_{18}H_{34}O_2$	282	51.33

TABLE 4. – Antimicrobial activities of methanolic extracts (1000 µg /ml) of the *Falkenbergia*- phase of *A. taxiformis.*

Test pathogens	Inhibition zone (mm)	Chloramphenicol (1000 µg)	Nalidixic acid (1000 µg)	MIC (µg)	MBC (µg)	MBC/MIC
V. vulnificus	31±2.65	31	-	750	90	0.1
V. parahaemolyticus	28±3.21	33	-	750	110	0.1
V. harveyi	26±2.85	30	-	750	60	0.1
V. alginolyticus	32±1.25	36	-	500	80	0.1
V. alcaligenes	33±2.34	32	-	500	50	0.1
S. epidermidis	21±2.31	-	33	1250	270	0.2
S. aureus	21±3.13	-	31	750	170	0.2
B. subtilis	23±1.86	-	35	750	180	0.2
P. aeruginosa	19±1.45	-	31	1250	420	0.3
K. pneumoniae	15±2.64	-	30	1250	380	0.3

can be considered as bactericidal. The most sensitive microorganism to the *Falkenbergia* extract was the shrimp pathogen, *V. alcaligenes,* which showed the highest mean zone of inhibition (33 mm) and the lowest MIC (500 µg/ml) and MBC (50 µg/ml) values. The MIC of the extract against shrimp pathogens was 500 µg/ml for *V. alginolyticus*; 750 µg/ml for *V. vulnificus, V. harveyi* and *V. parahaemolyticus*; 750 µg/ml for *S. aureus* and *B. subtilis* and 1250 µg/ml for *P. aeruginosa, K. pneumoniae* and *S. epidermidis*. These variations may be due to the chemical nature and quantity of bioactive metabolites present in the *Falkenbergia* extract and their mode of action towards different test organisms (Barbour *et al*., 2004).

CONCLUSION

Our results imply that the search for novel bioactive compounds by screening of different marine flora and fauna is an efficient sourcing method. Screening tactics based on the ecological knowledge of marine organisms are being increasingly deployed in the investigation of novel bioactive compounds. However, the results presented here indicate that only a few marine invertebrates show antimicrobial properties compared to the flora examined. Of the 47 species screened, the red alga, *Falkenbergia* stood out as a potential producer of marine bioactive metabolites that inhibit the growth of both shrimp *Vibrios* and multiresistant human pathogens. The main bioactive constituents of this alga were investigated using GC-MS analysis. Bioactivity guided purification of the active fraction resulted in the preliminary identification of a mixture of volatile compounds including fatty acids. This report shows the antimicrobial activity of the *Falkenbergia*-phase of *A. taxiformis* from the Indian coast for the first time. The large biomass produced by *Falkenbergia* along the Kollam coast could make exploiting these bioactives economically viable for shrimp disease management. Our preliminary results also reveal that many of these marine organisms produce more or less structurally diverse secondary metabolites which could be of agrochemical and pharmaceutical interest.

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