Annals of Microbiology, **59 (2)** 207-219 (2009)

Antimicrobial potential and seasonality of red algae collected from the southwest coast of India tested against shrimp, human and phytopathogens

Aseer MANILAL[1], Sugathan SUJITH[1], George SEGHAL KIRAN[2], Joseph SELVIN[1]*, Chippu SHAKIR[1], Ramakrishnan GANDHIMATHI[1], Aaron Premnath LIPTON[3]

[1]Department of Microbiology, [2]Department of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, India; [3]Department of Marine Biotechnology, Centre for Marine Sciences and Technology, Manonmaniam Sundaranar University, Rajakkamagalam, India

Received 28 October 2008 / Accepted 9 March 2009

Abstract - Fifteen seaweeds belong to 13 families and 6 orders of the rhodophyta were sampled for one year from April 2007 to March 2008 along the southwest coast of India (Indian Ocean). The species were examined for *in vitro* antimicrobial activity against six pathogenic *Vibrio* strains isolated from moribund tiger shrimp (*Penaeus monodon*), six type cultures (Microbial Type Culture Collection, MTCC) of prominent shrimp *Vibrio* pathogens, 10 multidrug resistant clinical pathogens, four species of *Candida* obtained from pulmonary TB patients and four species of plant pathogenic fungi to evaluate their potency to be used as natural antibiotics in pharmaceutical and agriculture field. Bioactivity was analyzed from crude extract of fresh and dried samples prepared from different polar and nonpolar solvents. Of these, four species of red algae (*Asparagopsis taxiformis, Laurencia ceylanica, Laurencia brandenii, Hypnea valentiae*) were found to be highly active. Broadest and highest activity was observed in the crude extract of *A. taxiformis*. Among the pathogens tested, shrimp pathogenic *Vibrios* were the most susceptible organisms while phytopathogens were found to be little resistant. In the present study, methanol was found to be the best solvent for extracting antimicrobial metabolites from dried samples rather than fresh. Seasonal variation in the antimicrobial activity was observed with higher level of activity recorded from *A. taxiformis* between December and January. The active principle of *A. taxiformis* was purified in column chromatography, TLC and reverse phase HPLC. The individual HPLC peaks were subsequently tested against a panel of pathogenic microorganisms and the active constituent was identified by GC-MS. The antimicrobial profile of *A. taxiformis* suggested that lipophilic compound which was primarily composed of pyrrole-2-carboxylic acid, pentadecanoic acid and octadecanoic acid might have functional role in the chemical defence against microbial invasion and these compounds could be utilized for the development of medically potential products.

Key words: seaweed extract; *Asparagopsis taxiformis*; antimicrobial activity; shrimp *Vibrios,* mycotoxic activity; phytopathogens.

INTRODUCTION

Marine algae (including microalgae) are one of the most primitive photosynthesizing (contribute nearly 40 percent of global photosynthesis) autotrophic groups of ecologically and economically important vegetation of oceanic ecosystem with unique life-cycle and physiology. Nearly fifty thousand species of seaweeds have been discovered in the marine environment (Filho-Lima *et al.,* 2002). A relatively small percentage (1 to 5%) of available seaweeds is used as foods by both humans and several animal species. Historically seaweeds provide essential economic, environmental, aesthetic, and cultural benefits to humanity (Dhargalkar and Neelam, 2005). In contrast to terrestrial vegetation, the marine flora constitutes valuable source for drug development (de Vries and Beart, 1995). For centuries, many of the seaweed secondary metabolites (SSM) have been used for traditional medicines due to their therapeutic potentials (Fitton, 2006). Recent studies have shown that marine algae are tremendous source of structurally novel and diverse array of marine secondary metabolites (Williams *et al.*, 1989; Williams and Maplestone, 1992). According to Kim and Park (2002) natural products have been regarded as important sources of potential chemotherapeutic agents. Marine algae are continuously exposed to many biotic and abiotic pressures which influence the organism's physiology, which in turn leads to the production of multifunctional natural secondary metabolites (Schmitt *et al.,* 1995). So far, more than 2400 SSM are described and many of the SSM are natural blueprints for the development of new

* Corresponding Author. Phone: +91-431-2407082; Fax: +91-431-2407084; E-mail: selvinj@rediffmail.com; seeshegal@yahoo.co.in

drugs (Munro and Blunt, 1999; Faulkner, 2001 and previous authors). Several of these compounds are constitutive, existing in biologically active forms in healthy seaweeds. The major secondary metabolites produced by seaweeds are halogenated compounds (Blunt *et al.,* 2007) displaying antibacterial, antifungal, antiviral, antifouling and antifeedent properties.

The abundance and diversity of secondary metabolites in seaweeds has made them prime material for pharmaceutical Industry. Numerous bioactive compounds were used in manufacturing of pharmaceutical intermediates and chemical entities for various medicines (Muñoz Crego and López Cruz, 1992). Seaweeds are ancient oceanic flora, holds a prodigious potential for the discovery of lead compounds for the future drug development. Seaweeds have been considered as potential source of marine medicinals including antimicrobial, cancer therapies (Yamamoto *et al.,* 1984) hypocholesterolemic and anithelminthic substances (Michanek, 1979). Although thousands of bioactive compounds have been discovered, the need for novel therapeutic compounds is still urgent in concern of number of new diseases and resistant strains of microorganisms.

Among all the algal divisions, rhodophyta are recognized as a species-rich phylum including > 2500 recognized species (Norton *et al.,* 1996). The Indian red algal species belong to 136 genera, 36 families and 16 orders. The collection site of present study (Kollam coast) alone witnessed 24 species of red alga belonging to 20 genera.

The aim of present study was to develop standard method for the extraction of algal bioactives and to evaluate the antimicrobial potency of red algae against a diverse range of pathogenic microorganisms including shrimp, human and plant pathogenic fungi, influence of sampling season on the activity of highly active seaweed and purification of active compounds.

MATERIALS AND METHODS

Collection of algae. The algae belonging to rhodophycea were randomly collected every month from April 2007 to March 2008 from the Kollam coast (southwest coast of India) (08°54′ N and 76°38′ E) during the ebb tide. The study area, Kollam, has a stretch of about 45 km coastal belt, lined uniquely with rocky intertidal habitat compare to other regions in the southwest coast India (Fig. 1). Its phytogeographical units are segregated on the basis of the distribution of intertidal hefty lithophilic algal assemblages. The Kollam coast is characterized by mixed tides and harbours a great diversity of species including micro and macroalgae, fishes, clams, crustaceans, sponges, sea anemones, sea cucumbers, sea urchins, soft corals and other sessile invertebrates. The intertidal and subtidal zone of the Kollam coast is distributed with extensive laterite rocky outcrops, scattered granite boulder reefs, mudstone platforms, tidal pools, clustered cobbles and pebbles which provide substrate for a diverse group of algae. Seaweed communities of Kollam coast remain almost pristine and represent a biogenic habitat structure. Throughout the year, the rocky intertidal zone is inhabited by extensive beds of mixed algal zone which is dominated by the red algal species. A list of red algae collected from Kollam coast is presented in Table 1. Among the red algal species, dense carpet of richly pigmented red alga *A. taxiformis* is very conspicuous during the winter season, inhabiting both horizontal and vertical rocky outcrops. To avoid ecological mutilation, the algal species were handpicked without removing the stem. Algal samples were transported to the laboratory in plastic bags containing seawater to prevent evaporation. Morphology and anatomical features of collected samples were analyzed and reconfirmed with the help of phycotaxonomist Dr. M.V.N Panikkar, Sree Narayana College, Kollam, Kerala, India.

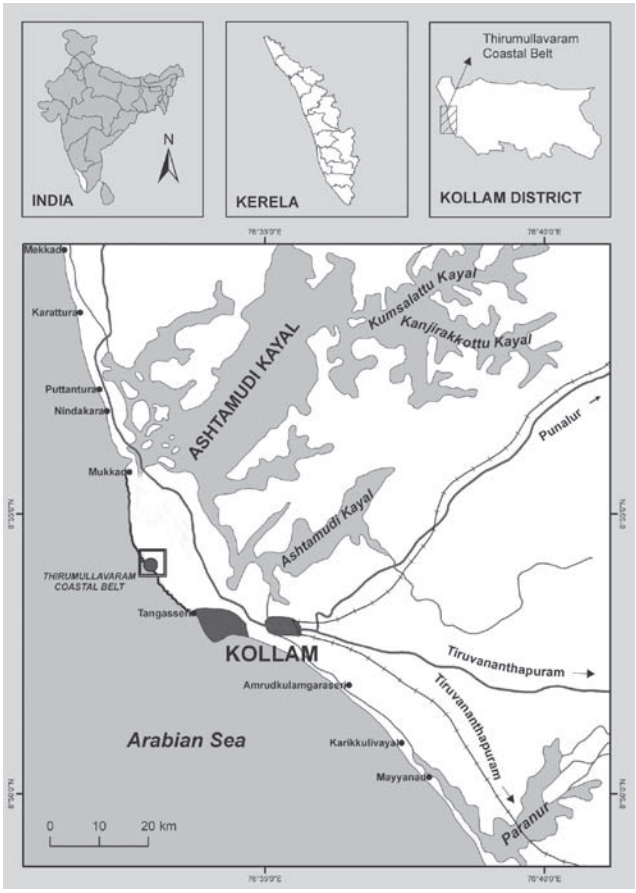

FIG. 1 - Map showing the study area, Kollam coast, southwest coast of India.

TABLE 1 - Red algae collected from southwest coast of India

	Red algae
1	*Myriogramme quilonensis* Anil Kumar & Panikkar
2	*Gelidium micropterum* Kützing
3	*Amphiroa anastomosans* Weber-van Bosse
4	*Ahnfeltiopsis pygmaea* (J. Agardh) P. Silva & DeCew
5	*Asparagopsis taxiformis* (Delile) Trevisan
6	*Porphyra suborbiculata* Kjellman
7	*Herposiphonia insidiosa* (Greville ex J. Agardh)
8	*Chondracanthus acicularis* (Roth)
9	*Hypnea musciformis* (Wulfen) Lamouroux
10	*Hypnea valentiae* (Turner) Montagne
11	*Centroceras clavatum* (C. Agardh) Montagne
12	*Laurencia brandenii* Saito & Womersley
12	*Laurencia ceylanica* J. Agardh
14	*Gelidiopsis variabilis* (J. Agardh) Schmitz
15	*Champia compressa* Harvey

Species description of highly active red alga *Asparagopsis taxiformis* (Delile) Trevisan. The unique feature of *A. taxiformis* include dull pink colour up to 15 cm tall attached to the rock surfaces by creeping rhizomatous portion bearing the hold fast, rhizoidal portion much branched, erect portion almost naked up to the middle, upper portion densely covered with branches and branchlets, delicate branches often tip curved, uniaxial. Central cavity is running all through the main stem and primary axis. Width of the thallus is 1000-1250 µm. Peripheral cells 30-43 µm long and 16-43 µm wide and covered with chromatophores. Cortex is pseudo-parenchymatous and colourless with 70-134 µm wide. Spermatangia are in dense cylindrical clusters with 333-500 µm width. Cystocarps terminal are on short branchlets, subspherical to pyriform 567-667 µm diameter with a small ostiole. Stalk of the cystocarp is 467-750 µm length and 133-167 µm width.

Extraction of fresh material. Ten grams of fresh tissue were pounded with 100 ml of solvents of increasing polarity, including, petroleum ether, ethyl acetate, dichloromethane, dichloromethane:methanol (1:1) and methanol respectively, vortexed and filtered through double folded muslin cloth and the filtrate was centrifuged at 8000 *x g* for 10 min (Eppendorf). The supernatant was concentrated in a rotary vacuum evaporator (Yamato) and used for antimicrobial assays.

Extraction of shade dried material. A definite quantity (20.0 g) of dried algal powder was submerged in Scott Duran flask containing 200 ml of solvents of increasing polarity and placed at 35 °C in a shaker at 120 rpm for two weeks to permit full extraction of the active compounds. After two weeks, algal material was filtered using Whatman filter paper No 1 fitted with a Buchner funnel using suction pressure followed by centrifugation (Eppendorf) at 8000 *x g* for 5 min at 20 °C. The supernatant was collected in a round-bottom flask and the solvent was concentrated in a rotary vacuum evaporator at 40 °C. The gummy extract was collected in airtight plastic vials and stored in the refrigerator for further studies.

Phycochemical analysis of highly active seaweed *Asparagopsis taxiformis*.

Biochemical analysis of active seaweed. The water content of the fresh material was calculated by subtracting the dried sample weight from wet weight of the total sample. Total lipid content in *A. taxiformis* was determined by Bligh and Dyer (1959) method. Total carbohydrates were determined as described by Dubois *et al.* (1956). The total protein content was determined after Lowry *et al.* (1951).

Fractionation and purification of bioactive compounds. The methanolic extracts of *A. taxiformis* (10 g) was loaded in a silica gel (60-120 mesh) (Merck) 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 assay (data not shown). The active fraction was further purified by preparative TLC using silica gel G as stationary phase with 1% methanol in dichloromethane as 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 spot were recovered by scrapping off the adsorbent at the appropriate place on the developed plate and eluted with methanol and centrifuged at 10000 *x g* for 5 min. The supernatant with antimicrobial activity was subjected to HPLC (Shimadzu chromatographic system, Kyoto, Japan) and GC-MS analysis (Hewlett Packard). Peak identification was carried out by comparison of the mass spectra with those available in the NIST and WILEY'S libraries.

Test microorganisms. Antimicrobial activity of seaweed extract were assessed using (i) six type cultures (MTCC, Microbial Type Culture Collection) of shrimp *Vibrio* pathogens, (ii) six shrimp *Vibrio,* isolated from diseased tiger shrimp *Penaeus monodon*, (iii) ten multidrug resistance pathogens obtained from clinical laboratory, (iv) four *Candida* species isolated from pulmonary TB patients, (v) four species of plant pathogenic fungi (Table 2). All the bacterial strains were maintained on nutrient agar slants (NB) (Himedia) at 37 ± 0.1 °C. The phytopathogens were maintained on Potato Dextrose agar (PDA) at 30 ± 0.1 °C while the yeast strains on Sabouraud Dextrose agar (SDA, Himedia) slants

TABLE 2 - Panel of pathogens used for antimicrobial assay

Group	Species
Shrimp *Vibrio* isolates	*V. harveyi* (Vb5)
	V. alginolyticus (Vb11)
	V. vulnificus (Vb14)
	V. fischeri (Vb17)
	V. parahaemolyticus (Vb21)
	V. damsela (Vb26)
Shrimp *Vibrio* (MTCC)	*V. parahaemolyticus* (MTCC 451)
	V. vulnificus (MTCC 1145)
	V. harveyi (MTCC 3438)
	V. alcaligenes (MTCC 4442)
	V. alginolyticus (MTCC 4439)
	Aeromonas hydrophila (MTCC 1739)
Clinical (MDR) pathogens	Gram negative bacteria
	Escherichia coli
	Proteus mirabilis
	Pseudomonas aeruginosa
	Klebsiella pneumoniae
	Gram positive bacteria
	non *Haemolytic Streptococcus*
	Staphylococcus aureus
	Staphylococcus epidermidis
	Enterococcus faecalis
	Micrococcus luteus
	Bacillus subtilis
Candida spp. (clinical)	*C. albicans*
	C. tropicalis
	C. glabrata
	C. krusei
Plant pathogenic fungi	*Fusarium oxysporum*
	Trichoderma sp.
	Aspergillus niger
	Aspergilus flavus

TABLE 3 - Overall percentage of activity of methanolic extract of dried red algae against test organisms

Red algae	Overall activity (%)*				
	Shrimp *Vibrio* isolates	Shrimp *Vibrio* (MTCC)	Clinical (MDR) pathogens	*Candida* spp. (clinical)	Plant pathogenic fungi
M. quilonensis	0	0	0	0	0
G. micropterum	0	0	0	0	0
A. anastomosans	16	0	10	0	0
A. pygmaea	0	0	0	0	0
A. taxiformis	100	100	100	100	100
P. suborbiculata	16	0	0	0	0
H. insidiosa	0	0	0	0	0
C. acicularis	0	0	0	0	0
H. musciformis	16	0	0	0	0
H. valentiae	50	33	60	25	0
C. clavatum	0	0	0	0	0
L. brandenii	100	100	70	50	0
L. ceylanica	66	33	60	25	0
G. variabilis	0	0	0	0	0
C. compressa	0	0	0	0	0

* Overall activity was expressed as relative antimicrobial activity of respective seaweeds against 30 tested pathogens including Shrimp *Vibrio* isolates (6 isolates), Shrimp *Vibrio* (MTCC) (6 isolates), Clinical (MDR) pathogens (10 isolates), *Candida* spp. (4 isolates), Plant pathogenic fungi (4 isolates).

at 4 °C. The shrimp *Vibrio* isolates were identified using standard biochemical, physiological, and morphological characterization and phylogenetic analysis.

Antimicrobial assays. The antimicrobial assay was carried out as per Selvin and Lipton (2004). Briefly, the base layer was prepared with 10 ml (1.5%, w/v) of Muller Hinton agar (Himedia). Five numbers of sterile porcelain beads were placed on the base layer at 60° angle 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 in triplicate were filled with 100 µl of appropriate algal extract. The well with solvent used for dissolution was taken as negative control. After 24 h of incubation at 37 °C, the diameter of inhibition around the wells was determined as average of triplicates.

The PDA and SDA were used for bioactivity screening and routine propagation of phytopathogens and yeast respectively. Cell suspensions containing 10^7 CFU/ ml cells for yeasts, and 10^5 spore/ml of fungi were prepared and evenly spread onto the surface of the agar plates of SDA medium for yeasts and fungi using sterile swab sticks. Appropriate extract (100 µl) was placed upto the brim of wells and the plates were incubated at 37 °C for 48 h for yeasts and at 30 °C for 72 h for fungi.

RESULT AND DISCUSSION

Antimicrobial activity of seaweeds

In the preliminary screening process, it was found that out of 15 red algae, the crude methanolic extract of four algae indicate significant antimicrobial activity: one species of genus *Asparagopsis*, two species of genus *Laurencia,* and one species of genus *Hypnea* (Table 3). But considering the broadest spectrum, highest activity and biomass availability, *A. taxiformis* was chosen for further phycochemistry and seasonality studies.

Zones of inhibition were used as an indication of antimicrobial activity. However, the diameters of inhibition zones varied according to the kinds and concentration of extracts and microbial strains tested (Table 4). These differences in efficacy at the *in vitro* level can be linked to the level of active substances in the alga and to purity of the extract. Of the solvents used, methanol was the most effective which showed significantly high inhibitory activity. The organic solvents usually preferred for antimicrobial activities compared to aqueous solvents (Masuda *et al.,* 1997; Filho-Lima *et al.,* 2002). Febles *et al.* (1995) reported that methanolic extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate. Choudhury *et al.* (2005) demonstrated the effect of methanolic extracts of *Gracilaria corticata* on the growth of *Pseudomonas aeruginosa*.

In this study, fresh algal extracts were less effective compare to dried algal extracts. In the extraction methods, the submerged extraction of dried samples using methanol at 37 °C ensured the isolation of metabolites with antimicrobial activity compared to fresh samples. The variation may be due to the fact that the submerged type extraction of dried samples leads to the complete extraction of bioactives. This can probably be due to higher dilution of the bioactive metabolites in the fresh algal material by 70-90% of higher water content (Jensen, 1993).

Earlier reports evidenced the lower activity in extracts from fresh tissue than in extracts from dried material (Rao *et al.,* 1986; Takaki *et al.,* 1988). As noted by Harper *et al.* (2001) among the three algal divisions, the members of rhodophyta contribute 51% of natural secondary metabolites. Among the red algal secondary metabolities, halogenated monoterpenes form a chemically complex group with diverse bioactivity (Maliakal, 2001). Numerous studies have documented the antibacterial activity of Rhodophyceae (Gao *et al.,* 2001; Fadhli *et al.,* 2006; Salvador *et al.,* 2007; Yavasoglu *et al.,* 2007; Ki-Bong *et al.,* 2008).

TABLE 4 - Inhibitory spectrum of active red algae collected from the southwest coast of India

Pathogens	Zone of inhibition (mm)*			
	A. taxiformis	*L. brandenii*	*L. ceylanica*	*H. valentiae*
Shrimp *Vibrio* isolates				
V. harveyi (Vb5)	27 ± 1.3	15 ± 1.2	12 ± 2.1	8 ± 1.8
V. alginolyticus (Vb11)	24 ± 2.2	20 ± 1.7	8 ± 1.8	9 ± 1.4
V. vulnificus (Vb14)	26 ± 1.5	14 ± 1.3	10 ± 1.5	3 ± 2.2
V. fischeri (Vb17)	26 ± 1.3	18 ± 1.2	9 ± 2.6	8 ± 1.5
V. parahaemolyticus (Vb21)	21 ± 2.5	20 ± 1.8	7 ± 1.1	4 ± 1.3
V. damsela (Vb26)	28 ± 1.1	12 ± 2.7	5 ± 1.2	7 ± 1.6
Shrimp *Vibrio* (MTCC)				
V. parahaemolyticus	22 ± 1.6	16 ± 1.6	6 ± 1.7	5 ± 1.8
V. vulnificus	24 ± 1.8	21 ± 1.23	19 ± 1.23	20 ± 1.4
V. harveyi	25 ± 2.2	14 ± 1.7	6 ± 2.2	5 ± 2.8
V. alcaligenes	30 ± 1.6	18 ± 2.4	18 ± 2.8	16 ± 2.2
V. alginolyticus	27 ± 2.3	20 ± 1.3	7 ± 1.4	3 ± 1.5
Aeromonas hydrophila	23 ± 1.7	15 ± 1.74	7 ± 1.65	5 ± 1.6
Clinical (MDR) pathogens Gram negative bacteria				
Escherichia coli	17 ± 1.4	9 ± 1.8	4 ± 2.8	6 ± 1.8
Proteus mirabilis	14 ± 1.3	6 ± 1.1	8 ± 1.9	5 ± 1.5
Pseudomonas aeruginosa	16 ± 1.2	7 ± 2.9	5 ± 1.5	2 ± 2.5
Klebsiella pneumoniae	20 ± 1.9	5 ± 1.7	6 ± 2.5	5 ± 1.4
Clinical (MDR) pathogens Gram positive bacteria				
non-haemolytic *Streptococcus*	20 ± 1.5	14 ± 1.3	9 ± 1.3	8 ± 2.8
Staphylococcus aureus	18 ± 2.7	10 ± 2.7	10 ± 2.2	11 ± 1.8
Staphylococcus epidermidis	22 ± 1.5	15 ± 1.5	9 ± 2.5	8 ± 2.5
Enterococcus faecalis	19 ± 2.1	13 ± 1.2	8 ± 1.9	10 ± 1.9
Micrococcus luteus	18 ± 1.4	12 ± 1.9	10 ± 2.1	11 ± 1.3
Bacillus subtilis	25 ± 1.1	16 ± 1.4	11 ± 1.6	10 ± 2.7
Candida spp. (clinical)				
C. albicans	24 ± 1.3	12 ± 2.7	8 ± 1.3	9 ± 1.4
C. tropicalis	21 ± 1.8	13 ± 1.5	3 ± 2.7	6 ± 1.8
C. glabrata	16 ± 1.1	5 ± 2.5	4 ± 1.4	4 ± 2.5
C. krusei	12 ± 1.6	6 ± 1.8	3 ± 2.6	1 ± 1.5
Plant pathogenic fungi				
Fusarium oxysporum	13 ± 1.7	-	-	-
Trichoderma sp.	12 ± 1.9	-	-	-
Aspergilus niger	13 ± 1.4	-	-	-
Aspergilus flavus	14 ± 1.2	-	-	-

* Values are mean ± standard deviation, n = 3 experiments, – denotes no activity.

Antibacterial activity of seaweeds against Shrimp pathogens

Of the 15 algae studied, dried methanolic extracts of four seaweeds showed considerable antimicrobial activity. Particularly, the extract of *A. taxiformis* has shown broader activity spectrum against all the MTCC cultures tested and produce significant zones of inhibition against *V. alginolyticus* (27 mm), *V. parahaemolyticus* (22 mm), *V. alcaligenes* (30 mm), *V. vulnificus* (24 mm), *V. harveyi* (25 mm), and *Aeromonas hydrophila* (23 mm). *Laurencia brandenii* showed considerable zones of inhibition against *V. alginolyticus* (20 mm), *V. parahaemolyticus* (16 mm), *V. alcaligenes* (18 mm), *V. vulnificus* (21 mm), *V. harveyi* (14 mm), and *A. hydrophila* (15 mm). However, *Laurencia ceylanica* and *Hypnea valentiae* exhibited weak antimicrobial activity against all tested bacteria except against *V. alcaligenes* (18 mm, 16 mm) and *V. vulnificus* (19 mm, 20 mm).

Methanolic extract of *A. taxiformis* was most active against all the tested shrimp isolates, its extract was being active against *V. damsela* (28 mm), *V. harvyei* (27 mm), *V. fischeri* (26 mm), *V. vulnificus* (26 mm) while *L. brandenii* showed high activity (20 mm each) against *V. alginolyticus* and *V. parahaemolyticus,* moderate activity (18, 15, 14mm) against *V. fischeri, V. harvyei* and *V. vulnificus* whereas *L. ceylanica* and *H. valentiae* exhibited less effect on the growth of shrimp *Vibrio.* This observation agrees with the previous reports of Bansemir *et al.* (2006) that methanolic extract of *A. taxiformis* show antibacterial activity against fish pathogenic *Vibrio*. Haloketones, dibromoacetic acid and bromoform isolated from genus *Asparagopsis* showed significant vibriocidal activity (Paul *et al.,* 2006). Liao *et al.* (2003) reported the vibriocidal activity of red algae against fish pathogen *V. vulnificus.* The significant finding of the present study include vibriocidal activity of *A. taxiformis*.

Thus, it is clearly evidenced that *A. taxiformis* is the best marine red algal candidate useful for aquaculture industry.

Bacterial infection is one of the threatening factor which crops up repeatedly in cultured shrimp and leads to considerable economic losses in shrimp aquaculture. The use of antimicrobial agents has increased significantly in aquaculture practices (Alderman and Michel, 1992) which give rise to multiple antibiotic resistance in bacterial pathogens (Moriarty, 1999). Moreover, there is a growing concern in the transfer of antibiotic resistance to human pathogens and the antibiotic accumulation in shrimp has been found to be hazardous to human consumers (Primavera, 1994). New antibiotics with high activity and without side effects for human and for environment are therefore urgently needed. Giant black tiger shrimp, *Penaeus monodon* is one of the most economically important fishery products of southern India with export viability. Unfortunately during 2007-08 shrimp production showed a decrease by 26 per cent over the previous year and overall shrimp production has stagnated at around 150000 tonnes a year. Diseases are considered to be crucial factor which masks the expansion of shrimp aquaculture. Shrimp farming has suffered great economic losses from various infections particularly Gram negative *Vibrionaceae* represent the most dreadful pathogenic bacteria causing disease in both grow-out ponds and hatcheries (Regunathan and Wesley, 2004). At present, application of bioactive natural products from marine source in mariculture industry appeared to be an alternate strategy for this knotty problem (Selvin and Lipton, 2003). Albeit the bioactive potential of seaweeds has been established long before, the application of seaweed-based products in shrimp disease management is a recently emerged approach (Selvin and Lipton, 2003; Huang *et al*., 2006). Therefore, the finding presented in this study and those of Selvin and Lipton (2003, 2004) again confirm the necessity of using marine natural products in shrimp disease management.

Antibacterial activity of seaweeds against multidrug resistant clinical human pathogens and *Candida* spp.

Of the 15 algae tested, red alga *A. taxiformis* exhibited broad spectrum of antimicrobial activity and showed zones of inhibition ranging from 14 to 25 mm (Table 4). The extracts of both *Laurencia* spp. showed significant microbicidal activity. Among the ten strains tested, the antibacterial activity of *A. taxiformis* was more pronounced against standard strains of Gram positive bacteria viz, *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus,* non-haemolytic *Streptococcus* and *Enterococcus faecalis,* showing zone of inhibition ranging from 18 to 25 mm of diameter respectively at 1 mg/ml concentration in comparison to Gram negative bacteria viz *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* showing zones of inhibition ranging from 14 mm to 20 mm at 1 mg/ml. However the activity was significantly higher against Gram positive bacteria than Gram negative bacteria. Our results agreed with the findings of Rao and Parekh (1981) and Padmakumar and Ayyakkannu (1997) that organic extract of Indian seaweed exhibit antimicrobial activity against Gram negative and Gram positive biomedical pathogens. The present findings will have immense potential on the control of clinical pathogens, since the strains used in the study were collected form hospital sources and most of the strains appeared as multidrug resistant and cannot be controlled with commercially prescribed antibiotics.

The Gram negative bacteria are more resistant to seaweed extract compared to Gram positive bacteria. This may be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Adwan and Abu-Hasan*,* 1998).

Previous studies reported the screening of seaweeds on human and plant pathogenic virus, bacteria and fungi (Robles-Centeno *et al.,* 1996; Arun Kumar and Rengaswamy, 2000). Hence, more studies pertaining to the use of seaweed as therapeutic agent should be emphasized, especially those related to the control of multidrug resistant microbes.

Opportunistic infectious diseases cause substantial morbidity, necessitate toxic and expensive therapies, result in hospitalization and shorten the survival of people with HIV infection (Moore and Chaisson, 1996). The genus *Candida* is opportunistic fungal pathogen that usually infects immuno-comprimised, immuno suppressed and diabetic patients causing spectrum of diseases including a secondary pathogen of HIV infection (Ashbee and Evans, 2000). The development of resistance in known *Candida* pathogens and emergence of new *Candida* pathogens intrinsically resistant to the currently available antibiotics demonstrate the urgent importance of identifying novel antifungal agents.

Methanolic extract of *A. taxiformis* showed remarkable anticandidal activity against all the species of *Candida* whereas the *L. brandenii* exhibited meager antifungal activity and a slight decrease, particularly against two *Candida* species viz *C. glabrata, C. krusei.* Among tested *Candida* spp., *C. albicans* was found to be most sensitive, *C. tropicalis* the next sensitive, and *C. krusei* the mild resistant pathogen. *A. taxiformis* produced a zone of inhibition of 24, 21, 16 and 12 mm diameter against *C. albicans, C. tropicalis, C. glabrata* and *C. krusei* respectively. Salvador *et al.* (2007) acknowledged the antifungal activity of *A. taxiformis* against *C. albicans*. Volatile extract of *A. taxiformis* also showed trace antifungal against *C. albicans* and *C. tropicalis* (El-Baroty *et al.,* 2007). The results suggest that *A. taxiformis* is a potential red alga for the management of human pathogenic yeast, *Candida* spp. which was established as secondary pathogen of HIV infections. However, further *in vivo* study is necessary to evaluate the clinical application of seaweed metabolites for *Candida* infection.

Mycotoxic activity of *Asparagopsis taxiformis* against phytopathogens

The fungal infections might be considered as the main factor influencing the health of plants at the early stages of their growth and development. Constant and broad use of synthetic pesticides is posing serious threat to the life supporting systems due to their residual toxicity (Andrea *et al.,* 2000; Harris *et al.,* 2001; Campos *et al.,* 2005). In the past two decades, a considerable amount of work has done on plant-derived compounds as environmentally safe alternatives to pesticides for plant disease control (Rice, 1984; Vyvyan, 2002). Utilization of natural products from plant origin, which retards the growth and propagation of undesirable phytopathogenic microorganisms, would be a

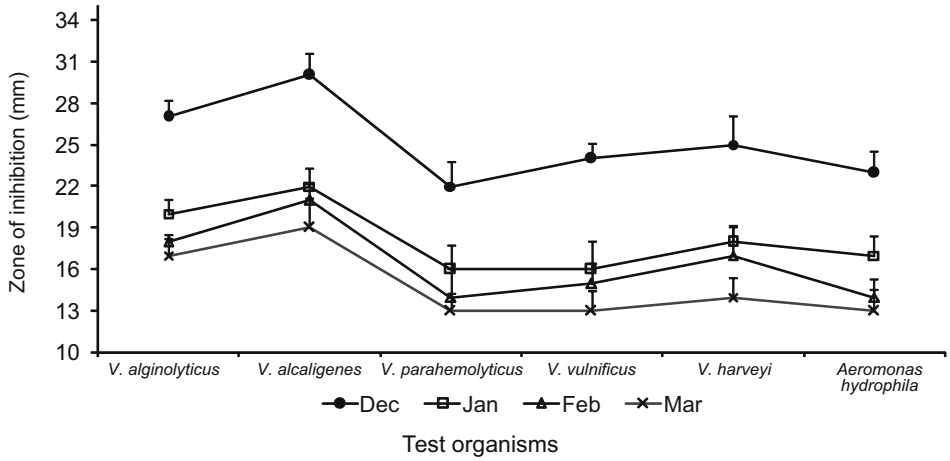

FIG. 2 - Seasonal variation of antimicrobial activity of methanolic extract of dried *Asparagopsis taxiformis* against MTCC cultures of shrimp *Vibrio* pathogens.

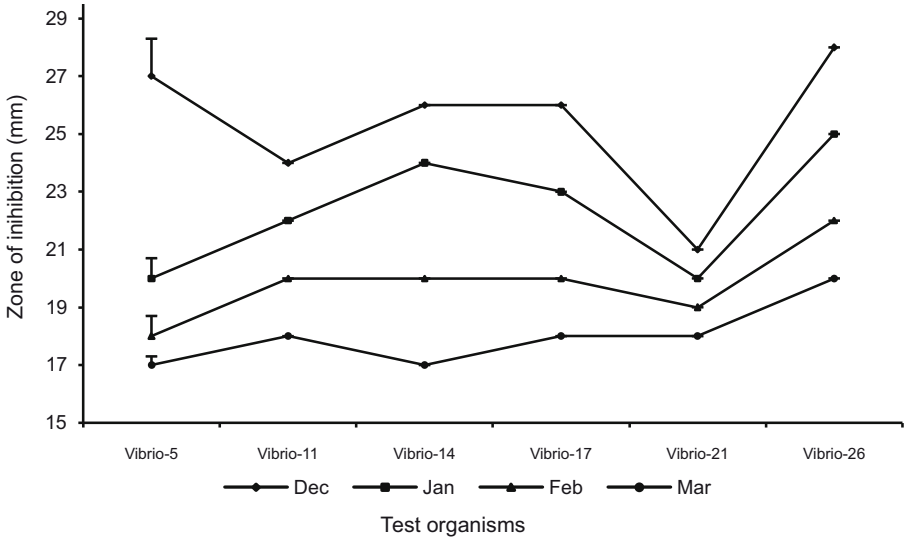

FIG. 3 - Seasonal variation of antimicrobial activity of methanolic extract of dried *Asparagopsis taxiformis* against shrimp *Vibrio* isolates. Vibrio-5: *Vibrio harveyi*, Vibrio-11: *Vibrio alginolyticus*, Vibrio-14: *Vibrio vulnificus*, Vibrio-17: *Vibrio fisheri*, Vibrio-21: *Vibrio parahaemolyticus*, Vibrio-26: *Vibrio damsela.*

more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial fungicides (Verma and Dubey, 1999; Gottlieb *et al*., 2002).

Antibacterial activity has been the most widely investigated in seaweeds, with less attention paid to antifungal activity (Stirk *et al.,* 2007). The diminishing bioactivity of natural products against fungal plant pathogens is a crisis throughout the world (Apers *et al.*, 2001). Different species of red algae have been reported to have antifungal activity (Ali *et al.,* 2000a, 2000b). The methanolic extracts of *A. taxiformis* were found to be the only seaweed which showed pronounced fungitoxicity against fungi viz *Fusarium oxysporum, Trichoderma* sp., *Aspergillus niger* and *Aspergillus flavus* which inhibit the growth of the tested fungi at 10 mg/ml of crude extract (Table 4). This study is the first report on the antimycotic activities of *A. taxiformis* from southwest of India against the phytopathogens. The active antifungal agents so far reported include acrylic acid, phlorotannins, terpenoids and steroids (Mtolera and Semesi, 1996). El-Baroty *et al.* (2007) demonstrated ethyl acetate and hexane fraction of *A. taxiformis* showed strong antifungal activity against *F. oxysporum*. As observed in the present study, the organic extracts of *A. taxiformis* from Canary Island showed marked antifungal activity against *Aspergillus fumigatus* (Val *et al.,* 2001). Our results prove that *A. taxiformis* can be used to defend plants against damages inflicted by fungal infections. The elucidation of the mode of action and interaction with microbes will be providing new tools for the management of plant pathogens.

Seasonal variation of bioactives from *Asparagopsis taxiformis*

Bioactivity of seaweeds varies with Geographical scale and seasonality (Crasta *et al.,* 1997). Albeit the samples were collected for one year from April 2007 to March 2008, the analysis of seasonal variation in antimicrobial activity of *A. taxiformis* was confined to one growth cycle (November-March). Since, the *A. taxiformis* samples were not available in the off season (April-October). The biomass of *A. taxiformis* was abundant during the winter season (December-February). Maximal activity was recorded during December to January and the activity was gradually reduced during

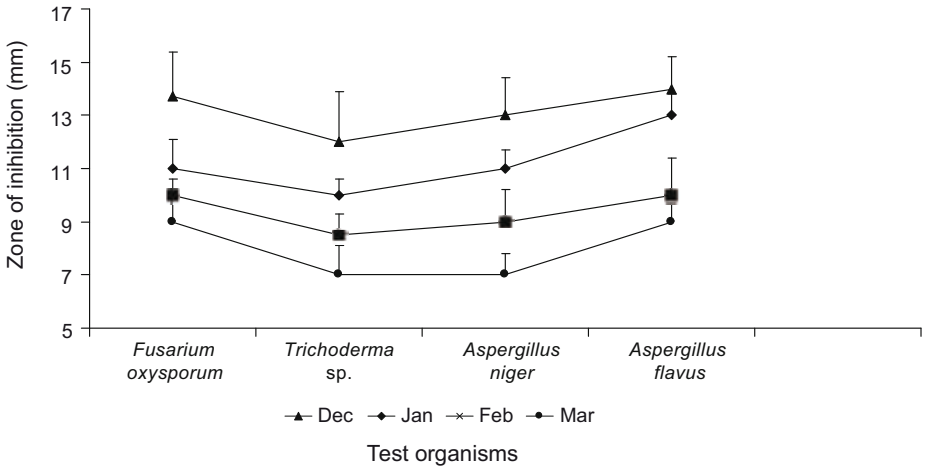

FIG. 4 - Seasonal variation of antimicrobial activity of methanolic extract of dried *Asparagopsis taxiformis* against phytopathogens.

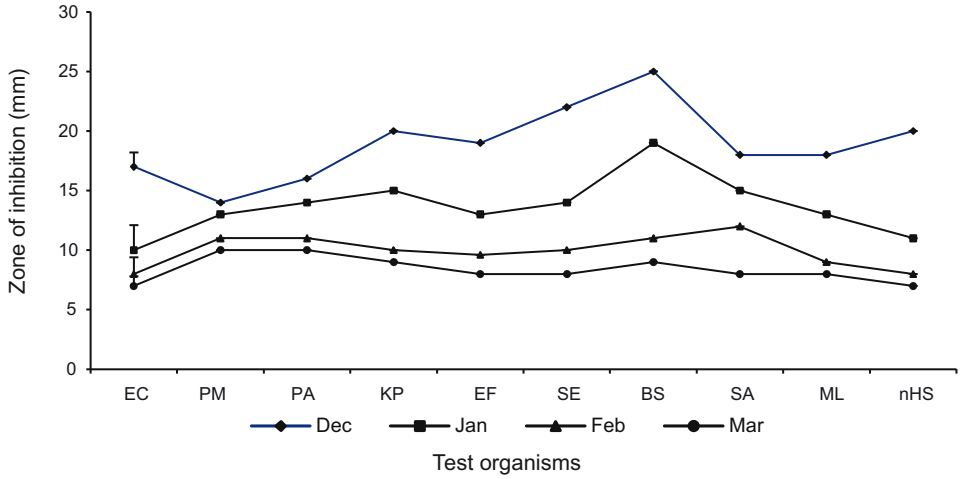

FIG. 5 - Seasonal variation of antimicrobial activity of methanolic extract of dried *Asparagopsis taxiformis against* multi drug resistant clinical pathogens. Gram negative group - EC: *Escherichia coli,* PM: *Proteus mirabilis,* PA: *Pseudomonas aeruginosa,* KP: *Klebsiella pneumoniae.* Gram positive group - EF: *Enterococcus faecalis,* SE: *Staphylococcus epidermidis,* BS: *Bacillus subtilis,* SA: *Staphylococcus aureus,* ML: *Micrococcus luteus,* nHS: non-haemolytic *Streptococcus.*

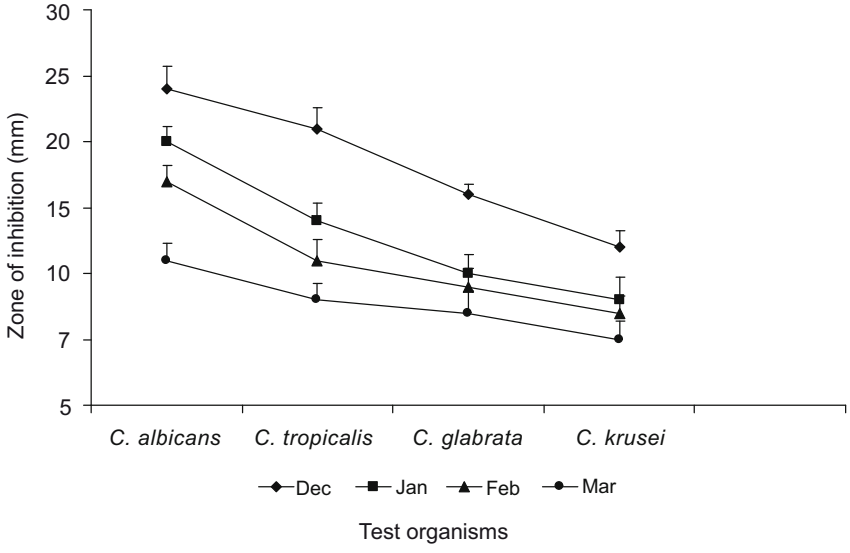

FIG. 6 - Seasonal variation of antimicrobial activity of methanolic extract of dried *Asparagopsis taxiformis* against *Candida* spp.

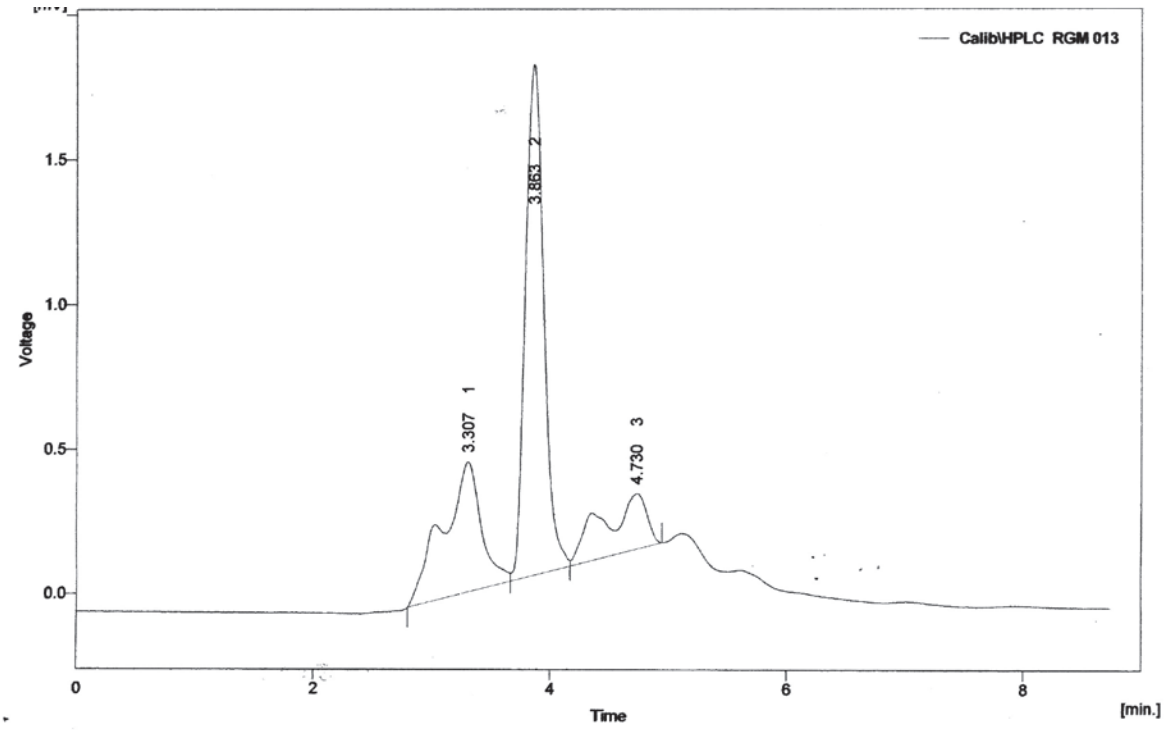

FIG. 7 - HPLC profile of active TLC fraction of *Asparagopsis taxiformis.*

February to March (Fig. 2 to Fig.6). Seasonal variation in the antimicrobial activity could be related to the fact that the content of secondary metabolite might be variable. Vlachos *et al.* (2001) detected the highest antimicrobial activity of seaweed collected during winter season. Similarly, seasonal variation in the antimicrobial activity of Indian seaweeds was reported by many authors (Rao and Parekh, 1981; Vidhyavathi and Sridhar, 1991; Arun Kumar and Rengaswamy, 2000). The present findings must be the first report evidenced *A. taxiformis* from the Southwest coast of India possesses different level of antimicrobial activity during different season. Seasonal variation in activity may be due to different quantities of a single compound, or the synthesis of different compounds due to varied growth conditions (Stirk *et al.,* 2007). Antimicrobial activity against different microbial strains may be due to a single chemical entity with a broad spectrum of activity or many different chemical entities.

Phycochemical composition of *Asparagopsis taxiformis*

Biochemical composition of *A. taxiformis* includes 23.68 mg/g of lipids, 62.82 mg/g of polysaccharide and 31.18 mg/g of protein. The water content of fresh material ranged from 87 to 91.3%. The polysaccharide content was higher when compare to protein and lipids. Normally, seaweeds are known to possess high level (50-60%) of polysaccharides and low level of lipids (Amsaki and Amsaki, 1983). The protein content of the seaweeds ranges between 4-25% of dry weight. Values of biochemical constituents of our result differed greatly in comparison to other published reports (El-Baroty *et al.,* 2007). The difference in the amount of biochemical constituents can be attributed to the influence of environmental factors.

Fractionation and purification of *Asparagopsis taxiformis* extract

The highly active extract of *A. taxiformis* was fractionated through column chromatography. The active fraction was purified using preparative TLC to obtain a single spot with *Rf* value of 0.487. The active TLC resolved spot was again purified with reverse phase HPLC at 254 nm 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 (25 °C) and three peaks with retention time (min) of 3.307, 3.863 and 4.730 respectively were attained at 254 nm (Fig. 7). The eluted HPLC peak that retained antimicrobial activity (data not shown) was chosen for GC-MS analysis.

A high resolution mass spectrum equipped with a data system in combination with gas chromatography was used for the chemical analysis of active fraction. Chemical characteristics of active fraction on the basis of spectral data by GC-MS were found to be a mixture of fatty acids with volatile compounds. The active fraction showed the presence of chlorobenzene (mol. wt 112), 14-methyl-pentadecanoic acid methyl ester (mol. wt 270), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (mol. wt 365), 9-octadecanoic acid, methyl ester (mol. wt 298), octadecanoic acid methyl ester (mol. wt 296), 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (mol. wt 167).

Based on the GC-MS results, the chemical constituents of main active fraction was extrapolated as low molecular weight lipophilic compound composed of mixture of volatile metabolites and fatty acids. The relative percentage and structure of identified compounds is summarized in Table 5 and Fig. 8. It was found that the main constituent of the purified fraction correspond to 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (56.012%), fatty acids, 14-methyl-pentadecanoic acid methyl ester (26.6%), octa-

TABLE 5 - GC-MS data of active fraction of *Asparagopsis taxiformis*

Systematic name	Retention time	Overall (%)	Fragmentation
Benzene, chloro	3.276	0.09	112 (M^+), 77
14-Methyl-pentadecanoic acid methyl ester	27.930	26.683	270 (M^+), 227, 213, 143, 129, 74
Octadec-9-enoic acid 2,3-dihydroxy-propyl ester	30.552	4.1195	265 (M^+), 211, 209, 151,111, 73, 55
9-Octadecenoic acid methyl ester	30.886	4.537	255 (M^+), 241, 209, 191, 143, 101, 55
Octadecanoic acid methyl ester	32.379	8.648	209 (M^+), 191, 151, 149, 73
4,5-Dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester	34.899	56.012	167 (M^+), 121

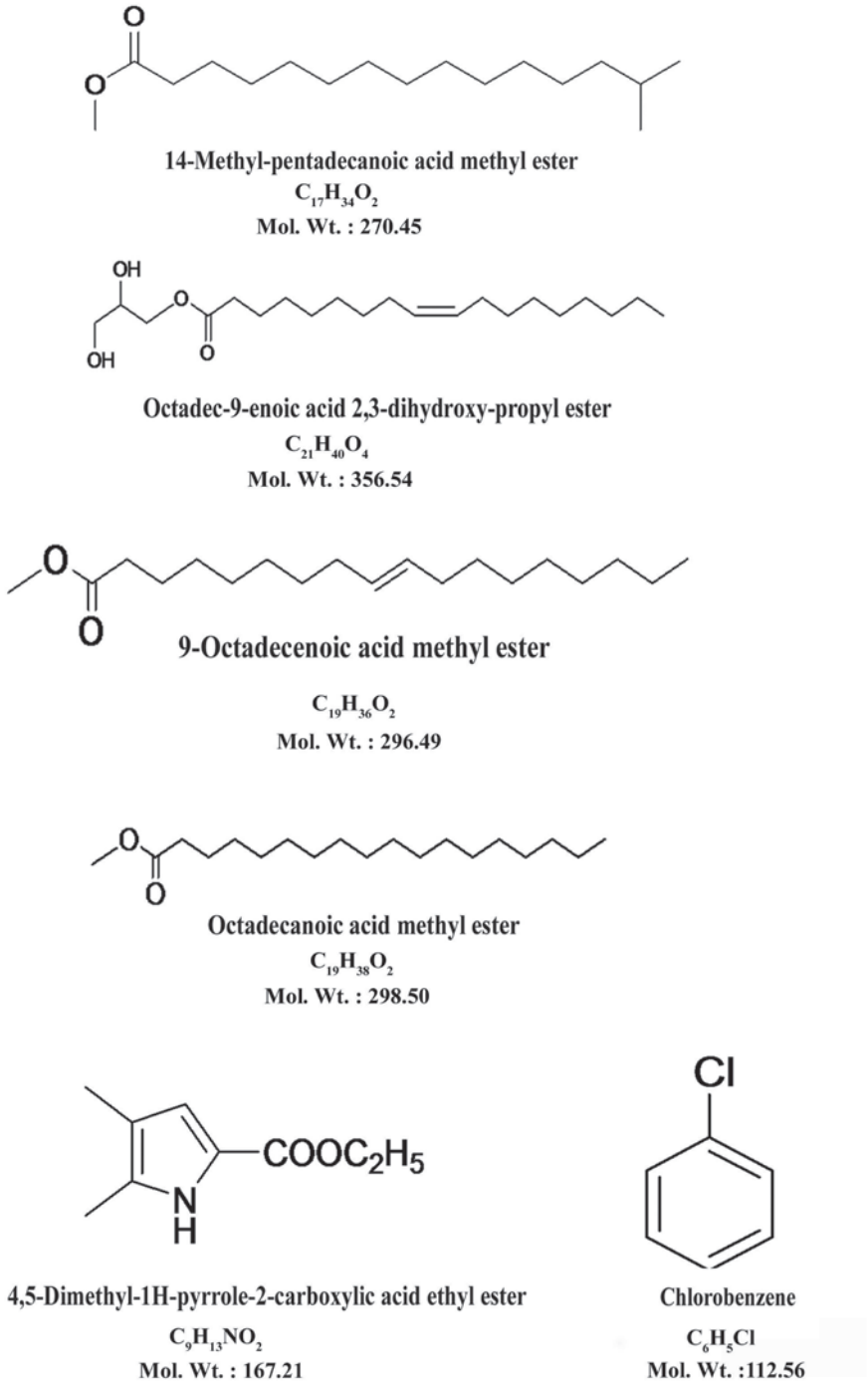

FIG. 8 - Structure of constituents identified from active fraction of *Asparagopsis taxiformis* by GC-MS analysis.

decanoic acid methyl ester (8.46%), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (4.11%), 9-octadecanoic acid, methyl ester (4.535%) and trace amount of chlorobenzene (0.09%).

Earlier studies have demonstrated the genus *Asparagopsis* as the richest bio-resource of potential secondary metabolites, especially halogenated metabolites which has been commonly occurring in this genus (Paul *et al.*, 2006; El-Baroty *et al.,* 2007). The most characteristic seaweed metabolites that have exhibited significant anti-HIV activity using various biochemical assays designed for chemotherapeutic strategies are sulphated polysaccharides extracted from seaweed, *A. taxiformis* (Haslin *et al.,* 2001). Kladi *et al.* (2004) acknowledged microbicidal property of *A. taxiformis* was due to volatile metabolites such as halomethanes, haloacetone and acrylates. Recently, many natural metabolites extracted from genus *Asparagopsis* are widely used as preservatives in cosmetics (Nash *et al.,* 2005).

Therefore, the microbicidal activity exhibited by volatile lipophilic compound may be useful in the development of alternative approaches to controlling shrimp, human and plant pathogens examined in the present study.

CONCLUSION

Red algal species were studied for the first time for antimicrobial activity from the Kollam coast (Kerala). Over 15 red algae from different order were evaluated. The most active marine algal candidate was selected for bioassay-guided isolation of active constituents. It was established that the methanolic extract of red alga *A. taxiformis* showed broadest and highest spectrum bioactivity as compared to other extracts and exhibited conspicuous peak of activity during winter season and highest extraction efficiency is achieved by using methanol as an organic solvent. The present findings revealed that activity profile of the seaweeds varied according to the different extraction methods and in the present study we found that the soaking of seaweeds in methanol was highly effective. Further fractionation and purification, of active fraction using different chromatographic system resulted in the isolation of lipophilic metabolites. It was found that this antimicrobial property was due to organic lipophilic compound composed of mainly of pyrrole-2-carboxylic acid, pentadecanoic acid and octadecanoic acid. This ultimately implicates that the red alga *A. taxiformis* represent an enormous source for natural secondary metabolites with diverse chemical structures and its activities could make it as a promising frontier for the discovery of new medicines in agriculture and pharmaceutical industry. Moreover of this compounds should be subjected to animal and human studies to ascertain their effectiveness in whole-organism systems, including in particular toxicity studies*, in vivo* data as well as an examination of their effects on beneficial normal microbiota. The dried seaweed could be used as a source of antimicrobial (100% shrimp vibriocidal activity) and polysaccharide substances (contain 62 mg/g of thallus dry weight) for formulation of natural shrimp/fish feed in aquaculture industry.

Acknowledgements

We thank Dr. Priyadharsan D. Prasad, R. Rajagopal, P. Jaya, H. Lalithambika, Dr. S. Sulekha, C. Thankachy and Dr. M. Jayakumari Professors, Department of Biotechnology, Botany and Zoology, Sree Narayana College, Kollam, Kerala for encouragements. AM and SS are gratefully acknowledged DBT for providing JRF. This paper is a part of the DBT project (BT/PR8064/AAQ/03/290/2006).

REFERENCES

Adwan K., Abu-Hasan N. (1998). Gentamicin resistance in clinical strains of Enterobacteriaceae associated with reduced gentamicin uptake. Folia Microbiol., 43: 438-440.

Alderman D.J., Michel C. (1992). Chemotherapy in Aquaculture Today. In: Michel C., Alderman D.J., Eds, Chemotherapy in Aquaculture from Theory to Reality, Office International Des Epizooties, Paris, pp. 3-4.

Ali M.S., Mazhar F., Saleem M., Jahangir M., Pervez K., Usmanghani K., Ahmad V.U. (2000a). Chemistry and biology of algae from seacoasts of Karachi. In: Ahmad V.U., Ed., Proceeding of National ONR Symposium on Arabian Sea as a Resource of Biological Diversity. HEJ Res Chem, Karachi Univ, Karachi, pp. 33-44.

Ali M.S., Jahangir M., Saleem M., Pervez M.K., Hameed S., Ahmad V.U. (2000b). Metabolites of marine algae collected from Karachi-coasts of Arabian Sea. Nat. Prod. Sci., 6: 61-65.

Amsaki S., Amsaki T. (1983). Vegetables from the Sea. Japan Publications, Inc Tokyo, pp. 2-16.

Andrea M.M., Peres T.B., Luchini L.C., Pettinelli A.Jr. (2000). Impact of long-term pesticide applications on some soil biological parameters. J. Environ. Sci. Heal., 35: 297-307.

Apers S., Baronikova S., Sindambiwe J.B., Witvrouw M., De Clercq E., Vanden Berghe D., Van Marck E., Vlietinck A., Pieters L. (2001). Antiviral, haemolytic and molluscicidal activities of triterpenoid saponins from *Maesa lanceolata*: establishment of structure-activity relationships. Planta Med., 67: 528-532.

Arun Kumar K., Rengasamy R. (2000). Evaluation of antibacterial potential of seaweeds occurring along the coast of Tamil Nadu, India against plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Dye. Bot. Mar., 43: 409-415.

Ashbee H.R., Evans E.G. (2000). Fungi and skin. Microbiol. Today, 27: 132-134.

Bansemir A., Blume M., Schröder S., Lindequist U. (2006). Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture, 252: 79-84.

Bligh E.G., Dyer W.J. (1959). A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol., 37 (10): 911-917.

Blunt J.W., Copp B.R., Hu W.P., Munro M.H.G., Northcote P.T., Prinsep M.R. (2007). Marine natural products. Nat. Prod. Rep., 24: 31-86.

Campos A., Lino C.M., Cardoso S.M., Silveira M.I.N. (2005). Organochlorine pesticide residues in European sardine, horse mackerel and Atlantic mackerel from Portugal. Food Addit. Contam., 22: 642-646.

Choudhury S., Sree A., Mukherjee S.C., Pattnaik P., Bapuji M. (2005). *In vitro* antibacterial activity of extracts of selected marine algae and mangroves against fish pathogens. Asian Fish. Sci., 18: 285-294.

Crasta J., Premila N.S., Raviraja, Sridhar K.R. (1997). Antimicrobial activity of some marine algae of Southwest Coast of India. Indian J. Mar. Sci., 26: 201-205.

de Vries D.J., Beart P.M. (1995). Fishing for drugs from the sea: status and strategies. Trends Pharmacol. Sci.,16: 275-279.

Dhargalkar V.K., Neelam P. (2005) Seaweed: Promising plant of the millennium. Sci. Cul., 71: 60-66.

Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350-3560.

El-Baroty G.S., Moussa M.Y., Shallan M.A., Ali M.A., Sabh A.Z., Shalaby E.A. (2007). Contribution to the aroma, biological activities, minerals, protein, pigments and lipid contents of the red alga: *Asparagopsis taxiformis* (Delile) Trevisan. J. Appl. Sci. Res., 3: 1825-1834.

Al-Fadhli A., Wahidulla S., D'Souza L. (2006). Glycolipids from the red alga *Chondria armata* (Kütz.) Okamura. Glycobiology, 6: 902-915.

Faulkner D.J. (2001). Marine natural products. Nat. Prod. Rep., 18: 1-49.

Febles C.I., Aris A., Gil-Rodriguez M.C., Hardisson A., Sierra Lopez A. (1995). *In vitro* study of antimicrobial activity in algae (Chlorophyta, Phaeophyta and Rhodophyta collected from the coast of Tenerife (in Spanish). Anuario del Instituto de Estudios Canarios, 34: 181-192.

Filho-Lima J.V.M., Carvalho A.F.F.U., Freitas S.M. (2002). Antibacterial activity of extracts of six macroalgae from the Northeastern Brazilian Coast. Brazil. J. Microbiol., 33: 311-313.

Fitton J.H. (2006). Antiviral properties of marine algae. In: Critchley A.T., Ohno M., Largo D.B., Eds, World Seaweed Resources. Windows and Macintosh. ETI Information Services, Workingham, UK, p. 7.

Gao D., Okuda R., Lopez-Avila V. (2001). Supercritical fluid extraction of halogenated monoterpenes from the red alga *Plocamium cartilagineum*. J. A.O.A.C. Int., 84: 1313-1331.

Gottlieb O.R., Borin M.R., Brito N.R. (2002). Integration of ethnobotany and phytochemistry: dream or reality? Phytochemistry, 60: 145-152.

Harper M.K., Bugni T.S., Copp B.R., James R.D., Lindasay B.S., Richardson A.D., Schnabel P.C., Tasdemir D., VanWagoner R.M., Verbitski S.M., Ireland C.M. (2001). Introduction to the chemical ecology of marine natural products. In: McClintock J.B., Baker B.J., Eds, Marine Chemical Ecology. CRC Bocca Raton FL, pp. 267-300.

Harris C.A., Renfrew M.J. Woolridge M.W. (2001). Assessing the risks of pesticides residues to consumers: recent and future developments. Food Addit. Contam., 18: 1124-1129.

Haslin C., Lahaye M., Pellegrini M., Chermann J.C. (2001). *In vitro* anti-HIV activity of sulfated cell-wall polysaccharides from gametic, carposporic and tetrasporic stages of the Mediterranean red alga *Asparagopsis armata*. Planta Med., 67: 301-305.

Huang X., Zhou H., Zhang H. (2006) The effect of *sargassum fusiforme* polysaccharide extracts on Vibriosis resistance and immune activity of the shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immunol., 20:750–757

Jensen A. (1993). Present and future needs for algae and algal products. Hydrobiology, 260/261: 15-23.

Ki-Bong O., Hye L.J., Soon-Chun C., Jongheon S., Jae S. H., Hye-Kyeong K., Hyi-Seung L. (2008). Antimicrobial activities of the bromophenols from the red alga *Odonthalia corymbifera* and some synthetic derivatives. Bioorg. Med. Chem. Lett., 18 (1): 104-108.

Kim J., Park E.J. (2002). Cytotoxic anticancer candidates from natural resources. Curr. Med. Chem. Anti-Canc. Agents, 2: 485-537.

Kladi M., Vagias C., Roussis V. (2004). Volatile halogenated metabolites from marine red algae. Phytochemistry Rev., 3: 337-366.

Liao W.R., Lin J.Y., Shieh W.Y., Jeng W.L., Huang R. (2003). Antibiotic activity of lectins from marine algae against marine *Vibrios*: J. Ind. Microbiol. Biotechnol., 30: 433-439.

Lowry O.H., Rosebrough N., Farr A., Randall R. (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Maliakal S., Cheney D.P., Rorrer R.L. (2001). Halogenated monoterpene production in regenerated plantlet suspension cultures of the macrophytic red alga *Octhodes secundiramea.* J. Phycol., 37: 1010-1019.

Masuda M., Abe T., Sato S. (1997). Diversity of halogenated secondary metabolites in the red alga *Laurencia nipponica* (Rhodomelaceae, Ceramiales). J. Phycol., 33: 196-208.

Michanek G. (1979). Seaweed resources for pharmaceutical uses. In: Hoppe H.A. *et al.,* Eds, Marine Algae in Pharmaceutical Science. Waiter de Gruyter, Ikrlin, New York, pp. 203-235.

Moore R.D., Chaisson R.E. (1996). Natural history of opportunistic disease in an HIV-infected urban clinical cohort. Ann Intern Med., 124 (7): 633-642

Moriarty D.J.W. (1999). Disease control in shrimp aquaculture with probiotic bacteria microbial biosystems: New frontiers. In: Bell C.R., Brylinsky M., Johnson-Green P., Eds, Proceedings of the 8th International Symposium on Microbial Ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Mtolera M.S.P., Semesi A.K. (1996). Antimicrobial activity of extracts from six green algae from Tanzania. Proc. Current Trends in Marine Botanical Research in East Africa, pp. 211-217.

Muñoz Crego A., López Cruz A. (1992). Drogas del mar. Sustancias Biomédicas de Algas Marinas. Universidad de Santiago de Compostela; Santiago de Compostela.

Munro M.H.G., Blunt J.W. (1999). Marinlit, version 10.4, Marine Chemical Group, University of Canterbury, Christchurch, New Zealand.

Nash R., Rindi F**,** Guiry M.D. (2005). Optimum conditions for the cultivation of the *Trailliella*-phase of *Bonnemaisonia hamifera* Hariot (Bonnemaisoniales, Rhodophyta), a candidate species for secondary metabolite production. *Bot. Mar.,* 48 (4): 257-265.

Norton T.A., Melkonian M., Andersen R.A. (1996). Algal biodiversity. Phycologia, 35: 308-326.

Padmakumar K., Ayyakkannu K. (1997). Seasonal variation of antibacterial and antifungal activities of the extracts of marine algae from southern coasts of India. Bot. Mar., 40: 507-515.

Paul N.A., de Nys R., Steinberg P.D. (2006). Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar. Ecol. Prog. Ser., 306: 87-101.

Primavera J.H. (1994). Shrimp farming in the Asia-Pacific: environmental and trade issues and regional cooperation. Nautilus Institute Workshop on Trade and Environment in Asia-Pacific: Prospects for Regional Cooperation East-West Center, Honolulu, pp. 23-25.

Rao S.P., Parekh K.S. (1981). Antibacterial activity of Indian seaweed extracts. Bot. Mar., 24: 577-582.

Rao P.S.P., Sreenivasa Rao P., Karmarkar S.M. (1986). Antibacterial substances from brown algae. II. Effeciency of solvent in the evaluation of antibacterial substances from *Sargassum johnstonni* Setchel et Gardner. Bot. Mar., 29: 503-507.

Regunathan C., Wesley S.G. (2004). Control of *Vibrio* spp. in shrimp hatcheries using the green algae *Tetraselmis suecica*. Asian Fish. Sci., 17: 147-157.

Rice E.L. (1984). Allelopathy, 2nd edn, Academic Press, New York, pp. 67-73.

Robles Centeno P.O., Ballantine D.L., Gerwick W.H. (1996). Dynamics of antibacterial activity in three species of Caribbean marine algae as a function of habitat and life history. Hydrobiology, 326/327: 457-462.

Salvador N., Gomez-Garreta A., Lavelli L., Ribera L. (2007). Antimicrobial activity of Iberian macroalgae. Sci. Mar., 71: 101-113.

Schmitt T.M., Hay M.E., Lindquist N. (1995). Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. Ecology, 76: 107-123.

Selvin J., Lipton A.P. (2003). Shrimp disease management using bioactive marine secondary metabolites: an ecofriendly approach. Naga, 26 (1): 11-13.

Selvin J., Lipton A.P. (2004). Biopotentials of *Ulva fasciata* and *Hypnea musiformis* collected from the peninsular coast of India. J. Mar. Sci. Technol., 12: 1-6.

Stirk W.A., Diana L., Staden R.J.V. (2007). Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. J. Appl. Phycol., 19: 271-276.

Takaki-Campos G.M., Diu M.B.S., Koening M.L., Peretra E.C. (1988). Screening of marine algae from the Brazilian northeastern coast for antimicrobial activity. Bot. Mar., 31: 375-377.

Val A.G. del., Platas G., Basilio A., Cabello A., Suay J.G.I., Vicente F., Portillo E., Rio M.J. del., Reina G.G., Pelaez F. (2001). Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). Int .Microbiol., 4: 35-40.

Verma J., Dubey N.K. (1999). Prospectives of botanical and microbial products as pesticides of tomorrow. Curr. Sci. India, 76: 172-179.

Vidyavathi N., Sridhar K.R. (1991). Seasonal and geographical variation in the antimicrobial activity of seaweeds from Mangalore coast in India. Bot. Mar., 34: 279-284.

Vlachos V., Critchley A.T., Holy von A. (2001). Effect of post collection storage time and season on the antibacterial activity of selected southern African marine macroalgae. In: Chen F., Jiang Y., Eds, Algae and their Biotechnological Potential. Kluwer Academic Publishers, The Netherlands, pp. 207-213.

Vyvyan J.R. (2002). Allelochemicals as leads for new herbicides and agrochemicals. Tetrahedron, 58: 1631-1646.

Williams D.H., Stone M.J., Hauch P.R., Rahman S.K. (1989). Why are secondary metabolites (natural products) biosynthesized? J. Nat. Prod., 52: 1189-1208.

Williams D.H., Maplestone R.A. (1992). Why are secondary metabolites synthesized? Sophistication in the inhibition of cell wall biosynthesis by vancomycin group antibiotics. In Chadwick D.J., Whelan J., Eds, Secondary Metabolites: Their Function and Evolutions. Ciba Foundation, pp. 45-63.

Yamamoto I., Takahashi M., Tamura E., Maruyama H., Mori H. (1984). Antitumor activity of edible marine algae: Effect of crude fucoidan fractions prepared from edible brown seaweeds against L-1210 leukemia. Hydrobiology, 116/117: 145-148.

Yavasoglu K., Ulku N., Atakan S., Guven O., Zerrin H. (2007). Antimicrobial activity of volatile components and various extracts of the red alga *Jania rubens*. Phytother. Res., 21: 153-156.