

***In Vitro* Antibacterial Activity of Extracts of Selected Marine Algae and Mangroves against Fish Pathogens**

S. CHOUDHURY¹, A. SREE^{1*}, S.C. MUKHERJEE³,
P. PATTNAIK² and M. BAPUJI¹

¹ Forest & Marine Products Division,
Regional Research Laboratory, Bhubaneswar, India.

² Aquatic Animal Health Division, C.I.F.A.,
Bhubaneswar, India.

³ Central Institution of Fisheries Education,
Versova, Andheri West, Mumbai, India.

* Corresponding author

Phone: 91-674-2581635, 36 Ext.308 (O), 2581957 ®
Fax:91-674-2581637
E-mail: asree@rrlbhu.res.in

Abstract

In vitro screening of organic solvent extracts of three marine algae viz., *Gracilaria corticata*, *Ulva fasciata* and *Enteromorpha compressa* and five mangroves viz., *Aegiceras corniculatum*, *Aegialitis rotundifolia*, *Aglaia cucullata*, *Cynometra iripa* and *Xylocarpus granatum* showed species specific activity in inhibiting the growth of six virulent strains of bacteria pathogenic to fish viz., *Edwardsiella tarda*, *Vibrio alginolyticus*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (2 strains). Three methanol extracts of *C. iripa* were active against all the six pathogens, whereas *A. corniculatum* and *A. cucullata* were active against four of the pathogens. The chromatographic fractionation of active extracts of *A. corniculatum*, *C. iripa* and *G. corticata* resulted in enriched fractions with wide spectrum activity and lowered values of minimum inhibitory concentration.

Introduction

Bacterial diseases are responsible for heavy mortality in wild and cultured fish. The problems in the farms are usually tackled by preventing disease outbreaks or by treating the actual disease with drugs or chemicals. The use of antimicrobial agents has increased significantly in aquaculture practices (Alderman and Michel 1992). Antibiotics used in both human as well as veterinary medicines have been tried experimentally to treat bacterial infections of fish. Problems including solubility, palatability, toxicity, cost, delivery and governmental restrictions have limited the available antibiotics to a select few, especially in food fish culture. Decreased efficacy and resistance of pathogens to antibiotics has necessitated development of new alternatives (Smith et al. 1994).

Many bioactive and pharmacologically active substances have been isolated from algae. For instance, extracts of marine algae were reported to exhibit antibacterial activity (Siddhanta et al. 1997, Mahasneh et al. 1995, Sachithanathan and Sivapalan 1975). Many authors had found antibacterial activities of microalgae due to fatty acids (Cooper et al. 1983; Findlay and Patil, 1984; Viso et al. 1987; Kellam et al. 1988). Changyi et al. (1997) opined that the fatty acids (PUFA) in litter fall of mangroves might have positive role on the growth of fishes and shrimps.

Scanty literature is available on the antibacterial activity of mangroves. However, studies of other biological activities in general are available. The study of Premnathan et al. (1992, 1996) revealed that the mangroves were found highly effective for antiviral activity as compared to seaweeds and sea grasses. Kokpal et al. (1990) had also reported the bioactive compounds from mangrove plants. Some mangroves had shown insecticidal activity (Miki et al. 1994, Ishibashi et al. 1993). Wu et al. (1997) reported the cytotoxic and antiplatelet aggregation activity of methanol extract of *Aglaia elliptifolia*.

Srinivasa Rao and Parekh (1981) showed that crude extracts of Indian seaweeds were active only against Gram positive bacteria. Ethanol extracts from 56 Southern African seaweeds from the divisions Chlorophyta (green), Phaeophyta (brown) and Rhodophyta (red) scored highest antibacterial activity for Phaeophyta (Vlachos et al. 1997). Similar results were reported by Caccamese and Azzolina (1979) and Pesando and Caram (1984) for screening studies on seaweeds of Mediterranean and Eastern Sicily coast respectively.

Though literature speaks diverse studies of bioactivity of marine flora, our work on testing the antibacterial efficacies of these on fish pathogens was comparatively a new concept and not much attempt had been made earlier in this line.

In previous studies, we evaluated the efficacy of sponges and coelenterates (Choudhury et al. 2002 and 2003) for their antibacterial activity against bacteria

pathogenic to fish. In this study, some marine algae and mangrove plant extracts were tested wherein we report their efficacy against six bacteria pathogenic to fish.

Materials and Methods

Marine algae were collected by hand from the submerged marine rocks of Kalingapatnam and Erramukkam, Srikakulam district of Andhra Pradesh state, India in low tide. Epiphytic and extraneous matter were removed by washing first in sea water and then in fresh water. The algae were transported to the laboratory in polyethylene bags at ice temperature. Specimens were preserved in 5% formalin. Mangroves were collected from Bhitarkanika wild life sanctuary of Orissa, India (during late winter season). Both algal and mangrove samples were identified by experts in the respective fields.

The samples (algae/respective mangrove plant parts) were shade dried, cut into small pieces and powdered in a mixer grinder. The extraction was carried out with different solvents in the increasing order of polarity, namely: hexane, chloroform, ethyl acetate, chloroform: alcohol (1:1) and methanol by soaking the material in the respective solvents thrice overnight at room temperature (1:3 v/v). The extraction with different solvents was carried out individually on samples. The extracts from three consecutive soakings were pooled and freed from solvent by evaporation under reduced pressure. The residues (crude extracts) obtained were finally dried under vacuum and were tested for their inhibitory effects on six species of fish pathogenic bacteria isolated from fresh water environment namely; *Vibrio alginolyticus* (VA), *Pseudomonas aeruginosa* (PA), *Aeromonas hydrophila* (strain-1, AHI), *Edwardsiella tarda* (ET), *Pseudomonas fluorescens* (PF) and *Aeromonas hydrophila* (strain-2, AHII). The test bacterial pathogen cultures were obtained from the stock cultures maintained in the Pathology Laboratory of Central Institute of Fresh Water Aquaculture, ICAR, Bhubaneswar (Vimala et al. 2000).

The antibacterial assays were done by disc-assay method (Casida 1986). In short, 500 µg of each extract dissolved in appropriate solvent (50 µl) was applied to sterile filter paper discs (6 mm). After allowing the solvent to evaporate, the discs were placed on nutrient agar test plates (Himedia, India) inoculated with 18 h culture of the test pathogen (10^6 bacteria /ml) in Brain Heart Infusion (BHI) broth. A disc loaded with only solvent was similarly prepared as a control. The plates were incubated overnight at 37°C. The zone of inhibition of bacteria around the disc was measured and the assay was scored positive (+) if it was < 2 mm, doubly positive (++) if the zone was ≥ 2 mm, triple positive (+++) if the zone of inhibition was ≥ 7 mm, and negative (-ve) if there was no inhibition of microbial growth (Thompson et al. 1985). The bacterial assay results were compared with those obtained when

challenging the bacterial pathogens with commercial antibiotics i.e., discs containing gentamicin (10 µg), streptomycin (10 µg) and polymyxin B (300 IU).

Selected active crude extracts (2g) were fractionated by column chromatography on silica gel (Acme, Mumbai, 100-200 mesh). Column (2 cm × 40 cm) was set up in n-hexane with silica gel (30-40 g). Based on the thin layer chromatography (TLC) study of the crude extracts, the order of solvent of elution in column chromatography was fixed as hexane, hexane:ethyl acetate (9:1), hexane:ethyl acetate (1:1) and finally acetone. The column fractions (100 ml each) were evaporated under vacuum and analysed by TLC. Fractions of similar TLC profile were combined to get the final fractions, which were freed from solvent, re-dissolved in appropriate solvent after weighing and screened for activity by disc diffusion method as described above (100 µg/ 20-50µl solvent/ 6mm disc). Determination of minimum inhibitory concentration (MIC) of the active crude extracts and of fractions was carried out in the manner previously described.

Results

In the present pilot screening of 5 algae and 6 mangroves, 3 extracts from 3 marine algae and 14 extracts from five mangrove species were found to show species specific activity against the six pathogens. The details of activity of different extracts of marine algae / mangrove plant parts along with activity profile with standard commercial antibiotics are presented in table 1.

The chloroform, methanol and ethyl acetate extracts of *C. iripa* (Leaf), *A. corniculatum* (Fruit) and *A. rotundifolia* (Leaf) exhibited activities quite comparable with the commercial antibiotic standards. *P. aeruginosa* (PA), *E. tarda* (ET) and *A. hydrophila* II (AHII) were not sensitive to any of the algal species. Three extracts of *C. iripa* (Me, EtA and Clf:Me (1:1)) were active against all the six pathogens tested. Extracts of *G. corticata* showed activity against only *P. fluorescens* (PF) and *V. alginolyticus* (VA). None of the extracts from two algae (*Amphiroa fragillissima* and *Cladophora* sp.) and one mangrove (*Xylocarpus moluccensis*) showed any activity.

Fractionation of four selected active extracts by column chromatography was carried out to yield various fractions. The results of antibacterial screening of fractions showed better efficacies of fractions compared to extracts. The MICs of the fractions compared to extracts are shown in figure 1 whereas the relative sensitivity of pathogens to algae and mangrove species is presented in figure 2. The MIC values of extracts showed a range of 37.5 to 400 µg with *C. iripa* showing the lowest MIC (37.5 µg), whereas the fractions exhibited 30-100 µg with better zones of inhibition (data not shown).

The fractions from *A. corniculatum*, ACF-1 and ACF-2 (eluted with hexane:

ethyl acetate 9:1) showed increased activity in comparison to extracts against all the pathogens (Fig. 1a).

Comparative data of MICs of extract and fractions of *C. iripa* is shown in (Fig. 1b). Three fractions (CIF2, 3 and 4 eluted with hexane:ethyl acetate 9:1) of *C. iripa* Me extract showed activity against all the six pathogens in the order CIF-4> CIF-3> CIF-2. More polar fractions (CIF-5 and CIF-6 eluted with hexane:ethyl acetate 1:1 and acetone respectively) showed less activity. The lowest MIC value (30 µg) was obtained with *C. iripa*, CRF-4 fraction.

In the case of *G. corticata* activity was more concentrated in the non-polar and moderately polar fractions (GCF-1 (eluted with hexane) and GCF-3 (eluted with hexane:ethyl acetate 1:1), which showed activity against all the six pathogens tested (Fig. 1c).

In the case of *A. rotundifolia* antibacterial activity was found in the polar fraction ARF-4 eluted with acetone. Fractions were not active against PF and AHII, whereas the extract had shown activity against PF and AHII.

Most of the fractions showed enhanced spectrum of activity showing the enrichment of specific active metabolites during the process. Eight out of 19 fractions of marine flora showed activity against all the six test pathogens. The overall study of the activity of fractions clearly showed enrichment of the active metabolites and their moderately polar nature.

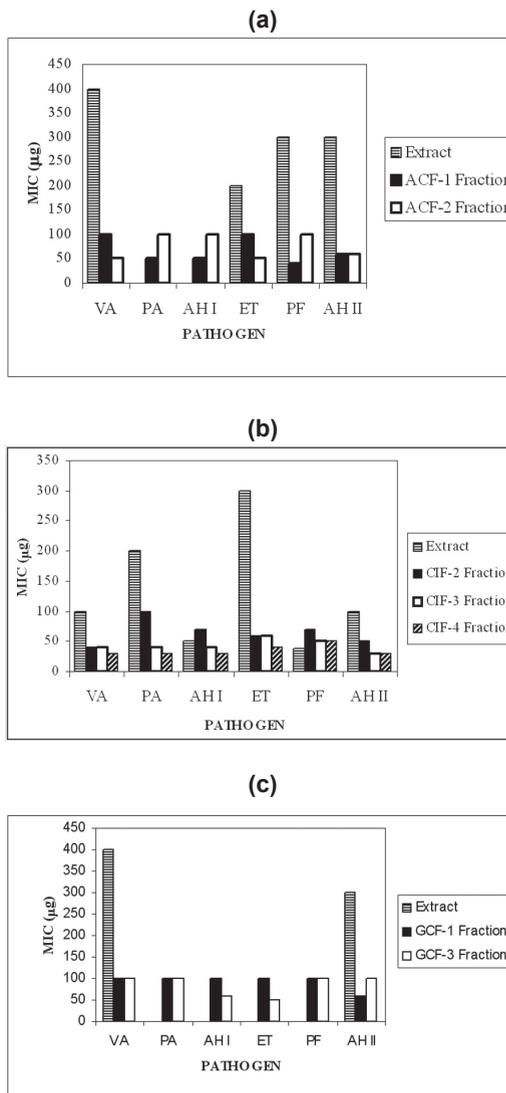


Fig. 1: MIC values of extract vs fractions of (a) *Aegiceras corniculatum*, (b) *Cynometra iripa* and (c) *Gracilaria corticata*

Table 1. Antibacterial activity of crude extracts of mangroves and algae

Name of organism (part) / commercial antibiotics	Solvent* of extraction	Activity against pathogen					
		VA	PA	AHI	ET	PF	AHII
<u>Mangroves</u> Aegiceras corniculatum (Fruit)	Me	++	+	-	-	+	+
Xylocarpus granatum (Stem) (Cuticle) (Leaf)	Me	-	-	-	+	-	-
	Me	-	-	-	+	-	-
	Hex	+	-	-	-	-	-
Aegialitis rotundifolia (Leaf)	Me	-	-	-	-	++	+
Aglaia cucullata (Leaf)	Me	+	+	-	++	-	+
	Cif:Me	-	+	-	+	-	-
	Cif	+	-	-	-	-	-
	Hex	-	-	-	+	-	+
Cynometra iripa (Leaf)	Me	++	++	++	++	++	++
	EtA	+	+	++	++	++	++
	Cif	++	-	+	+	+	++
	Cif:Me	+	+	+	+	+	++
	Hex	-	-	-	-	-	-
<u>Algae</u> Gracilaria corticata	Me	+	-	-	-	++	-
Enteromorpha compressa	Me	-	-	-	+	-	-
Ulva fasciata	Me	-	-	-	-	+	-
<u>Commercial Antibiotics</u> Gentamicin (10 µg)		+++	+++	+++	+++	+++	+++
Polymyxin B (300IU)		+	++	++	+	++	++
Streptomycin (10 µg)		++	++	+++	+++	+++	+++

* Hex = Hexane, Cif = Chloroform, EtA = Ethyl acetate, Me = Methanol

Discussion

Aglaia odorata (Ishibashi et al. 1993) and *Aglaia elliptifolia* (Wu et al. 1997) exhibited insecticidal and cytotoxic activities respectively. In our study of *A. cucullata* (Leaf) Me extract was highly active (Table 1) against *V. alginolyticus* (VA), *P. aeruginosa* (PA), *E. tarda* (ET) and *A. hydrophila* II (AH II). Thus, we can conclude that various species of *Aglaia* had varied bioactive potentialities.

Hornsey and Hide (1974) tested 151 species of British marine algae and found that, although antibacterial activity was more evident in some taxonomic

groups, it also varied seasonally. They found no activity was marked by *Gracilaria* sp., *Enteromorpha* sp. and *Cladophora dalmatica*. But, in our case the alcohol extract of *G. corticata*, *E. compressa* and *U. fasciata* showed moderate antibacterial activity (Table 1).

Our results clearly showed that the Clf:Me (1:1) and Me solvent systems were efficient in extracting the active compounds. The antibacterial activity found in three hexane extracts showed the success of the non-polar hydrophobic extracts independent of diffusion parameters in the assay method employed.

Padmakumar and Ayyakkannu (1986) reported toluene-methanol (1:3) extracts of species belonging to Rhodophyceae exhibited broad-spectrum activity when compared to Chlorophyceae and Phaeophyceae. Vidyavathi and Sridhar (1991) reported chloroform-methanol extract of fully grown *G. corticata* showed maximum activity against *S. aureus* compared to medium and young stages of growth. Srinivasa Rao and Parekh (1981) analysed *Enteromorpha intestinalis* and *G. corticata* collected from Gujarat coast of India for antibacterial activity and found that the algae were active throughout the year with a peak during the winter season.

Acetone and ethanol extracts of marine algae *Cladophora fascicularis*, *Caulerpa taxifolia*, *Chaetomorpha antennina*, *Ulva lactuca* and *G. corticata* collected from south-west coast of India in three seasons showed good inhibitory activity against *Bacillus subtilis*. The results differ from the findings of (Crasta et al. 1997) who had recorded significantly different inhibitory activity from season to season.

Our results also showed that the sensitivity of pathogens is more to mangrove extracts compared to algal extracts (Fig. 2) with *V. alginolyticus* and *A. hydrophila* II showing maximum sensitivity. *P. fluorescens* was moderately sensitive to the algal extracts. In their studies with algae, Padmakumar and Ayyakkannu (1997) found that *S. aureus* was the most susceptible bacterial pathogen followed by *Vibrio* sp. whereas *P. aeruginosa* was most resistant. Our findings fall in line with these observations.

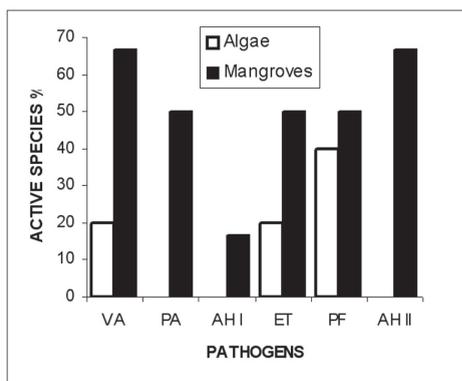


Fig. 2: Sensitivity of test pathogens vs percentage of active species of algae and mangroves

In our case, some of the bacterial strains did not respond to extracts, whereas the purified fractions showed broad-spectrum activity against multiple strains. This might be due to masking of antibacterial activity by the presence of some inhibitory compounds or factors in the extract as observed by Sastry and Rao (1994). The variation of antibacterial activity of our extracts might be due to distribution of antimicrobial substances, which varied from species to species as suggested by Lustigman and

Brown (1991). Similar observations were made by Vlachos et al. (1997) who found that fractionation of crude extracts tested enhanced their activity against both Gram negative as well as the resistant Gram positive pathogens.

Enrichment of the antibacterial activity of lipid extracts of marine algae *Laurencia obtusa* during fractionation process guided by TLC profiles has been obtained (Caccamese et al. 1981), which tallied with our results where *G. corticata* fractions were enriched with antibacterial activities in hexane eluent.

Sastry and Rao (1994) found the benzene extract of *G. corticata* showed antibacterial activity only against *Salmonella typhi* and *Escherichia coli* whereas the methanol and chloroform extracts had activity against *P. aeruginosa*. In our studies, fractions of methanol (Me) extract of *G. corticata* had shown good activity against *P. aeruginosa*.

Conclusion

Overall, the present study provides enough data to show the potential of mangrove and algae extracts for development of anti-pathogenic agents for use in aquaculture. Ecologically, there is likelihood that the secondary metabolites / PUFAs produced by the different marine flora may have a role in fish growth and protection from diseases. The renewable / cultivable nature of marine flora is another advantage for development of potential antibacterial products for use in feed or by other means administration in aquaculture. Economically feasible standard operating procedures can be developed in preparing the extracts/fractions in large scale with reproducible antibacterial efficiency.

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