Journal of Research in Biology

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Original Research Paper

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Biorestraining potentials of marine macroalgae collected from Rameshwaram, Tamil nadu

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ABSTRACT:

The marine ecosystem is the treasure place for many natural resources. In this study, about five different marine macroalgae were chosen to study the antibacterial activity and larvicidal activity of *Aedes sp.* About five different stranded strains of bacteria have been selected to detect the antibacterial activity of collected algae. Among the five algae collected, *Gracilaria crassa* and *Hypnea valentia* have shown maximum antibacterial activity in methanolic extraction by antibiosis of Kirby-Bauers method. The active biocompound from methanol was determined by using different solvent systems in TLC. The partially purified antibacterial compound was identified as saponins by phytochemical tests. Larvicidal bioassay was carried out with the two algae *Gracilaria crassa* and *Hypnea valentia*. The LC50 determined by the *Gracilaria crassa* and *Hypnea valentia* was noted as 52.2 and 53.4 respectively.

Keywords:
Larvicidal bioassay, LC50, antibiosis.

Web Address:
http://jresearchbiology.com/Documents/RA0100.pdf.

Article Citation:
Anandhan S and Sorna kumari H.
Biorestraining potentials of marine macroalgae ollected from Rameshwaram, Tamil nadu.
Journal of research in Biology (2011) 5: 385-392

Dates:
Received: 09 Sep 2011 /**Accepted:** 16 Sep 2011 /**Published:** 23 Sep 2011

INTRODUCTION

Man relied on natural products in general and plants in particular to promote and maintain good health and fight sickness, pain and disease since in time immemorial. India is an important country in world where ambient system of medicine such as ayurveda, siddha and unani has been in practice for many years. In common all the above mentioned system of medicine are directly dependent upon resources such as plants with the advances in experimental methods in phytochemistry and pharmacology, several medicinal plants were screened for active principles and biological activites (Prashant Kumar *et al*., 2006). Marine environment is a rich source of biological and chemical diversity. The diversity has been a unique source of chemical compounds of potential for pharmaceuticals, cosmetics, dietary supplements and agrochemicals (Chau Van Minh *et al*., 2005). In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms.

(Narisnh *et al*., 2005).Marine algae were also reported to have some antioxidant properties (Faten *et al*., 2009) Commercially available varieties of marine macro algae are commonly referred to as seaweeds. Seaweeds have some of valuable medicinal value compounds such as antibiotics, laxatives, anticoagulants, antiulcer products and suspending agents in radiological preparation (Rajasulochana *et al*., 2009).Fresh and dry seaweeds are extensively consumed by people especially living in coastal areas. Seaweeds are classified as rhodophyta (red algae) or phaeophyta (brown algae) or chlorophyta (green algae) depending on their nutrient and chemical composition (Cox *et al* ., 2010).As a consequence of an increasing demand in screening for new therapeutic drugs from natural products, there is a greater interest towards marine organisms. Several marine organisms produce bioactive metabolites in response to ecological pressure such as competition for space maintenance of unfolded surface deterrence of predation and the ability to successfully reproduce (Fangming Kong *et al*., 1994). The antibacterial agent found in the algae include terpenoid, phlorotannins, acryl acid, phenolic compounds, steroid, halogenated ketone and alkaline, cylic polysulphides and fatty acid. In a number of marine algae antimicrobial activites are attributed to the presence of acryl acid (James *et al*., 1975). Seaweeds provide a rich source of structurally diverse secondary metabolites. The secondary metabolites offer a defense against herbivores, fouling organism and pathogens. They also play a role in reproduction, protection from UV radiation and as allelopathic agent. There is an urgent need to search for alternatives to synthetic antibiotics. The revalution of the discovery of new groups antimicrobial peptides make natural antibiotics the basic elements of novel generation drugs for the treatment of bacterial and fungal infection. Marine algae have become recognized as potential source of antibiotic substances (Zheng Yi *et al*., 2001). The antibacterial activity of six marine algae belonging Rhodophyceae and *phaeophyceae* were studied against pathogenic microbes (*Staphylococcus aureus*, *E.coli*, *Micrococcus*, *Enterobacter aerogens*, *Enterococcus faecalis* were studied (Taskin *et al*., 2007). Mosquito transmits serious human diseases like Malaria, Filariasis, Japanese encephalitis, dengue, hemorrhagic fever and yellow fever causing millions of death every year. Extensive use of chemical insecticides for control of vector borne diseases has created problems related to physiological resistance to vectors, adverse environmental effects, high operational cost and community acceptance, numerous plant products have been reported either as insecticides for killing larva or adult mosquit oes or as repellent for mosquito biting and are one the best alternatives for mosquito control (Rajkumar *et al*., 2009). The revolution therapy of infection disease by the use of antibacterial drugs has certain limitation due to changing pattern of resistance in pathogens and side effects they produced. These limitation demands for search of new antimicrobial compounds for development of drugs. Seaweeds are used alternative and traditional remedies in many parts of the world.

The production on inhibitor substance by seaweeds has antibacterial actions and some of their substances have potential use in mosquito control (Nagi *et al*., 2010).Use of synthetic insecticides to control vector mosquitoes has caused physiological resistance and adverse environmental effects in addition to high operational cost (Virendra *et al*., 2009). The treatment of this disease becoming more difficult since *Plasmodium falciparum* parasite has developed resistance to wide range of anti malarial drugs (Anne Platt Mc Ginn *et al*., 2002). Secondary metabolites of many marine algae species isolated from Canaria Island were shown to have a wide antimicrobial activity (Antonio Gonzalez *et al.,* 2001). Thus the screening of marine organism for a

variety of biological activities with in aim identifying novel with interesting and potentially therapeutic activities continues till this date. (Sreenivasan Sasidharan *et al*., 2010).

MATERIALS AND METHODS

Collection of algae samples

Marine algae samples were collected from the coastal region of Rameshwaram. Algae were washed with sea water to remove extraneous materials and brought to the laboratory in plastic bag containing sea water to prevent evaporation.

Sample preparation

After collection of sample, it was brought to the laboratory. Algal samples were washed in running tap water to remove any associated debris and then with the distilled water. After washing, the samples were dried in a blotting paper for two weeks. After drying, the samples was grinded in to powder form, which was then stored in 4ºC for further studies. (Rajasulochana *et al*., 2009).

Preparation of different solvent extracts

One gram of each algal sample was extracted with different solvents systems 10ml of methanol, Hexane, and ethyl acetate in a beaker for 24 hours at room temperature. Then the solvent portion was centrifuge at 5000rpm for 10minutes. The supernatant was collected from the centrifuge tube and the solvent were evaporated. Finally crude extract was obtained. The extracts were collected in plastic vials and stored in the refrigerator for further studies. (Aseer *et al*., 2009)

Disc preparation

The solvent extracts were used for disc diffusion assay to test for antibacterial activity. The discs were prepared by using Whatman filter paper approximately 5mm in diameter and then it was soaked in extracts and then dried. Then the discs were stored and used for further works.

Antibacterial assay

The stranded strains of *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *E.coli* cultures were collected from the Department of Microbiology, Sri Sankara Arts and Science College, Enathur, Kanchipuram. The antibacterial activity of algae extracts were studied by disc diffusion methods by using Muller Hinton Agar (MHA). 18hours old broth culture was prepared and inoculated on Muller Hinton agar plates by using sterile cotton swab. After the swabbing, 20µl of crude extract disc were added in to sterile filter paper disc approximately 5mm in diameter and allowed at room temperature for few minutes. After that the disc were placed on the Muller Hinton agar plates by using sterile forceps. All the plates were incubated at 37ºC for 24 hours. After incubation of 24 hours at 37°C a clean zone around the disc was evidence of antimicrobial activity (Rajasulochana *et al*., 2009)

Partial purification of selected algae extract

Based on the antibacterial activity of potential algae extracts further investigations like partial purification by thin layer chromatography were done.

Partial purification of crude extract by thin layer chromatography (TLC)

Analytical TLC

The crude extract was purified by using thin layer chromatography. For determining the best solvent system for good separation of crude compound, solvents such as methanol, chloroform, n-hexane, diethyl ether, n-butanol, ethyl acetate and acetic acid were used in the following ratio. Butanol:Aceticacid:water [30:45:25,45:30:25,50:35:15],hexane:ethylacetate [90:10,80:20,70:30,60:40,50:50], hexane:diethylether:aceticacid [80:10:10,70:20:10,60:30:10], hexane:methanol [70:30,60:40,50:50], meth anol:ethylacetate:hexane [70:20:10,60:30:10,75:20:5].The crude extract was dissolved in 200µl of methanol. With the help of capillary tube the sample was spotted on the silica gel coated slide and placed in the developing chamber which contain solvent mobile phase, covered with the watch glass in order to prevent the evaporation of solvents. The solvent was allowed to run till it reaches about half a cm below the top of the plate. After running the slide was kept in room temperature for the complete drying of the plate. Then the slide was kept closed in iodine chamber to visualize the separated compound as clear spots (Jebakumar Solomon *et al*., 2008). The RF value were determine by RF=Movement of the solute from the origin /movement of solvent from the origin.

Preparative TLC

Preparative TLC was performed to get partially purified compound.TLC plate was prepared by spreading the slurry of silica gel evenly on the plate. The plate was activated at 100ºC for 15 minutes. Crude extract was applied on the plate as a single line and the chromatogram was performed with the solvent system Hexane:

ethylacetate solvent system (50:50).After separation, the active spot band was scrapped, mixed with methanol and centrifuged at 3000rpm for 15 minutes. Supernatant was collected in a pre weighed vial and kept for evaporation. The partially purified compound obtained from preparative TLC was tested for antibacterial activity against stranded organisms by disc diffusion method.

Antimicrobial Activity of TLC fractions

After the swabbing the stranded organism, 20µl of TLC extracts disc were added on to the sterile filter paper disc approximately 5mm in diameter and allowed at room temperature for few minutes. After that the disc were placed on the Muller Hinton agar plates by using sterile forceps. All the plates were incubated at 37ºC for 24 hours. After incubation of 24 hours at 37ºC a clean zone around the disc was evidence of antimicrobial activity.

Phytochemical test

The partially purified compound obtained from preparative TLC was subjected to qualitative phytochemical analysis for the identification of compound present in the purified extract. All the tests were carried out using standard methods (Aliyu *et al*., 2008).

Larvicidal bioassay

Mosquito larva was collected from ditch area near Enathur, in Kanchipuram district and it was examined by experts for the confirmation of mosquito larva of *Aedes sp*. The algal extracts of *Gracilaria crassa* and *Hypnea valentia* were volumetrically diluted to obtain the test concentrations of 25, 50 and 75 mg/10ml. Control was set up by 1 ml of methanol in 10 ml of water. Twenty five late third instar larvae were introduced to each of the test concentration as well as control. The larval mortality was recorded after 24 h of exposure, during which no food was given to the larvae. The lethal concentrations (LC50) were calculated by probit analysis (Rajkumar *et al*., 2009).

RESULTS AND DISCUSSION

Screening of algae extract for antibacterial activity

The macroalgae from the Moroccan coast are potential sources of bioactive compounds for investigating natural antibiotics(Chiheb *et al*., 2009).Different extracts of the brown algae *Sargassum cinereum* have different anti bioactive properties such as antibacterial and antifungal activity (Divya *et al*., 2011). Antibacterial activity of selected algae extracts were given in **table 1**.In the present study, among the five different algae with varying extracts tested by disc diffusion method, the methanolic extract of all algae have satisfactory inhibition properties. Stranded strain of *Bacillus subtilis* had shown maximum sensitivity to the algae *Gracilaria crassa* and *Hypnea valentia* with a maximum zone of inhibition of about 14mm.Similarly, in a study conducted on antimicrobial activities of microalgae *Trichodesmium erythraeum*, the hexane extracts have shown good inhibitory activities (Kasinathan thillairajasekar *et al*., 2009). The crude methanolic extract of different algae like *Asparagopsis*, *Laurencia,* and *Hypnea* showed significant antimicrobial activity(Nagi *et al*., 2010). It was observed that kappaphycus, a red sea weed algae showed maximum activity against *Pseudomonas flouresences*, *Staphylococcus aureus* and less inhibition on *Vibrio cholera* and *Proteus mirabilis* (Rajasulochana *et al*., 2009). In a study it was observed that the crude butanol extract of *Isochrysis galbana*, marine algae had shown a satisfactory inhibitory effect against the selected bacterial pathogens (Srinivasakumar *et al*., 2009).Among the sea weeds highest inhibitory activities was documented among the members of red, green and brown algae against both the gram positive and gram negative (Vallinayagam *et al*., 2009).In this study, the second highest sensitivity is shown in *Enteromorpha intestinalis* against *Pseudomonas* of an inhibition zone about 13mm as shown is table 1. Based on this result, *Gracilaria crassa* and *Hypnea valentia* were selected for further studies.

Partial purification of antibacterial compound by TLC

Among the various solvent systems analyzed in TLC, three well separated spots were observed in Hexane: ethylacetate solvent system for *Gracilaria crassa* and two well separated spots were seen for *Hypnea valentia*. RF values for *Gracilaria crassa* was calculated as 0.64, 0.78, and 0.84 and for *Hypnea valentia* it was calculated as 0.62 and 0.67 respectively. The antimicrobial effect of bioactive compound present in *Dictyota acutiloba* has been purified by TLC to examine the compound for further analysis.

Antimicrobial activity of partially purified TLC fractions

Among the three different spots that were observed in *Gracilaria crassa* the third band named G3 showed maximum inhibitory of 16mm while the *Hypnea valentia* showed inhibitory effects of 15 and 18mm.the results were presented in **table-2.**

Table .1.Antimicrobial activity of crude algae extract against selected pathogens

Algae	Solvent extract(mg/10ml)	Selected strains -zone in diameter (mm)				
		Staphylococcus aureus	*Bacillus subtilis*	*E.coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Acanthophora spicifera	Methanol	10	13	-	-	12
	Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Ethyl acetate	9	-	-	-	-
Enteromorpha intestinalis	Methanol	-	10	10	9	13
	Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-
Gracilaria crassa	Methanol	11	14	12	13	-
	Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Ethyl acetate	9	10	-	-	10
Gracilaria edulis	Methanol	-	11	-	-	-
	Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-
Hypnea valentia	Methanol	12	14	10	11	13
	Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-

Table.2.Effect of partially purified compound against Bacillus subtilis

S. No	Spots	Zone on inhibition (mm) in diameter
1	G1	-
2	G2	-
3	G3	16
4	G4	15
5	G5	18

Phytochemical analysis of partially purified antibacterial compound

Results of phytochemical analysis of partially purifies antibacterial compound were given in **table- 3**.Based on the results it was found that the partially purified compound consists of saponins. Similarly in a study, flavonoids and tannins extracted from the *Acacia albida and Pavetta crassipes* which possess the antimicrobial activity against MRSA (Aliyu *et al*., 2008).Phytochemical screening of *C.decorticatum* in methanolic and petroleum ether extracts revealed the presence of saponins, phytosterols and

Table.3. Phytochemical properties of partially purified active compound

S.no	Phytochemicals	Result
1	Alkaloids	-
2	Anthraquinones	-
3	Flavanoids	-
4	Catachols	-
5	Phenols	-
6	Saponins	+
7	Tannins	-

glycosides which showed a well documented antimicrobial activity (Anbu Jeba Sunilson *et al.,* 2009). The phytochemical analysis of *Gracilaria fergusonii* revealed the presence of coumerins, phenols, quinines and steroids, which ultimately may inhibit the microorganisms (Renuka Bai., 2010).

Larvicidal bioassay

Insecticides of botanical origin may serve as suitable alternative biocontrol techniques in the future (Kamaraj *et al*., 2009). An insecticide containing azadirachtin, a neem tree (Azadirachta indica) extract, was tested against mosquito larvae in the Islamic Republic of Iran under laboratory and field conditions. (Vatandoost *et al*., 2004). Certain species of green algae in the order Chlorococcales kill larvae primarily because they are indigestible (Gerald., 2007). The extracts of *J. curcas* and *E. tirucalli* were highly effective against the larvae of A. aegypti (LC= 8.79 and 4.25 ppm) and against *C. quinquefasciatus* (LC =11.34 and5.52 ppm). The LC values were 35.39, 256.77, 384.19, 703.76, and 13.14 ppm against *A. aegypti* (Abdul Rahuman *et al*., 2008). In this study also, *Gracilaria crassa* and *Hypnea valentia* in methanolic extract have shown good larvicidal activity with a LC50 of about 52.2 and 53.4 respectively.

Thus, the present study discussed about the marine algae as potential compound reservoirs which not only act as antibacterial agents but also used to control the mosquito larval population which is a biggest growing threat.

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