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Antibacterial Potential of Macroalgae Collected from the Madappam Coast, India

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Abstract: *In vitro* antimicrobial activity of two selected marine macroalgae has been evaluated in the present research, which lies in identifying certain seaweeds whose extract can act as an alternative to commonly used antibiotics hence possessing activity against human pathogens. Extract from two species of seaweed samples namely *Caulerpa racemosa* and *Grateloupia lithophila* were collected from different locations at Gulf of Mannar, Southeast Coastal Region, Mandapam, Tamil Nadu, India and were screened for antimicrobial activity. Extracts of Methanol, Ethanol, Butanol, Acetone, Chloroform and Dichloromethane were tested against selected human pathogens. Both the seaweeds collected had shown moderate antibacterial activity with <15 mm of zone of inhibition. Out of which only butanolic extract of has shown significant activity. Phytochemical screening revealed the presence of alkaloid and phenolic compounds in both the seaweeds whereas flavonoids and steroids were found to be present in only *Caulerpa racemosa.* The screening result confirms that these seaweeds can be further studied and used as possible source of antimicrobial compounds.

Key words: Alkaloids, antimicrobial activity, flavonoids, phytochemical analysis

INTRODUCTION

Seaweeds refer to any large marine benthic algae that are multicellular, macrothallic, and thus differentiated from most algae that are of microscopic size. These plants form an important renewable resource in the marine environment and have been a part of human civilization from time immemorial. Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals (Faulkner, 2000a, b; Schwartsmann *et al*., 2001). Seaweeds are the extraordinary sustainable resources in the marine ecosystem which have been used as a source of food, feed and medicine. It was estimated that about 90% of the species of marine plant are algae and about 50% of the global photosynthesis is contributed from algae (Dhargalkar and Neelam, 2005). The vast varieties of seaweeds were found to possess useful untapped biochemical compounds, which might be a potential source of drug leads in the future. Until now more than 2400 marine natural products have been isolated from seaweeds of subtropical and tropical populations. These natural products are known as secondary metabolites, which posses a broad range of ecological interactions in marine life (Khaleafa *et al*., 1975). India (08.04-37.06N and 68.07-97.25E), a tropical South Asian country has 7,500km of coastline with diverse habitats and rich biota. Southwest coast of India is a unique marine habitat infested with diverse seaweeds. Approximately, 841 species of marine algae found in both inter-tidal and deep water regions of the Indian coast (Oza and Zaidi, 2000). The Gulf of Mannar is a Marine Biosphere Reserve situated along the east coast of India and Sri Lanka, an area of about 10,500sq.km which has a luxuriant growth of about 680 species of seaweed belonging to the Rhodophyta, Pheaophyta and Chlorophyta, in both the inter-tidal and deep water regions.

As a consequence of an increasing demand for biodiversity in the screening programs seeking therapeutic drugs from natural products, there is now a greater interest in marine organisms, especially algae. Screening of seaweeds for antimicrobial activity and bioactive constituents is quite imperative. It is well known that many drugs can be prepared from marine source which could profitably be used in pharmaceutical industries. There is a growing demand and need for new bioactive drugs to control many bacterial and fungal diseases of plants and animals. Methods commonly applied are based on the agar diffusion principle using pour plate or spread-plate techniques. Antimicrobial effects are shown as visible zones of growth inhibition. Bacterial bioassays comprise different test bacteria. *Micrococcus luteus*,

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Bacillus subtilis, *B. cereus* and *Escherichia coli* are commonly used to detect antibiotic residues in food (McGill and Hardy, 1992). Therefore the present study was undertaken to evaluate antimicrobial activity of macroalgae.

MATERIALS AND METHODS

Collection and identification of seaweeds: Five different seaweed samples were collected (2009-2010) during the low tidal conditions at depths of 1 to 3m from different locations at Gulf of Mannar, southeast coastal region, Mandapam (9º16′47″N 79º7′12″E) India. Marine algae were collected by hand picking from the submerged marine rocks and transferred to the laboratory and were identified at Department of Marine and Coastal Studies, Madurai Kamaraj University, Mandapam, Tamil Nadu, India. The antimicrobial study was conducted in the Biomolecules laboratory at VIT University, Vellore, India.

Sample preparation: Algal samples were cleaned of epiphytes and extraneous matter, and necrotic parts were removed. Plants were washed with seawater and then in fresh water. The seaweeds were transported to the laboratory in sterile polythene bags at 0ºC temperature. In the laboratory, samples were rinsed with sterile distilled water and were shade dried, cut into small pieces and powdered in a mixer grinder.

Phytochemical screening of seaweed extracts: Seaweed extracts were subjected to various qualitative chemical tests to screen for phytochemical constituents.

Detection of alkaloids: Preparation of filtrate solvent free extract (50 mg) is stirred with 2 mL of dilute hydrochloric acid and filtered. To a 1 mL of filtrate a drop of Mayer's reagent was added by the side of tube and then observed for a white creamy precipitate (Evans, 1997).

Detection of carbohydrates: The extract (100 mg) is dissolved in 5 mL of water and filtered. To 2 mL of filtrate two drops of alcoholic solution of α-naphthol were added, the mixture is shaken well and 1 mL of concentrated sulfuric acid was added slowly along the sides of the test tube and allowed to stand, then observed for the formation of violet ring (Molish's test).

To 0.5 mL of filtrate 0.5 mL of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min and after that observed for characteristic red colored precipitate formation (Benedict's test).

Detection for saponins (Kokate, 1999): Two grams of the powdered sample was boiled in 20 mL of distilled water in a water bath and filtered. 10 mL of filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Detection of proteins and amino acids: The extract (100 mg) was dissolved in 10 mL of distilled water and filtered through Whatman no.1 filter paper and the filtrate is subjected to tests for proteins and amino acids. To 2 mL of filtrate, few drops of Millon's reagent were added and were observed for white precipitate (Millon's test).

Detection of phytosterols: The extract (50 mg) was dissolved in 2 mL of acetic anhydride. To this 1 or 2 drops of concentrated sulfuric acid was added slowly along the sides of the tube and observed for an array of colour (Finar, 1986).

Detection of phenolic compounds: The extract (50 mg) was dissolved in 5 mL of distilled water. To this few drops of neutral ferric chloride solution was added and observed for a dark green coloration (Mace, 1963).

Detection of flavonoids: To 5 mL of dilute ammonia solution a portion of the aqueous filtrate of each algal extract followed by addition of concentrated sulfuric acid. Then was observed for a yellow coloration. The yellow coloration disappears on standing (Harborne, 1973; Sofowara, 1993).

Detection of steroids: Two mililitre of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 mL of sulfuric acid and this was observed for colour change from violet to blue or green in some samples.

Detection of terpenoids: Salkowski test: 5 mL of each extract was mixed in 2 mL of chloroform and concentrated sulfuric acid was added to form a layer and then was observed for reddish brown coloration of the interface.

Test for cardiac glycosides: Keller-Killani test: 5 mL of each extract was treated with 2 mL of glacial acetic acid containing 1 drop of ferric chloride solution. This was underplayed with 1 mL of concentrated sulfuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring while in the acetic acid layer a greenish ring may form just gradually throughout thin layer.

Cultures and media: Microbial Pathogens used for testing antimicrobial activity were the following bacterial strains *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922),

Klebsiella pneumoniae (ATCC 10273), *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* Exactly 0.2 mL of overnight cultures of each organism was dispensed into 20 mL of sterile nutrient broth medium and incubated for about 3-5 h to standardize the culture to 10^6 cfu/mL. These standard cultures were further used for the antimicrobial assay. The antimicrobial activity of crude extract was evaluated against some selected microorganisms. The bacterial cultures were maintained in different medium.

Aqueous extraction of seaweeds: Aqueous extracts were prepared by transfer of 1 g of the powder to sterile wide-mouthed screw-capped bottles of 50 mL volume. 10 mL of sterile deionised distilled water was added to the powdered samples which were allowed to soak for 24 h at room temperature, after heating the extracts for an hour at 100ºC. The mixture were then centrifuged at 2000 rpm for 10 min at 4ºC.The supernatants were filtered through a sterile funnel containing sterile Whatman filter paper No. 1 and then the filtrate was sterilized using 0.2 µ membrane filter with 5 mL sterile syringe.

Solvent extraction of seaweeds: One gram of each seaweed sample was extracted with 10 mL of the solvents (methanol, ethanol, n-butanol, chloroform, acetone, dichloromethane). The dried sample were soaked in the solvents for 48 h in sterile wide-mouthed screw-capped bottles of 50 mL volume and then covered with aluminium foil. The mixture was then centrifuged at 2000 rpm for 10 min at 4ºC.The supernatants were filtered through a sterile funnel and sterile Whatman filter paper No. 1 and then the filtrate was sterilized using 0.2 µ membrane filter with 5 mL sterile syringe. Extract obtained was used for screening of their antimicrobial potential.

In vitro antibacterial assay: The antibacterial activity of crude extract (25 mg/mL) was tested by agar diffusion assay. The plates were incubated at 37ºC for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the crude extract when compared to the controls. Chloramphenicol was used as positive control.

RESULTS AND DISCUSSION

The metabolic and physiological capabilities of marine organisms that allow them to survive in complex habitat types provide a great potential for production of secondary metabolites, which are not found in terrestrial environments. Thus, marine algae are among the richest sources of known and novel bioactive compounds (Faulkner, 2002; Blunt *et al*., 2006).

Table 1: Phytochemical analysis of seaweeds collected

Test	*Caulerpa racemosa*	*Grateloupia lithophila*
Phenolics	+	+
Flavonoids	+	-
Sugars	-	-
Alkaloids	+	+
Glycosids	-	-
Phytosterol	-	-
Saponins	-	-
Proteins	+	+
Steroids	+	-
Terpenoids	ND	+

+: Positive; -: Negative

Sample collection: Two specific environmental requirements dominate seaweed ecology. These are the presence of seawater (or at least brackish water) and the presence of light sufficient to drive photosynthesis. Another common requirement is a firm attachment point. As a result, seaweeds most commonly inhabit the littoral zone and within that zone more frequently on rocky shores than on sand or shingle. Seaweeds occupy a wide range of ecological niches. Two samples collected for our current study i.e., *Caulerpa racemosa* and *Grateloupia lithophila*.

Phytochemical analysis: Alkaloids are commonly found to have antimicrobial properties (Omulokoli *et al*., 1997) against both Gram-positive and Gram-negative bacteria (Cowan, 1999). Presence of alkaloids in all the extracts and hence exerting a remarkable antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria fall in line with the above findings. An earlier study reported the antibacterial activity of methanol extract of six marine macroalgae including *C. decorticatum* which inhibited the growth of *S. aureus* and *Bacillus subtilis*. The results for phytochemical screening of seaweeds were tabulated in Table 1 and have revealed the presence of phenolics and alkaloids in both the extracts where as saponins, phytosterols and glycosides were not found in methanolic extracts. In addition methanol extracts also showed the presence of amino acids. In present studies alkaloids was found to be present in the seaweeds tested, which can be one of the key chemical exerting the antimicrobial activity and is supported by earlier presented research.

Seaweed extracts are considered to be a rich source of phenolic compounds (Athukorala *et al*., 2003; Heo *et al*., 2005). The large majority of these (about 60%) are terpenes, but fatty acids are also common (comprising about 20% of the metabolites), with nitrogenous compounds and compounds of mixed biosynthesis each making up only about 10% (Van Alstyne and Paul, 1988). Fatty acids are isolated from micro algae that exhibited antibacterial activity (Kellam and Walker, 1989). Many workers revealed that the crude extracts of Indian seaweeds are active against Gram-positive bacteria (Rao and Parekh, 1981). Methanolic extracts of fifty-six sea-weeds collected from South African coast, belonging to

Table 2: Antibacterial activity of *Caulerpa racemosa*

	Caulerpa racemosa			
Sample Test organism	MET Zone of inhibition (mm)	BUT Zone of inhibition (mm)	CHL Zone of inhibition (mm)	DICHL Zone of inhibition (mm)
E.coli	ND	12	12	ND
S.aureus	12	5	10	ND
S.epidermidis	10	10	ND	10
Klebsiella	10	10	ND	ND
Pseudomonas	ND	ND	ND	ND
Bacillus	ND	ND	ND	18

Table 3: Antibacterial activity of *Grateloupia lithophila*

	Caulerpa racemosa			
Sample Test organism	MET Zone of inhibition (mm)	BUT Zone of inhibition (mm)	CHL Zone of inhibition (mm)	DICHL Zone of inhibition (mm)
E. coli	ND	10	5	5
S. aureus	ND	10	ND	5
S. epidermidis	ND	8	5	10
Klebsiella	ND	8	12	5
Pseudomonas	ND	ND	ND	ND
Bacillus	ND	10	ND	ND

MET = Methanol, BUT = Butanol, CHL = Chloroform, DICHL = Dichloromethane

Chlorophyceae, Phaeophyceae and Rhodophyceae showed antibacterial activity.

***In vitro* Antimicrobial activity:** Antibacterial activities of two species of seaweed (*Caulerpa racemosa, Grateloupia lithophila*) tested against bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *S. epidermidis*, *Bacillus* Sp. The results of primary screening tests are summarized in Table 2 and 3, which shows that the extracts of algal species processed moderate antibacterial activity. For some species the anti bacterial activity we observed was similar to previous screening studies. We have evaluated the antimicrobial potential of from both aqueous and solvent extract. Different solvents: Methanol, Ethanol, Butanol, Chloroform, Acetone, Dichloromethane, water were used. Present work revealed the presence of moderate antimicrobial activity in almost both the seaweed extract and was compared to the standard drugs used.

Bacterial infection causes high rate of mortality in human population and aquaculture organisms. For an example, *Bacillus subtilis* is responsible for causing food borne gastroenteritis. *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* cause diseases like mastitis, abortion and upper respiratory complications, while *Salmonella* sp. causes diarrhea and typhoid fever (Leven, 1987; Jawetz *et al*., 1995). *P. aeruginosa* is an important and prevalent pathogen among burned patients capable of causing life-threatening illness (Boyd, 1995). Preventing disease outbreaks or treating the disease with drugs or chemicals tackles these problems. Nowadays, the use of antibiotics increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs is common due to in dis-criminate use of antibiotics. It becomes a greater problem of giving treatment against resistant pathogenic bacteria (Sieradzki *et al*., 1999).

Moreover the cost of the drugs is high and also they cause adverse effect on the host, which include hypersensitivity and depletion of beneficial microbes in the gut (Idose *et al*., 1968). Decreased efficiency and resistance of pathogen to antibiotics has necessitated the development of new alternatives (Smith *et al*., 1994). Many bioactive and pharmacologically important compounds such as alginate, carrageen and agar as phycocolloids have been obtained from sea-weeds and used in medicine and pharmacy (Siddhanta *et al*., 1997).

CONCLUSION

Marine macroalgae collected from the Mandappam coast of India have been shown to possess a number of biological activities. In our studies *Caulerpa racemosa*, *Grateloupia lithophila* were collected and checked for their antimicrobial and hemolytic activity. To the best of our knowledge, this is the first report demonstrating the antimicrobial activity of this species taken up in this study, with few exceptions. These seaweeds are currently undergoing preliminary investigations with the objective of screening of biologically active species. Furthermore, the encouraging biological activities seen in this study show that the Indian coastline is a potential source of variety of marine organisms worthy of further investigation.

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