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Screening for the Presence of Antimicrobial Activity in Few Indian Seaweeds

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ABSTRACT

Methanolic extracts of 17 commonly found seaweeds in the west coast of India were screened for the presence of antimicrobial activity against *Bacillus subtilis*, *Escherichia coli, Pseudomonas* sp., *Streptococcus pyrogenes, Staphylococcus aureus, Proteus vulgaris*, *Klebsiella pneumoneae, Serrratia marganii* and *Candida albicans*. The results of this study indicated that the extracts of *Padina tetrastomatica* and *Jania rubens*, brown and red alga respectively, were promising. Further purification of the active extracts followed by bioassay indicated that fraction of Ethyl Acetate (EtOAc) was more active as compared to other fractions. Phytochemical investigation revealed the presence of phenols, tannins, saponinis, cardiac glycosides, terpenoides, alkaloids, anthraquinones and flavonoides.

Keywords: Antimicrobial activity, phytochemical analysis, Indian seaweeds

INTRODUCTION

The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites. It also represents a great challenge which requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential. So far, many chemically unique compounds of marine origin, with different biological activities, have been isolated and a number of them are under investigation or are being developed as new pharmaceuticals (Faulkner, 2000a,b; Da Rocha *et al.*, 2001; Schwartsmann *et al.*, 2001).

Seaweeds are marine plants divided into three categories, based on their colours, such as red (4,500 species), green (900 species) and brown (1,000 species). Seaweeds have been used as food, fertilizer and for medicinal purposes for a long time. It has been reported that seaweeds contain high levels of minerals, vitamins, essential amino acids, indigestible carbohydrates and dietary fibre (Jiménez-Escrig and Goni, 1999). In food manufacturing, seaweeds have been developed as raw or semi-processed food products (Mabeau and Fleurence, 1993). In the present study, antimicrobial activity of the seaweeds and their phytochemical analyses were carried out. The methanolic extracts of different seaweeds were screened for the presence of potential antimicrobial activity against some medically important bacterial strains.

MATERIALS AND METHODS

In the present study, 17 species of seaweed were collected from the beaches of Goa and Maharashtra (India) on low tide from intertidal regions (Table 1). Seaweeds were collected and washed carefully for about 3 to 4 times with fresh water and kept for drying in shade. Methanol extracts were prepared from all the seaweeds. After this preliminary step, the same extracts were partially purified into hexane, ethyl acetate and aqueous fractions.

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TABLE 1
List of seaweed species and collection points

Sr. N	Seaweeds	Place	Sr. N.	Seaweeds	Place
	Phaeophyceae			**Rhodophyceae**	
1	*S. marginatum*	Marvel beach, Goa	9	*Asparagopsis taxiformis*	Anjuna beach, Goa
2	*St. marginatum*	Marvel beach, Goa	10	*Amphiroa fragilissima*	Anjuna beach, Goa
3	*P. tetrastromatica*	Baga beach, Goa	11	*Jania reubens*	Malvan, Maharashtra
4	*P. gymnospora*	Marvel beach, Goa		**Chlorophyceae**	
5	*C. implexa*	Malvan, Maharashtra	12	*Caulerpa racemosa*	Malvan, Maharashtra
6	*D. australis*	Malvan, Maharashtra	13	*Caulerpa peltata*	Marvel beach, Goa
7	*D. bartayresiana*	Malvan, Maharashtra	14	*Caulerpa taxifolia*	Malvan, Maharashtra
8	*S. aspermum*	Malvan, Maharashtra	15	*Codium fragile*	Anjuna beach, Goa
			16	*Chlorodesmis fastigiata*	Anjuna beach, Goa

The methanolic extracts of 17 seaweeds were subjected for preliminary phytochemical testing for the detection of major chemical groups. The details of the tests are as follows: Braemer's test for tannins, phosphomolybdic acid test for phenols, borntrigers test for anthraquinones and alkaloids, as described by Singleton and Rossi (1965). Forthing test for saponins, Keller - kiliani's test for cardiac glycosides of Harborne (1973). Salkowski test for terpenoides and shinoda test for flavonoids of Sofowara (1993) and Harborne (1973).

The pathogenic strains, such as 5 Gram-negative bacteria (*Klebsiella pneumonia*, *Pseudomonas sp., Escherichia coli, Proteus vulgaris* and *Serrratia marganii*) 3 Gram-positive bacteria (*Bacillus subtilis, Streptococcus pyrogenes, Staphylococcus aureus)* and one fungal strain (*Candida albicans),* were obtained from the Goa Medical College (India) from which, the strains were isolated from the patients admitted at the hospital. These isolated strains were maintained in the laboratory at NIO, Goa (India) and used for microbial assay for the present study.

The antimicrobial activity of the 17 seaweed extracts was screened using a standard disc diffusion method. Sterilized filter paper discs (5 mm diameter), loaded with 10 µl of seaweed extract (10 mg/100µl) along with one disc loaded with 10 µl (0.01 mg/ml) of Gentamycin (positive control) and another with 10 µl of sterilized DW/ methanol (negative control), were also placed on the nutrient agar. These plates were incubated at 32±1°C for 18 hrs. Zone of inhibition (measured in mm) was considered as an indicator for the presence of antimicrobial activity of a particular extract against the set of pathogenic micro-organisms. During the bioassay, zones of inhibition appearing above 8 mm diameter were considered as excellent, between 7–8 mm diameter as good, between 6 and 7 mm as moderate and less than that as poor indicators of the anti-microbial activity.

Column with Sephadex LH-20 (70 cm X 5 cm) was prepared and Ethyle acetate fraction of *P. tetrastromatica* (PTOH.2) was loaded onto this column. The fractions were eluted with methanol-chloroform (1:1) solvent. This yielded

180 fractions of 5 ml each and every fraction was analysed for the presence of antimicrobial activity.

RESULTS

The results of the assay, on the methanolic extracts of 17 seaweeds, confirmed the presence of phenols, cardiac glycosides, tannins, alkaloids, anthraqu-inones, saponins and flavonoids in all the seaweed species. Cardiac glycosides were absent in *S. marginatum*. Steroids and Terpenoids were *absent* in *S. marginatum, A. taxiformis, A. fragilissima, J. reubens* and *Cd. fastigiata* (Table 2).

In the present study, the methanolic extracts of 17 seaweeds were assayed for the presence of antimicrobial activity. They were mainly active against Gram negative bacteria as compared to Gram positive bacteria (Table 3). Seaweed (*P. tetrastromatica*) also showed a very good antimicrobial activity against the Gram positive bacteria (*S. pyrogens, B. subtilis and S. aureus*). On the basis of the preliminary results, the crude extract *of P. tetrastromatica* was further fractionated with hexane (PTOH.1), ethyl acetate (PTOH.2) and the remaining fraction was the aqueous part (PTOH.3). These fractions were concentrated and lyophilized. After that, the anti-microbial activity of these fractions was evaluated (Table 4). The ethyl acetate fractions of *P. tetrastromatica* (PTOH.2) were found to be active against *P. vulgaris*, *S. pyrogens* and *Pseud.* sp.

PTOH.2 were further loaded onto LH –20 column, with methanol-chloroform (1:1) solvent and fractions were merged into six fractions, based on the TLC results (PTOH.2.1, PTOH.2.2, PTOH.2.3, PTOH.2.4, PTOH.2.5, PTOH.2.6) and bacterial activities were checked. The findings indicated that the fraction of PTOH.2.2 was active against *S. pyrogens* and *P. vulgaris*, while the fraction of PTOH.2.6 was also active against *S. pyrogens* (Table 5).

TABLE 2
Preliminary phytochemical screening of methanolic extracts of seaweeds

Sr. No.	Phytochemical analysis								
	Seaweeds	Phenols	Tannins	Alkaloids	Anthraqu-inones	Flavonoids	Cardiac glycosides	Saponins	Steroids and terpenoids
1	*S. marginatum*	++	++	++	+++	++	-	++	-
2	*St. marginatum*	+++	+++	+++	+++	++	++	+++	+++
3	*P. tetrastromatica*	+++	+++	+++	+++	+++	++	+++	++
4	*P. gymnospora*	+++	+++	+++	+++	+++	+++	++	+++
5	*C .Implexa*	+++	+++	+++	+++	+++	+++	+++	+++
6	*D. australis*	+++	+++	+++	+++	++	++	+++	++
7	*D. bartayresiana*	++	+++	+++	+++	++	++	+++	++
8	*S. aspermum*	+++	+++	+++	+++	+++	++	+++	++
9	*A. taxiformis*	++	++	+++	+++	++	++	+++	-
10	*A. fragilissima*	++	++	+++	+++	++	++	++	-
11	*J. reubens*	++	++	+++	+++	++	++	+++	-
12	*C. racemosa*	+++	+++	++	+++	+++	+++	++	+++
13	*C. peltata*	+++	+++	+++	+++	+++	+++	+++	++
14	*C. taxifolia*	++	++	+++	+++	++	++	+++	++
15	*C. fragile*	++	++	+++	+++	+++	++	+++	++
16	*Cd. fastigiata*	++	++	+++	+++	+++	++	++	-

(- absent, + traces, ++ moderate, +++ abundance)

TABLE 3
Antimicrobial activity (zone of inhibition in mm) of the methanolic extracts of seaweeds extracts

S. No.	Seaweeds	Micro-organisms								
		S. aureus	*S. pyrogenes*	*B. subtilis*	*E. coli*	*Pseud.* sp	*S. marganii*	*P. vulgaris*	*K. pneumon*	*C. albicans*
1.	*S. marginatum*	6	-	6	-	-	-	-	7	-
2.	*St. marginatum*	-	6	-	-	-	6	6	7	-
3.	*P. tetrastromatica*	7	8	9	-	9	7	7	9	-
4.	*P. gymnospora*	8	-	-	-	-	-	-	9	6
5.	*C. implexa*	-	-	-	-	-	-	-	6	-
6.	*D. australis*	6	-	-	-	7	6	6	6	6
7.	*D. bartayresiana*	-	-	-	-	-	-	-	6	6
8.	*S. aspermum*	6	-	-	-	-	-	-	6	-
9.	*A. taxiformis*	-	-	-	-	-	-	-	6	6
10.	*A. fragilissima*	-	-	-	-	-	7	-	-	-
11.	*J. reubens*	-	9	6	8	-	8	6	6	9
12.	*C. racemosa*	-	4	-	-	6	6	-	-	6
13.	*C. peltata*	-	-	-	-	6	6	6	6	-
14.	*C. taxifolia*	6	6	6	-	-	6	-	6	6
15.	*C. fragile*	-	-	6	-	6	6	6	6	6
16.	*Cd. fastigiata*	-	-	6	-	-	6	-	7	6

TABLE 4
Antimicrobial activity (zone of inhibition in mm) of fractions of *P. tetrastromatica*

S. No.	Microorganisms	*P. tetrastromatica* (Fractions)		
		PTOH.1	PTOH.2	PTOH.3
1.	*S. pyrogenes*	8	9	-
2.	*B. subtilis*	-	-	9
3.	*P .vulgaris*	8	8	-
4.	*Pseud.* sp	-	8	-

The active fraction was again fractionated on Sephadex LH-20 column [methanol-chloroform (1:1) solvent]. For this, a total of 21 fractions were collected and after TLC, they were pooled in 8 fractions. Antimicrobial activity was checked against the active pathogens for all 8 fractions. The results indicated that fractions 1-5 (PTOH.2.2.1, PTOH.2.2.2, PTOH.2.2.3, PTOH.2.2.4 and PTOH.2.2.5) were active against *P. vulgaris*. Meanwhile, PTOH.2.2.5 was also found to be active against *S. pyrogens* (Table 6).

The preliminary NMR of the fraction PTOH.2.2.2 was carried out to find out the nature of the compounds responsible for the antimicrobial activity. The compounds appeared to be fatty acids. The major fatty acid appeared to be similar to stearic acid (*Fig. 1*). On the basis of the NMR, the antimicrobial activity of pure stearic acid (0.01 µg/ml) was checked against *S. pyrogens*, *B. subtilis*, *P. vulgaris* and *Pseud* sp. However, no activity was observed against these bacteria.

TABLE 5
Antimicrobial activity (zone of inhibition in mm) of gel fractions *P. tetrastromatica*

S. No.	Microorganisms	*P.tetrastromatica* (Fractions)					
		PTOH.2.1	PTOH.2.2	PTOH.2.3	PTOH.2.4	PTOH.2.5	PTOH.2.6
1.	*S. pyrogenes*	-	9	-	-	6	-
2.	*B. subtilis*	-	-	-	-	-	-
3.	*P .vulgaris*	-	8	-	-	-	-
4.	*Pseud.* sp	-	-	-	-	-	-

TABLE 6
Antimicrobial activity (zone of inhibition in mm) of gel fractions *P. tetrastromatica*

S. No.	Microorganisms	*P. tetrastromatica* (Fractions)					
		PTOH.2.2.1	PTOH.2.2.2	PTOH.2.2.3	PTOH.2.2.4	PTOH.2.2.5	PTOH.2.2.6
1.	*S. pyrogenes*	-	-	-	-	9	-
2.	*B. subtilis*	-	-	-	-	-	-
3.	*P .vulgaris*	9	10	9	9	9	9
4.	*Pseud.* sp	-	-	-	-	-	-

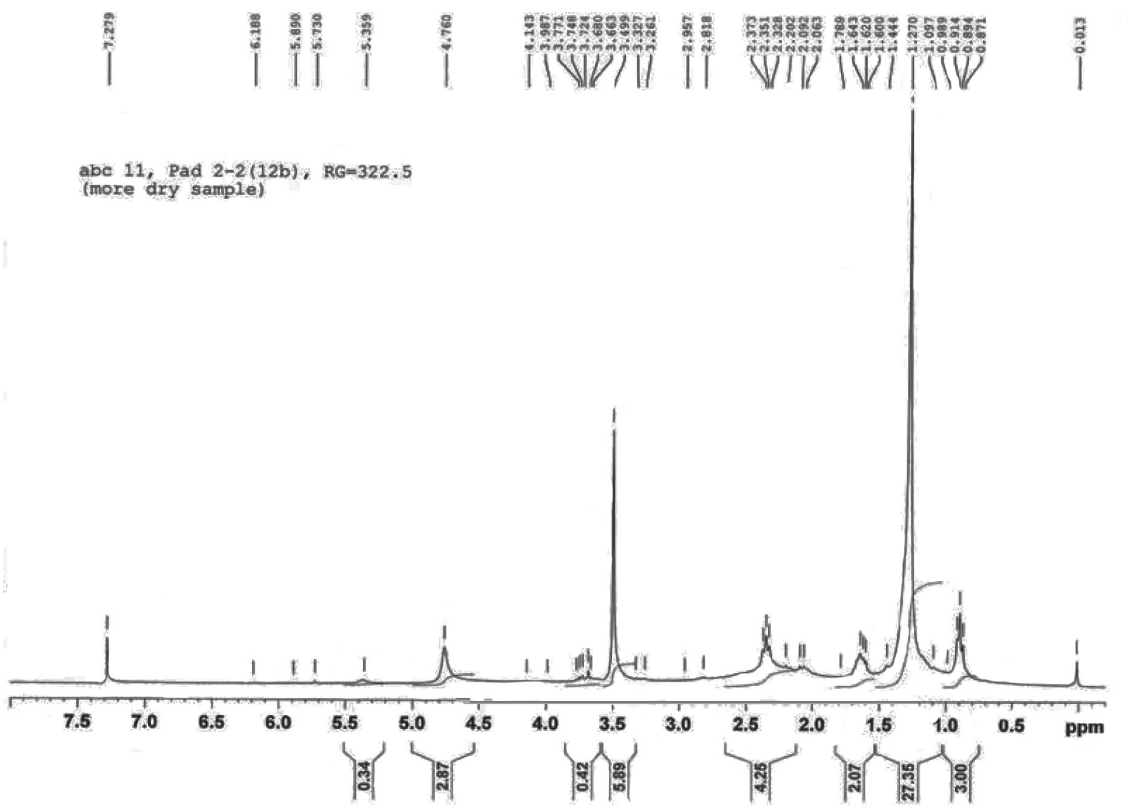

Fig. 1: NMR of the fraction PTOH.2.2.2

DISCUSSION

Recently, much attention has been directed towards extracts and biologically active compounds isolated from seaweeds. Marine algae have received comparatively less bioassay attention. On the contrary, there are a number of seaweeds with economic potential (Critchley *et al.*, 1998). Alternatively, findings from academic laboratories could result in new cultivation initiatives. It is important to highlight that the red alga *Sphaerococcus coronopifolius* has shown to possess antibacterial activity (Donia *et al.*, 2003) and the green alga (*Ulva lactuca)* has been indicated as an anti-inflammatory compound. An anti-tumour compound has been isolated from *Portieria hornemannii* (Faulkner *et al.*, 2002). *Ulva fasciata* produces a novel sphingosine derivative which has been found to have antiviral activity *in vivo* (Garg *et al.*, 1992). A cytotoxic metabolite, stypoldione, inhibiting microtubule polymerization and thereby preventing the formation of mitotic spindle, has been isolated from tropical brown alga - *Stypodium zonale* (Gerwick *et al.*, 1985). *P. hornemannii* has been found to be a novel source of cytotoxic; whereas penta, halogenated monoterpene, halomon, exhibit one of the most extreme examples of differential cytotoxicity in the screening conducted by the National Cancer Institute (NCI), USA. In specific, halomon has been selected for preclinical drug development since this compound shows toxicity to brain, renal and colon tumour cell-lines. The preliminary *in vivo* evaluations have been extremely encouraging (Carte *et al.*, 1996).

The review of literature in some studies shows that methanol extraction appears more effective, particularly in terms of antimicrobial activity than *n*-hexane and ethyl acetate (Febles *et al.,* 1995), whereas in others, chloroform has been shown to be better than methanol and benzene (Sastry and Rao, 1994). In the present study, the crude extract with methanol was prepared as it was evident from the experience of the previous study and the fact that the use of organic solvents always provides a higher efficiency in extracting antimicrobial activities, as compared to water extraction (Rosell and Srivastava, 1987). Methanol extract was further fractionated using *n*-hexane and ethyl acetate to separate compounds in increasing polarity.

The result from the antimicrobial assay of the methanolic extracts of 17 seaweeds in the present study showed the presence of biologically active compounds. The antimicrobial assay of the methanolic extracts of all the 17 seaweeds tested showed varying degrees of antibacterial activity, indicating that most of the active compounds are polar in nature. *P. tetrastromatica* and *J. reubens* showed activity against the maximum number of bacteria, whereas *A. fragilissima* and *C. implexa* showed activity against only one bacterium. Different bacterial species were found to differ in susceptibility to the methanol extract of different seaweeds, with the Gram positive organisms being generally more susceptible than the Gram negative bacteria (Rao and Parekh, 1981; Pesando and Caram, 1984; Reichelt and Borowitzka, 1984). Ethyl acetate extract (PTOH.2) exhibited a higher degree of antimicrobial activity as compared to water and hexane extract fractions.

The production of antimicrobial activities is considered to be an indicator of the capability of the seaweeds to synthesize bio-active secondary metabolites. The complexity of antimicrobial properties in seaweeds is due to their multiple inhibitory properties. In several cases, different substances have been found in the same seaweed (Olesen *et al.*, 1963). The pytochemical properties (phenols, tannins, alkaloids, anthraquinones, flavonoids, cardiac glycosides, saponins, steroids and terpenoids) of the screened seaweeds were observed to differ from traces to abundance. Therefore, it is not surprising that there are differences in the antimicrobial activity of different seaweeds.

The preliminary NMR study of fraction PTOH.2 indicated the presence of a number of fatty acids and one of the major compounds is similar to stearic acid. However, the antimicrobial assay of pure stearic acid on the tested pathogens did not show any activity.

The data presented in this study constitutes the initial results of a screening programme. The seaweed appears as a potential source of antibacterial drugs, and as about 720 species are common in Indian waters (Sajid and Satam, 2003), many more seaweeds could be investigated for the presence of potential bioactivities.

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