Research Paper

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Antibacterial Potential of Algae Sargassum Spp. and Chlorophyta Spp. from Ratnagiri, Maharshtra-An in Vitro Study

Biotechnology

KEYWORDS :
Antimicrobial activity, Chlorophyta, Sargassum spp, well diffusion technique, HPTLC

Vaidya Shruti	Department of Biotechnology, K.C.College, Churchgate, Mumbai – 400020. (India)
Shah Kruti	Department of Biotechnology, K.C.College, Churchgate, Mumbai – 400020. (India)
*** Harshal Anupma**	The Department of Biotechnology, Kishinchand Chellaram College, Dinshaw Wacha Road,Churchgate, Mumbai,Maharashtra 400020, India * Corresponding author

ABSTRACT

Antimicrobial formulations of plant origin are of great interest as certain antibiotics have undesirable effects while many lead to the development of multidrug resistance. The aim of the present work was to screen the antimicrobial potency of two locally collected green algae belonging to Chlorophyta from a water body in Ratnagiri (Maharashtra) and two commercially procured brown algae Sargassum (sexual and vegetative forms). The algal samples were extracted using the Soxhlet extraction and methanol as a solvent. The methanolic extracts were tested against various Gram positive and Gram negative microorganisms using the well diffusion technique for antimicrobial assay. The study revealed that green algal extracts were more effective against Gram Positive organisms such as S.aureus, Bacillus spp. and more effective against Micrococcus spp. While the brown algal extracts were effective against Gram Negative strains, none of the extracts could however inhibit Pseudomonas spp. The algal extracts were also subjected to HPTLC fingerprinting for detecting the presence of terpenes, coumarins and flavonoids, which will help in future to establish strong correlation of these phytoconstituents and their antimicrobial potency.

Introduction:
The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to known antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradzki, Robertz, Haber, Tomasz, 1999). Efforts are on to discover new antimicrobial compounds or screen the antimicrobial potential of various kinds of sources such as plants, animals and microorganisms. Systematic screening of them may result in the discovery of novel effective compounds (Tomoko, Takashi, Hiromu, Yuka, Hiroko, Munekazu, Totshiyuki, Tetsuro, Fujiyo, Iriya, Tsutomu N., Kazuhito, 2002).

Algae (Latin "seaweed", singular Alga) are a large and diverse group of simple, typically autotrophic organisms, ranging from unicellular to multicellular forms. Algae are found in diverse conditions in nature, in oceans, salt lakes, ponds etc. Algal cells produce certain phytoconstituents as defense mechanism which can act as an antagonist to certain other organisms. Hence, these can be tapped as source for production certain antibiotics. Presence of Dipeptides, Coumarins, Flavonoids and Sterols has also been reported in algae. The current work focuses on the antimicrobial activity of naturally collected green algae from water body and commercially available brown algae on microorganisms.

Many bioactive and pharmacologically active substances have been isolated from algae. For instance, extracts of marine algae were reported to exhibit antibacterial activity. Kashinath T., Veeramuthu Duraipandiyan, Pachiappan Permal, Savarimuthu Ignacimuthu, 2009 have also reported antibacterial activities of microalgae.

Though literature speaks diverse studies of bioactivity of marine flora, our work on testing the antibacterial efficacies and identification of various phytocontituents using HPTLC of these algal species was comparatively a new concept and not much attempt had been made earlier on this line.

Materials & Methods:
Algae samples A and B belonging to Chlorophyta were collected from two local ponds in Ratnagiri. The samples were shade dried, cut into small pieces and powdered in a mixer grinder.

Sexual and vegetative forms of commercially available alga, Sargassum belonging to Phaeophyta were purchased from Silvia Scientific Corporation, Alibaug. These were washed thoroughly to remove any debris using distilled water; shade dried and powdered using a blender.

Preparation of extract
An extract (1%) of each alga was prepared in methanol using the Soxhlet extractor for 24 hrs at 65°C (Boiling point of Methanol). Extracts prepared were concentrated, collected in sterile vials and refrigerated for further use. The extracts were diluted with methanol so as to obtain 500 µg, 250 µg, 100 µg & 50 µg concentrations of the extracts per 50 µL of methanol, at which the activity was evaluated. Protein content of all the extracts was determined by Folin Lowry technique and the absorbance was measured at 660nm using a spectrophotometer (Lowry O.H., Rosebrough NJ, Farr AL, Randall RJ, 1951).

Test Micro-organisms
The test bacterial pathogen cultures were obtained from the stock cultures maintained in the Biotechnology Laboratory at Kishinchand Chellaram College. The test cultures used for the study were Staphylococcus aureus, Bacillus spp., Micrococcus spp., Corynebacterium diphtheriae, Escherichia coli, Pseudomonas spp., Klebsiella pneumoniae and Proteus mirabilis, whose culture density was set to 0.1 O.D. at 540 nm.

Antibacterial testing
The antimicrobial activity of various algal extracts was checked by MIC (Minimum inhibitory concentration – by tube method) and well diffusion method at various concentrations and methanol was used as a control. For well diffusion assay, medium was seeded with the test organism (1ml culture + 20 ml Medium) and a 10 mm well was made using a cork borer. The wells were filled with different algae extracts of various dilutions and screened for their antimicrobial activity against respective test organisms. The crude extract was allowed to diffuse into the wells for 10-15min at 4ºC. The Petri plates were incubated at 37ºC for 24hours. The diameter of zone of inhibition of each well was recorded. Methanol control was used along with algal extracts A, B, C & D. The results of antibacterial activity were tabulated as reported in Table-1.

Fingerprint analysis was performed using the method described by Hildebert Wagner & Sabine Bladt, 1929.

Samples (A, B, Sargassum sexual and Sargassum vegetative) were screened for the presence of Flavenoids, Terpenes and Coumarins using HPTLC. Selected active crude extracts were loaded on silica gel 10.0 x 10.0 cm, HPTLC plates of silica gel 60 F 254, Manufacturer E. MERCK KGaA. The profile obtained was analysed using winCATS Planar Chromatography Manager. Appropriate solvent systems and spray reagents were used for the detection. Silica plates were scanned at 254nm, 366nm and 580nm, and observations made regarding presence of cou-

marins, flavonoids and terpenes.

Results:
Protein content of samples A, B, C and D were found to be 16mg%, 80mg%, 10.9 mg% and 24.2 mg% respectively by Folin Lowry assay.

Antimicrobial activity of the algal extracts:
Extract A was effective against Gram Positive organisms S.aureus followed by Micrococcus spp. and Bacillus spp., at both 250mg as well as 500mg. It did not display against Gram Negative organisms except K. pneumoniae against which it shows some inhibition at 500mg.

Extract B is ineffective at 500mg against Gram Negative organisms and C.diptheriae to a lesser extent. It shows variable results for inhibition action against other Gram Positive organisms. However, Micrococcusspp. is most sensitive at 250mg but not at higher concentration.

Inhibitory action of extract C against Gram Negative organisms is observed only at 500mg, however extract C is ineffective against C.diptheriae and Pseudomonas spp. Extract C shows very little inhibition action on Bacillus spp. and S.aureus while Micrococcus spp. appears to be most sensitive to it.

Extract D shows no inhibition against C.diptheriae and Pseudomonas spp. It is effective against Micrococcus spp. and K.pneumoniae at 100mg and 250mg respectively. Inhibition against E.coli, P.mirabilis and S.aureus is observed only at 500mg.

Control wells with methanol were maintained with all the test plates that showed very little or no inhibition of the test organisms in consideration.

HPTLC Analysis:
TLC plate for the separation of coumarins when scanned at 254nm without chemical treatment showed prominent quenching against green background which indicated presence of coumarins in algal extracts. As seen in Figure 1, the dark zones in extract B were quite prominent, hence, the concentration of coumarins in sample B may be higher than samples A, C and D.

As seen in Figure 2, blue, violet or grey bands at ~R_f 0.15 - 0.25 indicate presence of terpene alcohols. Bands at the solvent front indicate presense of terpene hydrocarbons.

Under UV – blue, violet pink fluroscent zones at R_f 0.2 – 0.75 showed possibile presence of essential oils, terpenes or sterols. As observed in Figure 3, the amount and types of terpenoids may be similar in the samples C and D. Also sample B may have higher amount of essential oils, terpenoid or sterols.

As observed in Figure 4, purple bands present at ~R_f 0.2 in extract C and D and at ~R_f 0.5 – 0.75 may be attributed to the presence of saponins or flavenoids.

Discussions
In this study, methanol extracts of algae were prepared and tested for their antimicrobial activity against various gram positive and gram negative microorganisms. The use of organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water-based extraction methods as reported by Lima-Filho JVM, Carvalho AFFU, Freitas SM et al. Extract A is the most effective amongst all the algal extracts considered in this study. None of the extracts inhibited all of the organisms tested. However, there appeared to be selectivity for gram-positive microbes. Extract B is ineffective against Gram Negative organisms. Extract C and Extract D are less effective as compared to extract A but show greater inhibition as compared to B.

Among the Gram Positive organisms, Micrococcus spp. is the most susceptible, while C.diptheriae is most resistant to these extracts upto 500mg concentration. On the other end, K.pneumoniae is inhibited by the various algal extracts as compared to the other Gram Negative organisms under this study. None of the algal extracts were found to inhibit the growth of Pseudomonas spp. upto 500mg.

In our study, some of the bacterial strains did not respond to extracts, highlighting the need for further purification of the extracts to remove inhibitory substances. This could also be due to masking of antibacterial activity by the presence of some inhibitory compounds or factors in the extract. The variation of antibacterial activity of our extracts might be due to distribution of antimicrobial substances, which varies from species to species Lustigman B, Brown C (1991). Similarly, it was found that fractionation of crude extracts tested enhanced their activity against both Gram negative as well as the resistant Gram positive pathogens Sastry V.M.V.S. and G.R.K. Rao (1994).

Conclusions:
Many marine algae produce antibiotic substances capable of inhibiting bacteria, viruses, fungi, and other epibionts. It appears that the antibiotic characteristic is dependent on many factors, including the particular alga, the microorganisms, the season, and the growth conditions (Centeno POR, DL Ballantine, 1999). It may be concluded from the above findings that extracts A, B, C and D act more effectively against Gram positive organisms and some Gram negative intestinal pathogens. All the samples may contain varying amounts of coumarins, sterols, terpenoids or essential oils and flavonoids. Sample B was found rich in coumarins and sample B, C, D have a greater amount of flavonoids. Hence compared to other algae, sample B shows less antimicrobial activity, thus antimicrobial activity may be attributed to presence of sterols, terperne and essential oils compared to coumarins or flavonoids. The findings of the pilot study do envisage that methanol extracts of the algal samples could be utilized as a good source of antimicrobial agent in the pharmaceutical industry.

The HPTLC fingerprint analysis was carried out to throw some light on the constituents of the crude extracts, it would help study the structure – function correlation of the antimicrobial agent in the extracts.

The antibacterial activity of marine algae is generally assayed using extracts in various organic solvents, e.g., acetone. Several extractable compounds, such as cyclic polysulfides and halogenated compounds are toxic to microorganisms and therefore responsible for the antibiotic activity of some marine algae as reported by Ohta (1979). However, an antibiotic assay of extracts in organic solvents probably does not reflect adequately the antibacterial activity of marine algae under natural conditions. Identification of the active antimicrobial molecule present in these algal samples by generating an HPTLC fingerprint may aid other researchers. Antimicrobial activity shown by these extracts may be because of presence of terpenoids, flavonoids or coumarins, but not proteins. Extract of Sargassum could serve as a potential candidate for purification and further in vivo studies.

Our study showed similar results with earlier observations made by Srinivasa Rao and Parekh (1981) who have demonstrated that crude extracts of Indian seaweeds were active only against Gram positive bacteria.

Overall, the present study provides enough data to show the potential of algal extracts for development of anti-pathogenic agents. 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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Algal Species	Conc. of extract (mg)	Zone of inhibition (mm)							
		Gram Positive					Gram Negative		
		B	Sa	M	Cd	Ec	Kp	Pm	P
A	500	14.67±0.58	20	19.67 ± 0.58	11.5 ± 0.5	-	15.67 ± 0.58	-	-
	250	15.67±1.53	17.67 ± 0.58	16.67 ± 0.58	11 ± 0.5	-	-	-	-
	100	12±1	15	15	10.5 ± 0.5	-	-	-	-
	50	12±1.73	13.33 ± 0.58	12.68 ± 0.58	-	12	-	-	-
B	500	15±1	14.33 ± 0.58	15.33 ± 0.58	-	-	-	-	-
	250	14±1	13	16	12.33± 0.51	-	-	-	-
	100	12.5±0.5	12	13.33 ± 0.58	-	-	-	-	-
	50	13.17±0.76	-	15	-	-	-	-	-
C	500	14.33±0.58	13.67 ± 0.58	18	-	14 ± 0.5	14.33 ± 0.58	14	-
	250	15	12.33 ± 0.58	16.33 ± 0.58	-	-	-	-	-
	100	-	-	13.33 ± 0.58	-	-	-	14.33 ± 0.58	-
	50	-	-	12.33 ± 0.58	-	-	-	-	-
D	500	14.67 ± 0.58	16.33 ± 2.3	18.68± 0.58	-	17 ± 0.5	18.33 ± 0.58	17 ± 1	-
	250	-	13 ± 0.5	17	-	-	16	13.67 ± 0.58	-
	100	13. ± 1	-	16.67± 0.58	-	-	-	-	-
	50	12.67 ± 1.53	-	12.33±0.58	-	-	-	-	-

Note – (Sa) Staphylococcus aureus, (B)Bacillus spp., (M)Micrococcus spp., (Cd)Corynebacterium diphtheria, (Ec)Eschericia coli, (P) Pseudomonas spp., (Kp)Klebsiella pneumonia, (Pm)Proteus mirabilis, (-) No antimicrobial activity

A - Ratnagiri sample 1, B- Ratnagiri sample 2, C - Sargassumsexual, D - Sargassumvegetative ±indicates standard deviations in the values zones of inhibition

Table 1: The results of antibacterial activity of algal extracts A, B, C and D against Gram positive and Gram negative microorganisms.

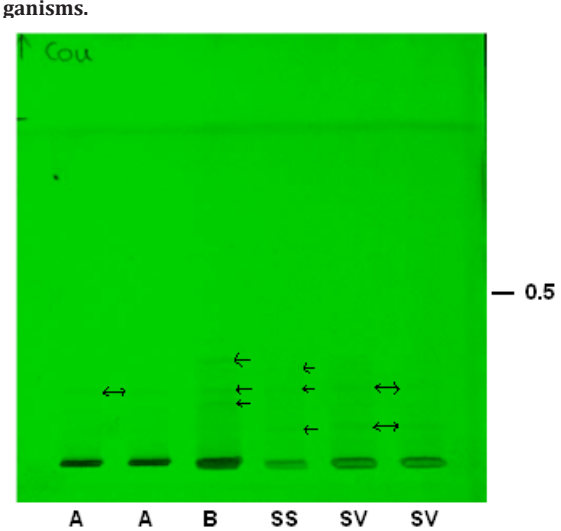

Figure 1: Silica plate scanned at 254 nm (without chemical treatment) showing promonent quenching against green background

Key:- A –sample A , B – sample B (A and B Chlorophyta spp.) SS – Sargassum Sexual and SV – Sargassumvegetative

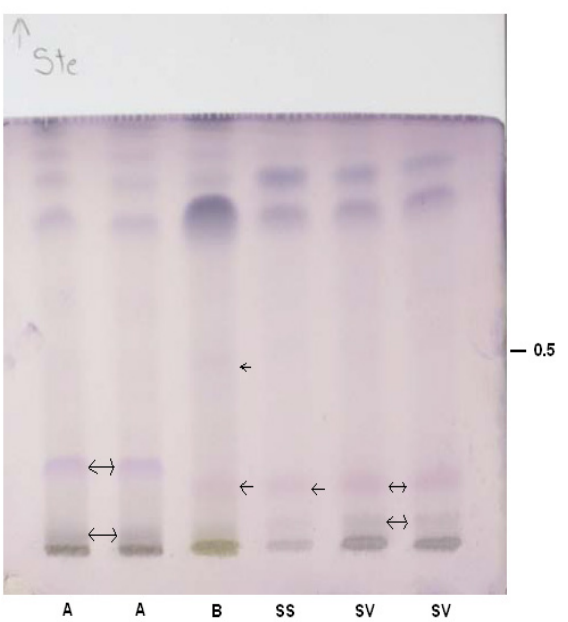

Figure 2: Silica plate at visible light (after derivatisation) for the detection of terpenes

Key:- A –sample A , B – sample B (A and B Chlorophyta spp.) SS – Sargassum Sexual and SV – Sargassumvegetative

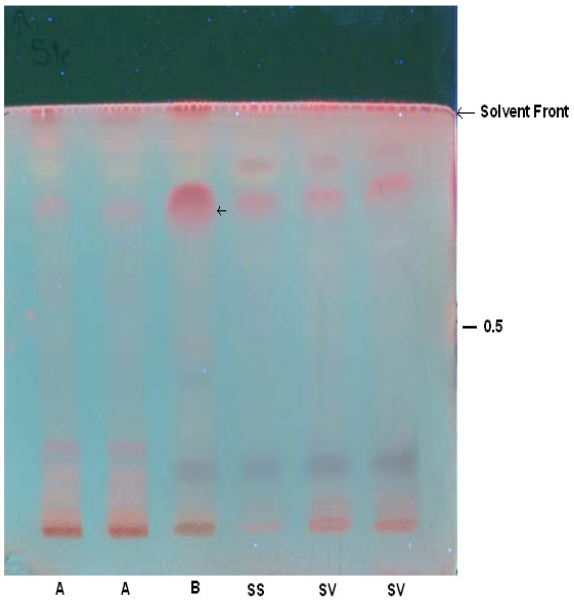

Figure 3: Silica plate scanned at UV for the detection of terpenes

Key:- A –sample A , B – sample B (A and B Chlorophyta spp.) SS – Sargassum Sexual and SV – Sargassumvegetative

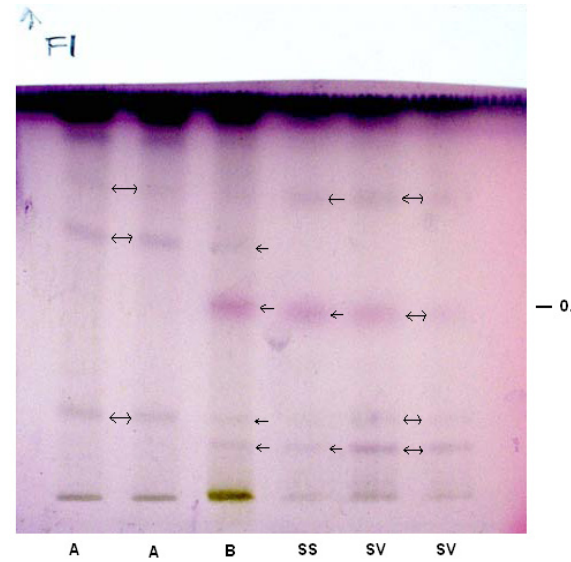

Figure 4: Silica plate at visible light (after derivatisation) for the detection of flavonoids

Key:- A –sample A , B – sample B (A and B Chlorophyta spp.) SS – SargassSum Sexual and SV – Sargassumvegetative

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