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Antibacterial activity of various solvent extracts of marine brown alga *Spatoglossum asperum*

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Abstract
Seaweeds offer a rich source of bioactive molecules; the present study was carried out to the antimicrobial activity of various organic solvent extracts (aqueous, methanol, chloroform, ethyl acetate and hexane) of the marine brown alga *Spatoglossum asperum* and test against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Salmonella typhi* by disc diffusion method. The zone of inhibition was compared. The chloroform and methanolic extract showed maximum activity against *Staphylococcus aureus* 86.88 and 84.94%, respectively, the aqueous extract showed moderate activity against *Bacillus cereus* (54.05%) and ethyl acetate showed minimum activity against *Bacillus cereus* (37.57%), whereas no activity was observed in hexane extract. This study established methanol extracts of brown seaweed *S.asperum* was highly effective, against most of the bacterial pathogens tested.
Keywords: Antibacterial activity, various solvent extracts, *Spatoglossum asperum*, pathogenic bacteria.

1. Introduction
Seaweeds are the renewable living sources, which have been screened extensively to isolate life saving drugs or biologically active substances all over the world. Selective utilization of marine algae as a potential source of pharmaceutical agents has been increasing in recent years. Natural products are a major resource for drug development. A large number of plants, microbes, and marine animals have been examined for bioactive secondary metabolites [1]. Marine algae such as harbor endophytes, like their terrestrial counterparts, are a potential source of new secondary metabolites [2]. Seaweeds are considered as a source of bioactive compounds with cystostatic, antiviral, antihelminthic, antifungal and antibacterial activities.

Marine sources are receiving much attention mainly because of the contents of functional ingredients such as polyunsaturated acids, carotene and their pigment carotenoids, sulphated polysaccharide and sterol. Among different compounds with functional properties, antioxidants and antibacterials are mostly widely studied. Despite this, the number of seaweed species studied and identified corresponds to only 2% of the 150,000 known[3]. Macroalgae and bacteria associated with surface 268 species worldwide [4]. From the species identified, approximately two thousand chemical compounds have been characterized [5].

Bacterial infection causes high rate of mortality in human population and aquaculture organisms [6]. The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and the side effects they produced. These limitations demand for improved pharmacokinetic properties, which necessitate continued research for new antimicrobial compounds for the development of drugs [7]. Pharmaceutical industries are giving importance to the compounds derived from traditional sources (soil and plants) and less traditional sources like marine organisms [8]. Hence, the interest in marine organisms as a potential and a promising source of pharmaceutical agents has increased during recent years [9].

Marine algae are rich and varied source of bioactive natural products, so it has been studied as potential biocidal and pharmaceutical agents [10]. There have been a number of reports of antibacterial activity from marine plants [11,12]. Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities [13] with antiviral, antibacterial and antifungal

activities [14] which acts as potential bioactive compounds of interest for pharmaceutical applications [8].

Most of these bioactive substances isolated from marine algae are chemically classified as brominated, aromatics, nitrogen-heterocyclic, nitrosulphuric-heterocyclic, sterols, dibutanoids, proteins, peptides and sulphated polysaccharides [12]. These limitations demand for improved pharmacokinetic properties which necessitates continued research in the search of new antimicrobial compounds for the development of drugs. Hence, the present study the antimicrobial activities of brown alga *Spatoglossum asperum* using different solvents were investigated.

2. Materials and Methods

2.1 Sample collection and preparation

Spatoglossum asperum was collected from the intertidal regions of the Mandapam coast (Lat. 09˚ 17.417'N; Long. 079˚ 08.558'E) of the Gulf of Mannar and was immediately brought to the laboratory in plastic bags containing water in order to prevent evaporation. An alga was identified and authenticated by the monograph of Phaeophyceae [15]. Then the alga was washed thoroughly with sterilized sea water to remove extraneous materials. The sample was shade dried to constant weight obtained and ground. The powdered samples were stored in an airtight container for future use.

2.2 Preparation of algal extracts

Seaweed powder was soaked in the organic solvents with the increasing order of polarity viz., hexane, ethyl acetate, chloroform, methanol and aqueous (1:3 w/v) and kept in hot air oven overnight at 60^{o}C and the extracts were collected and concentrated. The extract was then filtered through a Buchner funnel with Whatman No.1 filter paper. The filtrate was evaporated to dryness under pressure using a rotary vacuum evaporator at 50^{o}C and the crude extracts were weighed. The yield of powdered sample obtained was 7.5 g/100 g from methanol solvent, 5.6 g/100 g from aqueous extract, 4.8g/100g from chloroform extract, 3.3g/100g from ethyl acetate extract and 3.1 g/100 g from hexane extract. These crude extracts were then tested for their antibacterial activity against selected human pathogens.

2.3 Pathogens used for the assay

Three strains of gram positive bacteria, namely, *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* and three strains of gram negative bacteria, namely, *Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi* were obtained from Post Graduate and Research Department of Botany, Pachaiyappa's College, Chennai. The bacterial pathogens were maintained on Nutrient Agar (Hi Media, India).

2.4 Preparation of inoculums

Bacterial inoculums were prepared by transferring a loopful of bacterial culture from fresh culture plates to tubes containing 10 ml of Nutrient Broth (Hi-media) and incubated for 24 hours at 37^{o}C. The tubes were shaken occasionally to aerate and promote growth. These cell suspensions were diluted with sterile Nutrient Broth to provide initial cell counts of about 2 x 10^{3} CFU/mL.

2.5 Antibacterial assay

Antibacterial activity was carried out using the disc diffusion method [16]. The petriplates were prepared with 20 mL of sterile Mueller Hinton Agar (MHA), (Hi-media) and the test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. The plates were swab inoculated using sterile cotton buds with each of the previously mentioned bacterial pathogens in the concentration of 2 x 10^{3} CFU/mL. Sterile filter paper discs 6 mm in diameters (Whatman No.1) were loaded with different extracts (100 µg/mL) and air-dried. Discs containing streptomycin were used as controls (100 µg/mL). The discs were placed on Muller Hington Agar (MHA). Plates were incubated for 24 hours at 37^{o}C temperature, the antibacterial assay were done in triplicates. For each algal extract zone of inhibition was recorded in millimeters and it was compared with the control and results were expressed in percentage of inhibition, all the data were statistically analyzed.

3. Results and Discussion

The antibacterial activity of the selected marine brown alga *Spatoglossum asperum* was studied by using different solvents such as aqueous, methanol, chloroform, ethyl acetate and hexane by disc diffusion method against (three gram positive and three gram negative bacteria) human pathogens. We have used different solvents from a low polar to highly polar to extract the all the possible active components from the *S. Asperum.* Further, the best solvent extract possessing high anti-bacterial activity will be subject to chromatographic techniques such as GC-MS, HPTLC to identify the number and composition of components and the structure of the active components identified using ^{1}H and ^{13}C NMR, and mass spectroscopy technique. The bacterial cultures of *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Salmonella typhi* were used for the present study.

The antibacterial activity was assessed by measuring the zone of inhibition and compared with the standard antibiotic Streptomycin. The antibacterial activity of various solvent extracts of *S.asperum* on human pathogens was presented in the Table.1 and Figure I. The study showed that the aqueous extract showed moderate antibacterial activity

against *Bacillus cereus* 10.27 ± 0.002 mm (54.05%), *Klebsiella pneumoniae* 8.28 ± 0.001mm (51.75%) and *Salmonella typhi* 10 ± 0.001 mm (50%) followed by a lesser activity against *Staphylococcus* 8.05 ± 0.001 mm (47.35%), *Pseudomonas aeruginosa* 9.36 ± 0.003 mm (46.80%) and *Bacillus subtilis* 7.12 ± 0.003 mm (41.88%). Subba *et al* [17] reported that the aqueous extract of marine brown alga *Sargassum ilicifolium* showed higher antibacterial activity against the pathogenic bacteria such as *P. aeruginosa*, *Klebsiella pneumonia*, *S. aureus* and *B. subtilis.* They also reported higher antibacterial activity in the marine brown algae *Padina tetrastromatica* against the following species of *B. subtilis*, *S. aureus* and *Klebsiella pneumonia*. The present study shows lesser inhibitory activity against the above bacterial species, so this study was contrasted to Subba *et al* [17].

In the present study methanolic extract of *S. asperum* showed the highest activity against *S. aureus* 14.44 ± 0.004 mm (89.94%) and followed by *K. pneumoniae* 13.15 ± 0.001 mm (82.18%), *B.subtilis* 13.63 ± 0.001 mm (80.17%) and *P. aeruginosa* 13.63 ± 0.003 mm (68.15%). The methanolic extract of *S. asperum* also shows lesser antibacterial activity against the above organism. Subba *et al* [17] reported more activity in the methanolic extract of marine brown algae *S.ilicifolium* and *P. tetrastromatica* against *B. subtilis*, *Klebsiella pneumonia*, *S. aureus*, *Klebsiella pneumonia*, *B. subtilis* and *S. aureus*. The present study was contrasted to Xavier *et al* [18]; reported the methanolic extract of marine brown algae *Padina gymnospora* and *Sargassum wightii* showed no activity against the bacterial species of *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Salmonella typhi*.

The chloroform extract of *S. asperum* shows highest antibacterial activity among the tested extract against *S. aureus* by showing 14.77 ± 0.002 (86.88%) followed by moderate activity against *B.subtilis* 10.00 ± 0.001 (58.82%) and lesser activity against *B. cereus* 8.17 ± 0.004 (43.00%), *S. typhi* 8.05 ± 0.004 (40.25%) and *Pseudomonas aeruginosa* 7.17 ± 0.005 (35.85%). None of the activity was recorded *against* the bacterium *K. pneumonia* in the chloroform extract of *S. asperum*. The present study about *B.subtilis* and *S. aureus* correlated with the study of Subba *et al* [17] in the brown alga *S. ilicifolium*, they reported 10 mm against *B.subtilis* and 13mm against *S. aureus.* They also reported higher activity against *P. aeruginosa* and *K. pneumonia*, whereas in the present study, none of the result was observed in the chloroform extract against the bacterium *K. pneumonia.* The chloroform extract of *P.tetrastromatica* [17] showed higher activity than the present study against the bacterial species *B.subtilis* (19 mm), *S. aureus* (13 mm), *P. aeruginosa* (13 mm) and *K. pneumonia* (13 mm).

Table.1 Antibacterial activity of crude extracts of *Spatoglossum asperum* in various solvent

S. No	Name of the sample	Zone of inhibition in mm on human pathogen					
		Aqueous	Methanol	Chloroform	Ethyl acetate	Hexane	Streptomycin
1	*B.cereus*	10.27 ± 0.002 (54.05%)	14.77 ± 0.002 (77.73%)	8.17 ±0.004 (43.00%)	7.14 ± 0.004 (37.57%)	-	19.00 ± 0.002 (100%)
2	*B. subtilis*	7.12 ±0.003 (41.88%)	13.63 ± 0.001 (80.17%)	10 .00±0.001 (58.82%)	9.00 ±0.002 (52.94%)	-	17.00 ± 0.004 (100%)
3	*S. aureus*	8.05 ±0.001 (47.35%)	14.44 ± 0.004 (84.94%)	14.77 ±0.002 (86.88%)	7.00 ±0.003 (41.17%)	-	17.00 ± 0.001 (100%)
4	*K.pneumoniae*	8.28 ±0.001 (51.75%)	13.15 ± 0.001 (82.18%)	-	8.00 ± 0.004 (50.00%)	-	16.00 ± 0.003 (100%)
5	*P.aeruginosa*	9.36 ±0.003 (46.80%)	13.63 ± 0.003 (68.15%)	7.17 ±0.005 (35.85%)	8.00 ± 0.001 (40.00%)	-	20.00 ± 0.002 (100%)
6	*S.typhi*	10.00 ± 0.001 (50.00%)	15.00 ± 0.005 (75.00%)	8.05 ±0.004 (40.25%)	9.17 ± 0.005 (45.85%)	-	20.00 ± 0.001 (100%)

Values are expressed as Mean ± SEM, n=3

The ethyl acetate extract showed moderate activity against *B.subtilis* 9.00 ± 0.002 (52.94%) and *K. pneumoniae* 8.00 ± 0.004 (50.00%). All other species such as *S. typhi* 9.17 ± 0.005 (45.85%), *S. aureus* 7.00 ± 0.003 (41.17%), *P. aeruginosa* 8.00 ± 0.001 (41.17%) and *Bacillus cereus* 7.14 ± 0.004 (37.57%) showed lesser inhibitory activity. [18] observed none of the inhibitory activity in the ethyl acetate extract of marine brown algae *Padina gymnospora* and *Sargassum wightii* against *P. aeruginosa*, *S. typhi*, and *K. pneumonia.* When compared to the present study ethyl acetate extract of both the brown seaweeds showed higher activity against *S. aureus* (*S. wightii* 14.33 ± 0.58 mm, *P. gymnospora* 12.67 ± 0.58 mm) and *B.subtilis* (*S. wightii* 11.33 ± 0.58 mm, *P. gymnospora* 11.00 ± 0.00 mm).

In the present study hexane extract of *S. asperum* showed none of the activities against the entire tested organism. Whereas Xavier *et al*[18] reported 12.67 ± 0.58 mm of inhibition in the hexane extract of *S. wightii* and 10.00 ± 0.00 of inhibition in *P. gymnospora* against *S. aureus*. No other organism (*P. aeruginosa*, *S. typhi*, *K. pneumonia*, *B.subtilis*) were inhibited by the hexane extract of *S. wightii* and *P. gymnospora*, except *B.subtilis* 10.67 ± 0.58 mm of inhibition in *S. wightii* [18].

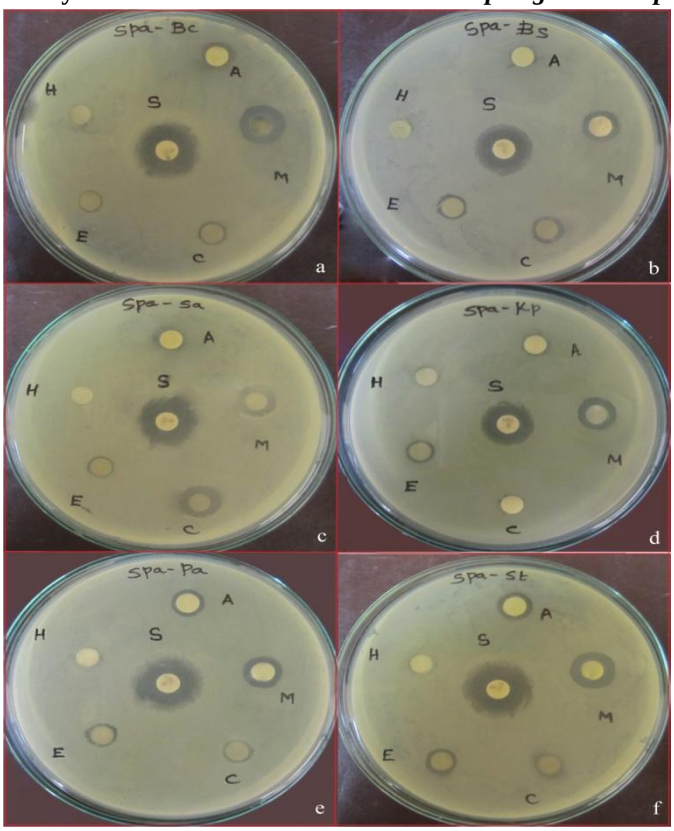
The antibacterial activity of various solvent extracts of marine brown alga *S. asperum* on six different human bacterial pathogens are presented in Table.1 and Figure I. The maximum activity against various human bacterial pathogens of the methanol extract. The main objective of this work was to evaluate and compare the ability of different solvent extract of marine brown alga *S. asperum* from the South East Coast of Tamil Nadu to produce bioactive compounds of potential therapeutic interest. The production of antibacterial activities was considered to be an indicator of the capability of the seaweeds to synthesize bioactive compounds. Because, marine natural products contain a wide range of novel bioactive compounds or antibiotics with distinctive complex structures because they developed unique metabolic and physiological capability.

The marine macro alga has an effective antibacterial activity against most of the human bacterial pathogens. It was reported that 151 species of macro algal crude extracts showed inhibitory activity against pathogenic bacteria [19]. There have been a number of reports that demonstrate the antimicrobial activity of marine algae or seaweeds [20-26]. Still, in India only limited information is available on marine algae. Hence it was intended to evaluate and compare the ability of some abundantly available marine algae in the coastal regions of Tamil Nadu, India in order to identify the bioactive potential of these seaweeds against selected human bacterial pathogens.

Seaweeds belonging to brown algae exhibit inhibitory action against both Gram-positive and Gram-negative bacteria [14,27,28]. Vallinayagam *et al* has reported that the brown alga showed higher activity than the green algae when tested against seven human pathogenic bacteria. The organic solvent chloroform and methanol extracts of some red and brown algae showed maximum activity against certain human pathogenic bacteria [28]. In our study it was reported that the methanolic extract of marine brown alga *S. asperum* showed highest antibacterial activity against both Gram-negative and Gram-positive bacteria when compared to other solvent which were studied.

Among the various organic solvents such as methanol, acetone, diethyl ether and ethanol extracts of eleven macroalgae screened for antimicrobial activity against human pathogens, the extracts of diethyl ether was found to possess bioactive compounds [29]. In another study, acetone was found best among several solvents used for extracting antibacterial substances [30]. Some other studies performed in the extraction of seaweeds using chloroform and ethyl acetate also exhibited good antibacterial activity [31,32]. It was reported that methanol extracts of seven different seaweeds tested showed broad spectrum antibacterial activity against human pathogenic bacteria[5]. This kind of less or more activity could also be attributed to the sequential extraction of marine algae using solvents from a low polar to highly polar.

Figure I: Antibacterial activity of the different solvent extracts of *Spatoglossum asperum*



Spa- *Spatoglossum asperum*, Bc-*Bacillus cereus*, Bs- *Bacillus subtilis*, Sa- *Staphylococcus aureus*, Kp- *Klebsiella pneumonia*, Pa- *Pseudomonas aeruginosa*, St- *Salmonella typhi*, A-Aquous, M-Methanol, C-Chloroform, E-Ethyl acetate, H-Hexane, S-Streptomycin

4. Conclusion

In the present study, the extract of brown alga *S. asperum* obtained from various solvents was tested for their antibacterial activity. Among, the extracts tested, the methanolic extract exhibited the highest activity, when compared to other solvent extracts which were studied. The present study concluded that the methanolic extract of marine brown alga *S. asperum* could be used for further investigation to identify actual components against human bacterial pathogens.

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