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Antibacterial Activity of Some Brown Seaweeds of Gulf Of Mannar, South East Coast of India

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Abstract:

Antibacterial activity of brown seaweeds viz, *Sargassum wightii, S.oligocystum, S.vulgare, Turbinaria ornata, Padina tetrastromatica and Stoechospermum marginatum* were tested against Gram positive (*Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis*) and Gram negative (*Aeromonas hydrophila, Pseudomonas fluorescens*) bacterial pathogens. The extracts indicated a stronger activity against Gram positive bacteria than Gram negative bacteria. Methanolic extracts displayed significantly higher antibacterial activity against all the bacterial pathogens followed by acetone extracts. Methanolic extracts of T.ornata and *P. tetrastromatica* exhibited the maximum antibacterial activitywith inhibition zone measuring of 13±0.2 mm and 12.1± 0.4 mm respectively against the Gram positive bacteria viz., B.subtilis and M.luteus. While the control or the water extracts of fucoidan revealeda weak or no activity against the microorganisms tested.

Keywords: Brown seaweeds, solvent extracts, fucoidan, antibacterial activity, pathogenic bacteria

INTRODUCTION

Seaweeds are being harvested by human for centuries, particularly in Japan, China and South Korea, where they form a part of the staple diet. Today, seaweeds and their products are a multibillion dollar industry and the demand for these products will continue to upsurge in future. World-wide at least 221 species of seaweed are being used for various purposes. In India, seaweeds are harvested from the natural beds along the coasts of Tamil Nadu and Gujarat since 1966 [1]. Brown seaweeds are the largest group of marine macroalgal species found in oceans. Gulf of Mannar located in Southeast coast of India is being flourished with brown seaweeds, particularly belonging to the group Fucales [2,3].

Seaweeds are considered as a source of bioactive compounds as they produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities [4]. A series of polyphenolic compounds such as catechins, flavonols and flavonol glycosides have been identified from methanol extracts of red and brown seaweeds and found to have antioxidant and antimicrobial activity [5]. The antibacterial substances in seaweeds can usually be extracted by water or organic solvents such as methanol, ethanol, acetone, ethyl ether, diethyl ether, ethyl acetate, chloroform, dichloromethane, benzene, hexane, chloroform: methanol (2:1), and chloroform: ethanol (1:1, 2:1) [6].

Recently, the occurrence of antibiotic resistant bacteria associated with fish diseases is a worldwide problem in aquaculture systems. To replace safer use of antibiotics, studies on the use of secondary metabolites from natural sources, including seaweeds have been increasing. Many studies indicated that seaweeds collected from Gulf of Mannar coast exhibits antibacterial activities against various human pathogens [7,8]. However, brown seaweeds have been reported to have bioactive property against a number of Gram-positive and Gram-negative microorganisms [9]. But literatures on antibacterial activity of seaweeds against fish pathogens are very few. Aeromonas hydrophilla, the most common bacterial pathogen in freshwater fish,

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has been recognized to be the etiological agent of several distinct pathological conditions including tail and fin-rot and hemorrhagic septicemia, especially in freshwater and ornamental fish [10].The ability of some seaweeds to inhibit activity of bacteria having potential interest as fish pathogens has also been documented6, [11,12]. However, comparative study on the use of various solvent extracts of different brown seaweeds available along this coast, against fish pathogens are very limited. Keeping this view,in the present study, the solvent extracts of various brown seaweeds and the water extract of the crude fucoidan were tested against selected fish pathogens to analyse their antimicrobial study so as to incorporate into the feed as dietary supplement to reduce the use of antibiotics in aquaculture systems.

MATERIALS AND METHODS

Seaweeds

Six species of brown seaweeds namely, *Sargassum wightii, S.oligocystum, S. vulgare, Padina tetrastromatica, Turbinaria ornata and Stoechospermum marginatum* were collected from Valinokkam (09˚ 13.684'N., 078˚ 47.194'E), Hare Island (N: 08˚ 047.254' E:078˚ 11.884') and Amalinagar (08˚ 29. 355, N, 078˚ 07. 2521'E)during the period from July 2012 to August 2013. The collected seaweeds were dried under shade overnight and then kept in oven at 60°C for 1 h to remove moisture and to prevent them from mould attack. The dried seaweeds were milled using food blender and sieved with the strainer (500µ mesh).

Solvent extraction

Extraction was performedas per the procedure described by Ganesan et al., [13] with minor modifications. Three solvents viz., dichloromethane, methanol and acetone were chosen based on their increasing polarity. Crude extract was prepared by mixing one gram of each dried seaweed with 10 ml of each solvent and soaking in respective solvents for 48 h in sterile screw-capped bottles. Since organic solvents are volatile in nature, the mixture was then centrifuged at 10,000 ×gfor 15 min and the solvent was evaporated to dryness under vacuum.

Fucoidan extraction

The extraction of fucoidan was performed as described by Yang et al method [14] with minor modification. The shade dried pulverized seaweed (20 g) powder was treated with 1 l of 85% ethanol with constant stirring for 12 h at room temperature in order to remove proteins and pigments. The ethanol treated seaweed was washed with acetone, centrifuged at 10000 ×g for 10 min. and then dried at room temperature. The dried biomass (5g) was extracted with 100 ml of distilled water at 65°C with continuous stirring for 1h twice, and the extracts were combined. The combined extract was centrifuged at 10000×g for 20 min. and the supernatant was treated with 1% of CaCl2 and kept at 4°C for overnight to precipitate alginic acid after centrifugation at 10000×g for 20 min. and the supernatant was collected. Ethanol was added into the supernatant to obtain a final ethanol concentration of 30%, and the solution was placed at 4°C for 4h in a chill cabinet. Again, the solution was centrifuged at 10000×g for 20 min. to remove the remaining impurities as residue. Finally, ethanol was added into the supernatant to obtain a final ethanol concentration of 70%, and then placed at 4°C overnight to precipitate out the intact fucoidan. After centrifugation at 10000×g for 15 min, the residue fucoidan was washed with ethanol and acetone, and again dried at room temperature. The yield was calculated based on the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of the obtained ucoidan(g)} \times 100}{\text{Weight of the dried biomass (g)}}$$

Microbial Culture Preparation

Type cultures of two Gram–negative fish bacterial pathogens viz., Aeromonas hydrophila(MTCC No.1739) and Pseudomonas fluorescens(MTCC No. 9856) and three Gram positive bacteria viz., Bacillus subtilis(MTCC No. 6633), Staphylococcus aureus(MTCC No.98) and Micrococcus luteus (MTCC No. 106 Type A) were obtained from Indian Institute of Microbial Technology

(IIMT), Chandigarh. Young cultures were prepared from type cultures by incubating them in nutrient broth for18 hours at 37°C. The cultures were again diluted with physiological saline in order to obtain an OD value of 0.14 at 540nm, which gives approximately 1x108cells/ml.

Screening of antibacterial activity

Antibacterial activity of solvent extracts of brown seaweeds and water extract of fucoidan extracted from seaweeds was performed by agar plate diffusion assay. For which, Muller Hinton agar was poured into sterile petri dishes and the diluted young type cultures were swabbed on agar medium using sterilized buds. Sterilized antibiotic discs (6mm dia) containing the solvent and crude extracts of fucoidan were individually placed onto the prepared petri dishes. A pre-diffusion for 3 h with all extracts were ensured. Oxytetracycline impregnated discs were used as positive control. The plates were then incubated at 37°C for 18 h. The antibacterial activity was observed by measuring the inhibition zones excluding the diameter of the disc (6 mm). Inhibition zones above 15 mm were declared as strong, from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities [6]. In all the experiment, a negative control with all the solvents (without extract)

Table 1. Antibacterial activity of solvent and water extracts of some brown seaweeds

Brown seaweed species	Solvent extraction / Water extraction (Yang et al.2008)	Activity against pathogens Zone of inhibition(mm)				
		AH	PF	ML	SA	BS
Sargassum wightii	Dichloromethane	2.1±0.2	-	-	-	-
	Methanol	-	**8.5±0.3**	8.1±0.1	**10.2±0.3**	8.6±0.4
	Acetone	7.5±0.3	7.3±0.1	6.8±0.4	8.4±0.4	8.6±0.3
	Water extraction	2.0±0.3	3.0±0.1	ND	ND	ND
S. oligocystum	Dichloromethane	-	-	-	2.1±0.2	2.1±0.2
	Methanol	2.1±0.1	6.2±0.3	4.0±0.1	**10.2±0.2**	5.9±0.2
	Acetone	3.09±0.3	7.0±0.2	6.2±0.3	**10.2±0.5**	8.2±0.4
	Water extraction	2.0±0.2	-	ND	ND	ND
S. vulgare	Dichloromethane	-	-	-	-	4.0±0.1
	Methanol	2.5±0.3	-	2.0±0.2	-	4.0±0.1
	Acetone	2.5±0.3	-	4.5±0.3	2.5±0.3	6.2±0.1
	Water extraction	ND	ND	ND	ND	ND
Turbinaria ornata	Dichloromethane	-	6.0±0.2	4.1±0.2	-	5.9±0.2
	Methanol	6.6±0.3	6.1±0.2	8.1±0.1	8.0±0.2	**13.0±0.2**
	Acetone	**9.0±0.2**	4.1±0.2	**11.5±0.3**	10.05±0.4	12.0±0.6
	Water extraction	4.7±0.4	4.4±0.4	6.0±0.1	4.6±0.3	4.2±0.4
Padina tetrastromatica	Dichloromethane	-	-	-	-	4.7±0.4
	Methanol	6.7±0.4	6.1±0.2	**11.5±0.4**	6.0±0.2	8.0±0.1
	Acetone	6.5±0.4	**9.6±0.4**	**11.3±0.4**	10.2±0.2	7.9±0.2
	Water extraction	4.4±0.3	4.5±0.3	6.2±0.3	6.1±0.4	6.3±0.2
Stoechospermum marginatum	Dichloromethane	2.1±0.2	-	-	-	2.1±0.4
	Methanol	2.0±0.3	2.2±0.3	6.5±0.3	6.1±0.3	8.1±0.3
	Acetone	6.5±0.3	4.6±0.3	6.0±0.2	6.1±0.3	6.7±0.4
	Water extraction	ND	ND	ND	ND	ND
Oxytetracycline (20µg/disc)	-	15.2±0.7	17.2±0.4	23.3±1.7	23.2±0.4	22.5±0.8

AH=Aeromonas hydrophlia(MTCC No: 1739);; PF=Psuedomonas fluorescens(MTCC No:9856); ML= Micrococcus luteusMTCC No:106; SA=Staphylococcus aureus(MTCC No:98); BF=Bacillus subtilis(MTCC No: 6633); ND=Not Determined; (1-8mm) equals low activity; (8-15mm) equals moderate activity ; (>15 mm) equals high activity ; (–)equals no visible zone.

was also used, but none of them showed activity.

RESULTS

Antibacterial activity of organic solvent extracts of brown seaweeds

The results of the antimicrobial activity of the organic extracts of brown seaweeds are summarized in Table.1. Of the six brown seaweeds tested, five exhibited antibacterial activities against all bacteria. The controls never indicated a positive activity. Organic extracts of seaweeds, in general had strong activity against Gram positive bacteria than Gram negative bacteria. Methanol and acetone extracts showed significant inhibitory effects against the pathogens viz., *Aeromonas hydrophila, Psuedomonas fluorescens, Micrococcus luteus, Staphylococcus aureus* and *Bacillus subtilis*. Dichloromethane extracts did not show good activity.

Fig.1 Water extract of fucoidan from *S. wightii*

Extracts of *T .ornata, P. tetrastromatica, Stoechospermum marginatum* exhibited strong, moderate and weak activity, respectively against all pathogens tested. In particular, the extracts derived from the organic solvents like methanol followed by acetone displayed significant activity against all microorganisms studied. Methanolic extracts of *T. ornata* had much higher activity against *M. luteus*. Methanol and acetone extracts of T.ornata, P.tetrastromatica exhibited promising inhibition effects against *P. fluorescens, M. luteus*, and *S. aureus*(Fig.2&3). Considerable activity against *A. hydrophlia, M. luteus, S. aureus* and *B. subtilis* were observed by methanol extracts of *T. ornata and P. tetrastromatica*. Moderate activity was expressed by methanol extracts of S.marginatum against B.subtilis (8.1±0.3mm diameter)

With respect to methanol extracts, five seaweeds exhibited moderate or weak antimicrobial activity, except Sargassum vulgare. Seaweed, Turninaria ornata revealed high activity against B.subtilis with diameter of inhibition greater than 13.0±0.2 mm while P. tetrastromatica exhibited only moderate activity against P.fluorescens and other two Gram positive bacteria viz, M. luteus and S.aureus.

Antibacterial activity of water extracts of fucoidan

Crude fucoidan extracted from four seaweeds viz. *Sargassum wightii, S.oligocystum, Padina tetrastromatica* and *Turbinaria ornata* were studied and result showed that only slight inhibition zone was noticed with fucoidan (4mg/ml) extracted from T.ornata and *P. tetrastromatica*, M. luteus and *B. subtilis*, respectively. All other species extract exhibited only poor or no activity against all test organisms. Therefore, it could be established that water extracts of fucoidan was not found to be suitable for antibacterial activities.

DISCUSSION

Seaweeds are used as a valuable source of bioactive compounds in food, pharmaceutical, nutraceutical and

Fig.2 Acetone and methanol extracts of *Padina tetrastromatica* against Gram positive bacteria *Micrococcus luteus* (D-Dichloromethane, A-Acetone, M-Methanol)

biomedicine industries. The results of the present study indicated that methanol extracts of Turbinaria ornata had strong antibacterial activities against Bacillus subtilis with inhibition zone of 13.0±0.2 mmthan the other extracts of brown seaweeds. Many compounds from derived from seaweeds viz.,polyphenols, flavonoids and polysaccharides having antioxidant and antimicrobial activities [15]. The higher antibacterial activity exhibited by T. ornata collected from Gulf of Mannar coast might by due to its high phenolic content (43.72 ± 1.63 mg GAE/gof extract) [16]. These phenolic compounds are secondary metabolites; characterized by an aromatic ring with one or more hydroxyl groups and the antimicrobial action is due to the alteration of microbial cell permeability and the loss of internal macromolecules or by the interference with the membrane function and loss of cellular integrity and eventual cell death [1]. Earlier studies also indicated the methanolic extract had a stronger antibacterial activity [17,18,19,20]. Methanolic and ethanolic extracts of *T. ornate* also contained higher phenolic content than Sargassum polycystum without chlorophyll removal [21] and they also observed that the antibacterial activity increased with increasing phenolic content. As phenols reacts primarily with the phospholipids component of bacterial cell membrane as constitutes for the antimicrobial activity of extract [22]. A series of polyphenolic compounds such as catechins, flavonols and flavonol glycosides have been identified from methanol extracts of red and brown algae [23] and found to have antioxidant and antimicrobial activity. Thus, the methanolic extractof *T. ornata* that contains the phenolic compounds was responsible for strong antibacterial activity than the other extracts of brown seaweeds in the study.

Moderate antibacterial activity was observed in methanol and acetone extracts of *P. tetrastromatica* against *M. luteus* with inhibition zone of 11.5±0.4 and 11.3±0.4 mm, respectively. The extracts of *T. ornate* and *P. tetrastromatica* exhibited strong and moderate activities against the growth of Gram positive than Gram negative (fish pathogenic) bacteria. Earlier studies revealed that extracts of brown algae were active against a number of Gram-positive and Gram-negative organisms [9]. Similarly it was observed that methanolic extracts of *T. ornata* exhibited the highest inhibition against *S. aureus* than *S. polycystum* [21]. Earlier report indicated that Gram positive bacteria, *B. cereus* were more susceptible to the seaweed extracts of *S. polycystum* than other bacterial strains [24]. Methanol extracts of various species of seaweeds isolated from Gulf of Mannar on multidrug-resistance bacterial strains like *E. coli, Klebsiella pneumoniae, P. aeruginosa, Salmonella typhi, Vibrio cholerae* and *Staphylococcus aureus* has exhibited differential activity [7,8]. It is clear from earlier reports that organic solvent always exhibit relatively higher efficiency in extracting some compounds for antibacterial activities compared to water or enzyme-based methods [6,25]. The extraction of antimicrobials from different species of seaweeds was solvent dependent and methanol was good for brown seaweeds and acetone for red, green seaweeds [15]. In the present study also it has been proved that methanol was found to be a better solvent for testing antibacterial activity.

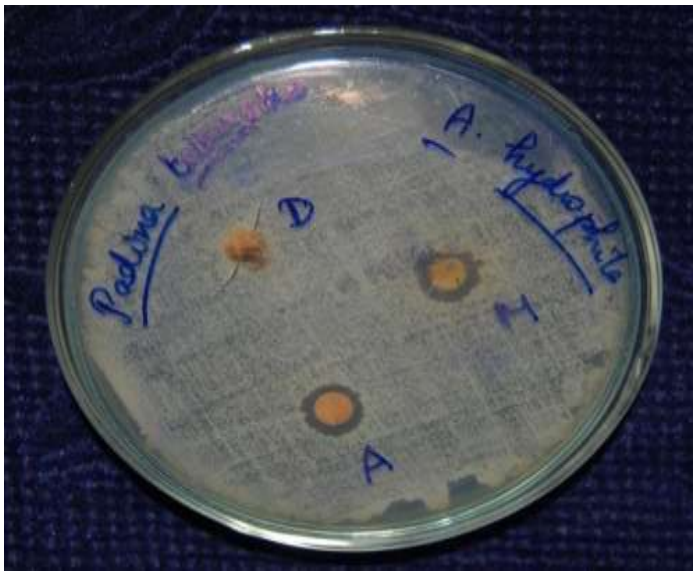

Fig.3.Acetone and methanol extracts of Padina tetrastromatica against Gram negative bacteria A.hydrophila

A series of small molecular volatile halogenated compounds (halomethanes and haloether) were observed to be effective against fish pathogenic

bacteria [26]. The substances isolated from green, brown and red algae showing potent antimicrobial activity belong to polysaccharides, fatty acids, phlorotannins, pigments, lectins, alkaloids, terpenoids and halogenated compounds [3]. Several extractable compounds such as cyclic polysulfides and halogenated compounds are toxic to microorganisms and they are responsible for the antibiotic activity of seaweeds. Therefore, halogenated compounds extracted by methanol-ethanol solvents present in the seaweeds could be responsible for the antibacterial activity against fish pathogenic bacteria in the present investigation. However, the influence of some natural factors, such as the environmental conditions viz., light, temperature, salinity, the life stage, reproductive stateand age of the seaweed, and the geographical location and seasonality, allowed for the consideration that this antimicrobial activity was not attributed to a single compound, but it could be related to some of them and to a combination of metabolites. Seaweed or macroalgae provide a great variety of metabolites and natural bioactive compounds with antimicrobial activity, such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds, and carotenoids [27].

Water extracts of fucoidan showed weak or no activity against all organisms tested and hence water extracts of fucoidan is not suitable for antibacterial activity.Thus, the present study suggests that the antibacterial activity of methanol and acetone extracts of *T. ornata* and *P. tetrastromatica* than other brown seaweed species from Gulf of Mannar makes it an attractive potential natural antimicrobial marine source to replace the use antibiotics in aquaculture systems. However, active compound responsible for antibacterial activity need to be studied in future.

CONCLUSION

From the study, it could be concluded that the brown seaweed species viz., *S. wightii, P. tetrastromatica, T. ornata* and *Stoechospermum marginatum* along the Gulf of Mannar coast in South east coast of India were found to have antibacterial activity against Gram positive (*Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis*) and Gram Negative fish pathogens (*Aeromonas hydrophila, Pseudomonas fluorescens*). The activity varies between the seaweed species and the solvents and its polarity. From the experiment, it could be inferred that the methanol was found to be the good solvent to get extract having better antibacterial activity against Gram positive bacteria than the other solvents for brown seaweeds. The results revealed that relatively higher inhibition zones in methanol - acetone extracts of *T. ornata* and *P. tetrastromatica* than other species. Further, water extracts of fucoidan is not suitable for antibacterial activity. The study therefore infers that, there is a tremendous seaweed resource potential in Gulf of Mannar coast of south east India shall be explored further to examine their bioactive potential to replace the use antibiotics in aquaculture practices.

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