

Bifunctional effect of fucoidan from *Padina tetrastromatica* against human pathogenic microbes and free radicals

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ABSTRACT

The antibacterial and antioxidant effect of fucoidan fractions isolated from brown algae *Padina tetrastromatica* was evaluated. Even though the polysaccharide was found to be a fucan, the composition of this polysaccharide is different from those reported, and the antibacterial and antioxidant effect has not been reported so far. Three fractions (F1, F2, and F3) were isolated by anion-exchange column chromatography. Chemical analysis suggested that the polysaccharide fractions contained a significant amount of sulfate and fucose, galactose xylose, and mannose as the major neutral sugars. Antibacterial activity was checked by disk diffusion method. Antioxidant activity was investigated by various *in vitro* systems, including 1,1-diphenyl-2-picrylhydrazyl radical scavenging, lipid peroxide inhibition, superoxide and hydroxyl radical scavenging activity, chelating ability and reducing power. Antibacterial and antioxidant assays suggested that the polysaccharide fraction F3 possessed good antibacterial activity and had stronger antioxidant properties than F1 and F2. Available data obtained by *in vitro* models suggested that the correlation between the sulfate content and pharmacological effect was positive. Fucoidan from *P. tetrastromatica* have the potential to be developed as an antibacterial and antioxidant agent, but further *in vivo* research for their mode of action are still needed to shed light on the effects. Overall, the present experiments showed fucoidan from marine brown algae as a potential therapeutic agent.

KEY WORDS: Antibacterial activity, Fucoidan, Marine algae, Sulfated polysaccharide, 1,1-diphenyl-2-picrylhydrazyl radical scavenging

INTRODUCTION

Humankind has known for the last several 1000 years that marine organisms contain substances capable of potent biological activity. However, the first serious investigation of marine organisms started only half a century ago. Today, research has been focused on finding therapeutic agents with selective pharmacology and less toxicity. As a consequence of increasing demand, serious research is on the way for seeking beneficial agents from natural sources, especially from marine organisms which are known to have a rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential (Ravikumar *et al.*, 2002). Among them, marine algae represent one of the richest sources of bioactive compounds, and algae-derived products are increasingly used in medical and biochemical research (Mayer and Lehmann, 2002). One particularly interesting feature of marine algae is their richness in

sulfated polysaccharides, the uses of which span from food, cosmetic, and pharmaceutical industries to microbiology and biotechnology. These macromolecules have been proven to show a wide range of biological activities such as antimicrobial, antitumoral, anti-inflammatory, antioxidant, and anticoagulant activity (Cumashi *et al.*, 2007 and Ghosh *et al.*, 2009).

Polysaccharides obtained from marine algae include fucoidan, ascophyllan, alginate, carragenean, and agar as phycocolloids have been used for decades in medicine and pharmacy. Fucoidan is a sulfated-fucan, one of a water-soluble non-starch polysaccharide (NSP) of complicated chemical structures, commonly found in brown seaweeds (Rioux *et al.*, 2007). Its structure and composition vary with the species, but it mainly consists of fucose molecules linearly linked by α -(1 \rightarrow 3), α -(1 \rightarrow 3)-(1 \rightarrow 4), or α -(1 \rightarrow 3)-(1 \rightarrow 2) and contains minor amounts of other

sugars such as xylose, galactose, mannose, and glucuronic acid (Chizhov *et al.*, 1999 and Duarte *et al.*, 2001). Since algae have been used in traditional medicine for a long time and also some algal substances have bactericidal and antioxidant activity, they have been extensively studied by several researchers.

Antioxidants are free-radical scavengers (FRS) which postpone the oxidation block the reaction chain initiated by high energy molecules and other consequent reactions (Halliwell and Aruoma, 1991 and Cespeles *et al.*, 2008). Although many synthetic antioxidants are promising for various human ailments, their pro-oxidant or cytotoxic nature at higher concentration prevents them from long term use. Similarly, a number of studies concerning the search for new antimicrobial agents from plants and antimicrobial screening of several compounds have been performed (Hamburger and Hostettmann, 1991). Antimicrobial peptides constitute a heterogeneous class of low molecular weight proteins, which are recognized as important components of the innate defense system of both animals and plants and they directly interfere with the growth, multiplication, and spread of microbial organisms (Garcia-Olmedo *et al.*, 1998 and Lehrer and Ganz, 1999).

Over the past decades, seaweeds produce a variety of compounds and some of them have been reported to possess the biological activity of potential medicinal value. Therefore, new interest has been developed to search natural and safe antibacterial and antioxidative agents from marine sources. A sulfated fucose-rich polysaccharide isolated from *Padina tetrastromatica*, marine brown algae widely distributed in the south east coast of India, especially Kerala was found to be a fucan. However, on a detailed study, it has been found that the composition of the polysaccharide is different from that reported so far. The uses and potential values of bioactive molecules from this species have not been well studied so far. So, in this study, the antibacterial and antioxidant effect of fucoidan from *P. tetrastromatica* was studied.

MATERIALS AND METHODS

Chemicals and Solvents

All biochemicals used in this study were purchased from Sigma Chemical Company, St. Louis, MO, USA and all other chemicals used were of the highest grade available.

Sea Weed Material

Samples of *P. tetrastromatica* were collected from the Vizhinjam coast of Kerala, located at 8°21'N and 77°0'E

on the west coast of India. The seaweeds were washed thoroughly with tap water, dried by forced air circulation, and pulverized in a waring blender. Depigmentation of 150 g of algal powder was done using sequential extraction with petroleum ether and acetone as a solvent in a Soxhlet apparatus. The residue material was placed in a plastic beaker and air-dried to yield an algal powder.

Extraction of Fucoidan

Fucoidan was extracted by the method as described by (Zvyagintseva *et al.*, 1999). Briefly, 10 g of algal powder was extracted thrice at 75°C with 250 mL of distilled water and adjusted the pH 3.0 by the addition of 2.0 mol/L HCl. The combined extract was concentrated to approximately one-tenth volume, and then twice the final volume of ethanol was added to the solution. Stirred well the resulting precipitate was collected by centrifugation at 3,000 rpm for 20 min, dissolved in distilled water, and the pH of the solution was adjusted to 2.0 with 2.0 mol/L HCl. The solution was left for 1 h, and the insoluble material was then removed by vacuum filtration. The filtrate was concentrated to one-tenth volume, and twice the final volume of ethanol was added. The precipitate was collected by centrifugation at 3000 rpm for 20 min and was washed with ethanol dried at 60°C for 24 h to get crude fucoidan fraction.

Anion-exchange Chromatography

The extracted fucoidan was purified by the method as described by (Worawattanamateekul *et al.*, 1993). Crude fucoidan was applied to a DEAE-Cellulose column (17 cm × 2.5 cm) equilibrated with 0.5 M sodium acetate (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate 15 mL/h with a linear gradient of 0.2-2.0 M NaCl containing 50 mM sodium acetate (pH 5.0). Fractions of 5 mL were collected and measured for polysaccharide by phenol-H₂SO₄ assay. Appropriate fractions were pooled, dialyzed, and lyophilized.

Chemical Analysis

The total polysaccharide was estimated by the phenol-H₂SO₄ method (Dubois *et al.*, 1956). After acid hydrolysis of the purified polysaccharide by hydrochloric acid, the sulfate content was determined using a modified BaCl₂-turbidimetric method (Dodgson, 1961) Total fucose was measured by the method (Dische and Shettles, 1948). The total uronic acid content was determined by the carbazole-sulfuric acid method (Bitter and Muir, 1962). Xylose was estimated by orcinol-sulfuric acid method

(Bruckner, 1952). Galactose content was estimated by modified phenol-H₂SO₄ method (Dubois *et al.*, 1956). Protein content was measured by the method (Lowry *et al.*, 1951).

Antibacterial Activity

Test bacteria

The bacterial strains were obtained from the stock culture collection maintained in the Department of Biotechnology, University of Kerala, Thiruvananthapuram, Kerala, India. *In vitro* antibacterial activity of the fucoidan fractions were evaluated against three Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* MTCC 1771, and the two Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* MTCC 441. All the strains were stored in the appropriate medium before use.

Inhibitory Effect by Disc Diffusion Method

Antibacterial activity of fucoidan fractions was determined by disc diffusion method (Bauer, 1966). Briefly, 100 µL of a suspension containing 10⁸ CFU/mL of bacteria were spread on the inoculated agar. Empty sterilized discs were impregnated with 20 µL of polysaccharide fractions of various concentrations (50-200 µg/mL). Ampicillin (10 µg/disc) was used as a positive reference standard. The inoculated plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition (ZOI) in mm against the test organisms. The experiments were repeated in triplicate, and the results were expressed as average values.

Determination of Minimum Inhibitory Concentration (MIC)

A broth microdilution method was used to determine the MIC (Mazzanti *et al.*, 2000). All tests were performed in Mueller-Hinton medium. Serial double dilutions were prepared with a mixture of fucoidan fractions:dimethylsulfoxide (95:5) in a 96-well microtiter plate over the range of 10-125 µL/L. Overnight broth cultures of each strain were prepared, and the final concentration of the microbe in each well was adjusted to 2 × 10³ cfu/mL. Plates were incubated at 37°C for 24 h and the absorbance was measured at 630 nm. The samples were analyzed in duplicate, and the assay was repeated twice. The antibiotic ampicillin was used as positive control. The wells showing complete absence of growth were identified, and 10 µL of each well were transferred to Mueller-Hinton agar plates and incubated at previously mentioned times and temperatures. The

concentration in which no growth was observed is the MIC.

FRS Activity of Fucoidan Fractions

The FRS activity of fucoidan fractions was evaluated by the following *in vitro* methods, and the results were compared with standard antioxidant quercetin.

1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The scavenging activity of the DPPH free radical by fucoidan fractions was assayed by the method described by (Karagozler *et al.*, 2008) with a slight modification. In brief, 2 mL of fucoidan fractions of different concentrations (1.5-1000 µg/mL) were added to 2 mL 0.2 mM ethanol solution of DPPH, the reaction mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance of the resulting solution was measured at 517 nm. The ability of scavenging the DPPH radicals was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where, A₀ is the absorbance of DPPH solution without the tested samples and A₁ is the absorbance of the tested samples with DPPH solution. Values of the concentration of samples required to scavenge 50% of free radicals or to prevent lipid peroxidation by 50% (IC₅₀) were calculated from the regression equations prepared from the concentration of samples and percentage inhibition of each system.

Liver Microsomal Lipid Peroxidation

The effects of fucoidan fractions on lipid peroxidation were determined by the method described by (Liu *et al.*, 1997). The liver microsomes were prepared from Wistar rats. The liver was homogenized in ice-cold 0.25 M sucrose and then centrifuged at 12,000 × g for 20 min at 4°C. The supernatant obtained was centrifuged at 15,000 × g for 60 min at 4°C. The microsomes were washed with ice-cold 0.15 M KCl and then stored at -20°C. The lipid peroxidation assay was performed in Fe²⁺/Vitamin C system. The microsomes (300 µg/mL) were incubated at 37°C for 60 min with varying concentrations of fucoidan fractions (1.5-1000 µg/mL), 10 mM FeSO₄·7H₂O, and 0.1 mM ascorbic acid in 1.0 mL potassium phosphate buffer (0.2 M, pH 7.4). The reaction was stopped by the addition of 20% trichloroacetic acid (1.0 mL) and 0.67% 2-thiobarbituric acid (TBA) (1.5 mL) in succession, and the solution was then heated at 100°C for 15 min.

The condensation reaction occurring between the malondialdehyde and TBA produces a pink compound, which has a strong absorption at 532 nm. The percentage of antioxidant activity of the samples was evaluated according to the following formula.

Inhibition (rate %) = $(A_0 - A) / (A_0 - A_c) \times 100$, where A_0 is the absorbance of the free-radical generation system, A is the absorbance of the test sample and A_c is the absorbance of the essential control.

Hydroxyl Radical Scavenging Activity

Hydroxyl radical-scavenging activity was measured by a modified method (Smironoff, 1989). The reaction mixture containing fucoidan fractions of different concentrations (1.5-1000 $\mu\text{g}/\text{mL}$) were incubated with 2 mM EDTA-Fe (0.5 mL), 3% H_2O_2 (1 mL), and 360 $\mu\text{g}/\text{mL}$ crocus in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4) for 30 min at 37°C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. In control, the sample was substituted with distilled water, and sodium phosphate buffer was replaced with H_2O_2 .

Superoxide Anion-Scavenging Activity

The effect of fucoidan fractions on scavenging superoxide radical was determined by the nitroblue tetrazolium (NBT) reduction method (Nishikimi *et al.*, 1979). Briefly, 1 mL of NBT solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL nicotinamide adenine dinucleotide solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4), and 0.1 mL of the samples (1.5-1000 $\mu\text{g}/\text{mL}$) were mixed. The reaction was started by adding 100 μL of phenazine methosulfate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. The positive control (the sample was replaced with quercetin) and the negative control (only the solvent was added) were subjected to the same procedures described above as the sample. All measurements were made in triplicate and averaged. The abilities to scavenge the superoxide radical were calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - A_1/A_0] \times 100$$

Where, A_0 is the absorbance of control without the tested samples and A_1 is the absorbance in the presence of the tested samples

Metal Chelating Assay

The ferrous ion-chelating ability of fucoidan fractions were investigated by slightly modified method (Decker and Welch, 1990). Samples in different concentrations (1.5-1000 $\mu\text{g}/\text{mL}$) were mixed with FeCl_2 (0.05 mL, 2 mM) and ferrozine (0.2 mL, 5 mM), shook well, stayed still for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. In the control, the sample was substituted with EDTA. The ferrous ion-chelating activity was given by the following equation:

$$\text{Chelating ability (\%)} = [1 - A_1/A_0] \times 100$$

Where, A_0 is the absorbance of control without the tested samples and A_1 is the absorbance in the presence of the tested samples

Reducing Power Assay

The reducing power of fucoidan fractions was determined by a modified method described by (Yen and Chen, 1995). Briefly, 0.13 mL of samples in different concentrations (1.5-1000 $\mu\text{g}/\text{mL}$) in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 mL of potassium ferricyanide (1%, w/v) and incubated at 50°C for 20 min. Afterward, 0.125 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. Then the solution was mixed with 1.5 mL ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm. Increased absorbance indicates increased reducing power.

Statistical Analysis

All experimental results were expressed as means \pm standard deviation. Analysis of variance was performed by ANOVA procedures. The correlation coefficient (R) was used to determine two variables. SPSS software was used for statistical calculations. The results with $P < 0.05$ were regarded to be statistically significant.

RESULTS

Three fractions were eluted during purification step of fucoidan fraction by anion-exchange chromatography with 0.5 M, 0.7 M, and 2.5M sodium acetate buffer. (Fraction-1, F1), (Fraction-2, F2), and (Fraction-3, F3) (Figure 1). The F3 fraction contains a significantly high amount of fucose, sulfate, and uronic acid when compared to other two fractions. The composition of the purified polysaccharide is given in Table 1.

Effect of Fucoidan on Bacterial Species

The results of antibacterial activity against bacterial species are summarized in Table 2 and Figure 2. Among the fractions of polysaccharide, F3 was found to be more active against the microorganism tested. The MIC of the polysaccharide fraction was determined at concentrations ranging from 16 to 55 µg/mL. The ZOI for most of the bacterial strains were in the range of 2.4±0.09 to 26.6±1.28 mm.

The F3 (200 µg/mL) of *P. tetrastromatica* exhibited highest zones of inhibition against *B. subtilis* and *S. aureus* (26.6±1.28 and 24.6±1.18 mm, respectively), whereas the F2 fraction (200 µg/mL) was found to be almost equally effective against *S. typhimurium* and *S. aureus* (21.3±1.05 and 23.4±1.13 mm, respectively). On the other hand, F1 fraction (200 µg/mL) was found to be effective against only to *S. typhimurium* (14.3±0.68 mm). MIC values were lowest for F3 against *B. subtilis* and *S. aureus* (9.6 µg/mL and 11.2 µg/mL).

One prominent fucoidan concentration (200 µg/mL) was taken for MIC test by two fold serial dilution method. MIC of fucoidan was tested against *S. typhimurium* and *B. subtilis*. The test organisms were inoculated in various concentrations of the F3 fractions (i.e., 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL). The ZOI values of the F3 fraction are shown in Figure 3. There was the appreciable growth of bacteria in all other concentrations except in 200 µg/mL and 100 µg/mL.

Table 1: Chemical composition of fucoidan fractions of *P. tetrastromatica*

Component (%)	Crude Fucoidan	F1	F2	F3
Sulfate	28.8	6.1	17.3	25.2
Fucose	21.5	9.8	16.1	31.5
Uronic acid	17.2	8.4	10.3	22.7
Xylose	15.8	10.8	13.3	25.2
Galactose	13.6	9.2	12.8	23.3
Mannose	16.4	5.6	9.2	20.5
Protein	8.1	2.2	1.6	0.82

P. tetrastromatica: *Padina tetrastromatica*

Table 2: Antibacterial activity of fucoidan fractions of *P. tetrastromatica*

Bacterial species	Zones of inhibition (mm)												Ampicilium (10 µg/ml)
	Fraction F1				Fraction F2				Fraction F3				
	5 µg/ml	10 µg/ml	20 µg/ml	MIC µg/ml	5 µg/ml	10 µg/ml	20 µg/ml	MIC µg/ml	5 µg/ml	10 µg/ml	20 µg/ml	MIC µg/ml	
<i>E. coli</i>	0±0.00	0±0.00	7.9±0.38	45	8.7±0.42	12.4±0.61	15.5±0.74	40	5.9±0.28	19.6±0.84	22.2±1.07	15	27.14±1.38
<i>S. typhimurium</i>	0±0.00	9.1±0.1	14.3±0.68	35	2.4±0.11	16.7±0.80	21.3±1.05	25	0±0.00	10.4±0.50	19.5±0.94	20	30.52±1.47
<i>B. subtilis</i>	0±0.00	0±0.00	9.7±0.46	40	0±0.00	0±0.00	20.2±0.97	30	2.4±0.09	19.8±0.95	26.6±1.28	10	25.57±1.23
<i>S. aureus</i>	0±0.00	0±0.00	5.4±0.26	50	9.8±0.47	15.4±0.74	23.4±1.13	25	9.2±0.43	21.2±1.02	24.6±1.18	10	24.64±1.19
<i>P. vulgaris</i>	0±0.00	0±0.00	10.2±0.49	40	0±0.00	0±0.00	4.6±0.22	40	0±0.00	6.6±0.32	18.5±0.89	20	27.21±1.31

E. coli: *Escherichia coli*, *B. subtilis*: *Bacillus subtilis*, *S. typhimurium*: *Salmonella typhimurium*, *S. aureus*: *Staphylococcus aureus*, *P. vulgaris*: *Proteus vulgaris*, MIC: Minimum inhibitory concentration, *P. tetrastromatica*: *Padina tetrastromatica*

Free-radical-scavenging Activity of Fucoidan Fractions on 2,2-diphenyl-1-picrylhydrazyl Radical

Figure 4 revealed the FRS activity of fucoidan fractions. The scavenging abilities of all samples were in a concentration-dependent manner. At a concentration of 10 µg/mL, F1 showed maximum inhibition of 26.89% and F2 fraction showed maximum scavenging ability of 55.98% at 25 µg/mL concentration on DPPH radicals. At 250 µg/mL concentration, F3 and quercetin attain the maximum scavenging effect of 86.06% and 75.461%. The present results proved that the sulfated polysaccharide isolated from *P. tetrastromatica* was good scavengers, and the sulfate group played an important role in the scavenging of DPPH radicals.

Inhibitory Effect of Fucoidan Fractions on Lipid Peroxidation

The dose-dependent inhibition of lipid peroxides by fucoidan fractions of *P. tetrastromatica* is shown in Figure 5. F1 and F2 fraction showed maximum inhibition of 14.71% at 1000 µg/mL and 19.62% at 500 µg/mL concentration. F3 showed its maximum inhibition of lipid peroxides of 86.06% at a concentration of 1000 µg/mL, whereas quercetin showed its maximum peroxides inhibition of 84.01% at 1000 µg/mL. The inhibition of lipid peroxidation by F3 might probably due to its FRS potential and thereby proves to be a good antioxidant and cytoprotective agent.

Hydroxyl Radical-scavenging Activity of Fucoidan Fractions

The results of hydroxyl radical scavenging ability of fucoidan fractions are shown in Figure 6. F1 and F2 fraction showed a maximum inhibition of 8.09% and 16.89% at a concentration of 50 µg/mL. The scavenging ability of F3 and quercetin on hydroxyl radicals was in a concentration-dependent manner. At a concentration of 1000 µg/mL, the scavenging activities were 86.06% and 97.41% for F3 and quercetin, respectively.

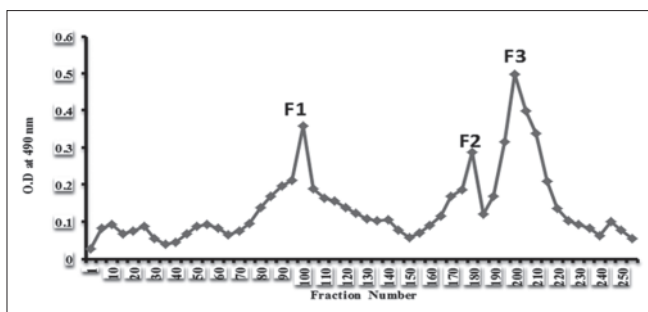


Figure 1: Purification of fucoidan by anion-exchange chromatography

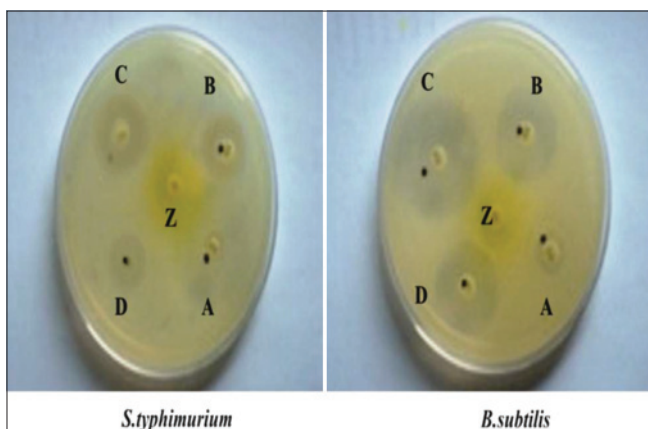


Figure 2: Effect of fucoidan fractions of *Padina tetrastromatica* on the growth of *Salmonella typhimurium* and *Bacillus subtilis* by disk diffusion method. A: F3 fraction (50 µg/mL), B: Ampicillin (100 µg/mL), C: F3 fraction (100 µg/mL), D: F3 fraction (200 µg/mL), Z: Phosphate buffer (10 µL)

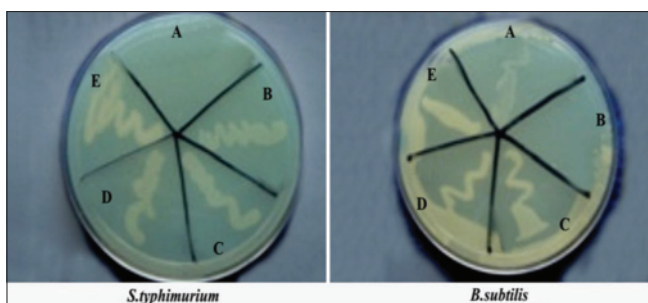


Figure 3: Determination of minimum inhibitory concentration of fucoidan fractions of *Padina tetrastromatica* against *Salmonella typhimurium* and *Bacillus subtilis*. A: F3 fraction 200 µg/mL, B: F3 fraction 100 µg/mL, C: F3 fraction 50 µg/mL, D: F3 fraction 25 µg/mL, E: F3 fraction 12.5 µg/mL

Superoxide Anion-Scavenging Activity of Fucoidan Fractions

The scavenging effects of fucoidan fractions on superoxide radicals were shown significantly in a concentration-dependent manner. A significant increase of the scavenging activity was observed at the concentration range (50-500 µg/mL) of the F3 fraction.

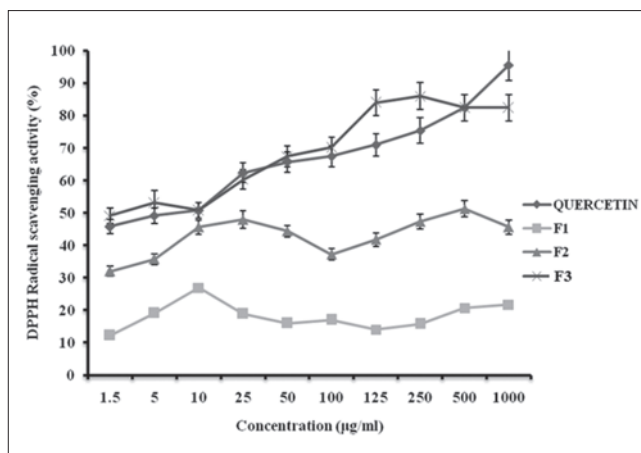


Figure 4: Scavenging effect of fucoidan fractions of *Padina tetrastromatica* on 1,1-diphenyl-2-picrylhydrazyl radical. Values are expressed as average of three samples ± standard error of mean

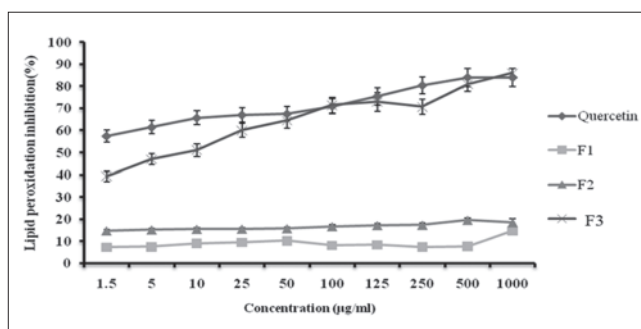


Figure 5: Inhibitory effect of fucoidan fractions of *Padina tetrastromatica* on lipid peroxides. Values are expressed as average of three samples ± standard error of mean

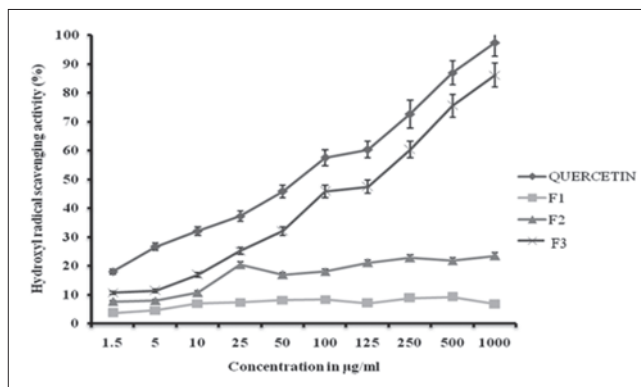


Figure 6: Scavenging effect of fucoidan fractions of *Padina tetrastromatica* on hydroxyl radicals. Values are expressed as average of three samples ± standard error of mean

At a concentration of 500 µg/mL, the F1 fraction showed 8.27% scavenging activity. The superoxide radical scavenging activity of F2 fraction is 9.09% at a concentration of 100 µg/mL. However, the scavenging effect of quercetin was only 71.01% at a concentration of 500 µg/mL (Figure 7).

Ferrous Ion-Chelating Effect of Fucoidan Fractions

The ferrous ion-chelating effects of fucoidan fractions were concentration dependent. At a concentration of 500 µg/mL, the chelating effect of F3 was stronger than that of F1 and F2, but it was not significant. At high concentration, the effects of quercetin were more pronounced than that of polysaccharide fractions (Figure 8).

Reducing Power of Fucoidan Fractions

Reducing power of fucoidan fractions is shown in Figure 9. Higher absorbance value means stronger reducing power of samples. The reducing power of F1 and F2 fractions at 1000 µg/mL were 0.056 and 0.07, respectively which were much weaker than those of F3 and quercetin. Although the reducing power of all samples was low in the tested concentration, F1, and F2 were concentration dependent. At 1000 µg/mL F3 and quercetin showed reducing power of 0.12 and 0.14, respectively.

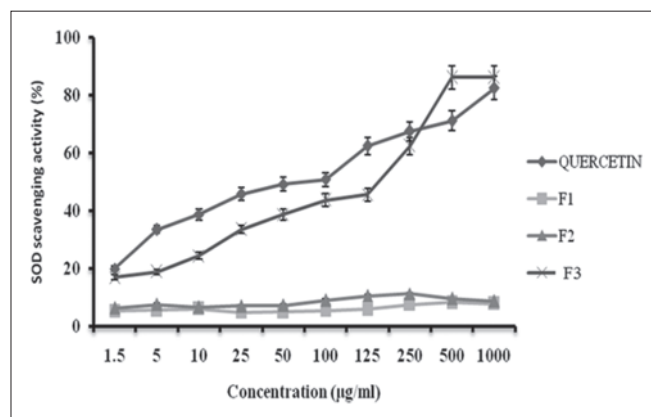


Figure 7: Scavenging effect of fucoidan fractions of *Padina tetrastromatica* on superoxide radical. Values are expressed as average of three samples ± standard error of mean

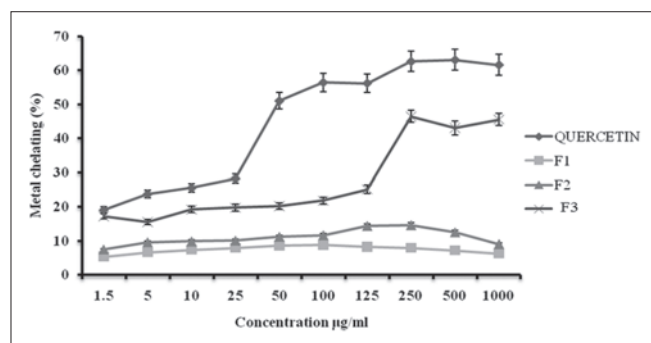


Figure 8: Chelating ability of fucoidan fractions of *Padina tetrastromatica* on ferrous ions. Values are expressed as average of three samples ± standard error of mean

DISCUSSION

Marine organisms are not only very important resources as food, feed, and energy, but they are also rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potentials. *P. tetrastromatica* has been traditionally used in India as a functional food for centuries. In this context, we isolated and characterized fucoidan fractions from *P. tetrastromatica* and examined its antioxidant and antibacterial activity. The algal powder was extracted with water, and polysaccharide was isolated by repeated precipitation with ethanol. Reports available indicate that the composition of the polysaccharides isolated from marine sources vary with tidal waves (Susan et al., 2006). Consequently, the biological activity will also vary.

The antibacterial activity of marine biopolymers has been demonstrated against foodborne Gram-positive and Gram-negative bacteria by many researchers (Tsai and Su, 1999). Agar diffusion assay showed that fucoidan fractions have significant antibacterial activity against Gram-positive bacteria and Gram-negative bacteria when compared to quercetin. The antibacterial activity was significantly high against Gram-positive bacteria *B. subtilis*. In Gram-positive bacteria, the cell membrane is covered by a cell wall made up of 30-40 layers of peptidoglycans, which contain GlcNAc and N-acetylmuramic acid as well as D and L-amino acids including isoglutamate and teichoic acid. Gram-positive bacteria do not have an outer membrane and cell wall structure. The sulfate content of fucoidan can form polyelectrolyte complexes of which can bind to the peptidoglycan layer resulting in cell wall disruption and exudation of the cytoplasmic contents. Antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and result in a

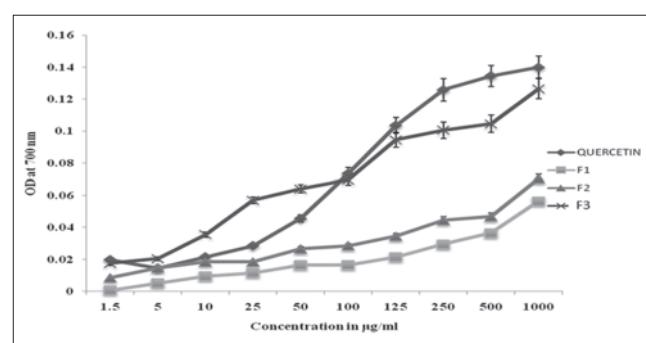


Figure 9: Reducing power of fucoidan fractions of *Padina tetrastromatica*. Values are expressed as average of three samples ± standard error of mean

leakage of the cytoplasm and its coagulation (Kalemba and Kunicka, 2003). The resistance of Gram-negative bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (Russell, 1991 and Gao *et al.*, 1999).

Reactive oxygen species (ROS) are an important part of the defense mechanisms against infection, but the excessive generation of free oxygen radicals may damage tissues. The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free-radical-scavenging activities of antioxidants (Huang *et al.*, 2005). When DPPH free-radical encounters an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical (Li *et al.*, 2007) The DPPH radical-scavenging activity of fucoidan fractions from *P. tetrastromatica* has been attributed to the ability of the polysaccharide fractions in pairing with the odd electron of DPPH radical (Park *et al.*, 2004). Among them, F3 fraction showed maximum scavenging activity.

Lipid peroxidation is a key process in many pathological events and is one of the reactions induced by oxidative stress. The unsaturated fatty acids in cell membrane on oxidation lead to the formation and proliferation of lipid peroxides. The oxygen uptake, structural rearrangements of unsaturated fatty acids and ultimate damage of membrane lipids leads to the production of malondialdehyde, which is known to be carcinogenic and mutagenic (Miyake and Shibamoto, 1997). The inhibition of lipid peroxidation by F3 might probably be due to its free-radical-scavenging potential and thereby proves to be a good antioxidant and cytoprotective agent.

The hydroxyl radical is considered to be a highly potent oxidant which can react with most biomacromolecules functioning in living cells and induce severe damage to the adjacent biomolecules. Thus, removing hydroxyl radical is important for antioxidant defense in cell or food systems. The result suggests that F3 had the strongest scavenging ability for hydroxyl radicals. Previous studies of the antioxidant activity of various algal biomolecules have reported that the scavenging activity for hydroxyl radicals is not due to direct

scavenging but due to inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^+ (Halliwell *et al.*, 1987).

Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in the initiation of oxidation reactions associated with aging (Wickens, 2001). Furthermore, it has been implicated in several pathophysiological conditions, due to its transformation into more reactive species such as hydroxyl radical that initiates lipid peroxidation. Superoxide has also been observed to directly initiate lipid peroxidation (Halliwell, 1978). Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which induces oxidative damage in lipids, proteins and DN (Gülçin *et al.*, 2006). Furthermore, superoxide anion is oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome C. Superoxide radicals were generated in PMS/NADH system by assaying the reduction of NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture (Okado-Matsumoto and Fridovich, 2001). The scavenging ability of the fucoidan fractions decreased in the order of $\text{F3} > \text{F2} > \text{F1}$ corresponding to the fucose and sulfate content of the samples.

Metal chelating activity is claimed as one of the antioxidant mechanisms since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction (Yen and Duh, 1994). The result of this study also suggested that the ratio of sulfate/fucose content of samples was obviously related with the chelating ability in low concentration. It was, therefore, supposed that any change in the ability might be due to the substitution position of the sulfate group. However, the exact mechanism yet remains unsolved.

It has been reported that reducing power serves as a significant reflection of the antioxidant activity. The presence of reductants in the antioxidant samples causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. The reducing properties were generally

associated with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free-radical chain by donating a hydrogen atom. Most non-enzymatic antioxidative activity, such as scavenging of free radicals or inhibition of peroxidation, are mediated by redox reaction (Zhu *et al.*, 2002). Our data show that reducing power of fucoidan fractions play a significant role as an antioxidant.

CONCLUSION

The antibacterial and antioxidant properties of algal polysaccharide fucoidan should be evaluated in a variety of model systems using several different indices to ensure the effectiveness of such antioxidant materials. The results obtained in this study clearly showed that sulfated-fucan has powerful antibacterial activity and antioxidant activity against various *in vitro* systems; moreover, the fucoidan from *P. tetrastromatica* can be used as an easily accessible source of natural antioxidant and as a possible antibacterial agent. Bioactive compounds found in seaweeds await a major breakthrough for a variety of medical applications as they have the potential for application of such compounds as natural antioxidants in different pharmaceutical products.

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