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## ***In vitro* Antioxidant Properties of Fucoidan Fractions From *Sargassum tenerrimum***

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**Abstract:** The aim of the present study is conducted to evaluate the antioxidant potential and toxicity effect of polysaccharide-fucoidan from the brown seaweed *Sargassum tenerrimum*. Fucoidan-a sulphated polysaccharide contains fucose and sulphate as major compounds. Interestingly, various studies reported that the presence of sulphate content of the sample plays a significant role in pharmacological activities. In this study, fucoidan was fractionated by Ion exchange chromatography method and its major chemical constituent sulphate and fucose was determined by the biochemical methods. The toxicity effect of fucoidan was analyzed by the brine shrimp toxicity assay. Three fucoidan fractions (F1, F2 and F3) were obtained from Intact Fucoidan (IF) through anion-exchange column chromatography. *In vitro* antioxidant capability was analyzed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide radical scavenging and total antioxidant assays and intact fucoidan showed the maximum activity  $83.66 \pm 0.35$ ,  $81.73 \pm 0.35\%$  and  $41.6 \pm 0.43 \text{ mg g}^{-1}$ , respectively. The finding of the present study was confirmed that the antioxidant property of fucoidan was depending upon the sulphate content of the fraction and these studies proved that fucoidan have non toxicity effect. Hence, fucoidan have the scope of being used as natural antioxidants in treating many human diseases.

**Key words:** Fucoidan, anti oxidant, *Sargassum tenerrimum*, brine shrimp toxicity

### **INTRODUCTION**

The various studies about the antioxidant activity have unveiled the path to pharmacological activities such as anti-aging, anti-inflammatory, anti-atherosclerosis, anti-cancer activities (Lee *et al.*, 2004; Middleton *et al.*, 2000). Commonly used synthetic antioxidants such as BHA and BHT have averted lipid peroxidation but they have been restricted by legislative rules because of their possible toxic and carcinogenic effects (Hu *et al.*, 2010). There has been a considerable interest by the food and pharmaceutical industries to develop nontoxic antioxidant compounds that demonstrate measurable health benefits. Sulphated polysaccharides from the brown seaweeds showed the excellent free-radical scavenging and antioxidants capacity (Costa *et al.*, 2010). Natural products with antioxidant activity may be used for human consumption considering their safety. Interestingly, sulfated polysaccharides from some brown algae such as *Turbinaria conoids*, *Laminaria japonica*, *Sargassum siliquastrum* and *Sargassum* spp. Have been flaunting to display antioxidant activity (Yan *et al.*, 1998; Lim *et al.*, 2002; Wang *et al.*, 2010; Chattopadhyay *et al.*, 2010).

Seaweeds are unanimously considered as a valuable source of bioactive compounds because of their ability to

produce a variety of secondary metabolites characterized by a broad spectrum of biological activities. They are also a rich source of polysaccharides (Smit, 2004). Brown seaweeds are known to contain more bioactive components than either green or red seaweeds.

Fucoidan isolated from the brown seaweeds and some other marine invertebrates. It contains fucose and sulphate as a major chemical constituent (Chizhov *et al.*, 1999; Bilan *et al.*, 2002). Pharmacological activities of fucoidan were extensively studied by various authors from different seaweeds, due its potential biological activity (Nagumo *et al.*, 1997; Beress, *et al.*, 1993; Usov and Kiryanov, 1994; Nishino *et al.*, 1991; McClure *et al.*, 1992; Painter *et al.*, 1983; Li *et al.*, 2008).

Based on the above fact, the present study was conducted for the evaluation of antioxidant potential of fucoidan fraction from brown seaweed *Sargassum tenerrimum*.

### **MATERIALS AND METHODS**

**Isolation and purification of fucoidan:** The brown seaweed *Sargassum tenerrimum* was collected by hand picking from the mandapam coast of Tamilnadu, India. The collected seaweeds are washed with sea water and then fresh water. Then the washed seaweeds were dried

at room temperature in the shadow, ground in a blender and sieved. The extraction of fucoidan was performed as described by Yang *et al.* (2008) with minor modification. Twenty gram of pulverized seaweed was treated with a liter of ethanol and stirred with a mechanical stirrer for about 12 h at room temperature in order to remove proteins and pigments. After washing with acetone, centrifugation is done at 1800×g for 10 min. Then the residue was left to dry at room temperature. After well drying a biomass, 5 g was taken and extracted in 100 mL of distilled water at 65°C with stirring for an hour. The extraction was done twice and the extracts were pooled. The combined extracts were centrifuged at 18500×g for 10 min and the supernatant was collected. Then the supernatant was mixed well with 1% CaCl<sub>2</sub> and the solution was kept at 4°C for overnight to precipitate alginic acid. The solution was then centrifuged at 18500×g for 10 min and the supernatant was collected. Ethanol (99%) was added in to the supernatant in order to arrive upon the final ethanol concentration of 30% and the solution was positioned at 4°C for 4 h. Again the solution was centrifuged at 18500×g for 10 min and the supernatant was collected. Again ethanol (99%) was added into the supernatant in order to arrive upon the final ethanol concentration of 70% and the solution was placed at 4°C for overnight. The intact fucoidan was then obtained through filtration of the solution with a nylon membrane 0.45 µm size. Fucoidan yield was estimated based on the dried biomass obtained after the treatment of the milled sample with 85% EtOH as a percentage of the algal dry weight (% dry wt.)

Then obtained intact fucoidan was further purified by ion exchange chromatography. 100 mg of intact fucoidan was dissolved in 10 mL of distilled water applied to a column (3.5×50 cm) of DEAE-cellulose. Pre equilibrated with water, flow rate at 2 mL min<sup>-1</sup> (pH 7.0 adjusted with 0.1 N NaOH). It was continued till the pH at the outlet matches with the pH of equilibration of water and stepwise elution with distilled water by passing NaCl in increasing concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 M) solution in turn at a flow rate of 1 mL min<sup>-1</sup>. Fractions were collected and the optical density was measured at 490 nm, until no more carbohydrate was detected. Each fraction was assayed for carbohydrates by phenol-sulfuric acid method (DuBois *et al.*, 1956). Carbohydrate-positive fractions were pooled together, dialyzed (for 24 h in distilled water and lyophilized.

**Chemical analysis:** The fucose was estimated by the phenol-sulfuric acid method by DuBois *et al.* (1956) using L-fucose as standard. Sulfate content was determined according to the gelatin-barium method by Saito *et al.* (1990), using sodium sulfate as standard.

### Antioxidant activity assays

**DPPH free-radical scavenging activity:** This method followed by Shimada *et al.* (1992) was adopted for measurement of free radical scavenging capability. To each 4 mL of sample solution contain the various concentrations of fucoidan (1-6 mg mL<sup>-1</sup>) and mixed well with freshly prepared methanolic DMSO solution of DPPH (0.5 mM). Then let stand for 30 min at room temperature in the dark. Absorbance was measured with a UV-VIS spectrophotometer at 517 nm, using MeOH as the blank. Butylated hydroxyanisole (BHA) was used as reference compound. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \left[ 1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}} \times 100 \right]$$

**Determination of total antioxidant activity:** Total antioxidant effect of fucoidan was performed as described by Prieto *et al.* (1999). Briefly, to each mL of sample solution was contained at different concentrations of fucoidan (10, 50, 250, 5000 and 1000 µg) and mixed with 1 mL of standard reagent solution (0.6 M Sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C. Absorbance of all the sample was measured at 695 nm against a blank. Ascorbic acid was used as a reference compound. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid in milligram per gram of sample. Each trial was repeated thrice.

**Superoxide radical scavenging assay:** The superoxide radical scavenging ability of the different fractions of fucoidan was assessed by the method of Nishikimi *et al.* (1972). Superoxide radical were generated in the PMS-NADH system containing 3 mL Tris-HCl buffer (16 mM, pH 8.0), 338 µM NADH (nicotinamide adenine dinucleotide, 72 µM NBT (nitro blue tetrazolium) and 30 µM PMS (phenazine methosulfate). The reaction mixture, containing various concentration of samples ranging from 100 to 400 µg mL<sup>-1</sup> were added to the PMS-NADH system for free radical scavenging. The mixture was incubated at room temperature for 5 min and the absorbance read at 560 nm against a blank. In the control, the sample was replaced with Tris-HCl buffer. Decreased absorbance of the reaction mixture showed increased superoxide anion scavenging activity. Each trial was repeated thrice. The capability of scavenging of superoxide radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \left[ 1 - \frac{\text{Absorbance of sample at 560 nm}}{\text{Absorbance of control at 560 nm}} \times 100 \right]$$

**Brine shrimp toxicity assay:** A brine shrimp (*Artemia*) bioassay was performed to evaluate the toxicity level of fucoidan. Brine shrimp (*Artemia salina*) eggs were hatched for 48 h in a conical flask containing 500 mL of filtered seawater. The flask was well aerated with the aid of an air pump and incubated at  $27\pm1^{\circ}\text{C}$  with constant illumination (2000lux light approximately) for 48 h. After hatching, the active nauplii were collected for this assay. The fucoidan was dissolved in 1 mL of aerated seawater at various concentrations (10, 50, 250, 500 and  $1000\text{ }\mu\text{g mL}^{-1}$ ). An aliquot of each concentration (1 mL) was transferred, into the aerated seawater (9 mL). Ten nauplii were transferred to each tube. Control group was treated identically without the addition of fucoidan. After 24 h the number of survivors was counted and the  $\text{LC}_{50}$  concentration is calculated in EPA Probit analysis software. The experiment was carried out in triplicate.

**Statistical analysis:** Unless stated otherwise all the experiments were performed in triplicate analysis. The statistical analysis was done by Origin 6.1 v software.

## RESULTS

In the present findings, fucoidan is extracted from brown seaweed *Sargassum tenerrimum* by the method was proposed by Yang *et al.* (2008) using ethanol and the yield of intact fucoidan  $3.6\pm0.07\%$ . Three fractions were obtained: F1 from 0.5 M NaCl elution, F2 from 1 M NaCl and F3 from 1.5 M NaCl. On a weight basis, F1, F2 and F3 accounted for  $33.4\pm0.7$ ,  $20.86\pm0.32$ ,  $43.66\pm2.33\%$  of the recovered polysaccharide, respectively. The major chemical constituent of the fucoidan, polysaccharide was shown in Table 1. The extracted Intact Fucoidan (IF) and fractions (F1, F2 and F3) were contained the fucose; sulphate in the order of percentage IF ( $59.3\pm0.43$ ;  $24.76\pm0.2$ ), F1 ( $43.55\pm0.3$ ;  $11.23\pm0.21$ ), F2 ( $32.8\pm0.22$ ;  $5.1\pm0.23$ ) and F3 ( $54.7\pm0.26$ ;  $16.36\pm0.24$ ).

The DPPH radical scavenging activities of fucoidan were depicted in Fig. 1. It was noted that the scavenging effect of the DPPH radical was increased with the amount of sulphate content present in the fractions and concentrations of the samples. An excellent scavenging capability ( $83.66\pm0.35\%$ ) on DPPH radicals at a dosage of  $6\text{ mg mL}^{-1}$  was found with the intact fucoidan and also scavenging effect of the fractions (F1, F2 and F3) showed the maximum activity at a dosage  $6\text{ mg mL}^{-1}$  ( $61.9\pm0.25$ ,  $50.93\pm0.11$  and  $77.3\pm0.32\%$ ), respectively.

Table 1: Yield and chemical composition ( $\pm\text{SD}$ ) (%) of intact fucoidan (IF) and fucoidan fractions (F1, F2 and F3) (dry weight)

	IF	F1	F2	F3
Yield	$3.6\pm0.0$	$33.4\pm0.7$	$20.86\pm0.32$	$43.66\pm0.13$
Fucose	$59.3\pm0.43$	$43.55\pm0.3$	$32.8\pm0.22$	$54.7\pm0.26$
Sulphate	$24.76\pm0.2$	$11.23\pm0.21$	$5.1\pm0.23$	$16.36\pm0.24$

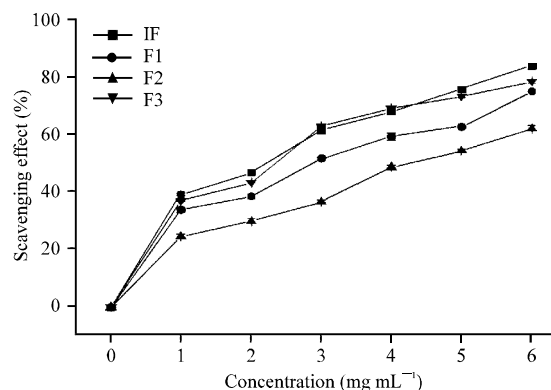


Fig. 1: Scavenging effect of fucoidan fractions isolated from the brown seaweed *Sargassum tenerrimum* on DPPH radicals. Each value is the Mean $\pm$ SD of three replicates

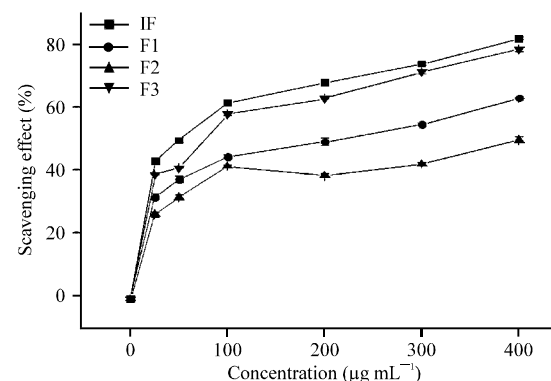


Fig. 2: Scavenging effect of fucoidan fractions from the *Sargassum tenerrimum* on superoxide radicals. Each value is the Mean $\pm$ SD of three replicates

The superoxide radical scavenging effect of fucoidan and their fractions was depicted in Fig. 2. All of them showed significant superoxide radical scavenging activity. The maximum activities were shown at  $400\text{ }\mu\text{g mL}^{-1}$  of IF, F1, F2 and F3 ( $81.73\pm0.35$ ,  $62.43\pm0.16$ ,  $49.9\pm0.33$  and  $78.3\pm0.27\%$ ). Intact Fucoidan (IF) had the strongest scavenging effect of super oxide radical, while F2 had the weakest effect. The total antioxidant activity of intact fucoidan showed the maximum activity ( $41.6\pm0.43\text{ mg g}^{-1}$ ) than other fucoidan fractions ( $24\pm0.3$  (F1),  $13.68\pm0.29$  (F2) and  $6.13\pm0.31\text{ mg g}^{-1}$  (F3) respectively).

There is no mortality was observed in brine shrimp toxicity assay during the incubation period.

## DISCUSSION

Recently, there has been considerable interest in the food industry and in the preventive medicine for the

development of antioxidants from natural sources. Marine macro algae represents one of the richest sources of bioactive compounds and also algae-derived products are increasingly used in the pharmacological industry. In the present study the yield of the intact fucoidan ( $3.6 \pm 0.07\%$ ) and fucoidan fraction was ranging from  $20.86 \pm 0.32$  to  $43.66 \pm 0.13\%$ . Usually the fucoidan content of various seaweeds is diverse much. Similarly, yield 1.1 to 4.8% of fucoidan was observed in some other brown seaweed, reported by Lee *et al.* (1995). However our observation had the comparative amounts of chemical constituent of fucoidan, polysaccharides from various seaweeds, reported by Bilan *et al.* (2006).

DPPH is a stable radical, extensively used as a model to evaluate antioxidant activities in a relatively short time compared with other methods. The DPPH radical is scavenged by an antioxidant through the donation of hydrogen to form a stable DPPH-H molecule. The DPPH carries the similar structural features such as -OH and -OSO<sub>3</sub>H groups. The excess -OH groups we replaced by the -OSO<sub>3</sub>H groups, thereby scavenging effect is exhibited. In the present results showed that the intact fucoidan have strong scavenging activities of DPPH radicals at a dosage of  $6 \text{ mg mL}^{-1}$  ( $83.66 \pm 0.35\%$ ) than other fucoidan fractions which may be attributed due to the high sulphate content of the intact fucoidan. In accordance with the present study, Zhang *et al.* (2003) correlated the antioxidant activity and sulphate content of the fractions of sulfated polysaccharide's from *Porphyra haitanesis*, they further stated that the sulfate content of F1, F2 and F3 fractions was 17.4, 20.5 and 33.5%, respectively. Among them, F3 showed a strongest scavenging effect on superoxide radical. Similarly, Yoshizawa *et al.* (1995) have reported the relationship between the sulfate content and macrophage stimulating activity of polysaccharides from *P. yezoensis*. These studies unveiled that sulfate group in polysaccharides led to differences in their biological activities. The present results also harmonize with these studies.

In the present study, maximum scavenging effect  $81.73 \pm 0.35\%$  was observed at  $400 \text{ } \mu\text{g mL}^{-1}$  of Intact Fucoidan (IF) rather than fucoidan fraction. It was suggested that sulphate content and concentration of the IF played a vital role in the superoxide radical scavenging activity. In consistence with the present study, Zhang *et al.* (2011) recently reported that the sulphated derivatives of polysaccharides obtained from fresh persimmon (*Diospyros kaki* L.) fruit, showed dose-dependent reducing power and free radical scavenging effect of 1,1-diphenyl-2-picrylhydrazyl, superoxide radical scavenging and hydroxyl radical scavenging activity. They further stated that the

sulphated modification of polysaccharides significantly increased their antioxidant activities.

Intact fucoidan showed the maximum total antioxidant activity ( $41.6 \pm 0.43 \text{ mg g}^{-1}$ ) than fucoidan fractions. In this phosphomolybdenum method, Mo (VI) is reduced to form a green phosphate Mo (V) complex. In a previous study, Chandini *et al.* (2008) reported the total antioxidant effect of extract from *Padina tetrastomatica* and *Turbinaria conoides*, the activity was 9.79 and  $9.65 \text{ mg g}^{-1}$  of ascorbic acid equivalent, respectively. The obtained result of total antioxidant capacity of fucoidan from *Sargassum tenerrimum* leading us to further testing of different antioxidant assays to determine their possible antioxidant mechanisms.

Several studies have shown that brine shrimp assay has been an excellent, rapid and cheapest method for preliminary investigations of toxicity, to screen medicinal plants popularly used for several purposes and for monitoring the isolation a great variety of biologically active compounds. Parra *et al.* (2001) reported, the toxicity in medicinal plants was positively correlated between the lethality to brine shrimp and the corresponding oral lethal dose in mice. In the present study the toxicity criterion was followed according Deciga-campos *et al.* (2007) and no mortality was observed during the incubation period. Interestingly our result was revealed that the fucoidan had non toxic effect against the brine shrimp (*artemia*). So far there are no reports on the brine shrimp toxicity of fucoidan.

## CONCLUSION

Finally in the present study, concluded that the extracted intact fucoidan from *Sargassum tenerrimum* having the significant antioxidant activity rather than other fucoidan fractions. Our result has proved that fucoidan is non - toxic polysaccharides. From these results, fucoidan have the scope of being used as natural antioxidants in treating many human diseases. Results of the present study, suggested that more findings, research and development activities are needed to improve the understanding of the sulphate content and antioxidant mechanism for the fucoidan.

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