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Determination of in vitro antioxidant activity of crude fucoidan extracted from *Sargassum wightii* by different methods

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ABSTRACT

Seaweeds are the most important source of sulphated polysaccharides. These sulphated polysaccharides exhibit potent therapeutic properties. Fucoidan is a complex sulphated polysaccharide found in many species of brown algae. In the present study, fucoidan was extracted from brown seaweed, *Sargassum wightii* via different procedures: alcohol extraction, hot buffer extraction, hot water extraction and acid extraction. Through the study, it was found that extraction with hot buffer results in the maximum yield (7.3%) of the polysaccharide. Measurement of fucose and sulphate content revealed that the extracted polysaccharide showed characteristics of fucoidan. The results revealed that the yield and composition of fucoidan are dependent on extraction methodology. Fucose and sulphate-rich crude fucoidan were then subjected to 1, 1-diphenyl-2-picrylhydrazyl (DPPH), radical scavenging activity, using ascorbic acid as standard and it showed higher free radical scavenging activity in a dose-dependent manner with an IC_{50} value of 133 µg/ml. Thus fucoidan can be exploited as natural antioxidants for protection against oxidative stress which is found to be the cause for the development of several diseases.

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INTRODUCTION

Seaweeds, also known as marine macro-algae, are saltwater-dwelling, simple organisms that are generally live attached to rock or other hard substrates in coastal areas. Historically, seaweeds have been used by humans as medicine and food. Seaweeds, particularly brown seaweeds, are rich in natural active ingredients (Abou-El-Wafa GSE *et al.*, 2011) and thus have generated an enormous amount of interest in the pharmaceutical industry. Brown algae are abundant in the sulphated polysaccharide, namely alginic acid (alginate), laminarins (laminarans) and sulphated fucans (fucoid) (S.L Vavilala *et al.*, 2005). Fucoidan designates a group of certain fucose-containing sulphated polysaccharides (FCSPs) that have their central chain built of (1→3)-linked α-l-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-l-fucopyranosyl residues (Jiao G *et al.*, 2011), and also has sulphated galactofuranose with backbones built of (1→6)-β-d-galactose- and/or (1→2)-β-d-mannopyranosyl units with fucose or fuco-oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions (Ale MT *et al.*, 2011). They offer several beneficial bioactive functions for humans including antitumor (Lei Wu *et al.*, 2016), immunomodulatory (Raghavendran *et al.*, 2011), antiviral (Ponce NM *et al.*, 2003), antithrombotic, anticoagulant (Cumashi A *et al.*, 2007), antioxidant (Huang CY *et al.*, 2015) and anti-inflammatory activities (Park HY *et al.*, 2011). These marine polysaccharides are well known for its antioxidant and antibacterial activities (Sellimi S *et al.*, 2016; Lee KY *et al.*, 2013; Marudhupandi T *et al.*, 2013;

Badrinathan S *et al*., 2012). *S.wightii,* a brown algae is one of the important species of the genus *Sargassum* that are rich in fucoidan (Antonisamy JM *et al*., 2012). It is widely distributed in many parts of Asia and India, on the southern coast of Tamilnadu. Numerous studies have been demonstrated to extract and characterize fucoidan and alginate from seaweed. The extraction procedure and extraction solvent seem to affect the yield and composition of the polysaccharide which indeed influence the biological activity (Junaidi L 2013; Do QD *et al*., 2014). Moreover, there are reports that fucose and sulphate ratio plays a significant role in determining the bioactivity of fucoidan (Li *et al*., 2008). Hence, the present study is focussed on obtaining crude fucoidan from *S.wightii* by different extraction procedures, analyzing fucose and sulphate content and determine the antioxidant activity of the fucose and sulphate-rich fucoidan.

MATERIALS AND METHODS

Collection and preparation

Seaweed, *S.wightii* was collected from intertidal regions of Mandapam (Lat. 9.28°N and Lon. 79.12°E), Tamilnadu, India. After collection, the seaweed was brought to the laboratory by keeping them in plastic bags with sea water. The collected seaweed was authenticated by Dr. P. Jayaraman, Plant Anatomy Research Center, Tambaram, Chennai. They were then washed with deionized water and air dried in the shade at room temperature. Dried samples were individually cut into small pieces (2–3 cm), homogenized and stored. The powdered seaweed was subjected to further analysis.

Preparation of fucoidan crude extract

Extraction-I (Alcohol extraction)

Extraction of fucoidan was carried out using alcohol (Wang CY *et al*., 2015). It is one of the classical methods. In this method, the milled sample was mixed with 95% ethanol in the ratio (1:2) and shaken well for 1 hour to remove pigments, proteins and lipids and then centrifuged for 10 minutes. The precipitate was collected, mixed with double distilled water (w/v=1:10) and placed in a water bath maintained at 40°C for 15 minutes with shaking. The mixture was centrifuged at 3870g for 10 minutes and the supernatant was collected. Ethanol (95%) was added to the collected supernatant to give a final ethanol concentration of 20%. The mixture was then centrifuged at 9170g for 30 minutes, the supernatant was collected and 95% ethanol was added until a final ethanol concentration of 50% was reached in order to obtain fucoidan. The precipitated fucoidan (F1) was then collected by centrifugation at 9170 g for 30 minutes and dried at 40°C.

Extraction-II (Hot buffer extraction)

The extraction process was carried out using McIlvain's buffer solution (Hifney AF *et al*., 2015). The seaweed sample was suspended in the buffer solution (0.1M citric acid; 0.2M Na_2HPO_4) of pH 4 in the ratio of 1:10 at 60°C for 3 hours. The mixture was then subjected to filtration to separate the extract from the residual alga. Subsequently, 3% (w/v) $CaCl_2.2H_2O$ was added to the filtrate (1:1 v/v) and left overnight at 4°C to precipitate alginate. The precipitated alginate was separated by vacuum filtration and then the double volume of absolute ethanol was added to the resultant filtrate. The mixture was maintained overnight at 4°C and then centrifuged at 6000 rpm for 15 minutes. The precipitated fucoidan (F2) was recovered by drying at 50°C and stored for analysis.

Extraction-III (Hot water extraction)

The extraction was done following the method of Giang *et al*., 2011 with desired modifications. The milled sample (10 g) was suspended in 300 ml of hot water and kept at 100°C for 6 hrs. Then the mixture was filtered and the filtrate was centrifuged at 4000 rpm for 10 minutes. The filtrate was added with the equal amount of ethanol (70%) until a precipitate was formed. Centrifugation was then performed once again to separate sediment from the liquid. The final precipitate (F3) was separated and dried and subjected to further analysis.

Extraction-IV (Acid extraction)

Black WAP *et al*., 1952 method was adopted to extract fucoidan from *S.wightii*. The milled sample was mixed with 0.1N HCl at pH 2-2.5 in the ratio 1:10 and kept at 70°C with constant stirring using a magnetic stirrer for 1 hr. The process was repeated thrice and each time, the filtrate was collected. The crude fucoidan was isolated by evaporation. This was followed by fractional precipitation by adding ethanol (80%). The precipitated fucoidan was further purified by adding formaldehyde. The obtained fucoidan (F4) was dried and subjected to further analysis.

Calculation of fucoidan yield

$$\text{Yield of fucoidan (\%)} = \frac{\text{Weight of fucoidan}}{\text{Weight of milled seaweed}} \times 100$$

Quantification of fucose and sulphate in fucoidan

The fucose content was determined by phenol-sulphuric acid method (Dubois *et al*., 1956) using

L-fucose as standard. Sulphate content was measured according to gelatin barium method using sodium sulphate as standard (Saito *et al*., 1990). The fucose and sulphate content was expressed in % dry wt. of fucoidan.

***In-vitro* antioxidant activity**

The scavenging activity of fucose and sulphate content rich fucoidan extract (F2) on DPPH radicals was determined according to the method of Brand-Williams W *et al*., 1995 with minor modifications. All reactions were carried out in triplicates and the degree of decolourization indicates the free radical scavenging activities of the crude fucoidan.

Scavenging of DPPH radicals by the crude fucoidan was calculated using the following formula,

$$\text{Percentage of scavenging} = \frac{A_1 - A_3}{A_1} \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of the test sample

RESULTS & DISCUSSION

The present study was undertaken to extract fucoidan by different methods and to determine its antioxidant activity.

Extraction and percentage yield of fucoidan

Fucoidan is a fucose-containing sulphated polysaccharide that can be easily extracted from the cell wall of seaweed. Several methods have been followed to extract fucoidan. Classical procedures include utilization of 10% TCA (Hoshino *et al*., 1998), HCl (Wang Zhae *et al*., 1985) and alcohol to carry out fucoidan extraction. Microwave- and ultrasound-assisted extraction, enzyme-assisted extraction (Hahn T *et al*., 2012), autohydrolysis (Rosa M. Rodriguez-Jasso RM *et al*., 2013), compressional-puffing-hydrothermal extraction (CPHE) process (Ale MT *et al*., 2012) and hot buffer extraction (Hifney AF *et al*., 2015) were found to be novel procedures to extract fucoidan. It has been reported that the yield of fucoidan was dependent on time, temperature, pH and extraction methods (Black WAP *et al*., 1952). In this study, four different methods were employed for the extraction of fucoidan from *S.wightii*. The results are summarised in Table 1. Among the four extraction methods, hot buffer extraction process was found to give a maximum yield of crude fucoidan (7.3%). This was followed by alcohol extraction process, hot water extraction process and acid extraction process with a yield of 3.5%, 1.35%, and 1.22% respectively. Recently, a comparative study was carried out on fucoidan yield and composition and found that fucoidan yield was significantly higher in composite enzyme method of extraction than hot water extraction (Dong X *et al*., 2017). A similar study was carried out by Eluvakkal T *et al*., 2010 extracting fucoidan from seaweeds by two methods and found that method I proposed by Velayutham and Jayachandran, 1991 maximum yield amount of crude fucoidan from *S.wightii* (7.15% alga dry wt.). In agreement with the previous reports, the present study emphasizes the role of extraction methods with the yield of fucoidan.

Table 1: Yield of crude fucoidan in different extraction methods

S.No	Extraction method	Yield of crude fucoidan (%)
1.	Alcohol extraction	3.5
2.	Hot buffer extraction	7
3.	Hot water extraction	1.35
4.	Acid extraction	1.22

Table 2: Content of fucose and sulphate in crude fucoidan

Crude fucoidan	% of Fucose content (±SD)*	% of sulphate content (±SD)*
F_1	35±1	11.3±1
F_2	38.3±2	15.3±0.57
F_3	36.2±1.7	14.1±0.1
F_4	33.8±1	10.2±0.9

Chemical composition of the crude fucoidan

Biochemical composition of fucoidan varies in relation to the seaweed species, the season of harvest (Honya *et al*., 1999), geographic location (Rioux, LE *et al*., 2003) and maturity of the plant (Zvyagintseva, TN *et al*., 2003). The fucose and sulphate content of fucoidan obtained from four different extraction processes is displayed in Table 2. Maximum of 38.3±2% of fucose was reported in F_2 obtained from hot buffer extraction process followed by F_3 (36.2±1.7%), F_1 (35±1%) and F_4 (33.8±1%). Similarly, the sulphate content of F_2 was found to be greater (15.3±0.57%) when compared to that of F_1 (11.3±1%), F_3 (14.1±0.1%) and F_4 (10.2±0.9%). This result suggests that the extraction procedures have not only an impact on yield of fucoidan but also the quality of fucoidan. The disparity in the chemical composition of fucoidan obtained by different extraction methods has been exemplified in various reports (Liu X *et al*., 2015; Baba BM *et al*., 2016). A recent study in the biochemical composition of fucoidan from *Sargassum polycystum* revealed that the extracted fucoidan has 46.8% of fucose and 22.35±0.23% of sulphate (Palanisamy S *et al*., 2017). In our study, fucoidan obtained by hot buffer extraction process has the maximum amount of fucose and sulphate compared to other methods.

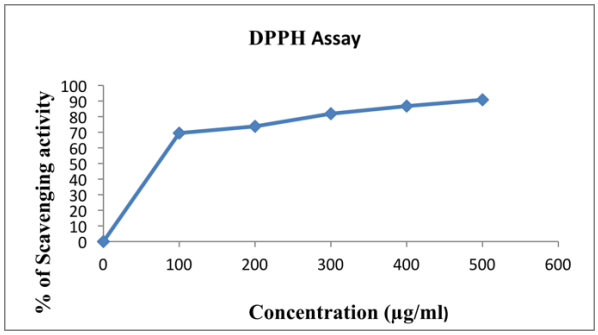

Figure 1: In vitro antioxidant activity of crude fucoidan (F2)-DPPH assay

In vitro antioxidant activity of fucose and sulphate-rich crude fucoidan (F2)

The antioxidant activities of the plant extract merely depend on the composition of the extract, extraction solvent and conditions. Studies have revealed that polysaccharide containing high-fucose content conferred maximum free radical scavenging efficiency *in vitro* (Roy Chowdhury S *et al*., 2014; Ruperez *et al*., 2002). Similarly, it has been suggested that sulphate and sulphate/fucose ratio of fucoidan might influence antioxidant activity (Huang CY *et al*., 2015; Guiyan QU 2014). In agreement with the above report, fucose and sulphate-rich crude fucoidan (F_2) was subjected to DPPH assay. The scavenging effects of F_2 are shown in Figure 1. It was found that the scavenging activity increases with the increased concentration of F_2 with an IC_{50} value of 133 µg/ml. Maximum scavenging (90.9%) was observed at the concentration of 500 µg/ml. Antioxidant property of fucoidan is closely related to its structure. It has been reported that the biological activities of fucoidan increase with the degree of sulfation (Schaeffer DJ *et al*., 2000; Cho ML *et al*., 2010). Ajisaka K *et al*., 2016 examined the relationship between the antioxidant activity and the structure of fucoidan extracted from the brown seaweeds *Cladosiphono kamuranus*, *Sargassum hornery*, *Kjellmaniella crassifolia* (*Saccharine Sculptra*), *Nemacystus decipiens*, and *Fucus vesiculosus* and suggested that the antioxidant activity of the fucoidans was possibly due to a combination of the factors involved, such as the amount of sulphate groups, the position of the sulphate groups, the kind of side chain sugar, the linkage of a side chain sugar, and the molecular weight. The metabolic process results in the synthesis of some reactive oxygen species (Mohanasundaram *et al*., 2016). These free radicals are highly reactive and when produced in excessive amounts cause an alteration in the oxidation-reduction system leading to oxidative stress. Oxidative stress promotes heart diseases, chronic inflammation, cancers, ageing and related degenerative processes. The results of the present study evidence indicate that fucoidan from *S.wightii* have beneficial effects as natural antioxidants and can combat free radical-related diseases.

CONCLUSION

In the current study, the thermal buffer extraction process provides higher fucoidan yield compared to hot water, alcohol and acid extraction methods. Moreover, the antioxidant activity of the fucose and sulphate-rich crude fucoidan was investigated. The results showed that the fucoidan exhibit anti-oxidant activities in a concentration-dependent manner. Thus, this study indicates that the fucoidan can act as a potent antioxidant. Further, the study implies that the biochemical composition of crude fucoidan depends on the extraction methodology. This creates a need to optimize the extraction procedure for obtaining fucoidan with maximum biological activity.

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