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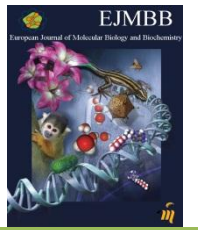

EXTRACTION AND ESTIMATION OF FUCOIDAN COMPONENTS FROM *PADINA DISTROMATICA* HAUCK (BROWN SEAWEED) IN HARE ISLAND, THOOTHUKUDI, TAMIL NADU, INDIA.

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ABSTRACT

Fucoidan extracted from brown seaweeds have been extensively studied in the past and interest in its pharmaceutical properties is growing. The present study reported the extraction and estimation of fucoidan components from *Padina distromatica* Hauck. Fucoidan was extracted by Rioux method and the fucoidan components such as fucose, sulphate, uronic acid and proteins were analyzed by standard methods. The result showed that the sulphate content was the highest percentage (31.68%) followed by fucose content (15.19%). Uronic acid content was significantly lower than the other components of fucoidan that was 5.47% followed by protein content (1.498%) from the tested fucoidan extracted from *Padina distromatica* Hauck in Hare island, Thoothukudi, Tamil Nadu, India.

INTRODUCTION

Research on fucoidan has so far been carried out in Japan, Korea, France, Australia, China and the United States. Studies have indicated that fucoidan is non-toxic, non-allergenic, and has no negative effects on the human body once consumed [1]. This statement is further supported by the fact that nutraceutical and food supplements containing fucoidan have been marketed for a number of years with no known adverse effects [2]. No toxicological changes were observed when rats were orally administered with up to 1000mg/Kg body weight per day of fucoidan for 28 days, but when the dose was increased to 2000mg/Kg body weight per day of fucoidan, the plasma ALT level, a biomarker of liver injury was increased indicating that the consumption of fucoidan up to 1000mg/Kg body weight per day was safe in rodents [3].

Fucoidan is known to exhibit a wide variety of biological activities. Among them are: anticoagulant, antioxidant, antiviral, antithrombic, and anticancer activities [4]. Many researchers have targeted the anticoagulant, anticancer, and antioxidant activities of fucoidan as being the most important activities in fucoidan. The effectiveness of these activities is related to the chemical composition of fucoidan [5]. Seaweed polysaccharides are usually heterogeneous and branched, it may contain monosaccharide components with acetyl groups and the amount of sulfation is irregular [6]. The structural complexity of fucoidan may vary from species to species, depending on the extraction method. For that reason, each type of fucoidan that may have unique structural features and possess varied bioactivities could potentially be a new drug [7]. The precise structure and backbone of fucoidans have been extensively studied for some time, but debate about its actual arrangement is still ongoing due to its complex structure. A reason for that is because fucoidan is difficult to extract in its pure form. Crude fucoidan is a polysaccharide made up of a complex mixture of fucose, sulphate and low uronic acid to a low sulphated fucan polysaccharide with high uronic acid content [8]. Some examples of the composition of crude fucoidan are summarized [9-13].

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Crude fucoidan can be purified into fractions using ion-exchange chromatography, a technique which separates molecules based on the overall charge of the molecule. As fucoidans generally have an overall negative charge due to their sulphate groups, they can bind with anion exchangers which contain positively charged functional groups such as diethylaminoethyl [14-15]. The chemical composition of fucoidan fractions obtained after subjecting crude fucoidan from *Sargassum swartzii* to DEAE Sephadex A-25 [10] and the fractionation of crude fucoidan from *Unandria pinnatifida* using DEAE Sephadex A-25 [16]. As mentioned earlier, fucoidan content, chemical composition, and its structural characteristics vary in relation to the seaweed species, season of harvest, and maturity of the plant. Maximum amounts of fucoidan can be found in the sporophylls compared to the blade, but the chemical compositions are quite similar within a given species [11]. Moreover, a study established a correlation between seasonality and fucoidan content, and reported that fucoidan content was highest during the reproductive stages of *Unandria pinnatifida* [16].

In spite of the increase in awareness regarding *Padina distromatica* Hauck, brown seaweed as a rich source of bioactive components including the polysaccharide fucoidan and the colour pigment fucoxanthin [17]. Fucoidan is a sulphated polysaccharide which gives seaweed its slippery texture [4]. It is found in the cell wall of several types of brown seaweed and protects them from harsh environmental conditions. Fucoidan has recently been reported to possess a wide range of bioactivities including antioxidant and antiviral properties, weight loss effects and blood thinning properties [18]. Therefore, an attempt has been taken to screen the components of fucoidan extracted from *Padina distromatica* Hauck in Hare Island, Thoothukudi, Tamil Nadu, India in the present study.

MATERIALS AND METHODS

Collection of Plant Materials

The collection of *Padina distromatica* Hauck (Figure 1) was made during the low tidal and subtidal regions (up to 1m depth) by hand picking from Hare island, Thoothukudi (Lat 8° 48'N; Long 78° 11'E) located in the south east coast of Tamil Nadu, India. The collected materials were washed thoroughly with marine water in the field itself to remove the epiphytes and sediment particles. Then the samples were packed separately in polythene bags in wet conditions and brought to the laboratory, then thoroughly washed in tap water followed by distilled water to remove the salt on the surface of the thalli. They were stored in 5% formalin solution. For drying, washed specimens were placed on blotting paper and spread out at room temperature in the shade. The shade dried samples were grounded to fine powder using a tissue blender. The powdered samples were then stored in the refrigerator for further use.

Figure 1. Natural Habit of *Padina distromatica* Hauck

Extraction of Fucoidan

Fucoidan was extracted by Rioux method [19]. Dried seaweed was mixed with 1% (w/v) $CaCl_2$ solution (1:30 ratio) and then stirred for 4 hours at 85℃ at 455 ± 5rpm using a stirrer RZR1 (Caframo Ltd. Canada). The supernatant was separated by centrifugation (16,887g, 20 min), and vacuum filtration on Whatman No. 4 filter. The filtered liquid was mixed with 2 volumes of 95% ethanol and 1 volume of 2% (w/v) NaCl and then stirred for 1 hour at room temperature for alcoholic precipitation of fucoidan. This solution was kept at -20ºC for 48 hours. The pellet containing fucoidan was recovered by centrifugation (16,887 g, 12 min). Then, it was resolubilized in 100 ml of fresh deionized water and dialyzed for 48 hr by using membrane of 15 KDa (Sigma, USA) to remove minor constituents and solvents. Fucoidan was recovered by freeze drying and preserved at -20℃ in a sealed tube to keep away from humidity.

Fucose content

Free fucose was determined in fucoidan by the Cysteine-sulphuric acid method for methyl pentoses. Three replicate samples were prepared in different concentrations with deionized water ranging from 20-100µg/ml. Commercial L-fucose (Sigma) was used as the standard. Each sample solution (1ml) was placed into separate test tubes and cooled in an ice water bath. 4.5ml of sulphuric acid (prepared by adding six volumes of concentrated sulphuric acid with one volume of water) was added into each tube and mixed. Tubes were warmed in a 25°C water bath for 3-4 minutes, and then placed into a boiling water bath for 3 minutes. Tubes were cooled under running tap water and 0.1ml cysteine hydrochloride solution (5% cysteine hydrochloride in deionized water) was added to each tube and mixed. Absorbance was read at 396nm and 427nm, after zeroing the spectrophotometer with a water blank treated in the same manner. Absorbance values were calculated by using the following equation: Absorbance = (A396nm – A427nm). This corrects for the presence of hexoses [20].

The measurement of sulphate in the fucoidan was based on the Barium sulphate ($BaSO_4$) determination using Barium chloride, whereby sulphate content was estimated turbidimetrically as $BaSO_4$. The conditioning reagent was prepared by mixing 50ml glycerol, 30ml concentrated

hydrochloric acid, 300ml deionized water, 100ml isopropyl alcohol and Sodium chloride into a large beaker with mechanical stirring overnight. Three replicate samples were prepared by weighing 15mg of dried fucoidan into separate closed test tubes containing 5ml 4M HCl. Samples were subjected to acid hydrolysis for 2 hours at 100°C. A solution of Potassium sulphate standards with concentrations ranging from 200-1000µg/ml of sulphate was prepared. Sample solution was added into a 100ml conical flask containing 15ml of deionised water. Conditioning reagent (5ml) was added and stirred mechanically at a constant speed. $BaCl_2$ (0.3g) was added, stirred for exactly 1 minute and then left standing for 4-6 minutes to allow the $BaSO_4$ precipitate to form. Absorbance was measured at 420nm using a spectrophotometer after zeroing with water blank that was treated in the same manner [21].

Uronic acid content

Tetraborate acid reagent (0.025M) was prepared by dissolving 0.503g of Sodium tetraborate into 100ml of concentrated sulphuric acid and stirring the solution mechanically overnight. 0.125% carbazole reagent was prepared by mixing 0.125g of carbazole with 100ml of absolute ethanol in a brown glass bottle and stored at 4°C until needed. Screw cap tubes were filled with 3ml of tetraborate acid reagent and cooled in an ice water bath. Three replicate samples with a concentration of 1mg/ml dissolved in deionised water saturated with benzoic acid was carefully added (0.5ml) to the acid and the tubes were closed. The tubes were shaken vigorously with constant cooling in the ice water bath for 5-10 seconds. Tubes were then heated for 10 minutes in a boiling water bath and then cooled to room temperature. Carbazole reagent (0.1ml) was added to each tube and heated for a further 15 minutes in the boiling water bath, and cooled to room temperature. Absorbance was measured at 530nm after zeroing the spectrophotometer with water blank treated in the same way. D-glucuronic acid was used as the standard [22].

Protein content

The Bradford protein assay was utilised in this study to determine the amount of contaminated protein present in crude fucoidan. It is based on the dye, Coomassie Brilliant Blue G-250, which changed initially from red to blue due to protein binding. The protein content was then estimated spectrophotometrically. Bradford reagent was made by dissolving 100mg of Coomassie brilliant blue G-250 into 50ml of 95% ethanol, followed by the addition of 100ml 85% phosphoric acid. This mixture was transferred into a one litre volumetric flask and diluted to the graduation mark with deionized water. Three replicate samples with a concentration of 20mg/ml dissolved in water was added into 1.6ml Bradford reagent in a test tube. Samples were incubated for 15 minutes at room temperature and the absorbance was measured at 595nm after zeroing the spectrophotometer with a water blank treated in the same way. Bovine serum albumin was used as the standard [23].

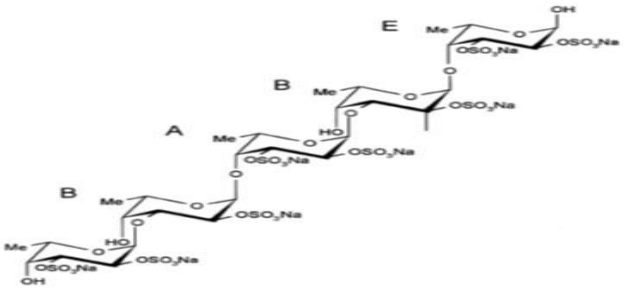
Statistical analysis

Minitab® (Version 15), analysis of variance (ANOVA) was carried out to test for differences between the composition of the extracted fucoidan. Where significant differences occurred, Tukey's HSD was employed to examine where that effect occurred.

RESULTS AND DISCUSSION

Fucoidan is a stored sulfated polysaccharides of brown seaweeds and is composed mainly of α-(1-2) or α-(1-3) linked L-fucose residues [24-25]. The precise structure of the fucoidans from *Fucus vesiculosus* and *Ascophyllum nodosum* [19] remains uncertain although the main repeating unit has been confirmed (Figure 2).

Figure 2. Structure of Fucoidan



Most fucoidans have very complex chemical composition and only little regularity in the structural components is known present. Fucoidan largely contains sulphated L-fucose residues. Hence fucose is the primary sugar in fucoidan. Sulphate groups also represent a large component of fucoidan and the biological activities of fucoidan are strongly related to its sulphate content [26]. Besides fucose and sulphate, other monosaccharides (glucose, mannose, galactose, xylose, etc), uronic acids, and even protein are present in detectable amounts. All these compounds have increased the difficulty in structural elucidation of fucoidan [27].

Fucoidan components Analysis

Chemical composition of the fucoidan derived from *Padina distromatica* Hauck are shown in Table 1. Extraction of seaweed with 1% $CaCl_2$ revealed with the highest percentage of sulphate, fucose and uronic acid content. The extraction was considered the best of the method employed in producing uncontaminated fucoidan, due to the low protein content in the derived fucoidan. Sulphate content showed the highest percentage (31.68%) followed by fucose content (15.19%) from fucoidan extracted from *Padina distromatica* Hauck in Hare island, Thoothukudi, Tamil Nadu, India. Uronic acid content was significantly lower than the other components of fucoidan that showed 5.47% followed by protein content (1.498%).

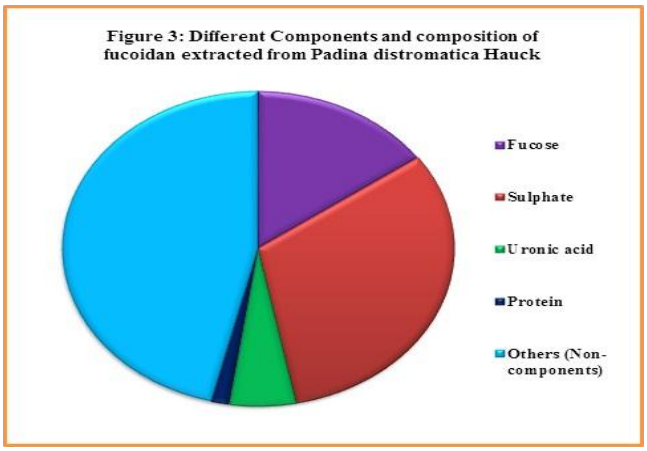
Chemical composition of fucoidan varies according to the season, geographic location, species, and

maturity of the plant. Fucoidan is a sulphated fucan and the regularity of the structural characteristics of fucoidan is minimal. Fucoidan is mainly composed of fucose, sulphate, uronic acid and small quantities of monosaccharides. The composition will vary between species and the extraction techniques used to extract the fucoidan also have a large impact on the determination of the final structure of fucoidan [19]. The method used to extract fucoidan may result in fucoidans that vary in chemical composition and structure. Fucoidan extracted at room temperature and at 70°C had completely different chemical compositions. Companies experienced in producing pure fucoidan that the colour of fucoidan is directly related to the amount of fucoidan in the powder. If the powder contained very small amounts of fucoidan, the colour should be close to white or creamy. However, a powder that contained high amounts of fucoidan should be close to a dark brown colour. Fucoidan have a white visual appearance and not brown while ranging from white to brown. Therefore, the perception of how fucoidan should look like is still being debated [28].

Table 1. Different Components and composition of fucoidan extracted from *Padina distromatica* Hauck

Components of Fucoidan	Percentage of Fucoidan Composition
Fucose	15.19±0.18
Sulphate	31.68±0.15
Uronic acid	5.47±0.09
Protein	1.498±0.04
Others (Non-components)	46.162±0.36

The fucose, sulphate, and uronic acid content of fucoidan obtained from the $CaCl_2$ extraction were comparable to the values reported. This supports the idea that the fucoidan extracted using the $CaCl_2$ method of Bilan *et al.* [6] was good quality. However, apart from the type of fucoidan extraction carried out, the season in which the seaweed was harvested, location and habitat in which the seaweed was grown in and the species of seaweed investigated, would all have an effect on the content and chemical composition of fucoidan and should be further investigated.



CONCLUSION

In this study, the fucose, sulphate, and uronic acid content of crude fucoidan extracted from *Padina distromatica* Hauck grown around the coastal waters of Hare Island, Thoothukudi, Tamil Nadu, India were comparable to other published studies. The yield and constituents of fucoidan was carried out with the use of $CaCl_2$ extraction method. $CaCl_2$ extraction appeared to be the best method to extract good quality fucoidan that had the highest yield, as well as highest sulphate, fucose and uronic acid content, possibly due to the nature of $CaCl_2$ where it reacts with sugar chain polymers and link them together. Calcium extracted fucoidan also was the darkest brown in colour, characteristic of a good quality fucoidan.

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