Influence of Species, Geographic Location, Seasonal Variation and Extraction Method on the Fucoidan Yield of the Brown Seaweeds of Gulf of Mannar, India

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Running title: Extraction Method on the Yield of Brown Seaweeds

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Brown seaweeds such as *Sargassum wightii, S. oligocystum, Padina tetrastromatica* and *Turbinaria ornata* were collected from two locations, Valinokkam and Hare Island in Gulf of Mannar, South East coast of India, to extract fucoidan and to analyze its variation in the yield for a period from July 2012 to January 2013. Water and acid extraction methods were followed to extract fucoidan. Water extraction yielded much higher fucoidan (9.46±0.18) than acid method (1.63±0.05). The yield of fucoidan also varied between the species, season and also geographic location. The highest yield was obtained from *P. tetrastromatica* (9.46±0.18%) followed by *T. ornata* (5.83±0.07%) and *S. wightii* (3.90±0.05%). The yield was higher during maturation stage of the algae, which was September for *T. ornata* and *S. wightii* and January for *P. tetrastromatica.* Seaweeds found in shallow region gave higher yield (Hare Island) than those collected from more than 1 m depth in Valinokkam region. Compositional analysis of fucoidan revealed that sulphate content was higher in *T. ornata* (24.27±0.85%) than *P. tetrastromatica* (22.70±0.53%) and *S. wightii* (18.73±0.82%). The fucose content was higher in *P. tetrastromatica* (54.51±1.08) and lower in *S. oligocystum*(43.16±3.21). Fourier transform infrared spectroscopy carried out for the two dominant species *viz. P. tetrastromatica* and *S. oligocystum,* showed a major broad band centered around 3448 cm^{-1} assigned to be due to hydrogen bonded O-H stretching vibration. Another two bands appeared at 723 cm^{-1} and 601 cm^{-1} seem to be the characteristic of C-O-S stretching of sulfate group and C=C-H stretching vibration, respectively in both the species. However, the major differences among the species were the presence of a minor band at 2103.73 cm^{-1} in *P. tetrastromatica* and slight shifts noticed in sulphate group band.

Key words: Brown seaweeds, fucoidan yield, species, season, extraction method, characterization

Seaweeds are widely distributed along the Indian coastal region and in particular, brown seaweeds belonging to the group *Phaeophyceae* are dominant along the coast of gulf of Mannar. Fucoidan is a sulfated polysaccharide found in the cell walls of several species of brown seaweed. In recent years, fucoidan has been isolated from different groups of brown seaweeds such as *Fucales, Laminariales, Chordariales* and Ectocapales[1]. Of which, brown seaweeds belonging to the group, *Fucales* are abundant in Indian coast and dominant in gulf of mannar region[2]. Many studies reveal that fucoidan exhibits versatile bio-active properties like antioxidative[3], anticoagulant[4], antimicrobial[5], antitumor[6] and immunomodulatory properties[7,8].

Fucoidan was first isolated and named so by Kylin[9]. It has been extracted from seaweeds mainly by acid, alkali and solvent extraction[10]. Brown seaweeds produce families of sulfated fucoidans among other polysaccharides. They contain other sugars besides L-fucose, *viz.*, D-xylose, D-galactose and D-glucuronic acid and additional sugars such as D-mannose and D-glucose have also appeared. However,

sulphate is the major constituent along with small amounts of uronic acid and other sugars such as galactose, mannose, xylose and glucose[11]. In general, extraction of sulfated polysaccharides from seaweed is usually performed by using hot water, dilute acid, or dilute alkali. But all of these methodologies involve long extraction time and high volume of diluents[12-16]. Although various extraction methods have been widely used, the use of organic solvents was the most common method practiced widely[17].

There are reports that the yield of fucoidan differ with the species, season and location of brown seaweeds; and also with the method of extraction. Further, the composition of fucoidan has been known to vary between the species of brown seaweeds. There is no report on the yield of fucoidan in the brown seaweed available along the coast of Gulf of Mannar at different species, season and location. Hence, in the present study; four different species of brown seaweeds predominantly available in two different locations of Gulf of Mannar during the peak season were analyzed for fucoidan by following two different extraction methods.

MATERIALS AND METHODS

Four species of brown seaweeds *viz. Sargassum wightii, S. oligocystum, Padina tetrastromatica* and *Turbinaria ornate*, collected from the two different locations, Valinokkam (N:09° 13.684, E:078° 47.194) and Hare Island (N:08° 047.254' E:078°, 11.884) of Gulf of Mannar during the peak season from July 2012 to January 2013, were used to extract fucoidan by water and acid extraction.

Water extraction method:

The extraction of fucoidan was carried out as described by Yang *et al.*[15] with a slight modification. The shade dried pulverized seaweed (20 g) was treated with 1 ml 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 10 000 g for 10 min and then dried at room temperature. The dried

biomass (5 g) was extracted with 100 ml of distilled water at 65° with continuous stirring for 1 h twice, and the extracts were combined. The combined extract was centrifuged at 10 000 g for 20 min and the supernatant was treated with 1% of $CaCl_2$ and kept at 4° for overnight to precipitate the alginic acid after centrifugation at 10 000 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° for 4 h in a chill cabinet. Again, the solution was centrifuged at 10 000 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° overnight to precipitate out the intact fucoidan. After centrifugation at 10 000 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 Eqn., yield (%)=weight of the obtained fucoidan (g)/weight of the dried biomass (g)×100.

Acid extraction method:

Acid extraction was followed as per the method described by *Aloe*[1] with a slight modification. Dried seaweed powder (120 g) was treated with the solvent mixture consisting of ethyl alcohol, chloroform and distilled water at the ratio of 4:2:1, with constant stirring at room temperature for 4 h in a magnetic stirrer. The supernatant was removed and the residue was dried overnight. Dried seaweed biomass was then treated with 0.03 M HCl (hydrochloric acid) at a ratio of 1:20 and heated at 90° for 3 h in a water bath. The content was then centrifuged at 10 000 g for 20 min at 4° and the supernatant was collected. The residue was washed again with distilled water and centrifuged again. The supernatants were pooled and 99% ethanol was added to obtain 60% ethanol concentration to precipitate out the crude fucoidan. The precipitate was washed with distilled water and immediately coagulated with 1 M $CaCl_2$ to remove the alginic acid. The sticky calcium alginate precipitate was discarded. The supernatant was again centrifuged at 10 000 g for 20 min at 4° to collect the fucoidan pellet. The pellet was again washed with alcohol and

diethyl ether and dried overnight at 50° to obtain fucoidan. The yield of fucoidan was calculated as described above.

Characterization of fucoidan:

For the characterization, fucoidan (10-15 mg) was hydrolyzed with 2 ml of 4 N HCl at 121° for 2 h and then neutralized with 4 N NaOH[8]. The sulphate content in the fucoidan was measured based on barium sulphate ($BaSO_4$) determination using barium chloride ($BaCl_2$) method described by Dodgson and Price[18]. Free fucose content in fucoidan was determined by cysteine-sulphuric acid method described by Dische and Shettles[19]. Total free sugars in the fucoidan were measured by the anthrone method[20]. The amount of protein in the fucoidan was determined by Bradford protein assay[21].

The active components of the fucoidan were qualitatively determined by Fourier Transmission Infra-Red spectroscopy (FT-IR; Brucker Optik GmbH). Fucoidan (10-15 mg) was treated with 2-N trifluroacetic acid (0.5 ml) at 121° for 2 h, in glass tubes sealed with N_2, cooled and neutralised with 2 N NaOH[22]. The frequency of the spectra analysis was set between 4000 and 400 cm^{-1} wave number and the vibration spectrum was recorded as a graphical chart.

Statistical analysis:

All data were subjected to analysis of variance (ANOVA) using SPSS (SPSS 17.0 for window, SPSS Inc., Chicago, IL, USA) and the difference between the yield of fucoidan and its compositions were compared.

RESULTS AND DISCUSSION

Although seaweeds are available throughout the year in Gulf of Mannar coastal region, large quantities are collected only during the maturation stages of the respective algae. Brown seaweeds are particularly dominant along this coast during July to January[23]. In this study, four selected brown seaweeds, *S. wightii, S. longifolium, P. tetrastromatica* and *T. ornate* that are dominant along this coast were collected from two different locations, Hare Island and Valinokkam to examine the variation in the yield of

fucoidan. Brown seaweed, *P. tetrastromatica* was dominant only in Hare Island, whereas *T. ornata* was abundant only in Valinokkam. The yield of fucoidan was found to be slightly higher from the respective seaweeds collected from Hare Island compared to those collected from Valinokkam, when subjected to water extraction ($P>0.05$), while significant variation was noticed with acid extraction ($P<0.05$) throughout the study period (figs.1 and 2). Seaweeds in shallow region, are exposed to sunlight during low tide and so would be expected to have more amount of fucoidan as they develop defense mechanisms against dryness[24]. Since, Hare Island is characterized with shallow coastal shore with the depth of less than 1 feet, where seaweeds are often exposed to sunlight during low tide, the weeds collected from this region gave higher yields when compared to those collected from Valinokkam, which has rocky shores with depth more than 1 m. Another fact that supports this theory is that seaweeds when exposed to sunlight, their sugar content would increase and the UV rys of sunlight might destroy cellular constituents[25].

Two extraction procedures were employed to extract fucoidan from brown seaweeds of Gulf of Mannar. The yield of fucoidan differed significantly ($P<0.05$) depending on the type of extraction procedure. Significantly higher yield of fucoidan was obtained through water extraction with a maximum of 9.46±0.18% in *P. tetrastromatica* followed by 5.83±0.07% in *T. ornata* and 3.28±0.04% in *S. wightii*. The yield of fucoidan extracted by acid method treatment was very low with a maximum yield of 1.63% in *P. tetrastromatica* and a minimum of 0.33% in *S. oligocystum*. It has been reported that the yield and chemical composition of fucoidan was strongly affected by the method of extraction[1]. In a similar study carried out by Mak[26], three extraction methods such as water, acid and $CaCl_2$ were compared, in which the highest yield was obtained with $CaCl_2$ and the lowest with acid extraction. The present study demonstrated that water extraction was the most suitable method for extracting fucoidan from brown seaweeds, irrespective of the species.

Four brown seaweeds were examined for the yield of fucoidan during July 2012 to January 2013 i.e., the period of maturation of seaweeds. The yield of fucoidan was high during September and October in all species extracted with water. At Valinokkam, the yield of fucoidan gradually increased from 2.79±0.06 to 3.28±0.04% for *S. wightii* during July to September and decreased to 2.81±0.06% in December. Similarly, fucoidan extracted from *T. ornata* was found to be 4.87±0.11 and 5.83±0.07% during July and September, respectively. A similar pattern was observed for other species, *S. oligocystum* and *S. longifolium*. Few studies have earlier reported that variations in the yield of fucoidan and its derivatives are affected by the season in which seaweeds are collected[25] and plant maturity[27]. The authors also reported a correlation between fucoidan yield and seasonality; and more fucoidan was obtained during the reproductive stages of the seaweed. The physical and chemical states of the seaweed change during the reproductive stage[16]. In the present study, *Sargassum* during maturation period (September and October) gave highest yield of fucoidan and *P. tetrastromatica* collected during January, recorded higher yield. The reproductive receptacles were observed during the month of October for *S. wightii* and *T. conoides*[28]. The highest yield of fucoidan was obtained during January at the maturation stage for *P. tetrastromatica* (9.46±0.18%) of Hare Island and during September, at the maturation stage of *T. ornata* (5.83±0.07%) of Valinokkam. The maturation stage of *S. wightii* was also September and the highest fucoidan yield of 3.9±0.05% at Hare Island. It was also reported that the highest yield of fucoidan from sporophyll of *Undaria pinnatifida* as 57.3 to 69.9% (d/w) during the maturation stage of alga and also found that the yield varied between the species, comparable to this study[29].

With respect to the yield of fucoidan from the different species of brown seaweeds, it was observed that the yield was higher in *P. tetrastromatica* (9.46±0.18%) collected from Hare Island irrespective of season, than the other species, during water extraction. The yield of fucoidan in *T. ornata* was (5.83±0.07%). The yield of fucoidan was very low in *S. oligocystum* collected from both the

locations. Nevertheless, following acid extraction, the yields of fucoidan from *S. wightii* and *P. tetrastromatica* were found to be more or less similar (0.87, 0.94, 0.79% and 1.14, 1.31, 1.22%) during the month of July, August and September, respectively. It was therefore clear that differences in the chemical structure played a role in the yield of fucoidan[12,30,31]. Earlier observations also indicated that the variation in the yield of fucoidan due to species, anatomical regions, growing conditions, extraction procedures and analytical methods[30,32]. This study also demonstrated that the yield of fucoidan from brown seaweeds varied with species, location, season and method of extraction.

In the present study, biochemical constituents of crude fucoidan such as fucose, sulphate, total free sugars and protein were analyzed (Table 1). It was found that fucose was the major constituent of the fucoidan extracted from all the four species. Among the four species, high fucose content was recorded in *P. tetrastromatica* (54.51±1.08%), followed by *T. ornata* (51.76±1.43%). The next major constituent in fucoidan was sulphate, which showed variation among the species similar to fucose. High sulfate content was recorded in *T. ornata* (24.27±0.85%) followed by *P. tetrastromatica* (22.70±0.53%). According to Bilan[31], the extracted fucoidan polymer consisted mainly of carbohydrates (54.9%) and sulfates (41.5%) and small amounts of proteins (2.8%). The sulfate content of crude fucoidan ranged from 5.6 to 42.3% and fucose from 49.5 to 54% for *S. swartzii*[33]. The values obtained for fucose and sulfate in this study were more or less similar to those ranges reported by Chattopadhyay[3] who have extracted fucoidan from Indian brown seaweed, *T. conoides* with fucose (56%) and other sugars such as glucose, galactose and xylose. According to Yang[15] monosaccharide composition analysis showed that fucose was the major sugar (78.8%) and considerable amount of galactose (21.2%) was also present in extracted fucoidan. The variation in the monosaccharide composition of fucoidan from *U. pinnatifida* and was analysed and reported that sulfated galactofucan varied from 19 to 38 mol. % galactose dependent on the maturity stage of algae[16].

In the present study, the content of total carbohydrates was estimated to be in the range of 10-11% in all the species of seaweeds. These values corroborate with the results of Rodriguez-Jasso *et al.*[22] who reported that the total free sugars in fucoidan polymers extracted from *T. turbinata, S. filipendula* and *P. perindusiata* were 9.0, 8.9 and 10.2%, respectively. There were no significant differences observed between the species with respect to total free sugars and protein content ($P>0.05$). The protein content ranged between 1.3 to 2.3%, which was quite higher than the value reported for pure fucoidan[34]. This study thus indicated that the biochemical composition of fucoidan is significantly different among the species. The variation in the composition of fucoidan also has a large impact on the final structure of fucoidan[12,30,31].

The characterization of the fucoidan structure of the two dominant brown seaweeds *viz., P. tetrastromatica* and *S. oligocystum* was performed by FT-IR analysis (figs. 3 and 4). A major band centered around 3448.03 cm^{-1} was assigned to be hydrogen bonded O-H stretching vibration in both the species. A weak signal at wavelength 2103.7 cm^{-1} indicating the presence of C=C=O was noticed in *P. tetrastromatica* only. Another major band at 1688 cm^{-1} corresponding to the asymmetric stretching of O-C-O vibration was noticed in the fucoidan of both the seaweeds. A minor band at around 1436.69 cm^{-1} and another two peaks around 1202.64 cm^{-1} and 140 cm^{-1} were characteristic of fucoidan in both the species. The peak at 1202 cm^{-1} corresponded to the C-H deformation vibration of β-manuronic residues. The presence of sulphate groups in fucoidan is very important as it is related to its bioactive properties[15]. The sulphate absorption bands at 800 cm^{-1} and above indicates that most sulphate groups are located at positions 2 and 3 of sugars[35].

FT-IR is frequently used to determine the position of a sulfate group in any bioactive molecule like fucoidan. In the present spectra, a band at 843.78 cm^{-1} corresponded to the S=O stretching vibration of *P. tetrastromatica* and at 842.18 cm^{-1} of *S. oligocystum.* The IR spectra of fucoidans from brown algae

such as *Laminaria cichorioides, Fucus evanescens* and *L. japonica* showed that most sulphate groups were in axial positions and the remainder were in equatorial positions according to a strong band at 842 cm^{-1} and a shoulder at 820 cm^{-1} in the spectra[27]. In the present study, the shoulder band appeared at around 803.4 cm^{-1} but was found at equal concentrations to that of the band at around 842 cm^{-1}, indicating that sulphate groups are present at equivalent amounts in axial and equatorial positions. Another two bands that appeared at 723.03 cm^{-1} and 601 cm^{-1} seem to be characteristic of C-O-S stretching of sulphate group and C=C-H stretching vibration, respectively in both the species of seaweeds. As discussed earlier, the major differences among the species were the presence of a minor band at 2103.73 cm^{-1} in *P. tetrastromatica* and slight shifts noticed in sulphate group band. Besides this, the structure of fucoidan extraction from the two seaweeds was more or less similar.

In conclusion, two brown seaweed species *viz., P. tetrastromatica* and *T. ornate* collected along the Gulf of Mannar coast were found to be the major source of fucoidan. Water extraction procedure provided high yield of fucoidan irrespective of the species. High yielding seaweed, *P. tetrastromatica* was dominant in Hare Island, while *T. ornata* was dominant in Valinokkam area. Yield of fucoidan was higher during maturation stage, which was September for *T. ornata* and *S. wightii*; and January for *P. tetrastromatica.* Fucoidan of *P. tetrastromatica* has high source of fucose and *T. ornata* has high sulphate. Structurally there is no significant difference in the functional groups of fucoidan extracted from these two major seaweed species. This study thus interferes that there is a tremendous resource potential for fucoidan yielding seaweeds in the Gulf of Mannar, which shall be explored further to examine their bioactive potential to suit for food and pharmaceutical applications.

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TABLE 1: CHEMICAL COMPOSITION OF FUCOIDAN

Composition (%)	***S. wightii***	***S. oligocystum***	***P. tetrastromatica***	***T. ornata***
Fucose	$49.34^{b}\pm2.78$	$43.16^{a}\pm3.21$	$54.51^{d}\pm1.08$	$51.76^{c}\pm1.43$
Sulphate	$18.73^{b}\pm0.82$	$16.63^{a}\pm0.62$	$22.70^{c}\pm0.53$	$24.27^{d}\pm0.85$
Free sugars	$11.39^{c}\pm1.03$	$10.96^{b}\pm0.99$	$11.83^{d}\pm1.53$	$10.17^{a}\pm0.23$
Protein	$1.30^{c}\pm0.31$	$0.96^{a}\pm0.04$	$1.14^{bc}\pm0.12$	$1.05^{ab}\pm0.15$

Each value is the mean of three observations. Mean bearing at least one common superscript within a row do not differ significantly ($P<0.05$)

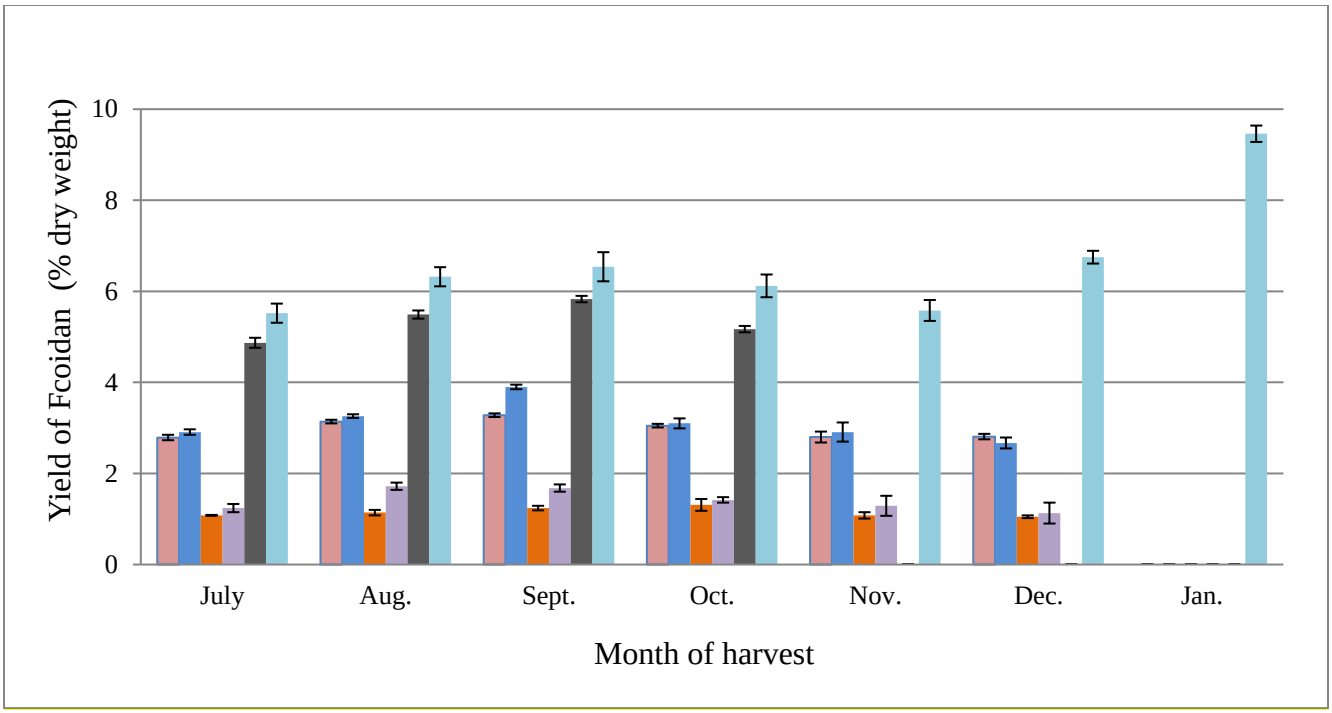


Fig. 1: Variation in the yield of water-extracted crude fucoidan content from brown seaweed species

Yield of water extracted crude fucoidan content (% d/w) from brown seaweed species collected at Valinokkam and Hare Island in the Gulf of Mannar. ■ *S. wightii* (V); ■ *S. wightii* (HI); ■ *S. oligocystum* (V); ■ *S. oligocystum* (HI); ■ *T. ornate* (V); ■ *P. tetrastromatic* (HI)

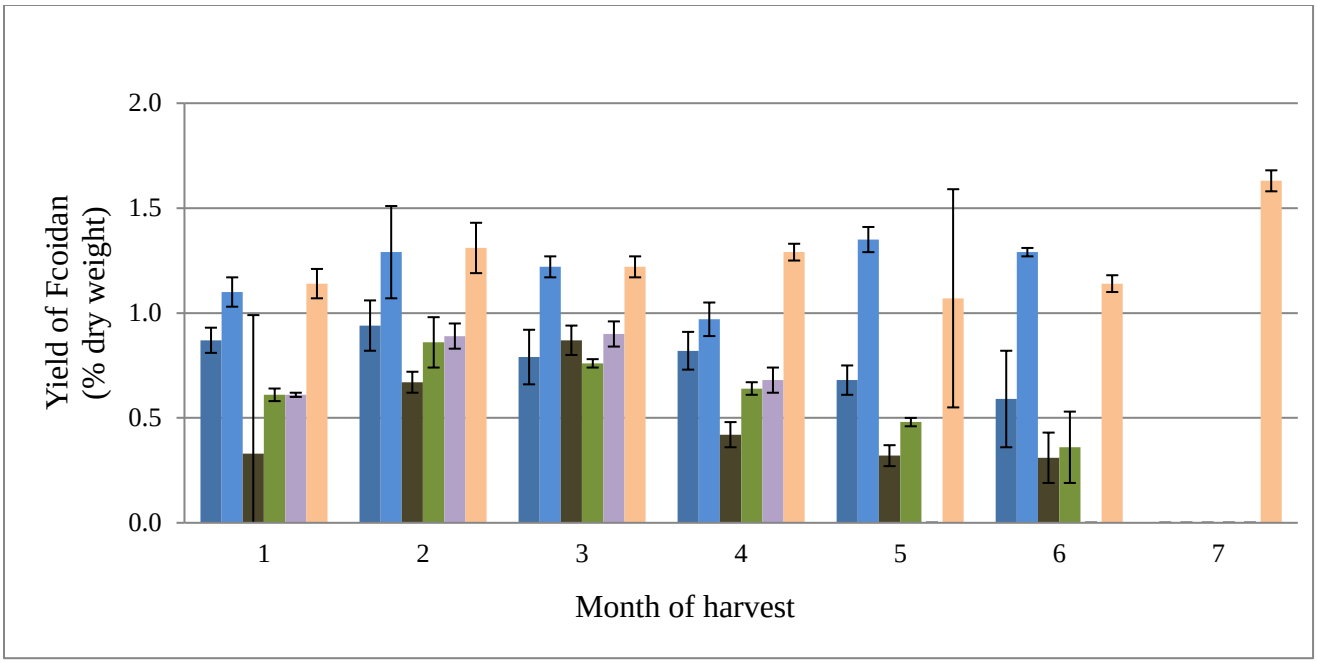
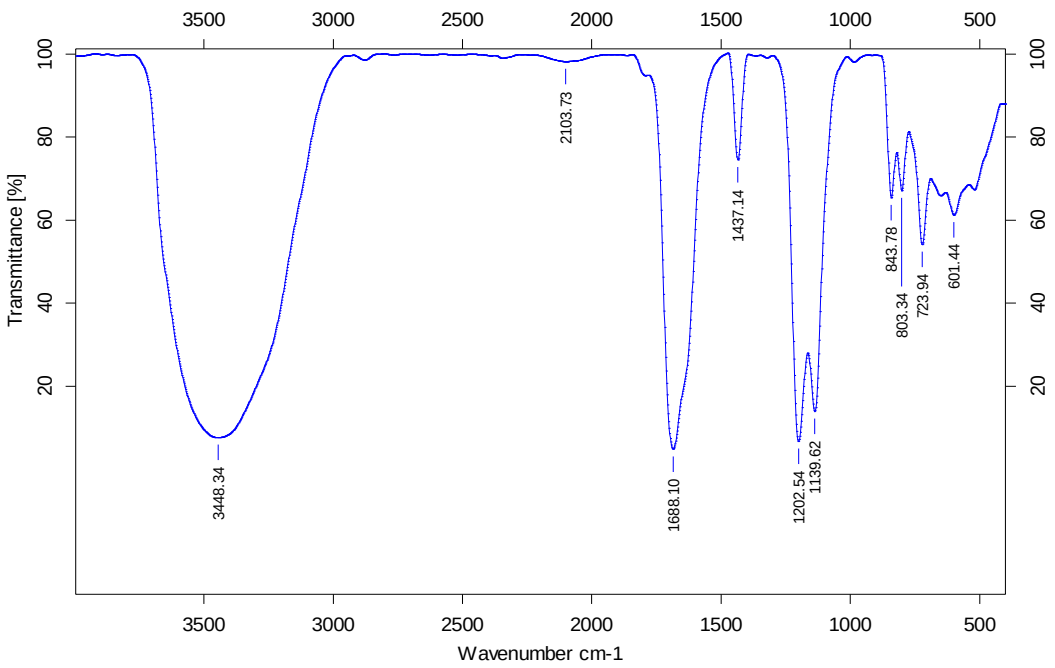


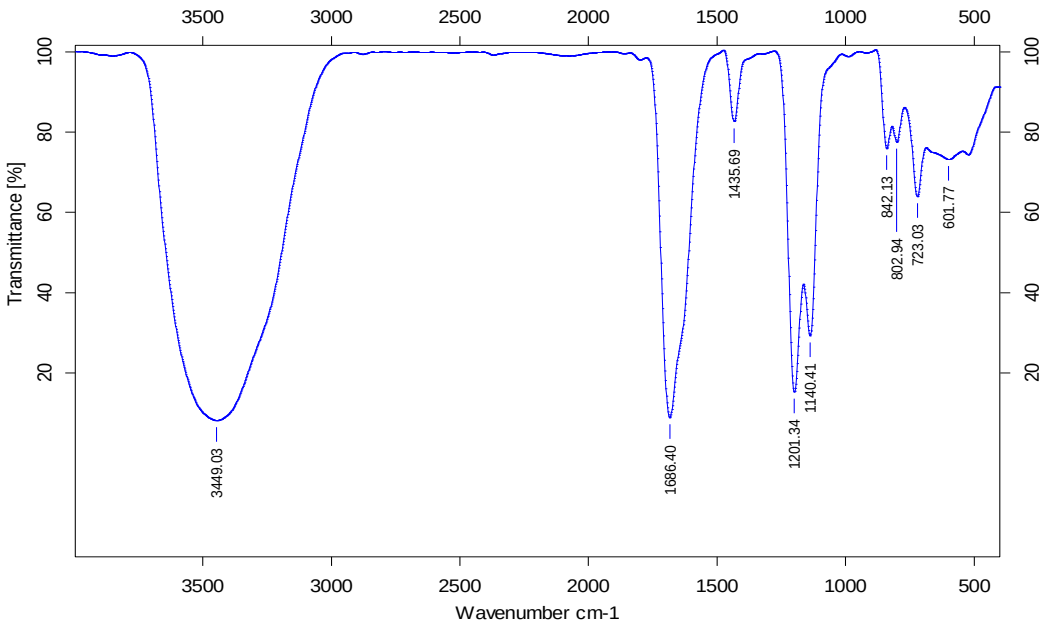
Fig. 2: Monthly variation in the yield of acid-extracted fucoidan content from brown seaweed species

Yield of acid-extracted fucoidan content (% d/w) from brown seaweed species collected at Valinokkam and Hare Island. ■ *S. wightii* (V); ■ *S. wightii* (HI); ■ *S. oligocystum* (V); ■ *S. oligocystum* (HI); ■ *T. ornate* (V); ■ *P. tetrastromatic* (HI).



Sample Name: PT-I

Fig. 3: FT-IR analysis of fucoidan extracted from brown seaweed *Padina tetrastromatica.*



Sample Name: SL-I

Fig. 4: FT-IR analysis of fucoidan extracted from brown seaweed *Sargassum oligocystum*.

Tables and figure titles and legend:

TABLE 1: CHEMICAL COMPOSITION OF FUCOIDAN (%)

Each value is the mean of three observations. Mean bearing at least one common superscript within a row do not differ significantly (P<0.05)

Fig. 1: Variation in the yield of water-extracted crude fucoidan content from brown seaweed species

Yield of water extracted crude fucoidan content (% d/w) from brown seaweed species collected at Valinokkam and Hare Island in the Gulf of Mannar. ■ S. wightii (V); ■ S. wightii (HI); ■ S. oligocystum (V); ■ *S. oligocystum* (HI); ■ *T. ornate* (V); ■ *P. tetrastromatic* (HI)

Fig. 2: Monthly variation in the yield of acid-extracted fucoidan content from brown seaweed species

Yield of acid-extracted fucoidan content (% d/w) from brown seaweed species collected at Valinokkam and Hare Island. ■ *S. wightii* (V); ■ *S. wightii* (HI); ■ *S. oligocystum* (V); ■ *S. oligocystum* (HI); ■ *T. ornate* (V); ■ *P. tetrastromatic* (HI)

Fig. 3: FT-IR analysis of fucoidan extracted from brown seaweed *Padina tetrastromatica*

Fig. 4: FT-IR analysis of fucoidan extracted from brown seaweed *Sargassum oligocystum*