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Extraction of antibacterial substances, galactofucoidan and alginate successively from the Gulf of Mannar brown seaweed *Sargassum wightii* Greville ex J. Agardh

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In the present study, galactose dominated fucoidan was isolated through successive extraction method in alkaline, acid and neutral pH from the brown seaweed *Sargassum wightii* Greville ex J. Agardh collected along the Gulf of Mannar (Pamban, India) during March 2011. In this study, crude extracts obtained in chloroform: methanol (1:1 v/v) and 85 % ethanol (at 23°C and 70°C) showed antibacterial activity against *Xanthomonas axonopodis* pv. *citri* and *X. oryzae* pv. *oryzae* which cause canker in citrus and blight of paddy, respectively. Low (< 3500 Da) and high molecular weight (> 3500 Da) fucoidans separated through DEAE cellulose 52 column were recorded same proportion of monosugars constituents dominating D-galactose over L-fucose. Spectroscopic studies (IR & NMR) of high molecular weight fucoidan (Fraction, A1H) with high sulphate showed higher anticoagulation activity than low molecular weight fucoidan under *in vitro* APTT assay. This investigation concludes that successive extraction method yields galactofucoidan (3.62 %), alginate (11.2 %) and antibacterial substances (13.34 %) in the same biomass of brown seaweed *S. wightii*.

Keywords: *Sargassum wightii*; Galactofucoidan; Antibacterial substances; Alginate.

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Introduction

Seaweeds (marine macroalgae) occurring intertidal and sub tidal regions of the coastal waters are classified as green, red and brown based on their pigmentation. Sulphated polysaccharides such as agar and carrageenan extracted from red seaweeds and alginate from brown seaweeds have been used as valuable additives in the food and pharmaceutical industries because of their rheological properties like gelling and thickening agents[1,2]. Fucoidan was first mentioned in the medical literature in 1970 for the characteristic slippery texture of brown algae thallus. This sulphated polysaccharide containing hemi-ester sulphate groups in their sugar residues commonly called fucan found in some *Phaeophyceae* members, marine invertebrates, scarcely in microbes and absent in higher plants[2]. Fucan of brown algal origin is known as fucoidan which is structural specific and its compositions are varied among the brown seaweeds and even unique within the species of the genus. It is present in the algal cell-wall matrix constituted with large proportions of L-fucose and sulphate together with other sugars like galactose, xylose, glucose, mannose, rhamnose and uronic acids[3]. This sulphated polysaccharide having structural and functional analog of heparin by displaying anticoagulation activity besides many biological activities such as antiviral and immune-inflammatory that might find relevance in nutraceutical, functional food, cosmetic/ cosmeceutical and pharmaceutical applications. Bioactivities of the fucoidan are depending on the proportion of L-fucose and other monosugars along with their degree of sulphation[2]**.** Indian coast is a tropical one bestowed with 844 species of seaweeds of which 194 species are brown recorded so far along the length of 7200 Km long coastal line[4]. As they endure wide range of climatic conditions, Indian brown seaweeds are promising candidates for the source of structurally unique fucoidans. In India, research done on the isolation and bioactivities of sulphated polysaccharides[5-9] are not mainly dealing with fucoidans[10, 11] of specific species. However, among the 11 Gulf of Mannar (India) brown seaweeds, a high amount of crude fucoidans is

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Plate 1-The brown seaweed *Sargassum wightii* collected along the Gulf of Manner (Pamban, 9°17′N 9°18′E/ 9.28°N 79.3°E, India)

extracted from *Sargassum wightii* Greville which is the major raw material for alginate industries (Plate 1). Hence, in this study, a method is reported not only for the isolation of fucoidans but also for the extraction of alginate and antibacterial substances successively from the same raw material of *S. wightii* Greville.

Materials and Methods

Sampling

Healthy specimen of 1.5 kg brown seaweed *S. wightii* Greville ex J. Agardh which remain immersed in the seawater during low tide were collected along the Gulf of Manner (Pamban, 9°17′N 79°18′E/9.28°N 79.3°E, India) during March 2011. Specimens were cleaned by washing thoroughly in seawater followed by tap water and distilled water. After air drying in dark for 3 days, specimens were pulverized into fine powder.

Extraction of non-fucoidan portion (pigments & protein) for evaluating antibacterial activity

For the successive extraction of fucoidan[12], powdered 500 g sample was initially extracted in 250 mL of chloroform: methanol (1:1 v/v) for 3 days at 30°C in dark in order to remove non-fucoidan portions such as pigments and lipophilic substances. After extraction, the extract was filtered through Whatman No.1 filter paper and collected separately. Then the sample was extracted in 200 mL of 85 % ethanol at 23°C [twice at 12 h and 70°C (twice at 5 h)] to remove protein. The extracts obtained in chloroform: methanol (1:1 v/v) and 85 % ethanol at 23° C and at 70°C concentrated separately using rotary evaporator at 60°C were evaluated for antibacterial activity against plant pathogenic bacteria *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al. AGACA 01 (canker in citrus) and *X. oryzae* pv. *oryzae* (Ishyama) Dye AGACA 02 (blight in paddy) which were obtained from the laboratory culture stock of Botany Department, Alagappa Government Arts and Science College.

Antibacterial activity assay[13]

The antibacterial assay was carried out by the agar diffusion technique using 6.0 mm diameter Whatman #1 filter paper discs in 1.5 % nutrient agar medium (Peptone – 10 g, Beef extract – 10 g, NaCl –5 g, Distilled H_2O – 1000 mL, Agar – 15 g, pH -7.0). Sterile paper discs loaded with 50 µL (100 µg) of crude extracts were impregnated onto Petri plates of 100 mm diameter containing ca. 20 mL of nutrient agar medium smeared with 100 µL of bacterial culture in exponential phase of 1.0 OD at 590 nm and incubated at 28°C for 48 h. The diameters of the agar clear zones of bacterial inhibition around the disc as a result of diffusion of active substances were measured as a measure of antibacterial activity. Three replicates were maintained for each experiment and the mean values expressed. The solvents used for reconstituting the crude extracts were loaded on paper discs treated as a solvent control which did not show any inhibition zones.

Extraction of fucoidan and alginate through successive method[12] as well as direct method[11, 14]

In the successive extraction, residue after removing the non-fucoidan portion was extracted thrice in 500 mL of 2 % $CaCl_2$ in distilled H_2O in pH 8.5 for 3 h at 70°C. Then the extract was filtered through muslin cloth and centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded and the pellet was considered as crude fucoidan A. Then the residue was adjusted to pH 2.0 using 0.01N HCl and again extracted thrice in 500 mL of 2% $CaCl_2$ solution as described above. The pellet thus obtained was crude fucoidan B. Then the residue was soaked in 500 mL of 3 % Na_2CO_3 in distilled H_2O thrice in pH 7.0 for 3 h at 70°C and the extract was filtered through muslin cloth and centrifuged. The pellet was treated as crude fucoidan C and alginate was precipitated from the supernatant by adding equal volume of acetone and stored overnight. The alginate yield was recorded. Shade dried specimens were subjected directly for the extraction of fucoidan[15] and alginate[14] separately and the yield was recorded.

Qualitative test of fucoidan[16]

Crude fucoidans such as A, B and C obtained through successive extraction and direct extraction were subjected to qualitative test for fucoidan. In a test tube, 400 µL of crude fraction and 1.8 mL concentrated H_2SO_4 were added and kept in boiling water bath for 10 minutes. The solution mixture was kept in ice bath for 5 minutes to cool and then 40 µL of L-cysteine HCl was added. Development of greenish yellow color persisted for 24 h indicating the presence of L-fucose in the test solution. For confirmation, above test was repeated by keeping the test solution in the boiling water bath only for 3 minutes which did not develop the greenish yellow colour confirmed the presence of L-fucose in the test solution. Crude fractions showing positive on L-fucose test were quantified gravimetrically and considered as crude fucoidans.

Composition of crude fucoidans obtained by successive extraction method

Monosugars such as L-fucose, D-galactose, D-glucose and D-mannose in the crude fractions (A, B & C) obtained through successive extraction were estimated by the phenol-sulphuric acid method[17] against the respective sugar standard. The sulphate content was determined by the Barium chloride and Gelatin method against Na_2SO_4 standard[18].

Isolation and purification of fucoidans

Based on the monosugars composition, crude fucoidan A and B were taken up for separation. Crude fucoidan A weighing 500 mg redissolved in 5 mL of distilled H_2O was loaded on the column (30 × 2.4 cm) packed with DEAE-cellulose 52 (SRL, India) and equilibrated in distilled H_2O at 25°C. The column was eluted stepwise by increasing concentration of 300 mL 0.5M, 1 M, 1.5 M, 2.0 M, 2.5 M and 3.0 M NaCl in distilled H_2O. The flow rate was maintained as 1 mL/minute. Eluvants of 50 mL were collected and L-fucose content in each eluvant was estimated by phenol-sulphuric acid method[17]. Based on the L-fucose content, eluvants were combined. After washing the column with 3 M NaCl, until no sugar was found in the eluvant, 500 mg of crude fraction B was loaded on the column and separated as described above.

Fractions of A1 and A2 and B1 and B2 obtained from the crude fucoidan A and B, respectively were dialyzed (MWCO3500, #21-152-9 flat width: 46 mm. vol/cm: 6.74 mL, wall thickness: 28µm dry cylinder diam: 29.3 mm Fisher, India) for 48 h in glass distilled water. Fucoidan substances retained in the membrane bag considered as high molecular weight (HMW > 3500 Da) and passed across considered as low molecular weight (LMW < 3500 Da) were lyophilized and stored at 4°C till further study. Composition of monosugars[17], sulphate[18] and yield of purified fucoidan were recorded.

***In vitro* anticoagulation activity by activated partial thromboplastin time[19]**

Activated partial thromboplastin time (APTT) was carried out using platelet poor plasma. Blood was obtained from two healthy individuals without having history of bleeding or thrombosis. Nine parts of blood collected by venipuncture were drawn into one part of 3.8% sodium citrate. Blood was centrifuged at 3000 rpm for 20 minutes and the plasma was collected and stored at 4°C until use. For APTT assay, citrated normal human plasma (90 µL) was mixed with 10 µL of different concentrations of fucoidan fractions such as A1L and A1H (0, 0.45, 0.9, 4.5 µg/10/µL) and incubated for 1 minute at 37°C. Then 100 µL anticoagulant reagent (Ethylene Diamine Tetraacetic Acid, EDTA) was added to the mixture and incubated for 5 minutes at 37°C. There after clotting was induced by adding 20 mM $CaCl_2$ (100 µL) and clotting time was recorded. All samples were dissolved in distilled water.

Characterization of fucoidan fraction, A1H

Based on the anticoagulation potential, A1H was read in the FTIR (Shimadzu, Japan) spectroscopy between 4000 and 400/ cm. A1H fraction was dissolved in 0.5 mL D_2O and ^1H and ^{13}C NMR spectra were recorded using a Bruker Biospin Avance 400 FT-NMR spectrometer (^1H frequency ¼ 400.13 MHz, ^{13}C frequency ¼ 100.62 MHz) at 298 K using 5 mm broad band inverse probe head equipped with shielded z-gradient and XWIN-NMR software version 3.5 using TMS as an internal reference. One-dimensional ^1H and ^{13}C spectra were obtained using one pulse sequence. One-dimensional ^{13}C spectrum using Spin Echo Fourier Transform (SEFT) and Quaternary Carbon Detection (QCD) 42 sequences were performed for structural determination of galactofucoidan.

Results and Discussion

Extraction of fucoidan and alginate by successive method as well as direct method

Results on antibacterial activity of non-fucoidan fraction, alginates yield and composition of crude fucoidan extracted by successive method and yield of

fucoidan and alginate obtained by direct methods are presented in the (Table 1). Fucoidan was isolated from the brown seaweeds by various methods[9, 20-22]. Yield and quality of fucoidan depends on the methods adopted for extraction and the species used[22,11].

Accordingly, in the present study, total crude fucoidan yield of 3.62 % in alga dry wt. was recorded in the successive extraction method whereas in direct method 5.73 % was obtained. But the recorded amount of monosugars and sulphate in the crude fucoidan obtained by successive method was higher than the direct method made in our earlier study in the *S. wightii*[11]. This result shows that removal of non-fucoidan portion in successive extraction provides crude fucoidan without impurities at the extraction level. Successive extraction yield 11. 2 % in alga dry wt. of alginate against 17.8 % obtained through direct extraction method. Since brown seaweeds are the only source for alginate as well as fucoidan our present study results suggested that adopting successive extraction is a alternate extraction method to extract not only fucoidan but also alginate from the Indian major alginophyte *S. wightii.*

Antibacterial activity of non-fucoidan fraction obtained by successive method

In the successive extraction, initial extraction was carried out to remove the pigments and proteins from the sample[12] and these extracts containing lipophilic substances[13] were evaluated for the antibacterial activity. The extracts were obtained in chloroform: methanol (1:1 v/v) followed by 85 % ethanol at 23°C and 85 % ethanol at 70° C. Maximum crude yield of 13.34 % in alga dry wt. was obtained in chloroform: methanol (1:1 v/v) exhibiting high antibacterial activity against *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri* followed by the extracts obtained in 85 % ethanol at 23°C and 85 % ethanol at 70° C. As a support to our present study, extracts prepared in chloroform: methanol (1:1 v/v) in *S. wightii* showed good antibacterial activity against the same bacterial plant pathogens[23]. Extract of 85 %

Table 1- Antibacterial activity of non-fucoidan fraction, yield of alginates and yield and composition of crude fucoidan

S No	Extract/ fraction	Yield (% alga dry wt.)	Total Sugar (mg g^{-1} crude fucoidan dry wt.)	*D-Glucose	*L-Fucose	*D-Galactose	*D-Mannose	Sulphate (mg g^{-1} crude fucoidan on dry wt.)	Antibacterial activity (zone of inhibition in mm diameter)	
									Xanthomonas axonopodis pv. *citri*	*X. oryzae* pv. *oryzae*
1	Chloroform: methanol (1:1 v/v)	13.344	-	-	-	-	-	-	11.0 ± 1.3	13.0 ± 1.2
2	85 % ethanol at 23°C C	7.321	-	-	-	-	-	-	9.0 ± 0.92	10.0 ± 1.1
3	85 % ethanol at 70 °C C	3.159	-	-	-	-	-	-	+	+
4	Crude fucoidan A	0.59	806.70	22.49	20.96	33.65	22.88	263.50	-	-
5	Crude fucoidan B	0.70	202.20	22.50	20.91	33.63	22.84	79.10	-	-
6	Crude fucoidan C	2.33	426.80	22.49	20.94	33.64	22.86	22.80	-	-
7	Crude fucoidan[1]	5.73								
	Alginate	11.2	-	-	-	-	-	-	-	-
8	Alginate[3]	17.8	-	-	-	-	-	-	-	-

3.62[2]

*% in Total sugar
[1] direct extraction
+ trace activity
[3] direct extraction
[2] total yield
- data not recorded

ethanol at 23°C made to remove protein displayed low antibacterial activity whereas the extract obtained in 85 % ethanol at 70° C showed only trace activity. These observations showed that crude extracts obtained in chloroform: methanol (1:1 v/v) and in 85 % ethanol at 23°C during successive extraction of fucoidan in *S. wightii* contained substances to inhibit the growth of bacteria causing canker in citrus and blight in paddy.

Composition, isolation and purification of fucoidans

Yield and constituents of crude fucoidans fractionated at alkaline, acid and neutral pH successively as three fractions are presented in the (Table 1). Maximum yield of 2.33 % in alga dry wt of crude fucoidan C was obtained in neutral pH followed by crude fucoidan B in acid pH and crude fucoidan A in alkaline. All the three samples (A, B & C) were confirmed as crude fucoidan by estimating the presence of L-fucose[15] which is the principle monosugar in the fucoidan polymer[24]. This heteropolysaccharide exhibit variety of biological activities including anticoagulation[25]. The bioactivity of fucoidan is mainly related to its constituents such as fucose, galactose and sulphate in the polymer which is varied depending upon the method adopted for extraction[26]. From this study, it was observed that pH influenced the yield, composition of monosugars and sulphate content of fucoidan. Low yield of crude fucoidan A fractionated in pH 8.5 was recorded with high amount of total sugar (806.70 mg/g crude fucoidan) and sulphate (263.50 mg/g crude fucoidan) whereas high yield of crude fucoidan B and C contained very less amount of total sugar and sulphate. Like this present study where fucoidan with high sulphate extracted in alkaline pH from *S. wightii*, previously such fucoidan obtained in *Undaria pinnatifida* showed good bioactivity[26]. Our study shows that proportion of monosugars in all the three crude fucoidans fractionated at alkaline, acid and neutral pH obtained in *S. wightii* was similar dominating galactose extracted by successive method[13] as recorded earlier in *Saccharina longicruris*[27], *Fucus evanescens*[28] and *Lobophora variegata*[29].

Fucoidans of LMW range from 500 Da to 3500 Da and HMW above 3500 Da[30, 31] isolated from the brown seaweeds collected at different coasts of the world[32] displayed different biological activities. In this study, LMW fucoidan fractions (< 3500 Da) such as A1L and A2L and B1L and B2L (Table 2) and

Table 2 -Yield and constituents of LMW fucoidans (< 3500 Da) isolated through the DEAE cellulose column

S.No		Crude fucoidan/ Eluvant number	Pooled volume (ml)	Purified fraction	Total fucoidan yield(mg g^{-1} alga dry wt.)	Total sugar yield (mg g^{-1} fucoidan)	Constituents of LMW fucoidans				
							*D-Glucose	*L-Fucose	*D-Galactose	*D-Mannose	Sulphate (mg g^{-1} fucoidan)
1.	Crude fucoidan A	1	50	-	-	-	-	-	-	-	-
2.		2-8	350	A1L	34.74	498.27	22.54	20.95	33.65	22.83	357.80
3.		9	50	-	-	-	-	-	-	-	-
4.		10-12	150	A2L	29.68	509.43	21.42	19.82	31.79	26.95	288.40
5.		13-36	1200	-	-	-	-	-	-	-	-
6.	Crude fucoidan B	1	50	-	-	-	-	-	-	-	-
7.		2-4	150	B1L	28.94	643.40	27.29	14.70	40.87	17.09	252.93
8.		6-8	150	-	-						
9.		9 & 10	100	B2L	25.85	643.71	19.12	15.80	35.14	23.88	162.86
10.		11-36	1300	-	-	-	-	-	-	-	-

% in total sugar - not recorded

HMW fractions (> 3500 Da) such as A1H and A2H and B1H and B2H (Table 3) were isolated from the crude fucoidan A and B, respectively. The yield of HMW fucoidan was more than LMW fucoidan. Among the fucoidan fractions, HMW A1H recorded maximum yield of 80.45 mg/g alga dry wt. The proportion of monosugars (fucose, galactose, gulucose and mannose) in both fucoidan (LMW and HMW) was similar dominating D-galactose over L-fucose but sulphate content was high in HMW fucoidan (A1H) isolated from the *S. wightii*. Similar type of fucoidan was isolated in *L. variegata*[29].

***In vitro* anticoagulation activity of isolated galactofucoidan**

Fucoidans exhibit structural and functional analog to heparin. Anticoagulation potential of fucoidans of algal origin is based on its molecular weight, proportion of monosugars and level of sulphation[26, 31]. Galactofucoidans with 50-100, 000 Da are considered as potential anticoagulants[8]. EDTA is commonly used *in vitro* anticoagulant in clinical laboratory inhibiting the clotting process by removing calcium from the blood considered advantageous over other anticoagulants as it does not distort blood cells, making ideal for hematology use[32]. In the present study, isolated HMW fucoidan (A1H) with high sulphate showed higher anticoagulation activity than LMW fucoidan (A1L) and the activity was increased with increasing concentration (Fig. 1) isolated from *S. wightii* as like this previously isolated in *L. variegata*[29].

Spectral characterization of galactofucoidan

The anticoagulation activity of sulphated polysaccharide mainly depends on molecular weight,

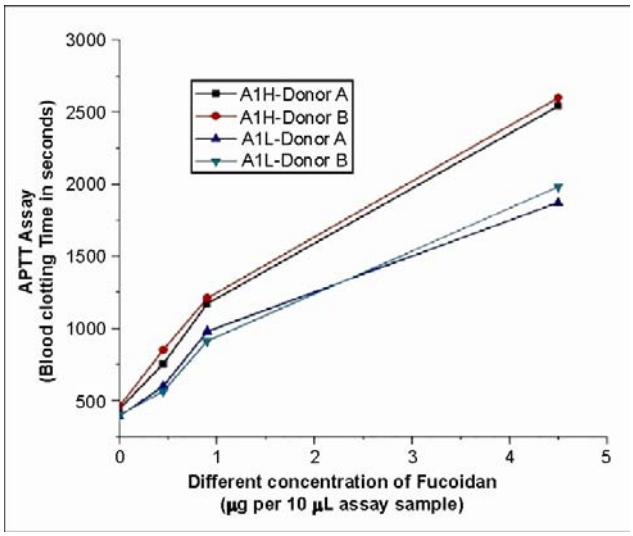

Fig. 1- *In vitro* Anticoagulation activity of fucoidans (A1H & A1L) isolated through the DEAE cellulose column

Table 3 - Yield and constituents of purified HMW fucoidans (3500< Da) isolated through the DEAE cellulose column

S.NO		Crude fucoidan/ Eluvant number	Pooled volume (ml)	Purified fraction	Fucoidan yield (mg g^{-1} alga dry wt.)	Total sugar yield (mg g^{-1} fucoidan)	Constituents of HMW fucoidans				
							*D-Glucose	*L-Fucose	*D-Galactose	*D-Mannose	Sulphate (mg g^{-1} fucoidan)
1.	Crude fucoidan A	1	50	-	-	-	-	-	-	-	-
2.		2-8	350	A1H	80.45	521.69	22.48	20.96	33.65	22.88	379.11
3.		9	50	-	-	-	-	-	-	-	-
4.		10-12	150	A2H	63.86	595.75	22.97	21.40	34.36	21.26	294.70
5.		13-36	1200	-	-	-	-	-	-	-	-
6.	Crude fucoidan B	1	50	-	-	-	-	-	-	-	-
7.		2-4	150	B1H	44.11	583.99	22.50	20.96	33.65	22.88	279.98
8.		6-8	150	-	-	-	-	-	-	-	-
9.		9 & 10	100	B2H	20.32	491.14	28.16	19.45	31.27	21.19	314.46
10.		11-36	1300	-	-	-	-	-	-	-	-

* % in total sugar - not recorded

sulphate content and level of 2-O-sulfation and 2, 3-O-disulphation which could be ascertained in FTIR spectrum. In the present study, FTIR spectrum of isolated fucoidan A1H (Fig. 2) having band at 1635/cm indicate the presence of C=O stretching vibration of O-acetyl groups. The band at 1454/cm assigned for CH_2 (galactose, xylose) and band at 1325/cm indicate the presence of CH_3 (fucose, O-acetyls). The stretching at 1072/cm for symmetric O=S=O vibration of sulphate esters is prominent. These bands are characteristics for sulphated polysaccharides. The level of sulphation in the compound is ascertained based on their peak intensity increase with the sulphate content. Beside band at 613/cm indicates the presence of C-O-S secondary axial sulphate at C-4 of fucopyranose residue. Based on the FTIR spectrum, it is clear that the main sulphate groups occupy positions of C-2 or C-3. The other major absorption band at 3433/cm indicates the presence of O-H stretching. The above characteristic features of FTIR spectrum (Fig. 2) shows that the isolated polysaccharide was confirmed as fucoidan as reported by Immanuel *et al*[33]. ^{1}H NMR spectrum (Fig. 3) shows resonance signals in the ring protons H-2 to H-5 between 3.612 and 4.247 ppm characteristic of α-1-fucopyranosyl and methyl signals appeared around 0.728-1.897 ppm. The sharp signals appearing at 3.853 and 3.635 ppm in the proton NMR spectrum recognized the methoxy group of 2-O-methyl-(1→4)-linked-3, 6-anhydrogalactose and 6-O-methyl-(1→3)-

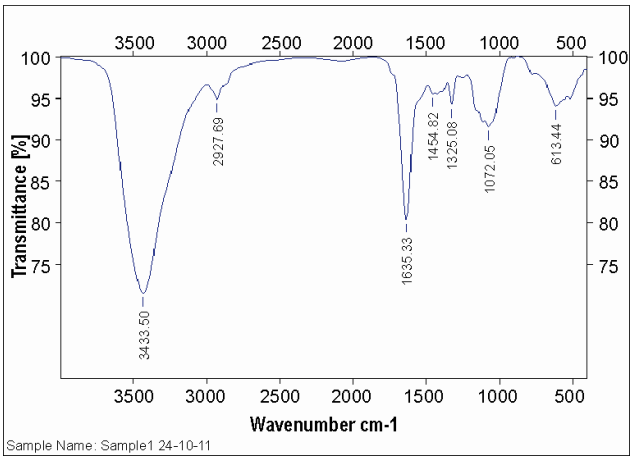

Fig. 2- FTIR characterization of A1H isolated from the Gulf of Mannar brown seaweed *Sargassum wightii*

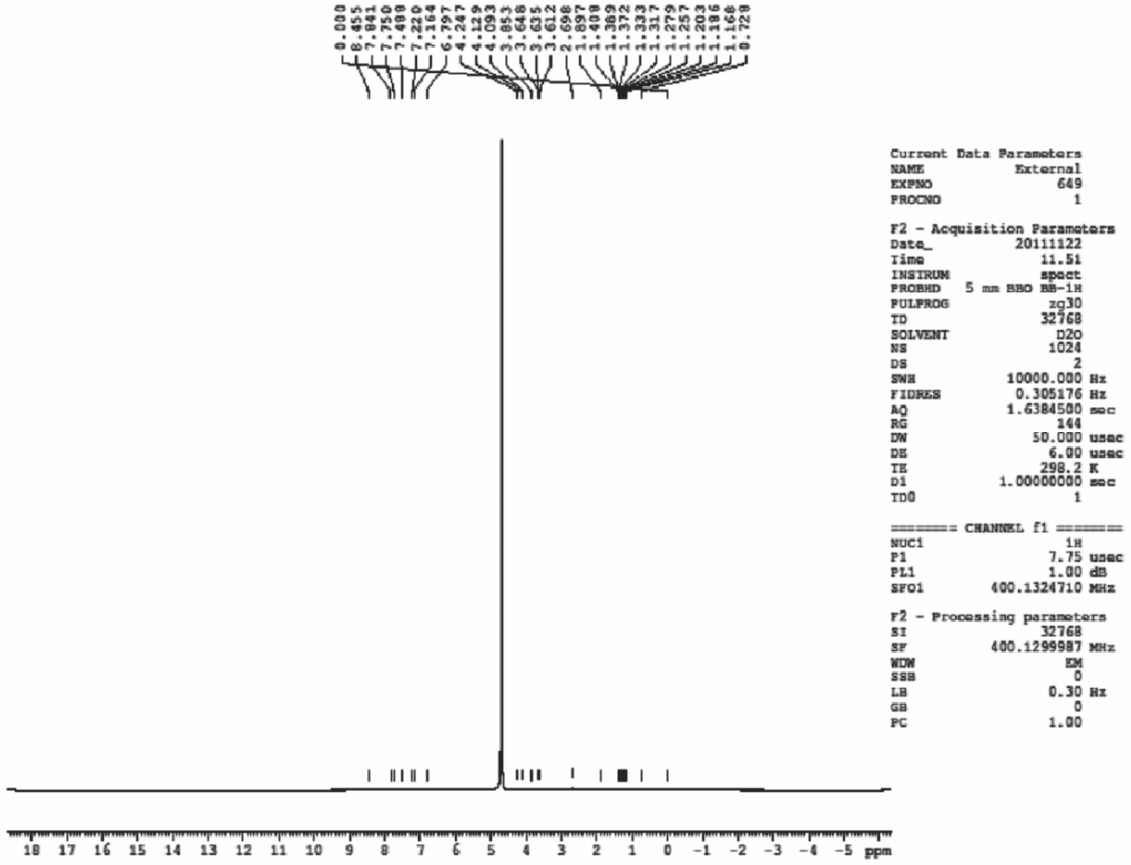

Fig. 3- ^{1}H NMR spectrum of fucoidan A1H

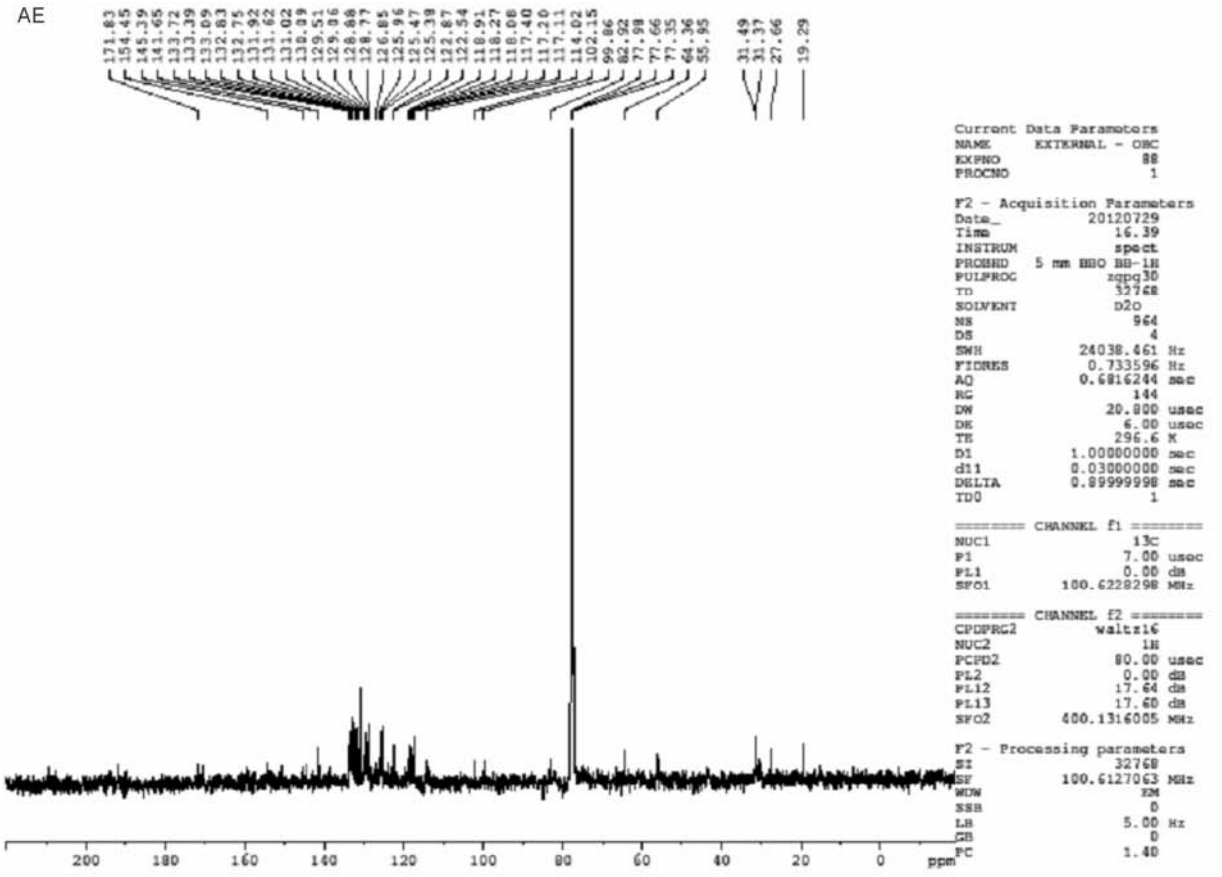

Fig. 4- ^{13}C NMR spectrum of fucoidan A1H

linked-galactose residue, respectively. In the ^{13}C NMR spectrum (Fig. 4), signals around 125 ppm recognize β-D-galactopyranosyl residues and the signal around 64.36 ppm shows some non-6-linked galactose presence in the sample whereas the signals around 77.35 ppm describe the C-6 of 6- linked galactose units. Absence of signals around 82.92-99.86 ppm suggest no C-3 or C-4 linked galactose units which indicate that the isolated fucoidan polymer of *S. wightii* contains C-1 and C-6 linked galactose[33].

Conclusion

It is concluded that galactose dominated fucoidan with high sulphate found in the Indian brown seaweed *S. wightii* extracted by successive method was isolated through DEAE cellulose column. Even though composition of monosugars remained same dominating galactose, fucoidan with high molecular weight (> 3500 Da) showed good anticoagulation activity than the low molecular weight fucoidan. This indicate that bioactivity of fucoidan in *S. wightii* is related with molecular mass. The successive extraction method suggested as suitable alternate to extract antibacterial substances, fucoidan and alginate in the same raw material. Extracts obtained in chloroform: methanol (1:1 v/v) and 85 % ethanol at 23°C by successive extraction to remove pigments and protein during fucoidan extraction possess antibacterial potential to control the plant pathogens that cause canker in citrus and blight in paddy.

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