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Extraction and purification of sulfated polysaccharide from brown algae and its efficacy in preventing blood clotting

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Abstract

The three brown algae *Sargassum wightii, Padina tetrastromatica and Dictyota sp.*, were collected from CS-MCRI, CSIR Institute, Mandapam coast, Rameshwaram, India. Extraction was carried out using HCl extraction method .The maximum yield of sulfated polysaccharide (3.91g) was obtained from *Sargassum wightii*. The Lyophilized powders of all the three sulfated polysaccharides (SPs) were further processed for estimation of carbohydrate, sulfate and protein content. The highest content of carbohydrate (240µg/mL), sulfate (127 µg/mL) and protein (176 µg/mL) were observed in *Sargassum wightii*. These SPs were characterized by high-performance liquid chromatography and FTIR which verified the presence of fucose and galactoseas main components (70:30% mol ratio in average).The HPLC profile of the three SPs showed prominent peaks and the retention time was obtained in 5.2 to 5.4. The FTIR spectrum of the three samples showed characteristic absorbance bands of sulfated polysaccharide. The *in vitro* anticoagulant activity of the SPs were investigated and compared. The high APTT values indicated that the crude from *S.wightii* (31.3±1) have high anticoagulation activity followed by *P. tetrasromatica* (29.7±1.1) and from *Dictyota sp.* (28 ± 1.03).

Key words : Sulfated Polysaccharide, Brown algae,Extraction, HPLC, FTIR, Anticoagulant

INTRODUCTION

Algae are said to be a large and diverse group of plant like organisms ranging from unicellular to multi cellular forms. Seaweeds are a group of macroscopic marine algae that form the biomass in the intertidal zone[1]. Brown seaweeds (Phaeophyta) are known to be very important. A variety of polysaccharides such as alginic acids, laminarans and sulfated polysaccharides (fucoidans) are abundantly present in Brown seaweeds. Fucoidans are well known to possess diverse *in vitro and in vivo* biological effects such as anticoagulant, antiviral, immuno modulating and antitumor.

It provides blood thinning, anti-inflammatory and antioxidant polysaccharides, which lets the blood from the heart to easily pass through the blood vessels, prevents clot forming, prevents free radical damage to the blood vessels and keeps plaques from clotting the blood vessels that feed the body. Edible seaweeds contain significant quantity of polyphenols[2], which are efficient antioxidants and may have particular biological activities. Polyphenol rich extracts and isolated phlorotannin components have been shown to inhibit proliferation of cancer cells and influence anti-inflammatory responses. Compounds with cytostatic, antiviral, antihelmintic, antifungal, and antibacterial activities have been detected in brown algae. Nearly over 15,000 novel bioactive metabolites have been isolated from a varied range of algae and their commercial application is vast [3].

Heparin has been used for more than fifty years as a commercial anticoagulant and it is widely used for the prevention of venous thromboembolic disorders. However, numerous side effects of heparin have been reported such as ineffectiveness in congenital, development of thrombocytopenia, acquired antithrombin deficiencies, hemorrhagic effect and incapacity to inhibit thrombin bound to fibrin [4].

Moreover, heparin is presented in very low concentrations in the intestine of pig or bovine lungs from where it can be extracted. After the analysis of blood anticoagulant properties of marine brown seaweed[5] it was reported that SPs derived these algae are alternative basis for creation of novel anticoagulant drugs[6, 7, 8]. Unfractionated heparins and low molecular weight heparins are only sulfated polysaccharides currently used as anticoagulant drugs. The Seaweed used in SPs production have been known to possess a higher anticoagulant activity similar to heparin [9].

MATERIALS AND METHODS

Collection and processing of brown algae

Three brown algae samples *Sargassum wightii,Padina tetrastromatica* and *Dictyota* sp. were collected and identified based on the morphology and structure from CS-MCRI, CSIR Institute, Mandapam coast, Rameshwaram, India. The collected samples were cleaned well with sea water to remove the dust, pebbles and shells and brought to the laboratory for further processing. The samples were washed using tap water followed by distilled water and allowed for shade drying. After the samples were well dried, they were cut into small pieces and powdered in a mixer grinder. The powder obtained was preserved at 4°C for further use.

Extraction of sulfated polysaccharides

25g of dried algal powder of *Sargassum wightii, Padina tetrastromatica* and *Dictyota* sp. were taken and soaked separately in an acetone - methanol solvent system in the ratio of 7:3 for two days in a shaker at 200 rpm. The process was repeated twice to ensure the complete decoloration and defatting of dry biomass. This biomass was then dried into powder and dispersed in 1.0 L of 0.1 M HCl for 24 h with constant stirring at room temperature. Then this was centrifuged and the pellet obtained was re-extracted as above. The resultant supernatants were pooled, kept at 4°C overnight and precipitated with two volumes of absolute ethanol 1:1 (v:v). The precipitate was collected and air dried. The air dried samples were lyophilized and stored for

further study[10].

Chemical analysis of crude sulfated polysaccharide

The crude extracts of sulfated polysaccharide (*Sargassum wightii, Padina tetrastromatica* and *Dictyota* sp.) were characterized by determining the carbohydrate, sulfate and protein content. The total carbohydrate content of the crude extract obtained was estimated by phenol sulphuric acid method proposed by Dubois[11] with D- glucose as standard. The sulfate content was determined after acid hydrolysis (2 N HCl at 100°C for 2 h) of the polysaccharides, using the gelatin-barium method, using potassium sulfate as the standard. The amount of protein present in the extract was estimated with bovine serum albumin as standard[12].

HPLC analysis

The High Performance Liquid Chromatography (HPLC) analysis of crude sulfated polysaccharides was performed in Shimadzu LC solution 20 AD, Japan and SPD 20 A, an instrument equipped with a Shimadzu LC solution No: 20 AD, UV detector in order to determine the peak purity. LCGC C18 column was used for isocratic resolution using the mobile phase at a flow rate of 1.0 ml/min.

Fourier transform IR spectrophotometer (FT-IR) analysis

ART (Attenuated Total Reflectance) model FT-IR Spectrophotometer (Bruker, United States) was used for the structural analysis of the sulfated polysaccharide and fraction. Five milligrams of the sample was mixed with 100 mg KBr (FT-IR grade) and then compressed, in order to prepare translucent salt discs (3mm diameter). The disc was immediately kept in the sample holder and FT-IR spectra were recorded in the absorption range between 4000 and 400cm^{-1} at room resolution 4cm^{-1} with scans using Thermo Nicolet FT-IR Nexus spectrometer coupled with TGS (Tri-glycine sulphate) detector by the KBr pellet technique. The spectrum was recorded using Attenuated Total Reflectance (ART) technique beach measurement.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to confirm the presence of polysaccharides in the extracted crude samples. In the present study three different buffers were used viz.,0.05 M 1,3-diaminopropane/acetate buffer, pH 9.0; discontinuous buffer containing 0.04 M barium acetate, pH 4.0; and 0.05 M phosphate buffer, pH 8.0[13].

Agarose gel electrophoresis of the sulfated polysaccharides was performed on 0.6% agarose gel (7.5 x 10 cm, 0.2 cm thick) prepared using four different buffers. Aliquots of the fractions (about 50 µg) were applied to the gel and run for 1 h at 100 V. The gel was fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide and kept undisturbed for 4 h. Then the gel was dried and stained for 15 mins with 0.1% Toluidine blue in acetic acid: ethanol: water in the ratio of (0.1:5:4.9) v/v and destained with the same solution without Toluidine blue added.

Anti-coagulant assay of sulfated polysaccharides

Activated partial thromboplastin time (APTT) assay

APTT assay of the sulphated polysaccharides was carried out [14]. Citrated normal human plasma (90 µl) was mixed with a solution of 10 µl of SPs at the concentration of 500µg/ml and incubated for 1 min at 37°C. Then 100 µl of APTT reagent was added to the mixture and incubated for 10 min at 37°C. Clotting was induced by adding 0.025M $CaCl_2$ (100 µl) and clotting time was recorded. The anticoagulation activities were expressed as relative clotting factor (R.C.F) which was calculated as follows:

Relative clotting factor (R.C.F) = clotting time of test sample/clotting time of control under similar condition.

The reaction mixture containing citrated normal human plasma (90 µl) was mixed with a solution of 10 µl of SPs at the concentration of 500µg /ml and incubated for 3 min at 37°C. Then pre-warmed PT reagent was added and the time for clot formation was recorded. Heparin and water were used as positive and negative controls, respectively[15].

RESULTS

Extraction of sulfated polysaccharide

The extraction of the sulfated polysaccharide from three dried algal powder was carried out using HCl extraction method. Among the three brown algal species, *Sargassum wightii* showed maximum yield of polysaccharide i.e. 3.91g (15.64%), followed by *Padina* sp.with 3.79g (15.16%) and *Dictyota sp.* with 1.85g (7.4%). The yields obtained in all the three algae are shown in (Table 1) and (Fig 1,2 & 3)

Chemical analysis of sulfated polysaccharide

Carbohydrate estimation

The carbohydrate estimation of the three sulfated polysaccharides was performed using Phenol sulphuric acid method with D - glucose as standard. The standard graph was plotted using various concentration of glucose and the absorbance (OD) measured. The R^2 value of 0.962 was attained. The analysis of the three SPs showed the highest amount of carbohydrate in *Sargassum sp.* as 240 µg/mL (Table 2 & Fig4).

Sulfate estimation

The sulfate estimation of the three SPs from *Sargassum*

Table 1: Yield of sulfated polysaccharides after extraction and lyophilization

S.No	Name of the brown algae	Yield in (g)	% Yield
3.	*Sargassum wightii*	3.91	15.64
4.	*Padina tetrastromatica*	3.79	15.16
5.	*Dictyota sp.*	1.85	7.4

a. Sargassumwightii *b. Padina tetrastromatica* *C. Dictyota sp*

Figure 1 : Macroscopic appearance of the seaweeds collected from Madapam, Rameshwaram, India.

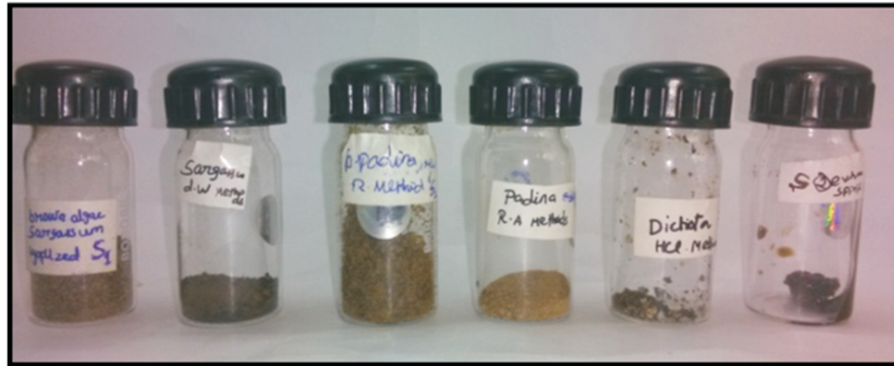


Figure 2 : Lyophilized sulfated polysaccharides obtained using HC1 extraction method

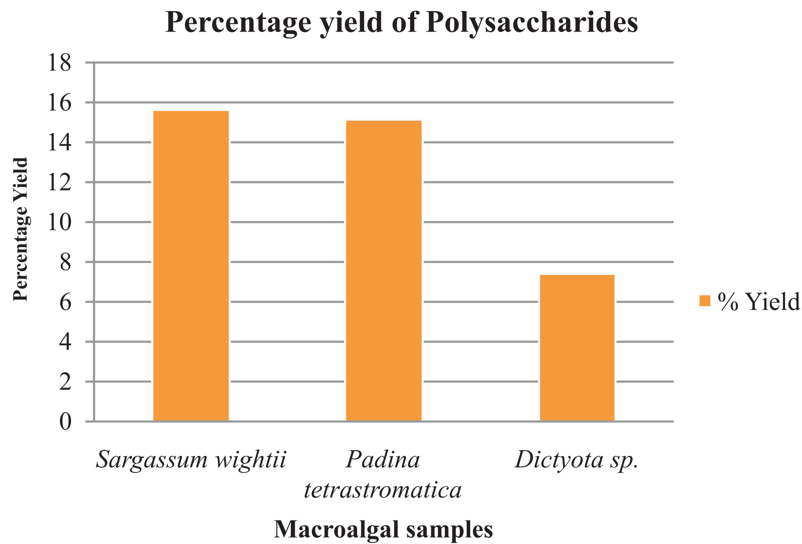


Figure 3 : Percentage yield of polysaccharide from Macroalgae

Table 2: Bio-Chemical analysis of crude sulfated polysaccharides

S.No	Name of the Brown algae	Protein (μg/mL)	Carbohydrate (μg/mL)	Sulphate (μg/mL)
1.	*Sargassum wightii*	176	240	127
2.	*Padina tetrastromatica*	86	69	68
3.	*Dictyota sp.*	59	20	72

wightii, *Padina sp.* and *Dictyota sp.* was performed by Barium chloride - gelatin method using potassium sulphate (K_2SO_4) as standard. The sulfate content of the SPs extracted from three seaweeds are tabulated in Table 2.The analysis showed the highest amount of sulphate in *Sargassum sp.* as127 µl/mL (Fig.4).

Protein estimation

The protein content of the three SPs was estimated by lowry's method using BSA as standard. Protein reacts with folin ciocalteau reagent to give a blue colored complex. The intensity of color depends on the amount of the aromatic amino acids present. The standard graph was plotted with concentration of bovine serum albumin against the UV spectrophotometer readings and a R^2 value of 0.985 was attained. Among the three SPs, *Sargassum wightii* showed maximum amount (176 µg/ml) of protein content (Table 2 & Fig.4).

HPLC analysis

On analysis the methanolic extracts of *S. wightii* showed five compounds (peaks) with varied retention time viz., 5.102, 6.797

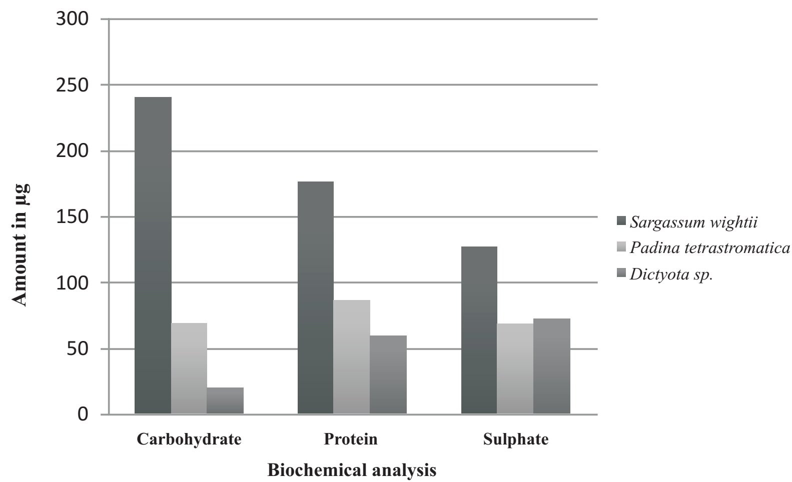


Figure 4 : Biochemical analysis of Sulphated polysaccharies from Macroalgae

Figure 5 : HPLC profile of different seaweeds

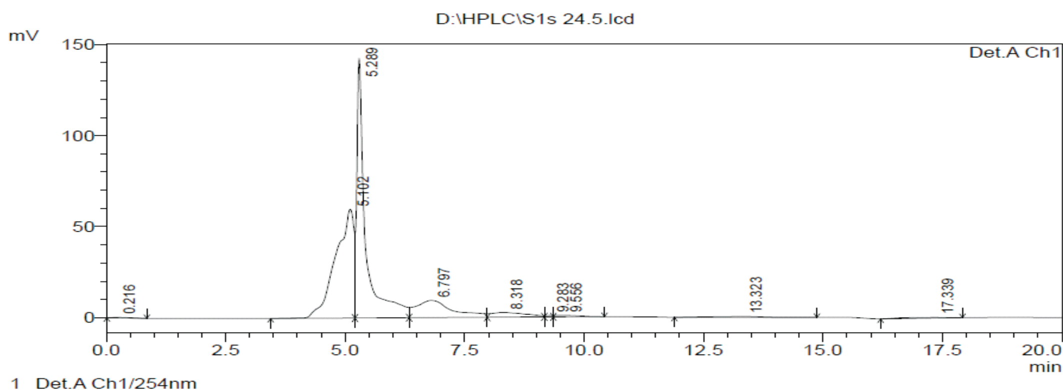


Fig 5 a) : HPLC profiles of *Sargassum wightii* – Ethanolic Extract

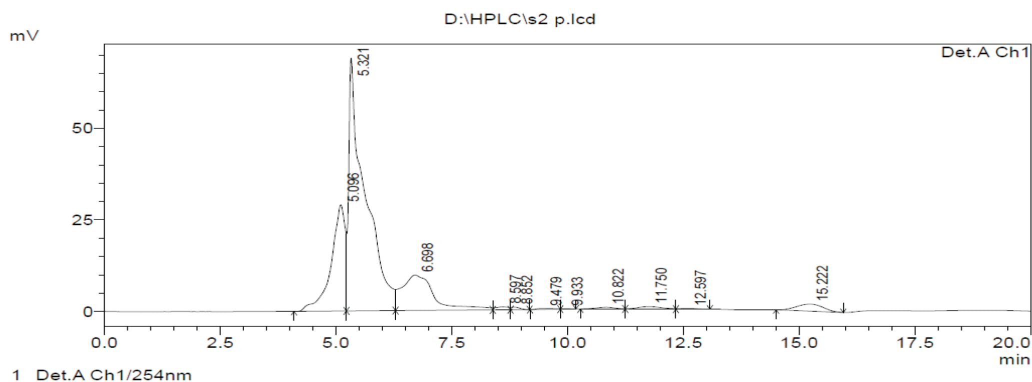


Fig 5 a) : HPLC profiles of *Sargassum wightii* – Ethanolic Extract

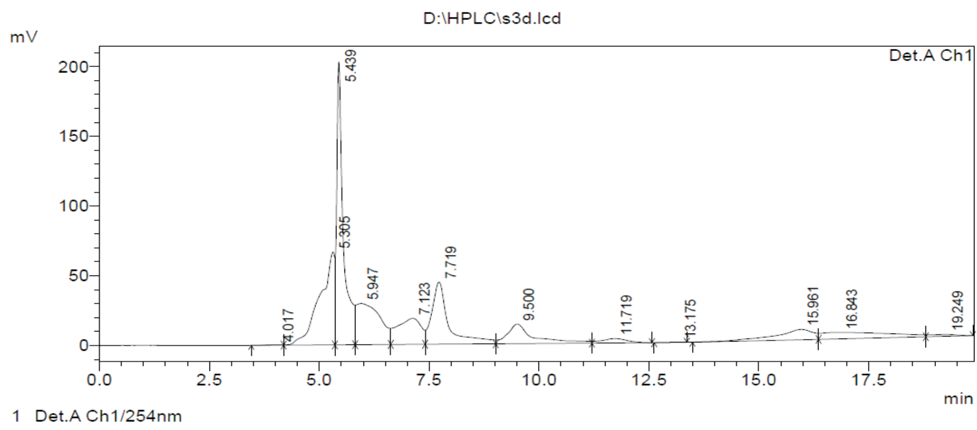


Fig 5 C: HPLC Profiles of *Dictyota* sp. – Ethanolic extract

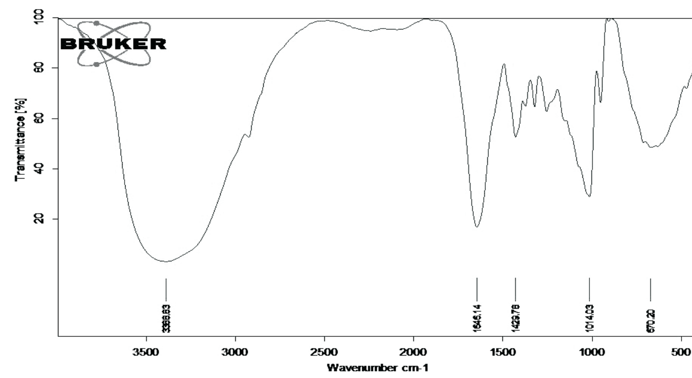


Figure 6 a) : FTIR analysis of *Sargassam wightii*

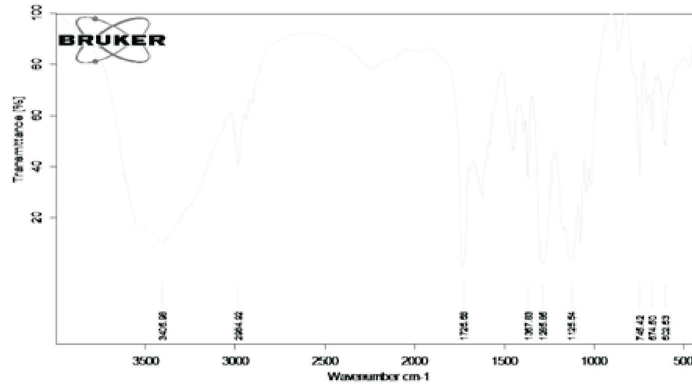


Figure 6 b) FTIR analysis of *Padina tetrastromatica*

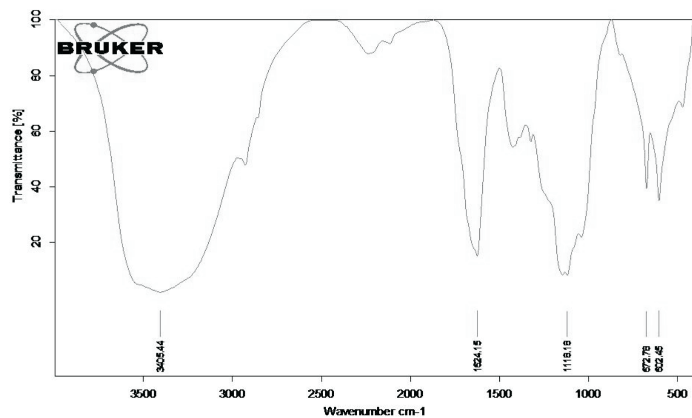
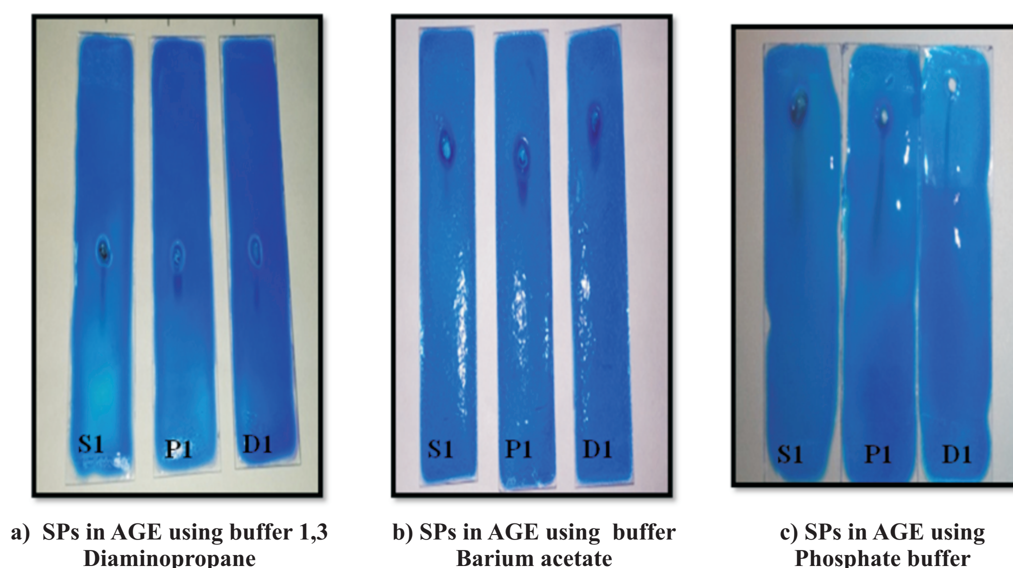


Figure 6c) FTIR analysis of *Dictyota* sp



a) SPs in AGE using buffer 1,3 Diaminopropane **b) SPs in AGE using buffer Barium acetate** **c) SPs in AGE using Phosphate buffer**

Fig 7: Agarose gel electrophoresis of sulphated polysaccharides extracted from Brown algae : S1- Sargassum wightii , P1- Padina tetrastromatica , D1-Dictyota sp.

Table 3: FTIR analysis of crude sulfated polysaccharides

Crude Sulfated Polysaccharides	IR (cm^{-1})							
Sargassum wightii	3388.83	1646.14	1129.78	1014.03	670.20	-	-	-
Padina tetrastromatica	3406.98	1726.68	1125.54	1286.86	674.50	602.63	745.42	1387.83
Dictyota sp.	3405.44	1624.44	1118.18	-	672.78	602.45	-	-

and 8.318min .The HPLC chromatogram of ethanolic extracts of *S. wightii* displayed a single prominent peak with a retention time of 5.289 min. (Fig.5 a).

The HPLC chromatogram of methanolic SPs extract from *Padina sp.* displayed seven compounds (Peaks) with varied retention time 5.096, 5.321, 6.698, 8.597, 10.822 and 15.222 min. The HPLC profile of ethanol extracts of *Padina sp.* demonstrated a single prominent peak at a retention time of 5.321min and few other moderate peaks at 8.852, 9.497, 11.750 min (Fig. 5 b).

The HPLC chromatogram of methanolic extract from *Dictyota sp.* displayed ten compounds (Peaks) with varied retention time 5.305, 5.493, 5.947, 7.123, 7.719, 9.500 and 15.961min.The HPLC profile of ethanolic extracts of *Dictyota sp.* demonstrated a single prominent peak at a retention time of 5.439 min and few other moderate peaks at 11.719, 13.175,16.843, 19.249 min (Fig 5c). HPLC analysis of the three sulfated polysaccharides showed that they are heteropolysaccharide majorly composed of fucose and galactose.

FT-IR analysis

The FTIR analysis of crude SPs from *S. wightii*, *P. tetrastromatica and Dictyota sp.* were carried out and it further confirmed the presence of sulfated polysaccharide. The FTIR spectrum showed characteristic SP absorbance bands and the results are shown in Table 3. The absorption bands in the range 900 and 1740 cm^{-1} was observed in crude SPs from all species, which indicated the presence of polysaccharides in all crude SPs from all species under study. The vibration near 600 cm^{-1} was present in crude sulfated polysaccharide fraction from *S. wightii*, *P. tetrastromatica and Dictyota sp.* which indicate more alkylene C-H bond. In the case of *P. tetrastromatica*, absorption band near 1726 cm^{-1} was found which indicates aldehyde/ester and shows the presence of polysaccharide (Table 3) (Fig 6. a,b and c). The absorption peak of *Sargassum wightii* showed a peak at 1429.78 and 670 cm^{-1} which indicates the presence of organic sulphates and disulphide bonds. In addition, sulfated polysaccharide from most of the species showed absorbance band around 3383 to 3420 cm^{-1} and near 2925 cm^{-1} due to vibration of hydroxyl (O-H) and Aldehyde(C-H), respectively. The bands near 1620 cm^{-1} were due to the vibration of carboxylic acid groups. [1720 to 1750 cm^{-1} and 900 cm^{-1} for polysaccharides, 1680-1620 for alkene CDC stretch; 1387 cm^{-1} for methyl (-CH_3 gem-dimethyl or iso-doublet) , 700-600 cm^{-1} for aliphatic bromo compounds, C-Br stretch; 3383 to 3420 cm-1 for O-H bond; 1608 to 1625 cm^{-1} for COO- groups].

Agarose gel electrophoresis

The electrophoretic profile in agarose gel, using three buffer system of 1,3-diaminopropane, barium acetate and phosphate

Table 4: The anticoagulant activity of the three polysaccharides extracted from brown algae

Crude Sulfated Polysaccharides	APTT (seconds)	RCF	PT (seconds)	RCF
Sargassum wightii	sample= 31.3 Control= 24.6	1.27	sample = 112.9 Control= 13.5	8.36
Padina tetrastromatica	sample = 29.7 Control= 24.6	1.20	sample = 102.3 Control= 13.5	7.58
Dictyota sp.	sample = 28 Control= 24.6	1.13	sample = 40.3 Control= 13.5	2.99

buffers and toluidine blue staining, confirmed SPs as polydisperse and highly metachromatic fucans and thereby proving that SPs are sulfated polysaccharide (Fig 7)

Anticoagulant Activity of crude SPS

The anticoagulant activities of three crude SPs were studied using APTT and PT tests; it was observed that considerable difference exists among crude SPs obtained from different algae. The high APTT values indicated that the crude from *S. wightii* (31.3 ± 1) have high anticoagulation activity followed by *P. Tetrasromatica* (29.7 ± 1.1) and BDP from *Dictyota sp.*(28 ± 1.03). The prolongation of APTT may be attributed to the interference by anticoagulant agent in the intrinsic coagulation pathway.

The prolongation in prothrombin time by SPS is not significant though our laboratory control showed 13.5 s as PT. However standard value for PT ranged from 12 to 14 s and the PT value shown by crude SPs extracted from *S. wightii*, *P. tetrastromatica*, and *Dictyota sp.* were in the range of 40 to 112 s (Table 4). The prolongation of coagulation time (PT) is by delayed extrinsic coagulation pathway.

DISCUSSION

The main focus of this study was to extract and characterize the sulfated polysaccharides present in three brown algae namely *Sargassum wightii*, *Padina tetrastromatica* and *Dictyota. sp.* and to study the anticoagulant activity of extracted polysaccharides. There has been an increasing interest in seaweeds, mainly due to their bioactive components such as polysaccharides and glycoproteins with immune stimulating, anticoagulant antitumor and/or antiviral activity[16].Among these, brown algae contain large amounts of cell-wall polysaccharides, most of which are sulfated polysaccharides and fucoidans[17]. The rich diversity of Seaweeds at Rameshwaram attracted the interest on collection of brown algae from Mandapam coastal area, Rameshwaram. After the collection of the seaweeds the polysaccharides were extracted using HCl extraction method. The yields of the polysaccharides obtained from HCl extraction process gave varying to usual yield of crude polysaccharides with 15.64% (w/w) as maximum from the dry weight source of *Sargassum wightii.* Two edible seaweeds, *Sargassum polycystum* and *Sargassum wightii,* were investigated for their antidiabetic potential using in vitro enzyme inhibitory assays [18].

In the present work it was found that carbohydrate content was maximum in all the three SPs. Carbohydrate content of *Sargassum wightii* (240 µg/mL) was found to be more than *Padina tetrastromatica* (69 µg/mL) and *Dictyota sp.* (20 µg/mL). All these samples were collected after the monsoon season. Almost 60% of the dry weight of seaweeds comprises of carbohydrate.The use of electrophoresis for identification of the algal acidic polysaccharides has been reported by several authors[19-22]. With the use of buffers at different pH they were able to differentiate some polysaccharides. In the present study the individualization of the polysaccharides was essentially due to the properties of the usage of buffers in the electrophoresis system, For e.g.1,3- diaminopropane. This diamine forms different complexes with the polysaccharides by their sulfate residues as it was previously observed for the sulfated glycosaminoglycans[13].

The electrophoretic profile in agarose gel and HPLC analysis of the three sulfated polysaccharides showed that they are heteropolysaccharides composed mainly of fucose, galactose, xylose, glucuronic acid and mannose, thereby confirming it as a fucan or heterofucan. Several investigations have demonstrated that chemical compositions and structures of fucans varies from from species to species and are very complex.

The extracted polysaccharides were analyzed for FTIR results and the data revealed absorption bands near 900 and 1740 cm^{-1} in crude SPs from all species, that shows the presence of polysaccharides in all crude SPs from all species under study. The vibration near 600 cm^{-1} was present in crude sulphated polysaccharides fraction from *Sargassum wightii, Padina tetrastromatica* and *Dictyota sp.,* which indicates more alkylene C-H bond. Signals at $3420\text{-}3450^{-1}$ and 1050-1070 cm^{-1} correspond to stretching vibration of OH hydroxyl group and CO, respectively.

The anticoagulant activity of the three SPs was examined and the thrombin and prothrombin activities were determined. This was measured with the APTT test, which evaluates the action of compounds present in the intrinsic and common pathway of blood coagulation and PT test which evaluates clotting time by extrinsic pathway. The results suggested that BSP promoted a maximum increase in clotting time, detected by APTT at a concentration of 500µg/ml. The high APTT values indicate that the crude SPs from *Sargassum wightii* (31.3 ± 1) have high anticoagulation activity followed by *Padina tetrastromatica* (29.7 ± 1.1) and *Dictyota sp.* (28 ± 1.03). The prolongation of prothrombin time by SPs is not significant though our laboratory control showed 13.5 s for PT. However the standard value for PT range from 12 to 14 s and the PT value shown by crude SPs extracted from *S. wightii*, *P. tetrastromatica* and *Dictyota sp.* were in the range of 40 to 112 sec. Providentially, the crude sulfated polysaccharide of

Sargassum wightii presented a discreet inhibition of coagulation in relation to heparin activity at 25g/ml for both APTT and PT assay.

CONCLUSION

Brown macroalgae have been identified as easily accessible producers of sulfated polysaccharides. In the present study the extraction of SPs using HCl extraction method was performed and a maximum yield of 15.64% was obtained from *Sargassum wightii*. The analysis of carbohydrate, sulfate and protein was carried out and the presence of carbohydrate was high in *Sargassum wightii* showing the presence of polysaccharides. As this is the crude form of polysaccharide the proteins were found in the extracted and lyophilized crude polysaccharide. The sulfate content was determined which proves the presence of sulfate group in the polysaccharides. The peaks in FTIR analysis also correlated with sulphur groups. FTIR results showed clearly the presence of functional groups of polysaccharides. The electrophoresis and HPLC analysis confirmed the presence of major sugars fucose and galactose of SPs. The anticoagulant activity of the sulfated polysaccharides was checked using the blood samples. The Prothrombin time and Activated Partial prothrombin time were determined using Heparin as control. From the study carried out, the results signify that the sulfated polysaccharide from *Sargassum wightii* could be utilized as a persuasive anticoagulant that might replace the existing anticoagulants.

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