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Anticoagulant property of sulphated polysaccharides extracted from marine brown algae collected from Mandapam Island, India

Manoj Saravana Guru M., Mahesh Kumar P. S., Vasanthi M. and Anant Achary*

Centre for Research, Department of Biotechnology, Kamaraj College of Engineering and Technology, Virudhunagar-626001, Tamil Nadu, India.

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The marine brown algae: *Sargassum tenerrimum, Sargassum wightii, Turbinaria conoides, Turbinaria ornata* and *Padina tetrastromatica* were collected from Mandapam Island, India. The crude sulphated polysaccharides (SPS) were extracted using hot water and examined for anticoagulation activity. The sugar, sulphate and protein in crude SPS were analyzed. The presence of sulphated polysaccharide was confirmed by agarose gel electrophoresis and further characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR analysis of crude SPS showed characteristic band of polysaccharides at 900, 1740 cm^{-1} and ester sulphate at 1250 to 1260 cm^{-1}. Moreover, the absorbance band at 820 cm^{-1} for *S. tenerrimum, S. wightii, T. conoides* and *T. ornata* denotes sulphation at equatorial position, but in the case of *P. tetrastromatica*, sulphation at axial position is denoted by absorbance band at 850 cm^{-1}. The heparin like activity of crude SPS was determined by metachromatic assay. The anticoagulant activity of crude SPS was evaluated by activated partial thromboplastin time (APTT) and prothrombin time (PT) assays. The metachromatic, APTT and heparinoid activities of crude SPS from *S. tenerrimum, S. wightii, T. conoides, T. ornata* and *P. tetrastromatica* were in the range of 0.045 to 0.0347, 134 to 89 s and 25.47 to 14.5 USP units/mg, respectively. The prolongation of prothrombin time by crude SPS was not found.

Key words: Brown algae, sulphated polysaccharides, Fourier transform infrared spectrometer (FTIR), agarose gel, metachromatic activity, activated partial thromboplastin time (APTT), heparin.

INTRODUCTION

The sulphated polysaccharides (SPS) from marine algae was demonstrated to have many biological activities such as anticoagulant (Shanmugam et al., 2000), antioxidant (Rocha de Souza et al., 2007), antitumor (Zhuang et al., 1995), anti-aging (Fujimura et al., 2002) and antiviral activities (Mohsen et al., 2007). The diverse nature of SPS from marine algae, the similarities between their structures with that of heparin and their anticoagulant nature were first reported by Chargaff et al. (1936). The SPS are widely distributed in marine algae. Sulphated galactans and sulphated fucoidans are found in red and brown algae, respectively; while sulphated arabinan and

*Corresponding author. E-mail: achyanant@yahoo.com. Tel: 0091-4549-278171.

Abbreviation: APTT, Activated partial thromboplastin time; **PT,** prothrombin time; **FTIR,** Fourier transform infrared spectrometer.

rhamnan are from green algae which are reported to have anticoagulant properties (Shanmugam et al., 2000). Anticoagulant and antithrombotic activities are the most widely studied properties of SPS. Unfractionated heparins and low molecular weight heparins are currently used as anticoagulant drugs and it is widely used for the prevention of venous thromboembolic disorder (Walenga et al., 1998). However, several side effects of heparin have been reported such as thrombocytopenia, hemorrhagic effect and incapacity to inhibit thrombin bound to fibrin (Kakkar et al., 1896; Liaw et al., 2001; Pereira et al., 2002). Moreover, heparin is extracted from internal organs of higher animal and purified; hence their recovery is poor. The SPS derived from marine brown algae has been suggested as an alternative source of blood anticoagulant (Killing et al., 1913). Anticoagulant activity of SPS has been identified from several brown algae such as *Padina gymnospora* (Silva et al., 2005), *Dictyota menstrualis* (Albuquerque et al., 2004), *Sargassum stenophyllum* (Duarte et al., 2001), *Spatoglossum schroederi* (Leite et al., 1998) and sulphated fucans from marine brown algae (Zhang et al., 2008; Chevolot et al., 1999; Colliec et al., 1991; Nishino et al., 1989). Therefore, there is necessity of research to find alternative sources of anticoagulants and safer anticoagulant therapy. In the present study, an attempt was made to screen anticoagulant activity, of partially characterized crude SPS preparation from brown algae: *Turbinaria conoides, Turbinaria ornata. Sargassum tenerrimum, Sargassum wightii and Padina tetrastromatica* collected from Mandapam Island, Ramanathapuram district, Tamil nadu, India.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade and obtained from Himedia Chemical India. APTT and PT reagents were obtained from Diagnostic Enterprises (Biomed industries, India).

Sample collection and its storage

Marine brown algae were collected from the sea shores of Mandapam Island, Ramnad District, Tamil Nadu, India (Lat.09°17.417'N;Long.079°08.558'E). The algae were thoroughly washed with tap water to remove sand and epiphytes (foreign substances). Finally, the samples were rinsed with deionised water and stored at -20°C for further experiments. The collected brown algae were identified as *S. tenerrimum, S. wightii, T. conoides, T. ornata* and *P. tetrastromatica.*

Extraction of crude SPS

The crude SPS were extracted using hot water at 90°C for 1 h according to the method described by Black et al. (1952).

Screening of phytochemical constituents in crude extract

Phytochemical tests were carried out for crude SPS. Qualitative estimation of phenol was done according to the method of Akenga et al. (2005). Presence of cardiac glycosides and saponins were qualitatively evaluated according to the methods described by Sofowora et al. (1982). Presence of tannins were confirmed according to the method described by Edeoga et al. (2005).

Estimation of total sugar, sulphate and protein in crude SPS

The crude SPS was hydrolysed with 2 M trifluoroacetic acid at 100°C for 3 h. Total sugars were assayed using phenol-sulphuric method of Dubois et al. (1956). The sulphate content was determined turbidimetrically using gelatine/$BaCl_2$ method of Dodgson et al. (1961). The protein content in the crude SPS was estimated by the method of Bradford et al. (1975).

Characterization of crude SPS

Agarose gel electrophoresis for crude SPS

The crude SPS extracted from different brown algae were analyzed on 0.5% agarose gel electrophoresis according to the method described by Dietrich et al. (1976) and compared with standard heparin.

FTIR analysis

The IR spectra of the crude SPS were determined using FTIR and scanned in the wavenumber range of 600 to 4000 cm^{-1}.

Metachromatic activity

Screening of heparin-like compounds can be made by comparing the sample with the standard heparin by metachromatic assay. The metachromatic assay was done using Azur A dye according to the method of Grant et al. (1984).

Anti-coagulation assay

Activated partial thromboplastin time (APTT) assay

APTT assay was carried out according to the protocol of Anderson et al. (1976). Citrated normal human plasma (90 µl) was mixed with a solution of 10 µl of SPS at the concentration of 500 µg/ml and incubated for 1 min at 37°C. Then, APTT reagent (100 µl) was added to the mixture and incubated for 10 min at 37°C. Clotting was induced by adding 0.025 M $CaCl_2$ (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.

Prothrombin time (PT)

Prothrombin time was determined according to the method of Quick et al. (1940). The reaction mixture containing citrated normal human plasma (90 µl) was mixed with a solution of 10 µl of SPS at the concentration of 500 µg/ml and incubated for 3 min at 37°C, then pre-warmed PT reagent was added and the time for clot formation was recorded. Water and heparin were used as negative and positive controls, respectively.

Heparinoid activity

Heparinoid activity was calculated with the standard curve drawn

Table 1. Screening of phytochemical constituents in crude extract from brown algae.

Crude SPS	Glycoside	Cardiac Glycoside	Phenol	Tannins	Saponins
S.tennerimum	+	+	+	+	+
S.wightii	+	+	+	+	+
T.ornata	+	+	+	+	-
T.conoides	+	+	+	+	+
P.tetrastromatica	+	-	+	+	+

+ Presence, - absence

Table 2. Analysis of total polysaccharide, sulphate and protein in crude sulphated polysaccharides.

Crude SPS	Percentage of total sugar in crude SPS (w/w)	percentage of total sulphate in crude SPS (w/w)	percentage of total protein in crude SPS (w/w)	Molar ratio of sulphate/total polysaccharides
S. tennerimum	26.27±1.33	15.22±0.67	2.25±0.46	1.11
S. wightii	35.27±5.15	15.52±1.19	0.96±0.03	0.81
T. ornata	56.42±3.75	18.85±0.18	4.17±0.66	0.62
T. conoides	62.1±1.98	14.74±0.65	2.21±0.01	0.45
P. tetrastromatica	50±0.44	13.37±0.33	2.99±0.37	0.50

between RCF vs. heparin concentration (µg/ml) (R^2 = 0.995, y = 0.040x + 0.832). The heparin units were calculated using the formula:

Heparin units = (concentration of heparin at a R.C.F/concentration of algal sample at a R.C.F) × heparin units (140.3 USP units).

RESULTS AND DISCUSSION

Screening of phytochemical constituents in crude extract from brown algae

The phytochemical analysis for the presence of tannin, phenol, saponins and glycosides were carried out in crude SPS extract from brown algae *T. ornata, T. conoides, S. wightii, S. tenerrimum* and *P. tetrastromatica.* The studies indicated the presence of tannin, phenol and glycoside in all the preparation of crude SPS extract (Table 1). Saponin was detected in all the preparation of crude SPS extract except *T. ornata* and cardiac glycoside was absent in crude SPS of *P. tetrastromatica*. The role of tannin and phenol (Cox et al., 2010; Rastian Zahra et al., 2007), and saponins (Huong et al., 1998) as antioxidants have been reported. However, no report on anticoagulant activity by these molecules is found. The presence of sulphated fucans and alginate in brown algae have been reported by various researchers (Killing et al., 1913; Bird et al., 1931; Haug et al., 1962; Rivera-Carro et al., 1984) and probably these constituents of the crude SPS might be responsible for the anticoagulant property (Nishino et al.,1989; Pereira et al., 2002; Silva et al., 2005).

Analysis of total sugar, sulphate and protein

The result of the present study as shown in Table 2 indicates that total sugar content varied in different species. Our results show that the species belonging to *Turbinaria* genus namely *T. conoides* and *T. ornata* contains higher amount of polysaccharides, 62.1 and 56.42% (w/w), respectively, followed by *P. tetrastromatica* 50% (w/w) and the species belonging to *Sargassum* genus viz. *S. wightii* and *S. tenarrimum* were found to contain lowest amount of polysaccharides, 35.27 (w/w) and 26.27% (w/w), respectively. However, *S. wightii* and *S. tenarrimum* showed high content of sulphate in SPS 15.22 and 15.52% (w/w), respectively. The molar ratio of sulphate to sugar is also high in *S. tenarrimum* and *S. wightii*, 1.11 and 0.81, respectively, when compared with other species (Table 2). Nishino et al. (1989, 1992) reported three sulphated fucan from *E. kurome* with sulphate to sugar molar ratio of 1.28, 1.38 and 1.98. Pereira et al. (2002) reported sulphate to sugar molar ratio of 1 in sulphated fucan from brown algae *S. franciscanus* and *S. droebachiensis*. Our finding on sulphate to sugar ratio in SPS from *S. tenarrimum* and *S. wightii* is comparable with the reported result on *S. franciscanus* and *S. droebachiensis by* Pereira et al. (2002).

Agarose gel electrophoresis

The presence of SPS was confirmed by agarose gel electrophoresis. The formation of a purple band stained

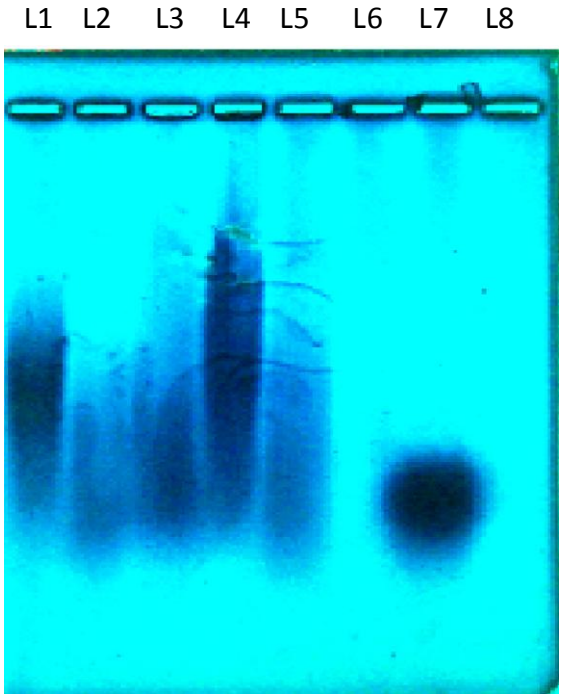

Figure 1. Agarose gel electrophoresis of crude sulphated polysaccharides. L1, *T. ornata*; L2, *T. conoides*; L3, *S. wightii*; L4, *S. tennerimum;* L5, *P. tetrastromatica*; L7, heparin.

Table 3. FTIR analysis of crude sulphated polysaccharides.

Crude SPS	IR (cm^{-1})							
S. tennerimum	1730	900	1270	1033	820	2924	3383	1608
S. wightii	1740	900	1260	1033	820	2926	3394	1625
T. ornata	1740	900	1260	1033	820	2926	3420	1620
T. conoides	1740	900	1259	1033	820	2926	3417	1620
P. tetrastromatica	1750	900	1275	1033	850	2924	3398	1620

1730 to 1750 cm^{-1} and 900 cm^{-1} for polysaccharides; 1259 to 1275 cm^{-1} for S = O stretching; 1033 cm^{-1} for symmetric C-O vibration associated with a C-O-SO_3 groups; 820 cm^{-1} for sulphation at equatorial position; 850 cm^{-1} for sulphation at axial position; 2924 cm^{-1} for C-H bond ; 3383 to 3420 cm^{-1} for O-H bond; 1608 to 1625 cm^{-1} for COO^- groups.

by toluidine blue confirms the presence of SPS. Diamine forms different complexes with SPS due to the spacing between the negative charges, which is caused mainly by sulphate substitutions in a specific sugar residue conformation (Dietrich et al., 1976). The electrophoretic mobility of crude SPS depends on their charge and molecular weight of the SPS. The result of the electrophoretogram shown in Figure 1 reveals polydisperse band that indicates presence of combination of different molecular weight SPS.

FTIR analysis

The FTIR analysis of crude SPS from *T. ornata*, *T. conoides*, *S. wightii*, *S. tenerrimum* and *P. tetrastromatica* were carried out and it further confirmed the presence of SPS. The FTIR spectrum showed characteristic SPS absorbance bands and the results are shown in Table 3.

The absorption bands near 900 and 1740 cm^{-1} were present in crude SPS from all species, which indicated the presence of polysaccharides in all crude SPS from all species under study. The absorption band near 1250 to 1260 cm^{-1} and bands around 1033 cm^{-1} were present in all crude SPS, which is due to the presence of asymmetric S=O stretching vibration (Zhang et al., 2009) and of symmetric C-O vibration associated with a C-O-SO_3 groups (Zhang et al., 2010), respectively. The vibration near 820 cm^{-1} was present in crude SPS fraction from *T. ornata, T. conoides, S. tenerrimum* and *S. wightii*,

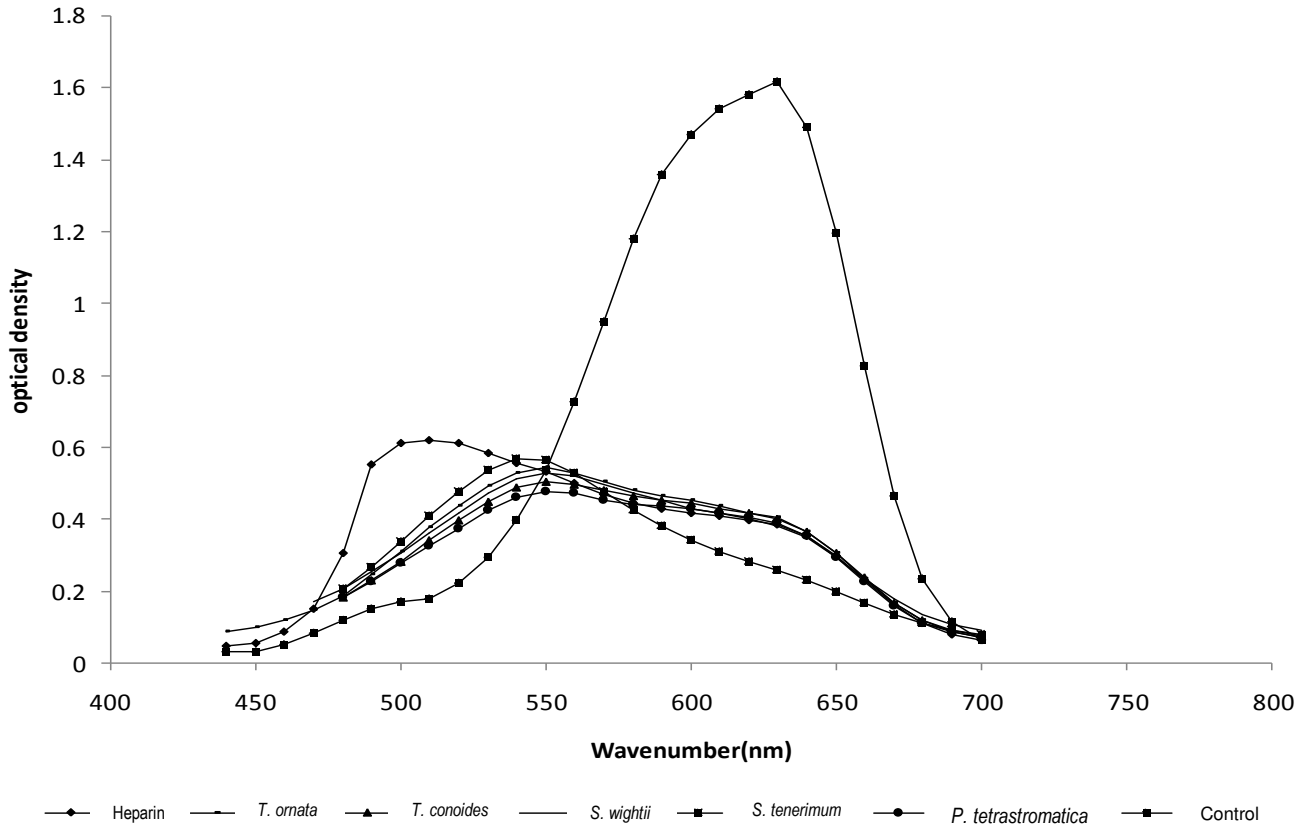

Figure 2. Metachromatic shift of crude SPS in azur A dye (crude SPS and heparin in the concentration of 50 and 10 µg/ml, respectively).

which indicates more sulphation at equatorial (C2 and C3) position. In the case of *P. tetrastromatica*, absorption band near 850 cm^{-1} was found which indicates more sulphation at axial (C4) position (Zhang et al., 2008). In addition, SPS from all species showed absorbance band around 3383 to 3420 cm^{-1} and near 2925 cm^{-1} due to vibration of O-H and C-H, respectively (Sekkal et al., 1993; Marc Lahaye et al., 1986). The bands near 1620 cm^{-1} were due to the vibration of COO^{-} groups (Silva et al., 2005).

Metachromatic activity of crude SPS extracted from brown algae

The metachromansia effect was produced by loose electrostatic reaction of polyanionic SPS with dimer dye. The dimerization results in the decrease of π electron delocalization in dye molecules. The metachromatic activity was used to determine the presence of heparin or heparin like molecule in the sample (Grant et al., 1984). The study on metachromatic activity using crude SPS from all species under study showed a shift of absorbance from 620 nm. The shift in the absorbance was also observed in heparin. In this study, 50 µg/ml sample of crude SPS was taken for assay. The crude SPS of *S. tenerrimum* showed a shift from A_{620nm} to A_{540nm}, whereas the crude SPS of *T. ornata*, *T. conoides*, *S. wightii* and *P. tetrastromatica* showed a shift from A_{620nm} to A_{550nm}. The heparin was used as positive control which showed shift in absorbance from A_{620nm} to A_{508nm}. The metachromatic effect was calculated and maximum metachromatic effect was found in the case of *S. tenarrimum* followed by *S. wightii*, *T. ornata*, *T. conoides* and *P. tetrastromatica* as shown in Figure 2 and Table 4.

Anticoagulation activity of crude SPS

Blood coagulation comprises a series of enzymatic reactions whereby an inactive proenzyme is converted to the active enzyme form which in turn, converts another proenzyme to its active form. The final step is the conversion of fibrinogen to fibrin and polymerization to cross-linked fibrin and clot forms (Hougie et al., 1980). The *in vitro* anticoagulant activity of the crude SPS was evaluated by APTT and PT coagulation assays. APTT assay was used to determine the action of crude SPS in

Table 4. Metachromatic activities of crude sulphated polysaccharides.

Crude SPS	Metachromatic activity	Relative metachromatic activity
S. tennerimum	0.0450	0.287
S. wightii	0.0382	0.243
T. ornata	0.0380	0.242
T. conoides	0.0377	0.240
P. tetrastromatica	0.0347	0.221
Heparin	0.157	1

Metachromatic activity was expressed as negative slope of standard curve of sample at A_{620nm} in a 0.02g/L of azur dye. Relative metachromatic activity was calculated from slope of sample/slope of heparin.

Table 5. APTT assay for crude sulphated polysaccharides.

Crude SPS	APTT (seconds)	RCF	PT (seconds)	RCF
S. tennerimum	134±1.73	4.47	16	1.33
S. wightii	122±1	4.07	15	1.25
T. ornata	117.6±1.52	3.92	15	1.25
T. conoides	108±1.41	3.6	15	1.25
P. tetrastromatica	89.3±1.52	2.98	15	1.25
H1	33	1.1	13	1.08
H2	55.8	1.86	15	1.25
H3	82.8	2.76	18	1.5
H4	115.8	3.86	21	1.75
H5	148.8	4.96	24	2
control	30	1	12	1

H1 to H5 = heparin (10, 25, 50, 75, 100 µg/ml respectively); Sample 500 = µg/ml.

intrinsic coagulation pathway and it is a very sensitive test to analyze any change in blood coagulation pattern and inhibition of the factors like VIIIa, IXa, XIa and XIIa and sometimes anticoagulant may inhibit the factors Xa, Va and IIa (Smythe et al., 2004: Tripodi et al., 2004; Hoffman et al., 2005; Koch et al., 2007). PT assay monitors the integrity of coagulation proteins especially factor VIIa involved in the extrinsic pathway (Duxbury et al., 2001). The anticoagulant activities were carried out using APTT; it was observed that considerable difference exists among crude SPS obtained from different algae. The high APTT values indicate that the crude SPS from *S. tenerrimum* have high anticoagulation activity (134 ± 1.73) followed by *S. wightii* (122 ± 1) > *T. ornata* (117.6 ± 1.52) > *T. conoides* (108 ± 1.41) > *P. tetrasromatica* (89.3 ± 1.52).

The prolongation of APTT may be attributed to the interference by anticoagulant agent in the intrinsic coagulation pathway. The inhibition in prothrombin time by SPS is not significant though our laboratory control showed 12 s for PT, however standard value for PT ranged from 12 to 16 s and the PT value shown by crude SPS extracted from *S. wightii*, *T. conoides*, *P. tetrastromatica*, *S. tenerrimum* and *T. ornata* were in the range of 14 to 16 s (Table 5). The prolongation of coagulation time may not be through extrinsic coagulation pathway.

Our results on anticoagulant activity suggests that crude SPS inhibits the molecular targets belonging to the intrinsic and/or common coagulation pathways but, do not act on the extrinsic coagulation pathways.

It is generally assumed that anticoagulation property is related to the pattern of sulphation allowing interaction with target proteins of the coagulation cascade (Church et al., 1989). Our results on anticoagulant activity shown by these crude SPS is in good correlation with the metachromasia effect and sulphate to sugar molar ratio. The sulphate/sugar molar ratio of crude SPS of *S. tenerrium* is high (Table 2) and also shows highest APTT activity (134 s) among the five species of brown algae under the study. The anticoagulant activity of crude SPS extract follows the order of sulphate/sugar molar ratio. The higher the sulphate/sugar molar ratio, the higher the anticoagulant activity. Similar finding was also reported by Nishino et al. (1992) while working on *E. kurome*. Qiu et al. (2006) reported that the anticoagulation activity improve with increase in sulphate content of the native fucoidan. However, in the case of *P. tetrastromatica*,

Table 6. Anticoagulation activities of crude SPS expressed in heparin units.

Crude SPS fraction	Anticoagulation activity [heparin units]	Percentage of heparin activity of crude SPS
S. tennerimum	25.46±0.42	18.15
S. wightii	22.52±0.24	16.05
T. ornata	21.46±0.37	15.29
T. conoides	19.18±0.28	13.61
P. tetrastromatica	14.53±0.37	10.34

Heparin activity calculated from the following formula (heparin units of sample/140.3) x 100.

though the sulphate/sugar molar ratio was higher than *T. conoides*, the anticoagulant activity is low. The FTIR analysis of crude SPS indicates vibration of sulphate at 820 cm^{-1} for *S. tenerrimum, S. wightii, T. conoides* and *T. ornata* which reveals that sulphation is at equatorial position, whereas in the case of *P. tetrastromatica*, the vibration of sulphate was found at 850 cm^{-1} which indicates sulphation at axial position. The position of sulphate groups on sugar is also important for the anticoagulant activity by SPS. The anticoagulant activity relates to the concentration of C2 sulphate and C-2,3 disulphate (Chevolot et al., 1999), moreover 2,3-disulphate sugar residue is the common structural feature in anticoagulant activity of fucoidan (Yoon et al., 2007; Chevolot et al., 2001). In the case of *P. tetrastromatica*, the sulphation was found at axial position and may be attributed to the low anticoagulant activity.

The heparinoid activity of crude SPS was calculated by relative clotting factor and substituted in heparin standard. The heparinoid activity of crude SPS is shown in Table 6. *S. tennerimum* showed maximum heparinoid activity (25.47 heparin USP units/mg), followed by *S. wightii* (22.52 heparin USP units/mg), *T. ornata* (21.45 heparin USP units/mg), *T. conoides* (19.1 heparin USP units/mg) and *P. tetrastromatica* (14.51 heparin USP units/mg). The anticoagulation activity of these crude SPS were compared with the anticoagulant activity of heparin which ranged from 10 to 18% of heparin activity (Table 6). Similar studies on anticoagulant activity of fucoidan from brown algae was done by Ushakova et al. (2009) and they reported 33, 24.2, 26.9, 19.1, 9.4 and 13.4 USP units/mg for fucoidan from *Laminaria sacchrina* and *Laminaria digitata*, *Fucus distichus*, *Fucus seratus* , *Fucus vesiculosus* and *Ascophyllum nodosum*, respectively. From our results on heparinoid activity, the SPS from *S. tennerimum* (25.47 heparin USP units/mg) and *S. wightii* (22.52 heparin USP units/mg) is in par with the heparinoid activity of fucoidan from *L. digitata* (24.2 heparin USP units/mg). Similarly, the SPS from *T. ornata* (21.45 heparin USP units/mg), *T. conoides* (19.1 heparin USP units/mg) is comparable with fucoidan from *F. seratus* (19.1 heparin USP units/mg) and also SPS from *P. tetrastromatica* (14.51 heparin USP units/mg) is in par with fucoidan from *A. nodosum* (13.41 heparin USP units/mg).

Currently, heparin and their derivatives are used as anticoagulant agent; however, they cause serious side-effects. The prolonged use of heparin induces heparin-induced thrombocytopenia, resulting in the degradation of platelets. The other side effects are elevation of serum aminotransferase levels, and hyperkalemia, alopecia and osteoporosis, with chronic use (Walenga et al., 1998; Farooq et al., 2004). The development of antithrombotic algal SPS would be advantageous, since their use would avoid the possibility of contamination with prions or viruses in commercial heparins, which are obtained from pig and bovine intestine. The SPS are extracted from natural source like brown algae and have no side effects. Moreover, with more specific activities and/or targets, the algal SPS could find application as complementary to heparin. SPS have many biological activities like antitumor, antiviral, antioxidant, anti-complimentary activity and immunomodulatory activity. Our current studies on anticoagulant activity encourage the potential application of SPS from brown algae in the development of anticoagulant agent and as an alternative drug for blood coagulation disorder. Further studies regarding the purification and characterization of SPS from brown algae are in progress.

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