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Anticoagulant activities of sulfated polysaccharides from the edible marine algae *Padina tetrastromatica* and *Ulva fasciata*: A combined *in vitro* and *in vivo* approach

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Abstract
The scientific validation of the bioactivities of sulfated polysaccharides (SPSs) from the edible marine algae glorifies the traditional aspects of their medicinal properties. The SPSs are the major structural ingredient of the algal cell wall composition. The aim of the present study is to evaluate the combined anticoagulant efficacy of SPSs purified from the edible marine algae *Padina tetrastromatica* and *Ulva fasciata*. After *in vitro* analysis, the results were confirmed by *in vivo* studies in SD rats induced with venous thrombosis. During the study, both compounds were found to have heparin-like anticoagulant properties. That is, these heparinoid-active compounds exert antithrombotic activity through the modulation of the intrinsic coagulation pathway. The overall results established that the combined effects of algal sulfated polysaccharides are more beneficial than the individual effects and they also showed excellent antiplatelet aggregation properties. In short, the study enlightened the pharmacological potential of the edible algae and their novel compounds sulfated polysaccharides.

Keywords: algal sulfated polysaccharides; anticoagulation; antiplatelet aggregation

1. Introduction
The marine algae are naturally enriched with structurally diverse bioactive compounds with promising health benefits and biomedical applications. Among marine algal compounds, sulfated polysaccharides (SPSs), the potent bioactive polymers containing hemi-ester sulfate groups in the simple monosaccharide units linked together by glycosidic bonds, are well reported for their various biological activities such as antioxidant, anticoagulant, antimicrobial, anticancer and anti-ageing properties [1,2,3]. The functional properties of these polysaccharides are reported to be attributed to the interrelation between physicochemical and biochemical characteristics which include sulfate content, it's positioning, monomeric units and molecular size [4]. The sulfated polysaccharides obtained from brown algal species are generally called as fucoidan and from green algae, they are called ulvan. SPSs account for the 10-20% of the total algal dry weight. Fucoidan composed of glucose, sulfated fucose, and small proportions of galactose, mannose, xylose and uronic acid [5] while ulvans contain rhamnose as the major sugar component [6]. The anticoagulant nature of SPSs and their similarities with heparin, the anticoagulant drug purified from the internal organs of higher animals, were first reported by Chargaff and co-workers in 1936 [7]. Even though low molecular weight heparin and un-fractionated heparins are widely used for the prevention of venous thrombosis, its life-threatening side effects such as heparin-induced thrombocytopenia (HIT) and haemophilia limits its usage and strengthens the possibility of using marine algal SPSs as alternative medicine [8].

In the present study, *in vitro* anticoagulant and antiplatelet aggregation properties of the SPSs, alone and in combinations, from the edible Phaeophyta *Padina tetrastromatica* and Chlorophyta *Ulva fasciata* were determined and the results were confirmed by *in vivo* studies against Wessler's thrombotic model using Sprague-Dawley rats as experimental animals. Similar types of sulfated polysaccharides were isolated previously from these algae in our lab and only antithrombotic activities were determined [1, 9]. These compounds were found to be different from our SPSs in chemical, structural and biological characteristics. So our study, for the first time, evaluating the combined anticoagulant activities of the sulfated polysaccharides from *P. tetrastromatica* and *U. fasciata* in the Wessler's thrombotic rat model through assessing the antithrombotic as well as antiplatelet aggregation properties.

2. Materials and Methods

2.1 Test materials

The edible brown algae *Padina tetrastromatica* and green algae *Ulva fasciata* were collected from the coastal rocks of Vizhinjam, Thiruvananthapuram, Kerala, India. Fig 1 shows the pictures of the algae used for the study. The algae were identified and herbarium specimens were deposited in the Department of Botany, University of Kerala with voucher number KUBH 9922 and 9921 respectively. From the algae, sulfated polysaccharides (PSPS; SPS from *P. tetrastromatica*, USPS; SPS from *U. fasciata*) were extracted and purified by DEAE (Diethylaminoethyl) cellulose-52 column chromatography. The compound PSPS and USPS were identified as fucoidan and ulvan respectively and were used for further experiments.

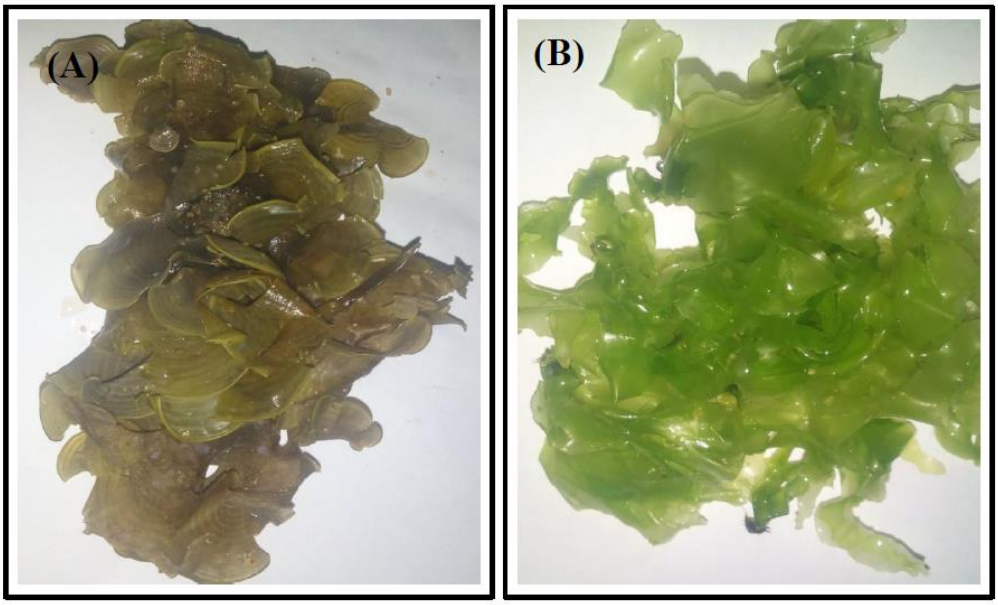

Fig 1: Photographs of the edible marine algae of Kerala coast. **(A)** Edible brown algae *Padina tetrastromatica* Hauck, **(B)** Edible green algae *Ulva fasciata* Delile

2.2 *In vitro* antithrombotic studies by APTT and PT Assays

For the *in vitro* anticoagulant assays, blood sample was collected from a healthy human subject and the experimental protocol was approved by University level Ethics committee on research involving human subjects (University of Kerala, Thiruvananthapuram) under project No. ULECRIHS/ UOK/ 2018/34.

The anticoagulant activities of SPSs were determined by APTT (Activated Partial Thromboplastin Time) and PT (Prothrombin Time) assays using kits purchased from Agappe Diagnostics (Mumbai, India), as per manufacturers' instructions. Briefly, platelet poor plasma (PPP) was obtained by centrifuging blood (Blood and sodium citrate in the ration 9:1) for 15min at 3000rpm. To 100µl of PPP, pre-incubated with different concentrations of SPSs for 3 min, 100µl pre-warmed APTT reagent 2 (Rabbit brain cephalin, Ellagic acid activator, Buffer) was added and incubated for 3min at 37ºC. Forcibly pipetted 100µl pre-warmed APTT reagent 1 (0.020 M/L Calcium chloride) to the reaction mixture and started a timer simultaneously to record the clotting time (CT) in seconds. For PT assay, 200µl PT reagent (Rabbit origin TF and Ca^{+}) was added to 100µl PPP and CT was recorded as described above. The results were then compared with the results of standard drug heparin (0.1mg/ml).

2.3 *In vitro* antiplatelet aggregation studies of PSPS

The antiplatelet aggregation properties of SPSs were determined by a spectrophotometric method and compared with standard drug aspirin (0.1mg/ml) [10]. The platelet-rich plasma (PRP) was separated by centrifugation of anticoagulant added blood at 1850rpm for 7min. From the supernatant, platelets were sedimented by centrifugation at 4500rpm for 18min. The platelets were dispersed in washing buffer (113mM NaCl, 4.3mM KH_2PO_4, 4.3mM Na_2HPO_4, 24.44mM NaH_2PO_4 and 5.5mM dextrose (pH 6.5) and recovered after centrifugation. The platelets were then suspended in a buffer composed of 109mM NaCl, 4.3mM KH_2PO_4, 16mM Na_2HPO_4, 8.3mM NaH_2PO_4 and 5.5mM dextrose (pH 7.5). After adjusting the optical density of the suspension to 0.5 at 600nm, they were pre-incubated with different concentrations of SPSs at room temperature for 3min. After incubation, 20µl 1mM ADP agonist was added to 1ml platelet suspension. The OD at 600 nm was measured at 1min intervals for 5 minutes and the % inhibition of platelet aggregation was calculated using the formula:

$$\% \text{ Inhibition} = (\text{Absorbance}_{\text{untreated}} - \text{Absorbance}_{\text{treated}}) / \text{Absorbance}_{\text{untreated}} \times 100$$

2.4 *In vivo* studies in Sprague-Dawley (SD) rats

Male Sprague-Dawley rats (200-250) were used for the study and the protocol was approved by the Institutional Animal Ethics Committee (IAEC-KU-06/2017-18-BCH-AAR (11). For the experiment, the animals were divided into five groups of 4 rats each as follows: Group I: Normal control, Group II: Thrombus control, Group III: PSPS (100µg/Kg BW), Group IV: USPS (100µg/Kg BW), Group V: P1U1 (PSPS & USPS in the ratio 1:1, 100µg/Kg BW) and Group VI: Heparin (50µg/Kg BW). The animals were given laboratory chow (Hindustan Lever Lab diet, India) and water ad *libitum* throughout the experimental period and were housed in a room with temperature mainlined at 26ºC. They were also provided with 12hr light and dark cycles and IAEC guidelines for the use and care of laboratory animals were strictly followed throughout the experiment period.

2.4.1 *In vivo* antithrombotic studies

Antithrombotic activities were investigated in adult male Sprague-Dawley rats using a modified stasis model described by Wessler, adapted for rat, using human serum as thrombogenic stimulus [11]. Briefly, following anaesthesia (12 % urethane, 10 ml/kg, i.p.) and mid-abdominal laparotomy, a segment of the inferior vena cava (IVC) was isolated between the left renal vein and the iliac bifurcation of male SD rats. The collateral vessels were carefully ligatured and two cotton threads, 2cm apart, were placed around the IVC and loosely tied. 0.1ml of human serum was injected via the tail vein and 30sec later the two ligatures around the vena cava were tightened. The stasis was maintained for 10min after which, the venous segment was opened and the thrombus removed, blotted of excess blood and weighed. 5min before thrombogenesis, 0.1ml of sulfated polysaccharides and standard drug heparin were injected intravenously to study their antithrombotic effect. The control rats underwent the same surgical procedure by administering saline instead of the drug. The platelet poor plasma (PPP) was collected after the drug administration and before the conduction of surgical procedure and then anticoagulant activities was determined.

2.4.2 Quantification and scoring of thrombus

Thrombus removed from the isolated IVC were weighed and the thrombus formation was scored on a 0-4 scale as follows: A score of 0 is given for complete fluid blood, 1-3 represented progressively larger clots, and a score of 4 indicated a complete thrombus formed in the IVC. The average score of 4 animals represented the mean thrombus score of the respective group and the results were expressed as % of the total possible score (% thrombus) [12]. Thrombus index was calculated using the formula:

Thrombus index (w/w) = Weight of thrombus in the IVC/ Total weight of IVC

2.4.3 Determination of Biochemical parameters

The tail transection bleeding time (BT) and clotting time (CT) were determined and results were expressed in seconds. The tail veins of SD rats were transected at 5mm from the tip and the bleeding time was determined as the time from the tail transection to the moment the blood flow stopped whereas the CT was determined by the capillary method. The antithrombotic activities were determined by APTT and PT assays as described earlier.

2.5 Statistical Analysis

The results are expressed as mean ± standard deviations of three experiments (n=3) for *in vitro* studies and four experiments (n=4) for *in vivo* studies. For the statistical evaluation, data were subjected to one-way ANOVA and the significance was accepted at $p<0.05$ calculated using Duncan's multiple range tests using the software SPSS Statistics 17.

3. Results and Discussion

3.1 Effect of SPSs on *in vitro* APTT and PT assay

The antithrombotic effects of PSPS, USPS and their combinations in the ratio 3:2 (P3U2), 1:1 (P1U1) and 2:3 (P2U3) were determined by APTT assay and the results are given in Fig.2. The results showed that both PSPS and USPS have the ability to extend the clotting time (CT) in APTT assay in a concentration-dependent manner with more efficacy by USPS. At a concentration of 1mg/ml, PSPS provided a CT comparable to heparin standard. The combination of PSPS and USPS in the ratio 1:1 (P1U1) exhibited prolonged CT than heparin standard. The results showed that the combined anticoagulant activities of algal SPSs are more beneficial than their individual effects. For prothrombin time test (PT), a significant increase ($p<0.05$) in CT was noted in all the experimental groups when compared to control group. Here also the combined effect of PSPS and USPS (P1U1) exhibited more potent activity than the standard drug heparin. Even though the algal SPSs treatment significantly increased the CT, not much change was observed in PT assay when compared to the APTT assay. The overall results showed that algal SPSs exhibit antithrombotic activities through the modulation of intrinsic coagulation pathway rather than extrinsic pathway.

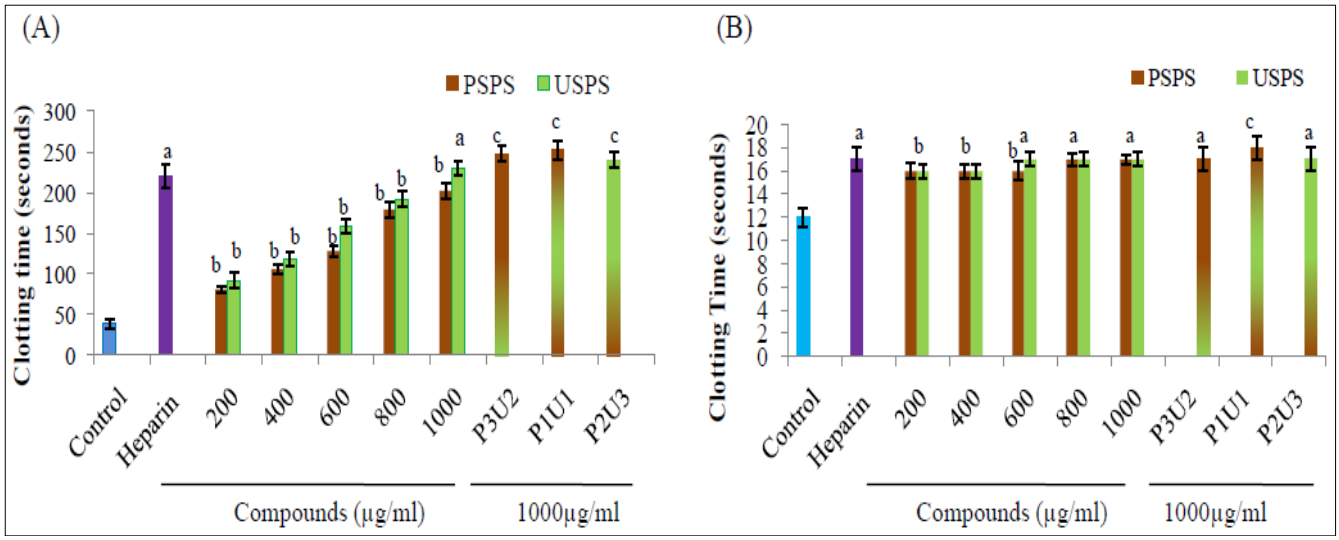

Fig 2: *In vitro* antithrombotic activities of marine algal SPSs (A) CT in APTT assay (B) CT in PT assay. Results are expressed as mean ± SD (n=3). Control has been compared with Heparin group ('a' indicates values that were significantly different from control), Heparin group has been compared with PSPS and USPS ('b' indicates values that are significantly different from Heparin group), USPS has been compared with combinations of PSPS and USPS ('c' indicates values that were significantly different from USPS). Significance accepted at $p<0.05$. *SPS; Sulfated polysaccharides, PSPS; SPS from brown algae Padina tetrastromatica, USPS; SPS from green algae Ulva fasciata, P3U2, P1U1 and P2U3; PSPS and USPS in the combination 3:2, 1:1and 2:3 respectively (1mg/ml)*

Activated Partial Thromboplastin Time assay (APTT) determines the anticoagulant activities of compounds through the activation of intrinsic and common pathways of coagulation cascades which include the factors like VIIIa, IXa, XI a and XII a [13]. PT assay monitors the coagulation proteins involved in the extrinsic pathway of coagulation cascade especially factor VIIa [14]. The antithrombotic activities of SPSs from other algal species substantiated our results [15]. They have also reported that antithrombotic activities of these compounds are through the APTT associated intrinsic coagulation pathway rather than extrinsic pathway [16]. The previous reports suggested that the antithrombotic potential of algal SPSs is correlated with the amount and position of sulfate groups present in the samples [17].

3.2 Effect of SPSs on ADP-induced *in vitro* platelet aggregation

In vitro antiplatelet aggregation properties of the algal compounds were determined and compared with standard antiplatelet drug aspirin. The results were expressed as % inhibition of platelet aggregation by assuming that 100% platelet aggregation has occurred in the control group induced with ADP. The results are given in Fig.3.

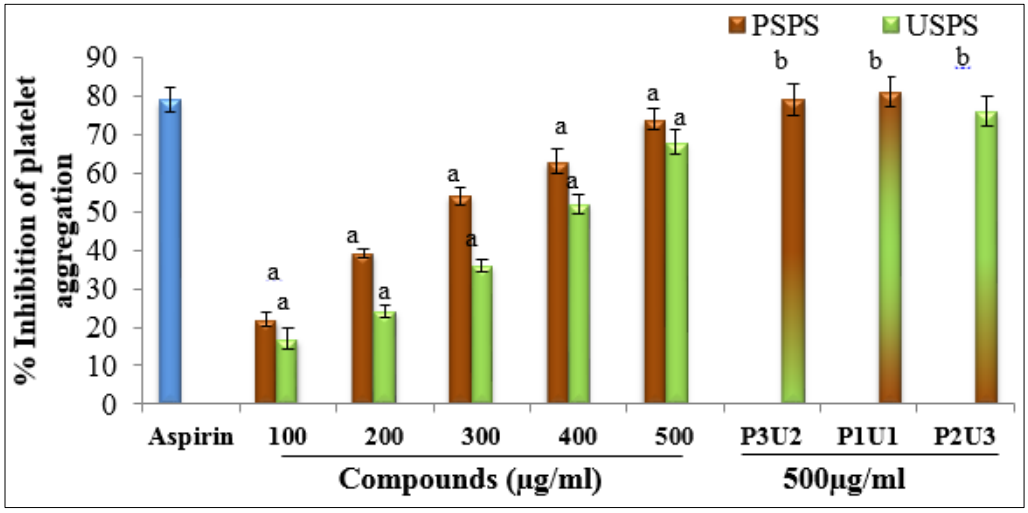

Fig 3: *In vitro* antiplatelet aggregation properties of marine algal SPSs. Results are expressed as mean ± SD (n=3). Aspirin group has been compared with PSPS & USPS ('a' indicates values that were significantly different from Aspirin group), USPS has been compared with combinations of PSPS and USPS ('b' indicates values that were significantly different from USPS). Significance accepted at p<0.05. *SPS; Sulfated polysaccharides, PSPS; SPS from brown algae Padina tetrastromatica, USPS; SPS from green algae Ulva fasciata, P3U2, P1U1 and P2U3; PSPS and USPS in the combination 3:2, 1:1and 2:3 respectively (1mg/ml)*

Adenosine diphosphate (ADP) is known to induce platelet aggregation in plasma and is an important tool for validating the antiplatelet aggregation properties of medicinal compounds [18]. The results of the study proved the antiplatelet aggregation properties of marine algal SPSs. During the analysis, PSPS exhibited more inhibition against platelet aggregation when compared to USPS, but both compounds showed significantly higher (p<0.05) antiplatelet aggregation properties in a concentration-dependent manner. Here also the combined effect of PSPS and USPS in the ratio 1:1 (P1U1) was found to be more effective as in the case of anticoagulation assays and hence this particular combination was selected for further *in vivo* studies. Platelet aggregation is an important event in thrombogenesis which may further lead to pathological conditions associated with cardiovascular diseases (CVDs). Anticoagulant drugs include antithrombotic and antiplatelet medications which could prevent and treat these complications related to CVDs. Heparin and aspirin are well known for their antithrombotic and antiplatelet aggregation properties at the cost of increased bleeding in patients on its long-term usage [19]. Since the algal sulfated polysaccharides are naturally occurring compounds they have the ability to exhibit potent anticoagulant and antiplatelet aggregation properties without causing any life-threatening side effects.

3.3 Effect of SPSs on thrombus formation in Wessler's rat thrombosis model

The effect of individual SPSs and their combinations on the thrombus formation in rats were evaluated by % thrombosis occurred in inferior vena cava (IVC) of experimental rats and by determining thrombotic index. The results are given in Fig.4. The % thrombosis in thrombus control is taken as 100 %. Thrombus scoring determines the extent of occurrence of thrombus in the IVC segments after the induction of experimental thrombosis. For thrombus control group, the thrombus score was obtained as 4 which confirmed complete thrombus formation in the IVC by the surgical procedure. PSPS, USPS and P1U1 treatments significantly reduced (p<0.05) the thrombotic index and thereby reduced the % thrombosis.

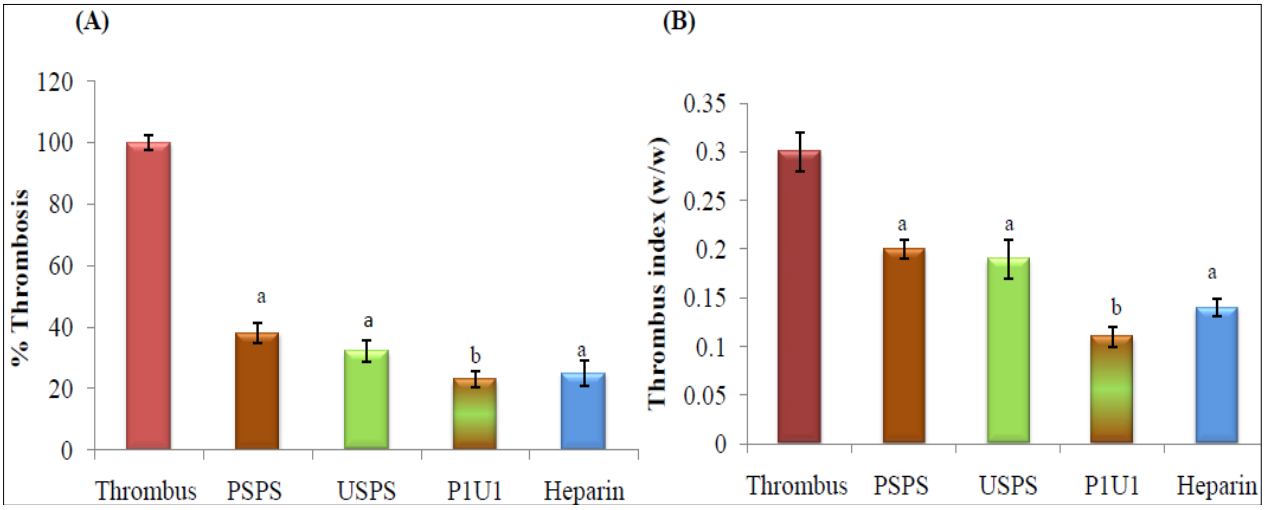

Fig 4: Effect of SPS on thrombus formation in Wessler's Rat model (A) % thrombosis (B) Thrombus index. Results were expressed as mean ± SD (n =4). Thrombus control has been compared with heparin and SPS groups ('a' indicates values were significantly different from Thrombus control), and heparin group was compared with PIU1 ('b' indicates values were significantly different from heparin group). Significance accepted at $p < 0.05$. *SPS; Sulfated polysaccharides, PSPS; SPS from brown algae Padina tetrastromatica, USPS; SPS from green algae Ulva fasciata, P1U1; PSPS and USPS in the combination 1:1 (1mg/ml)*

The treatment with PSPS and USPS significantly reduced ($p<0.05$) the thrombus size induced by thrombogenic stimuli in Wessler's rat model. As in the *in vitro* study, the combined effect of both SPSs dominates over the activity of individual compounds and standard drug heparin. The results of the study confirmed the anticoagulant efficacy of the marine algal SPSs through the antithrombotic mechanism. The study results substantiated the data in the article which discussed the perspectives on the use of sulfated polysaccharides from the marine world as a new source of antithrombotic drugs [20].

3.4 Effect of SPSs on *in vivo* coagulation parameters

The effect of SPSs on coagulation parameters such as CT, BT, APTT and PT assays were determined and compared with the result of standard drug heparin. The results are given in Table.1. In this study also, the combined effect of PSPS and USPS (P1U1) exhibited more potent antithrombotic activities than the standard drug heparin.

Table 1: Effect of SPS on *in vivo* coagulation parameters

	Control	Thrombus control	PSPS (100µg/ml)	USPS (100µg/ml)	P1U1 (100µg/ml)	Heparin (50µg/ml)
CT (sec)	130±3	129±4	290±5[a]	296±2[a]	321±4 [a]	312±4 [a]
BT(sec)	80±1.52	81±2.30	185±2.82[a]	187±3.01[a]	214±2.46 [b]	202±1.82 [a]
APTT(sec)	24±0.72	23±0.52	96±1.26 [a]	94±0.86 [a]	113±1.42 [b]	107±1.76 [a]
PT(sec)	16±0.01	16±0.01	17±0.01 [a]	17±0.03[a]	18±0.02 [a]	18±0.02 [a]

Results are expressed as mean ± SD (n=4). Control has been compared with treated groups ('a' indicates values that were significantly different from normal control), P1U1 has been compared with heparin ('b' indicates values are significantly different from heparin). Significance accepted at $p<0.05$. *SPS; Sulfated polysaccharides, PSPS; SPS from brown algae Padina tetrastromatica, USPS; SPS from green algae Ulva fasciata, P1U1; PSPS and USPS in the combination 1:1 (1mg/ml)*

In all the coagulation parameters studied, PSPS, USPS and heparin significantly increased ($p<0.05$) the clotting time when compared to the normal and thrombus controls. For PT assays slight variations in results were observed between groups when compared to APTT assay. Moreover, the antithrombotic activity of the combination of PSPS and USPS (P1U1) was found to be significantly higher ($p<0.05$) to that of heparin activities in terms of BT and APTT. These results further confirmed the results of *in vitro* anticoagulant studies. There are reports validating the distribution of heparinoid-active sulfated polysaccharides along the coastal sides of India [21]. So we could corroborate that the sulfated polysaccharides isolated from the edible brown algae *Padina tetrastromatica* and green algae *Ulva fasciata*, collected from the coastal rocks of Kerala, are heparinoid-active and exert anticoagulant activities superior to the heparin.

4. Conclusion

The present *in vitro* and *in vivo* pharmacognostic studies on the anticoagulant activities of the sulfated polysaccharides from the edible marine algae *Padina tetrastromatica* and *Ulva fasciata* proved the cardioprotective properties of these compounds through antithrombotic and antiplatelet aggregation dependent mechanisms. Since these SPSs are natural products from the edible marine algal species, they could overcome the life-threatening side effects of the currently used synthetic drugs. The core findings of the study ascertained the suitability of using these edible marine algae as functional food ingredients and their novel bioactive SPSs as lead compounds for the management of cardiovascular diseases.

5. Conflict of interest

We wish to declare that there are no conflicts of interest associated with this study.

6. Acknowledgement

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