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Effect of anticoagulative sulfated polysaccharide purified from enzyme-assistant extract of a brown seaweed *Ecklonia cava* on Wistar rats

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ABSTRACT

In this study, the anticoagulant activity of a sulfated polysaccharide purified from enzyme assistant extract of brown seaweed *Ecklonia cava* (ECSP) was investigated both *in vitro* and *in vivo*. The sulfated polysaccharide purified from AMG assistant extract of *E. cava* showed anticoagulant activity comparable to that of a commercial fucoidan in activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) clotting assays *in vitro*. In Fourier transform infrared spectroscopy (FT-IR), similar transmittance was observed for the commercial fucoidan and ECSP with an intense absorption band at 1240 and 820 cm^{-1} indicating its high sulfate content. The administration of the anticoagulant drug in rats clearly extended the coagulation time in a dose dependent and time dependent manner in both APTT and TT assays, especially a clear effect was observed at 30 min after the initial sample treatment. In the tail bleeding assay, ECSP showed prolonged bleeding time (>1800 ± 05.1 s) than the control (900 ± 20.1 s) at the dosage of 300 μg/kg. With the current findings ECSP might be a promising candidate in pharmaceutical applications as a natural anticoagulant.

1. Introduction

A group of pharmaceuticals called anticoagulants can be used *in vivo* as a medication for thrombotic disorders. Heparin, a sulfated polysaccharide is the first compound used clinically as an anticoagulant and antithrombotic agent (Fachin & Verli, 2008). Over the years, anticoagulant activity is among the most widely studied properties of sulfated polysaccharides. Anticoagulant activity of sulfated polysaccharides isolated from different seaweed species had been widely reported (De Zoisa, Nikapitiya, Jeon, Jee, & Lee, 2008). Seaweeds are rich resources for sulfated polysaccharides possessing anticoagulant activity (Desai, 2004). Therefore, as an alternative source, seaweed polysaccharides gain much attention in the pharmaceutical industry to develop better and safe drugs with low or less side effects (Athukorala, Lee, Kim, & Jeon, 2007).

Sulfated polysaccharides are widespread in nature. The structures of algal sulfated polysaccharides are complex and heterogeneous. They also vary among species (Yoon, Pyun, Hwang, & Mourao, 2007). The profound functional properties of the sulfated polysaccharides are probably due to the presence of sulfate groups in varying amounts and different positions along the macromolecular backbone. The existence of structural similarities between sulfated polysaccharides from marine algae and heparin has also been reported (Lee, Athukorala, Lee, & Jeon, 2008). Hence, over the recent years, extensive studies have been made on the preparation and characterization of natural or semi-synthetic bioactive polymers exhibiting heparin like properties (Huynh, Chaubet, & Jozefonvicz, 2001).

Seaweeds contain a significant amount of soluble polysaccharides, and have potential function as dietary fiber. Specially, brown seaweeds contain large amounts of cell wall polysaccharides, most of which are sulfated polysaccharide fucoidans (Eldeen, Ahmed, & Zeid, 2009). Enzymatic degradation of cell wall polymers has received attention for many years and is becoming a more and more attractive alternative to chemical and mechanical processes. Therefore, use of enzyme assistant extraction technique could be a useful step in classical extraction procedures (Athukorala, Jung, Vasanthan, & Jeon, 2006).

In the present study, an edible brown seaweed *Ecklonia cava* was taken as the target material to purify a bioactive sulfated polysaccharide. This brown seaweed is found abundantly in the subtidal regions of Jeju Island of South Korea (Kim et al., 2006, 2008). It has long been utilized as a traditional food and also as a

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traditional folk herb (Shim, To, Lee, & Kim, 2009). In addition, *E. cava* has a variety of unique compounds including sulfated polysaccharides showing different biological activities (Heo et al., 2009). In this study, first, a sulfated polysaccharide was purified from AMG assistant extract of *E. cava* and the anticoagulant activity was studied. *In vitro* activity of ECSP was compared with a commercial fucoidan and heparin, prior to *in vivo* experiment. Then, the anticoagulants were introduced into rats and the time taken to blood coagulation process was assessed by APTT, TT and PT assays. In addition, a tail bleeding time experiment was conducted to assess compounds on bleeding implications. The potential anticoagulant activity of ECSP could be developed to use in pharmaceutical fields.

2. Materials and methods

2.1. Plant material and chemicals

The brown seaweed *E. cava* was collected along the cost of Jeju Island in South Korea. Salt, epiphytes, and sand were removed from the samples using tap water. Then the *E. cava* samples were carefully rinsed with fresh water and stored at −20 °C. The frozen samples were lyophilized and homogenized with a grinder prior to extraction.

AMG 300 L (an exo1, 4-alpha-d-glucosidase) was purchased from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). Activated partial prothrombin time reagent (APTT, ellagic + bovine phospholipid) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan). Prothrombin time reagent (PT, rabbit thromboplastin) and thrombin time (TT) reagents were purchased from Fisher Scientific Company (USA). All the other chemicals used in this study were of analytical grade.

2.2. Purification of sulfated polysaccharide from E. cava (ECSP)

The purification of sulfated polysaccharide was followed as previously published (Athukorala et al., 2006; Matsubara, Matsuura, Hori, & Miyazawa, 2000). Briefly, the dried algal sample was ground (MFC SI mill, Janke and Kunkel Ika-Wreck, Staufen, Germany) and sieved through a 50 standard testing sieve. A 100 g of the sample was homogenized with water (2 L), and then 1 mL of enzyme (AMG 300 L) was mixed. The enzymatic digestion was performed for 12 h to achieve an optimum degree of the digestion. Before the digestion pH of the homogenate was adjusted to its optimal pH value, and after the digestion it was boiled for 10 min at 100 °C to inactivate the enzyme. The reactant was clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. The enzymatic digest (240 mL) was well-mixed with 480 mL of 99.5% ethanol. The mixture was allowed to stand for 30 min at a room temperature, and then the crude polysaccharides were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C. Freeze dried crude polysaccharide from the digest was introduced to diethylaminoethyl cellulose (DEAE-cellulose) ion exchange chromatography. Then high anticoagulant fractions were separated according to activated partial thromboplastin time (APTT) assay. And then the sample was further purified on a new DEAE cellulose column to improve the purity of the sample. Thereafter, the sample was applied into a gel permeation chromatography on Sepharose-4B to purify the sample according to its molecular weight. The purity of the sample was confirmed by agarose gel electrophoresis, and the molecular weight of the sample was determined by gel filtration chromatography (GFC) system. The sulfated polysaccharide (0.92 sulfate/total sugar) showed 1381 kDa molecular weight and comprised mainly of fucose (82%) and a small amount of galactose.

2.3. IR-spectrum study

Freeze dried ECSP (approximately 0.1 g) was mixed with potassium bromide powder (100–200 g), then pressed into thin disc under hydraulic power and used as the sample for FT-IR measurement. The spectrum was arranged at mildest infrared region.

2.4. In vitro blood coagulation assay

Normal pooled plasma was obtained from 10 healthy individuals, without a 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 for 20 min at 2400 × *g*, and the plasma was stored at −60 °C until use. All coagulation assays were performed with four individual replicates using Dual-channel clot-2 (SEAC, Italy) and mean values were taken. For APTT assay, citrated normal human plasma (90 μL) was mixed with a solution of sample (10 μL) and incubated for 1 min at 37 °C, then APTT reagent (100 μL) was added to the mixture and incubated for 5 min at 37 °C. Thereafter, clotting was induced by adding 0.025 mol/L $CaCl_2$ (100 μL) and clotting time was recorded. In PT assay, citrated normal human plasma (90 μL) was mixed with a sample solution (10 μL) and incubated for 10 min. Then, PT reagent (200 μL) pre-incubated for 10 min at 37 °C was added and clotting time was recorded. For TT measurement, citrated normal human plasma (190 μL) was mixed with a sample solution (10 μL) and incubated for 2 min. Then pre-incubated TT reagent (100 μL) was added into the mixture and clotting time was recorded. All the samples including heparin were dissolved in water.

2.5. In vivo determination of anticoagulant activity in Wistar rats

The *in vivo* anticoagulant experiment was conducted using male rats (~300 g). Before starting the experiment the rats were anesthetized by injecting a mixture of Ketamine and Xylasine (intraperitoneally) with a dose of 100 and 16 mg/kg respectively. The sample ingestion was done according to the previously reported method (Martinichen-Herrero, Carbonero, Gorin, & Lacomini, 2005; Martinichen-Herrero, Carbonero, Sassaki, Gorin, & Lacomini, 2005). Briefly, the carotid artery of a rat was dissected and carefully separated from the surrounding tissues and each of heparin, ECSP and saline was administrated. During the each time interval (5, 30 and 60 min) the blood samples were taken out into sodium citrated tube and the plasma was immediately separated by centrifugation at 2500 × *g* at room temperature. Then, the APTT, TT and PT assays were done as described above.

2.6. Tail bleeding time in Wistar rats

The samples were administrated through the carotid artery and the bleeding time of rats was measured after cutting 3 mm from the tip of the tail. The tip of the tail was blotted using a tissue paper at 30 s intervals and the time taken to stop the bleeding was recorded. (Martinichen-Herrero, Carbonero, Gorin, et al., 2005; Martinichen-Herrero, Carbonero, Sassaki, et al., 2005). Each test component was administrated five min prior to tail dissection.

2.7. Statistical analysis

All the data were expressed as mean ± standard deviation (SD) of three determinations. Statistical comparison was performed via a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). *P* values of less than 0.05 ($P < 0.05$) were considered as significant.

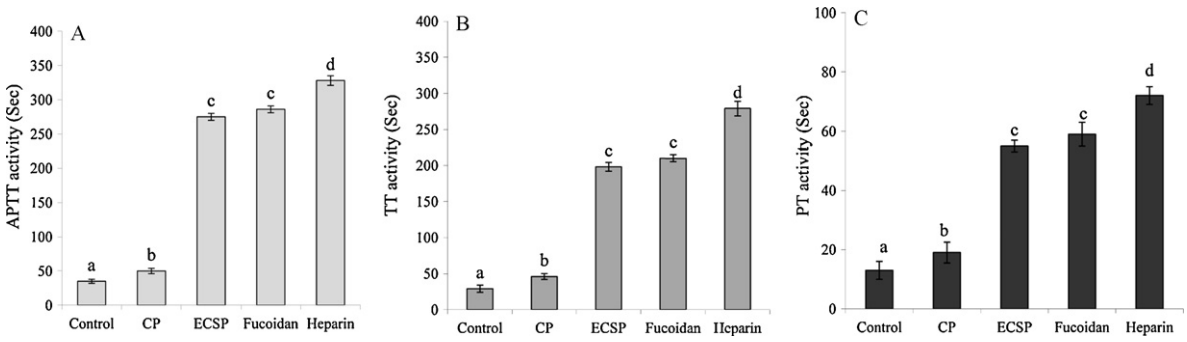

Fig. 1. The effect of crude polysaccharide fraction (CP), purified sulphated polysaccharide (ECSP), commercial fucoidan and heparin on the prolongation of (A) APTT, (B) TT and (C) PT *in vitro*. Sample concentration 1.5 μg/mL. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test (DMRT).

3. Results and discussion

In recent years, some bioactive polysaccharides isolated from natural sources have attracted much attention in the field of pharmacology (Yang & Zhang, 2009). With today's interest in new renewable sources of chemicals and polymers, the marine algae represent potential source to be explored. Marine algae often contain bioactive substances with novel functional structures. Especially sulfated polysaccharides of edible algae attracted extensive interest due to their numerous biological activities including anticoagulant activity. (Pereira, Silva, Valente, & Mourao, 2002). Here, we report the isolation and purification of a natural sulfated polysaccharide responsible for anticoagulant activity from enzyme-assistant extract of brown seaweed *E. cava*.

3.1. In vitro anticoagulant activity

ECSP was purified and confirmed its anticoagulant activity *in vitro* before conducting *in vivo* experiments in rats. Results showed anticoagulant activity of ECSP comparable to that of the commercial fucoidan in APTT, TT and PT assays (Fig. 1). As shown in Fig. 1A, the plasma sample treated with ECSP successfully prolonged coagulation time compared to that of the saline treated control sample. Interestingly, the commercial fucoidan and ECSP had similar activities at the same concentrations. Of the tested samples heparin showed the best anticoagulant activity, while the crude polysaccharide fraction of *E. cava* showed the least activity. The ability of sulfated polysaccharides to interfere with biological systems has a longstanding record, as illustrated with heparin, an anticoagulant drug that has been used clinically for more than 50 years (Huang, Du, Yang, & Fan, 2003; Huynh et al., 2001). Therefore, the sulfated polysaccharides having improved action profile are of special interest (Alban, Schauerte, & Franz, 2002). APTT is a performance indicator measuring the efficacy of both the intrinsic and the common coagulation pathways. Apart from detecting abnormalities in blood clotting, it is also used to monitor the treatment effects with anticoagulants. Similarly, tested samples had similar trend in TT and PT assays (Fig. 1B and C). But, according to the results quite low activity showed in the PT assay. Algal polysaccharides are poor in modulating prothrombin time (Athukorala et al., 2006); therefore the tested samples had lower activity in the particular assay. One of the previous studies demonstrated that sulfated polysaccharides purified from an enzymatic hydrolysate of the brown alga *E. cava* strongly and selectively (FVII, FX, and FII) enhanced ATIII-mediated coagulation factor inhibition in both the extrinsic and common coagulation pathways (Jung et al., 2007). Further, we suggested that this may contribute to its high anticoagulant activity *in vitro*. However, the profound anticoagulant activity of ECSP should be exercised in evaluating the *in vivo* activity of the sample.

3.2. FT-IR analysis of anticoagulant polysaccharides

Fourier transform spectroscopy is a measurement technique whereby spectra are collected based on measurements of the coherence of a radioactive source, using time domain or space-domain measurements of the electromagnetic radiation or other type of radiation. FT-IR spectroscopy is used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of polysaccharides, such as monosaccharide types, glucosidic bonds and functional groups, can be analyzed using FT-IR spectroscopy (Yang & Zhang, 2009). The spectra obtained from the wave number 400–4000 cm^{-1} give some inside information of the tested compounds; therefore, the method is popular in identifying vibrational structure of the materials (Patankar, Oehninger, Barnett, Williams, & Clark, 1993).

In this study, to have an idea of the main spectral features of the polysaccharides, FT-IR study was carried out (Fig. 2). The crude polysaccharide fraction and ECSP were compared with that of the commercial fucoidan. The crude polysaccharide fraction showed completely different pattern with that of the other two samples (Fig. 2A). This may be due to its complex nature and impurity. However, ECSP showed infrared absorption properties similar to that of the commercial fucoidan (Fig. 2B and C). As it has been reported, intense absorption band at 1240 cm^{-1} common to sulfate esters (S=O), meanwhile band at 820 cm^{-1} indicate the presence of C–O–S secondary axial sulfate. Therefore, it is assumed that the main sulfate groups occupy positions C-2 or C-3 and lesser part of sulfate in located at C-4 of fucopyranose residue. The other major absorption band at 3500 and the sharp absorption peak at 1030–1200 cm^{-1} are belong to O–H and COO− stretching respectively (Bernardi & Springer, 1962; Qui, Amarasekara, & Doctor, 2006). Taken together, these results clearly illustrate the similarity of purified ECSP with that of the commercial fucoidan. Their slight intensity differences in FT-IR may be due to origin and technique of the isolation procedure. The anticoagulant activity of sulfated polysaccharide mainly depends on to molecular weight, sulfate content but also to the levels of 2-O-sulfation and 2,3-O-disulfation (Qui et al., 2006; Suwan et al., 2009). However, deep structural information on sulfated polysaccharides is limited due to their structural heterogeneity (Kloareg & Quatrano, 1988; Patankar et al., 1993).

3.3. In vivo anticoagulant activity

After confirming the *in vitro* anticoagulant activity, the *in vivo* anticoagulant activity of the ECSP was studied by introducing samples into male rats at different concentrations (50–300 μg/kg). The highest APTT activity of ECSP was recorded after 30 min of application time at the concentration of 300 μg/mL (Table 1). However, heparin showed much higher activity than that of ECSP at the

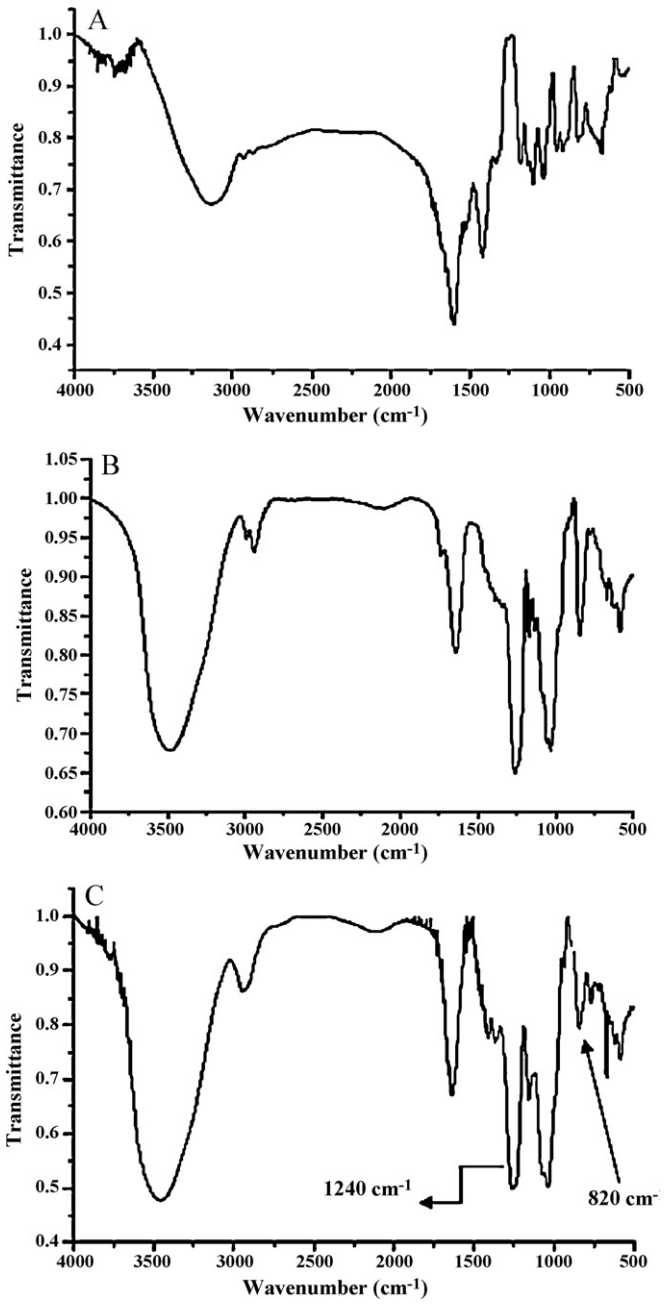

Fig. 2. FT-IR frequencies of bands for (A) crude polysaccharide fraction, (B) purified ECSP and (C) commercial fucoidan.

Table 1
The effect of the sulfated polysaccharide purified from *E. cava* (ECSP) and commercially available heparin on activated partial thrombin time (APTT) in Wistar rats.

Compound	Dose (μg/kg)	APPT (min)			
		0	5	30	60
ECSP	50	20 ± 1.0	36 ± 1.2	38 ± 0.6	45 ± 0.1
	100	20 ± 0.5	58 ± 1.2	67 ± 0.9	62 ± 0.9
	300	18 ± 0.8	128 ± 1.1	156 ± 0.2	144 ± 0.7
Heparin	50	19 ± 1.3	70 ± 0.8	211 ± 0.6	189 ± 1.2
	100	23 ± 0.7	>300 ± 0.1	>300 ± 0.1	>300 ± 0.1
Saline		23 ± 0.3	25 ± 0.2	23 ± 0.1	22 ± 0.2

For each treatment group the mean APTT time ± SD was determined for $n = 4$/group.

Table 2
The effect of the sulfated polysaccharide purified from *E. cava* (ECSP) and commercially available heparin on thrombin time (TT) in Wistar rats.

Compound	Dose (μg/kg)	TT (min)			
		0	5	30	60
ECSP	50	16 ± 0.3	17 ± 0.1	19 ± 0.2	19 ± 0.8
	100	17 ± 0.4	21 ± 1.2	29 ± 1.0	20 ± 0.5
	300	14 ± 0.9	85 ± 0.2	92 ± 0.4	89 ± 0.6
Heparin	50	16 ± 0.8	58 ± 1.2	70 ± 0.8	68 ± 0.4
	100	18 ± 0.2	>300 ± 0.1	>300 ± 0.1	>300 ± 0.1
Saline		16 ± 0.2	14 ± 1.1	16 ± 0.1	16 ± 0.2

For each treatment group the mean TT time ± SD was determined for $n = 4$/group.

similar concentrations. According to one of the previous studies, moltodapoh, a sulfated tetrasaccharide, exhibits good anticoagulant and antithrombotic activity in rabbits (Martin, Toce, Anevski, Tollefsen, & Abendschein, 1999). In contrast, with a single oral dose of maltodapoh (3 mg/kg), anticoagulation persisted for at least 24 h.

TT value indicates the ability of the ECSP to prolong the blood coagulation by controlling the fibrinogen to fibrin conversion, the addition of TT reagent (human thrombin) to plasma stimulate the conversion but the presence of anticoagulants block the conversion by extending the coagulation time (Table 2). It was previously reported that the sulfated polysaccharide purified from *E. cava* had high affinity on factor II (prothrombin) (Athukorala et al., 2006) therefore; the ECSP may have an effect on fibrin polymerization which explain the extended TT activity in ECSP treated rats. ECSP result of *in vivo* TT at 300 μg/mL was comparable with the results of heparin at 50 μg/mL. ECSP as well as heparin showed comparatively lower activity in PT assay than that of TT and APTT (Table 3). In PT assay, bio-molecular interaction of the tested sample on II, V, VII, X and fibrinogen is estimated; these factors of the coagulation pathway are in conjunction with the intrinsic pathway. As it has been documented, abnormalities of VII are much responsible for the extending the PT. Since the ECSP has a strong effect on VII, a considerable PT activity was observed in this study. Taken together, anticoagulant activity of ECSP was relatively lower than commercially available heparin. However, unlike heparin, the sulfated polysaccharides stimulates t-PA induced plasma clot lysis and thereby decrease the rate of fibrin polymer formation, therefore the sulfated polysaccharides have better fibrinolytic and anticoagulant activities which useful to develop thrombolytic agents (Soeda, Sakaguchi, Shimeno, & Nagamatsu, 1992). Moreover, ECSP showed high anti proliferative effect on human leukemic monocyte lymphoma (U-937) cell line through apoptosis related protein regulation (Athukorala et al., 2009). Therefore, ECSP has added advantages over heparin.

Bleeding implications can be taken place with the treatment of anticoagulant drugs. Bleeding time is an indicator of the platelets functioning. Animal experiments are not a suitable way of evaluat-

Table 3
The effect of the sulfated polysaccharide purified from *E. cava* (ECSP) and commercially available heparin on prothrombin time (PT) in Wistar rats.

Compound	Dose (μg/kg)	PT (min)			
		0	5	30	60
ECSP	50	10 ± 0.3	13 ± 0.5	12 ± 1.2	12 ± 1.1
	100	12 ± 0.8	14 ± 0.3	13 ± 1.0	15 ± 1.0
	300	11 ± 0.3	22 ± 0.8	28 ± 0.4	25 ± 0.9
Heparin	50	10 ± 0.7	55 ± 0.9	69 ± 1.2	57 ± 0.2
	100	13 ± 1.3	100 ± 0.2	120 ± 0.2	189 ± 0.4
Saline		12 ± 0.2	11 ± 1.0	10 ± 0.1	10 ± 1.2

For each treatment group the mean PT time ± SD was determined for $n = 4$/group.

Table 4
Anticoagulant activity of the sulfated polysaccharide purified from *E. cava* (ECSP) on tail bleeding time of Wistar rats.

Compound	Dose (μg/kg)	Bleeding time (s)
ECSP	50	960 ± 10.1
	100	990 ± 30.1
	300	>1800 ± 05.1
Heparin	50	1200 ± 15.2
	100	>1800 ± 05.0
Saline		900 ± 20.1

Tail bleeding time of sample treated rat was examined after the dissection of the tail extremely 3 mm from the tip. The tail was blotted with a tissue paper in every 30 s and the time to stop bleeding was noted. For each treatment group the mean bleeding time ± SD was determined for $n = 4$/group.

ing bleeding problems, however tail bleeding is still the choice of many scientists to investigate the antihemostatic effect of anticoagulant compounds. After 5 min of the sample administration, exactly 3 mm from the tip of the tail of mice was dissected and the time to cessation of bleeding was noted. According to the results, injection of ECSP and heparin dose-dependently extended the bleeding time (Table 4).

4. Conclusion

This communication discussed the purification of a sulfated polysaccharide from AMG-assistant extract of brown seaweed *E. cava* and its anticoagulation activity both *in vitro* and *in vivo*. In conclusion, ECSP exhibited good anticoagulant properties comparable with commercially available fucoidan *in vitro*. In addition, *in vivo* studies demonstrated the successful use of ECSP as an anticoagulative agent. However, it is less effective than heparin. Findings of this study could be beneficial to development of pharmaceutical preparations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Alban, S., Schauerte, A., & Franz, G. (2002). Anticoagulant sulfated polysaccharides. Part I. Synthesis and structure-activity relationships of new pullulan sulfates. *Carbohydrate Polymers*, *47*, 267–276.

Athukorala, Y., Ahn, G. N., Jee, Y. H., Kim, G. Y., Kim, S. H., Ha, J. H., et al. (2009). Antiproliferative activity of sulfated polysaccharide isolated from an enzymatic digest of *Ecklonia cava* on the U-937 cell line. *Journal of Applied Phycology*, *21*, 307–314.

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydrate Polymers*, *66*, 184–191.

Athukorala, Y., Lee, K. W., Kim, S. K., & Jeon, Y. J. (2007). Anticoagulant activity of marine green and brown algae collected from Jeju Island in Korea. *Bioresource Technology*, *98*, 1711–1716.

Bernardi, G., & Springer, G. F. (1962). Properties of highly purified fucan. *Journal of Biological Chemistry*, *237*, 75–80.

De Zoisa, M., Nikapitiya, C., Jeon, Y. J., Jee, Y., & Lee, J. (2008). Anticoagulant activity of sulfated polysaccharide isolated from brown seaweed *Sargassum fulvellum*. *Journal of Applied Phycology*, *20*, 67–74.

Desai, R. U. (2004). New antithrombin-based anticoagulants. *Medicinal Research Reviews*, *24*, 151–181.

Eldeen, A. M. G., Ahmed, E. F., & Zeid, M. A. A. (2009). *In vitro* cancer chemopreventive properties of polysaccharide extract from the brown alga, *Sagassum latifolium*. *Food & Chemical Toxicology*, *47*, 1378–1384.

Fachin, L. P., & Verli, H. (2008). Depiction of the forces participating in the 2-O-sulfo-α-L-iduronic acid conformational preference in heparin sequences in aqueous solutions. *Carbohydrate Research*, *343*, 1435–1445.

Heo, S. J., Ko, S. C., Cha, S. H., Kang, D. H., Park, H. S., Choi, Y. U., et al. (2009). Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicology In Vitro*, *23*, 1123–1130.

Huang, R., Du, Y., Yang, J., & Fan, L. (2003). Influence of functional groups on the *in vitro* anticoagulant activity of chitosan sulfate. *Carbohydrate Research*, *338*, 483–489.

Huynh, R., Chaubet, F., & Jozefonvicz, J. (2001). Anticoagulant properties of dextram-ethylcarboxylate benzylamide sulfate (DMCBSu); A new generation of bioactive functionalized dextran. *Carbohydrate Research*, *332*, 75–83.

Jung, W. K., Athukorala, Y., Lee, Y. J., Cha, S. H., Lee, C. H., Vasanthan, T., et al. (2007). Sulfated polysaccharide purified from *Ecklonia cava* accelerates antithrombin III-mediated plasma proteinase inhibition. *Journal of Applied Phycology*, *19*, 425–430.

Kim, M. M., Ta, Q. V., Mendis, E., Rajapakse, N., Jung, W. K., Byun, H. G., et al. (2006). Phlorotannins in *Ecklonia cava* extract inhibit matrix metalloproteinase activity. *Life Sciences*, *79*, 1436–1443.

Kim, S. K., Lee, Y., Jung, W. K., Kim, J. H., Choi, I., Park, S. G., et al. (2008). Effect of *Ecklonia cava* ethanolic extracts on airway hyper responsiveness and inflammation in a murine asthma model: Role of suppressor of cytokine signaling. *Biomedicine & Pharmacotheraphy*, *62*, 289–296.

Kloareg, B., & Quatrano, R. S. (1988). Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanography and Marine Biology Annual Review*, *26*, 259–315.

Lee, S. H., Athukorala, Y., Lee, J. S., & Jeon, Y. J. (2008). Simple separation of anticoagulant sulfated galactan from marine red alga. *Journal of Applied Phycology*, *20*, 1053–1059.

Martin, D. J., Toce, J. A., Anevski, P. J., Tollefsen, D. M., & Abendschein, D. R. (1999). Anticoagulant and antithombotic activity of maltodapoh, a novel sulfated tetrasaccharide. *Journal of Pharmacology and Experimental Therapy*, *288*, 516–521.

Martinichen-Herrero, J. C., Carbonero, E. R., Gorin, P. A. J., & Lacomini, M. (2005). Anticoagulant and antithrombotic activity of a sulfate obtained from a glucan component of the lichen *Parmotrema mantiqueirense* Hale. *Carbohydrate Polymer*, *60*, 7–13.

Martinichen-Herrero, J. C., Carbonero, E. R., Sassaki, G. L., Gorin, P. A. J., & Lacomini, M. (2005). Anticoagulant and antithrombotic activities of a chemically sulfated galactoglucomannan obtained from the lichen *Cladonia ibitipocae*. *International Journal of Biological Macromolecules*, *35*, 97–102.

Matsubara, K., Matsuura, Y., Hori, K., & Miyazawa, K. (2000). An anticoagulant proteoglycan from the marine green alga, *Codium pugniformis*. *Journal of Applied Phycology*, *12*, 9–14.

Patankar, M. S., Oehninger, S., Barnett, T., Williams, R. L., & Clark, G. F. (1993). A revised structure for fucoidan may explain some of its biological activities. *Journal of Biological Chemistry*, *29*, 21770–21776.

Pereira, M. S., Silva, A. C. E. S. V., Valente, A. P., & Mourao, P. A. S. (2002). A 2-sulfated, 3-linked α-L-galactan is an anticoagulant polysaccharide. *Carbohydrate Research*, *337*, 2231–2238.

Qui, X., Amarasekara, A., & Doctor, V. (2006). Effect of oversulfation on the chemical and biological properties of fucoidan. *Carbohydrate Polymers*, *63*, 224–228.

Shim, S. Y., To, L. Q., Lee, S. H., & Kim, S. K. (2009). *Ecklonia cava* extract suppresses the high-affinity IgE receptor, FcRIε expression Food & Chemical. *Toxicology*, *47*, 555–560.

Soeda, S., Sakaguchi, S., Shimeno, H., & Nagamatsu, A. (1992). Fibrinolytic and anticoagulant activities of highly sulfated fucoidan. *Biochemical Pharmacology*, *43*, 1853–1858.

Suwan, J., Zhang, Z., Li, B., Vongchan, P., Meepowpan, P., Zhang, F., et al. (2009). Sulfonation of papain-treated chitosan and its mechanism for anticoagulant activity. *Carbohydrate Research*, *344*, 1190–1196.

Yang, L., & Zhang, L. M. (2009). Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. *Carbohydrate Polymers*, *76*, 349–361.

Yoon, S. J., Pyun, Y. R., Hwang, J. K., & Mourao, P. A. S. (2007). A sulfated fucan from the brown alga *Laminaria cichoriodes* has mainly heparin cofactor II-dependeant anticoagulant activity. *Carbohydrate Research*, *342*, 2326–2330.