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Evaluating the possible anticoagulant and antioxidant effects of sulfated polysaccharides from the tropical green alga *Caulerpa cupressoides* var. *flabellata*

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Abstract Seaweeds are a source of several biopolymers widely used in cosmetics, food, and pharmaceuticals. Among them are sulfated polysaccharides, which have several biological/pharmacological activities, such as antioxidant and anticoagulant activities. In the present study, four sulfated polysaccharides, denominated CCB-F0.3, CCB-F0.5, CCB-F1.0, and CCB-F2.0, were obtained from the chlorophyte *Caulerpa cupressoides* var. *flabellata* through proteolytic digestion, followed by acetone fractionation and molecular sieving in Sephadex G-100. Chemical analyses showed that CCB-F0.5 had the highest sulfate/sugar ratio (0.73), whereas CCB-F1.0 exhibited the lowest ratio (0.23). Polysaccharides from *C. cupressoides* displayed a heterogeneous constitution of monosaccharides, with galactose as the main sugar unit (except for CCB-F2.0). The presence of sulfated polysaccharides was confirmed by electrophoretic and infrared analyses. Sulfated polysaccharides showed no activity in superoxide and hydroxyl radical scavenging; however, they did demonstrate total antioxidant capacity and ferrous chelating activity. *Caulerpa* polysaccharides also exhibited anticoagulant activity in the intrinsic (activated partial thromboplastin time (aPTT) test) and extrinsic pathway (prothrombin time (PT) test). In the aPTT test, all polysaccharides displayed considerable dose-dependent activity. A significant result was the aPTT activity of the polysaccharides CCB-F0.3 and CCB-F0.5, which was similar to that of Clexane®, a commercial low molecular weight heparin. In addition, CCB-F0.3 and CCB-F0.5 showed PT activity. Sulfated polysaccharides from *C. cupressoides* are therefore promising antioxidant agents in preventing the formation of reactive oxygen species and for their possible use in anticoagulant therapy.

Keywords Sulfated galactans · Biological activities · Green seaweed · Chlorophyta

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Introduction

Seaweeds are a rich source of various compounds widely used in medicine, pharmacy, biotechnology, cosmetology, and the food industry. Sulfated polysaccharides extracted

from seaweeds occupy a special place among them, since they have a broad range of activities when tested in different biological systems, displaying anti-adhesive, antioxidant, antiviral, antitumor, anti-ulcerogenic, antithrombotic, and anticoagulant properties (Rocha et al. 2001; Bilan and Usov 2008; Cumashi et al. 2007; Athukorala et al. 2007; Ohta et al. 2009). However, the most extensive studies have been conducted on the anticoagulant activities of several algal sulfated polysaccharides (Albuquerque et al. 2004; Bilan and Usov 2008; Zoysa et al. 2008).

The world's leading causes of death are diseases involving the heart and blood vessels, including thrombosis. The incidence of death caused by thrombosis is almost twice that of the next deadliest disease, cancer (WHO 2008). Most thromboembolic processes require anticoagulant therapy. In addition, the mainly anticoagulant drug, a sulfated polysaccharide named heparin, has several side effects such as the development of thrombocytopenia and hemorrhage (Nader et al. 2004). This explains current efforts to develop specific and potent anticoagulant agents from several sources, including sulfated polysaccharides from seaweeds.

Recently, the antioxidant activity of sulfated polysaccharides has gained considerable attention. These polymers have been shown to play an important role as free-radical scavengers in vitro and as antioxidants to prevent oxidative damage in living organisms (Rupérez et al. 2002; Souza et al. 2007; Wang et al. 2008; Lim and Ryu 2009). The relationship between structure and anticoagulant or antioxidant activities of algal sulfated polysaccharides has not yet been clearly established (Bilan and Usov 2008). Nevertheless, it is most likely that some structural features are required for biological activities, particularly sulfate clusters to ensure interactions with cationic proteins (Mulloy 2005). Indeed, the importance of molecular size has been reported (Li et al. 2008). On the other hand, qualitative and quantitative sulfated polysaccharides of seaweeds depend on many factors and are primarily species specific (Dietrich et al. 1995). Thus, seasonal variations also impact structure, composition, and consequently biological activities of sulfated polysaccharides (Honya et al. 1999; Rioux et al. 2009).

The green alga *Caulerpa cupressoides* var. *flabellata* is abundant on the Brazilian northeast coast. We previously demonstrated that the polysaccharide-rich extract from *C. cupressoides* exhibited diverse biological activities, including anticoagulant and antioxidant activities (Costa et al. 2010). However, the biological activities of the purified sulfated polysaccharides from *C. cupressoides* have not been determined. As such, the main objective of this research was to obtain sulfated polysaccharides from *C. cupressoides* and evaluate their antioxidant and anticoagulant activities in vitro.

Material and methods

Extraction of sulfated polysaccharides

The green alga *Caulerpa cupressoides* var. *flabellata* Børgesen was collected on Búzios beach (05°58′23″ S, 35° 04′97″ W), Rio Grande do Norte State (Northeast of Brazil), in November 2009. Immediately after collection, algae were identified by Dr. Eliane Marinho-Soriano from the Centro de Biociências/UFRN, Natal, Rio Grande do Norte, Brazil.

Extraction of sulfated polysaccharide fractions was performed as described by Rocha et al. (2005). Immediately after collection, the seaweed was dried at 50°C under ventilation and ground in a blender. It was then treated with acetone to eliminate lipids and pigments. One hundred grams of defatted, dried, and powdered alga were suspended in 500 mL of 0.25 M NaCl, and pH was adjusted to 8.0 with NaOH. Twenty milligrams of maxatase, an alkaline protease from *Esporobacillus* (Biobras, Minas Gerais, Brazil), was added to the mixture for proteolytic digestion. Following 18 h incubation at 60°C with agitation, the mixture was filtered through cheesecloth. The filtrate was fractionated by precipitation with acetone as follows: 0.3 volumes of ice-cold acetone was added to the solution under gentle agitation and maintained at 4°C for 24 h. The precipitate formed was collected by centrifugation (10,000×*g*, 20 min), dried under vacuum, resuspended in distilled water, and analyzed. The operation was repeated by adding 0.5, 1.0, and 2.0 volumes of acetone to the supernatant. Four polysaccharide-rich fractions were obtained and denominated according to the volumes of acetone used (F0.3, F0.5, F1.0, and F2.0). These fractions were further purified by molecular sieving on Sephadex G-100 (140×2.6 cm). About 200 mg of each fraction, dissolved in 2 mL water, was applied to the column and eluted with a solution of 0.2 M acetic acid and 0.15 M NaCl. Fractions of 1 mL were collected and assayed by the phenol/H_2SO_4 reaction (Dubois et al. 1956), and by metachromatic assay using 1.9-dimethylmethylene blue (Farndale et al. 1986). Fractions containing sulfated polysaccharides were pooled, dialyzed against distilled water, and lyophilized.

Chemical analysis, monosaccharide composition, and molecular weight determination

Total sugars were estimated by the phenol–H_2SO_4 reaction (Dubois et al. 1956) using D-galactose as standard. After acid hydrolysis of polysaccharides (4 M HCl, 100°C, 6 h), sulfate content was determined according to the gelatin-barium method (Dodgson and Price 1962), using sodium sulfate as standard. Protein content was measured by Spector's method (Spector 1978). To determine the best polysaccharide acid hydrolysis using HCl, that is, where polymer degradation occurs without destroying monosaccharides released by this

degradation, polysaccharides from *C. cupressoides* were hydrolyzed with 0.5, 1, 2, and 4 M HCl, at 30 min, 1 h, 2 h, and 4 h. A temperature of 100°C was maintained in all hydrolysis conditions. The material was later neutralized, dried, and resuspended in water, and reducing sugars were determined by the Somogyi–Nelson method (Somogyi 1952). In all cases, the best hydrolysis condition was 2 M HCl for 2 h. Thus, polymers were hydrolyzed (2 M HCl, 100°C, 2 h) and their sugar composition was determined by a LaChrom Elite® HPLC system from VWR-Hitachi with a refractive index detector (RI detector model L-2490). A LichroCART® 250-4 column (250×40 mm) packed with Lichrospher® 100 NH_2 (5 μm) was coupled to the system. Sample mass used was 0.2 mg and analysis time was 25 min; as references, the following sugars were analyzed: arabinose, fructose, fucose, galactose, glucose, glucosamine, glucuronic acid, mannose, mannuronic acid, rhamnose, and xylose.

In order to determinate the sulfated polysaccharide molecular weight, they were subjected to gel permeation chromatography on Sephadex G-100 (140×1 cm) using 0.2 M acetic acid/0.15 M NaCl as an eluent. The elution was monitored for total sugar (Dubois et al. 1956) and metachromasia (Farndale et al. 1986). In order to estimate the molecular weight of polysaccharides, dextrans of different molecular weights were used as standards.

Agarose gel electrophoresis

Agarose gel electrophoresis of acidic polysaccharides was performed in 0.6% agarose gel (7.5×10×0.2 cm thick) prepared in 0.05 M 1.3-diaminopropane acetate buffer pH 9.0 (Dietrich and Dietrich 1976). Aliquots of the polysaccharides (about 50 μg) were applied to the gel and subjected to electrophoresis. The gel was fixed with 0.1% cetyltrimethylammonium bromide solution for 2 h, dried, and stained for 15 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol. It was then destained using the same solution without the dye.

Fourier transformed infrared spectroscopy

Sulfated polysaccharides (5 mg) were mixed thoroughly with dry potassium bromide. A pellet was prepared and the infrared spectrum was measured on a Thermo Nicolet spectrometer instrument, model Nexus 470 Fourier transformed infrared spectroscopy (FT-IR), between 500 and 4,000 cm^{-1}. Thirty-two scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

Antioxidant activity

The determination of total antioxidant capacity was based on the reduction of Mo(VI) to Mo(V) by sulfated polysaccharide and subsequent formation of a green phosphate/Mo(V) complex at acid pH (Costa et al. 2010). Tubes containing sulfated polysaccharides and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, absorbance of each solution was measured at 695 nm against a blank. Antioxidant capacity was expressed as mg of ascorbic acid/g of polysaccharide, denominated ascorbic acid equivalent.

Scavenging activity of seaweed sulfated polysaccharides against the hydroxyl radical was investigated with Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$). Results were expressed as an inhibition rate. Hydroxyl radicals were generated using 3 mL sodium phosphate buffer (150 mM, pH 7.4), which contained 10 mM $FeSO_4{\cdot}7H_2O$, 10 mM EDTA, 2 mM sodium salicylate, 30% H_2O_2 (200 μL), and varying sulfated polysaccharide concentration. In the control, sodium phosphate buffer replaced H_2O_2. Solutions were incubated at 37°C for 1 h, and the presence of the hydroxyl radical was detected by monitoring absorbance at 510 nm (Costa et al. 2010).

The superoxide radical scavenging activity assay was based on the capacity of sulfated polysaccharides to inhibit photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system (Costa et al. 2010). Each 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM), and 1 mL of sample solution. Production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes containing the reaction mixture were kept in the dark and served as blanks.

The ferrous ion-chelating ability of samples was investigated according to previous studies (Costa et al. 2010). Briefly, the reaction mixture containing samples of $FeCl_2$ (0.05 mL, 2 mM) and ferrozine (0.2 mL, 5 mM) was well shaken and incubated for 10 min at room temperature. Absorbance of the mixture was measured at 562 nm against a blank.

Anticoagulant activity

Both the prothrombin time (PT) and activated partial thromboplastin time (aPTT) coagulation assays were performed with a coagulometer as described earlier (Albuquerque et al. 2004) and measured using citrate-treated normal human plasma.

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA

using SIGMAStat version 2.01 software. Student-Newman-Keuls post-tests were carried out for multiple group comparison. In all cases, statistical significance was set at $p<0.05$.

Results and discussion

Isolation and chemical analysis of sulfated polysaccharide from C. cupressoides The defatted powdered alga was extracted at 60°C in the presence of proteases. Then, using acetone precipitation, we obtained four fractions of sulfated polysaccharides from *C. cupressoides* denominated F0.3, F0.5, F1.0, and F2.0, respectively. The addition of increasing volumes of acetone gradually decreased the dielectric constant of water, thereby promoting different sulfated polysaccharide precipitation rates. Acetone separates polysaccharides by the way in which the charges of these polymers interact with water. As such, those that interact more with water are the last to precipitate. Each fraction was subjected to molecular sieving on Sephadex G-100, monitored for total sugar and metachromasia (Fig. 1). Each had a single peak, indicating it is composed of a unique population of sulfated polysaccharide. Tubes containing sulfated polysaccharides were pooled and lyophilized, and sulfated polysaccharides were also named CCB-F0.3, CCB-F0.5, CCB-F1.0, and CCB-F2.0, respectively.

The yield and chemical composition of sulfated polysaccharides is shown in Table 1. Yield ranged from 0.43% (CCB-F2.0) to 46.91% (CCB-F0.5). CCB-F0.5 showed the highest sulfate/sugar ratio (0.73) whereas CCB-F1.0 exhibited the lowest ratio (0.23). In addition, we did not observe protein contamination in all sulfated polysaccharides, since proteolytic enzyme was used during polysaccharide extraction.

The monosacharide composition was also examined (Table 1). Polysaccharides from *C. cupressoides* showed a heterogeneous constitution monosaccharide. For all polysaccharides (except CCB-F2.0), galactose was the main sugar unit. In addition to galactose, other monosaccharides were also present in polysaccharides, although the amount of these sugars in each polymer was different. CCB-F0.3 was composed of galactose and a small amount of glucose, mannose, and xylose. CCB-F0.5 contained galactose, mannose, and traces of xylose. CCB-F1.0 exhibited galactose, mannose, xylose and traces of glucose and rhamnose. CCB-F2.0 presented higher heterogeneity of monosaccharide content in comparison with other polysaccharides, since six monosaccharides were found. The presence of sulfated heteropolysaccharides was also common in other Chlorophyta species, as observed for *Ulva conglobata* (Mao et al. 2006) and *Ulva pertusa* (Qi et al. 2005), *Caulerpa* species such as *Caulerpa okamurai* (Hayakawa et al. 2000) and *Caulerpa racemosa* (Ghosh et al. 2004), and even for Codiales such as *Codium fragile* (Ciancia et al. 2007). For instance, Codiales polysaccharides were mainly composed of arabinose or galactose, and the Caulerpales, such as *C. cupressoides* and Ulvales

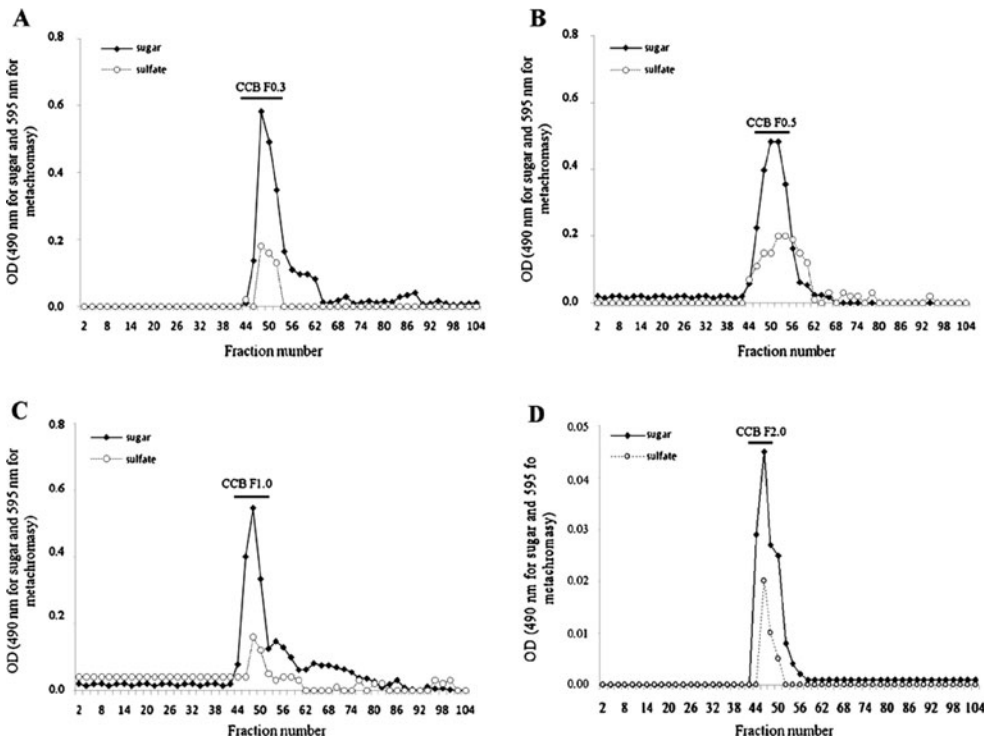

Fig. 1 Gel permeation chromatography of sulfated polysaccharides from *C. cupressoides*. The fractions precipitated with volumes of acetone F0.3 (**a**), F0.5 (**b**), F1.0 (**c**), and F2.0 (**d**) were applied to a Sephadex G-100 column (140×1 cm). The column was eluted with 0.2 M acetic acid, 1 mL fractions were collected, and the effluent was analyzed for the presence of sugars by the phenol-H_2SO_4 method and metachromasia

Table 1 Chemical composition and average molecular mass of the sulfated polysaccharides from the alga *C. cupressoides*

Polysaccharide	Yield (%)[a]	Total sugar (%)[b]	Sulfate (%)[b]	(SO_4)/total sugar (%/%)[b]	Molecular weight (kDa)[c]	Molar ratio of monosaccharide composition[d]					
						Gal	Glc	Man	Xyl	Rha	Fuc
CCB-F0.3	13.43	54.91	34.63	0.63±0.02	155±10	1.0	0.1	0.2	0.1	–	–
CCB-F0.5	46.91	52.38	38.05	0.73±0.04	130±10	1.0	–	0.1	tr	–	–
CCB-F1.0	39.23	76.47	17.95	0.23±0.01	155±10	1.0	tr	0.1	0.6	tr	–
CCB-F2.0	0.43	59.60	31.64	0.53±0.02	170±10	1.0	0.6	1.8	1.0	0.5	1.0

Gal galactose, *Glc* glucose, *Man* mannose, *Xyl* xylose, *Rha* rhamnose, *Fuc* fucose, – not detected, *tr* traces

[a] All polysaccharides obtained by molecular sieving on Sephadex G-100 were dried and weighted and total polysaccharide amount corresponded to 100%

[b] The total sugars and sulfate of polysaccharides were determined by the phenol/H_2SO_4 and $BaCl_2$/gelatin reactions, respectively

[c] The average molecular weight (kDa) was estimated by gel permeation chromatography on Sephadex G-100 (three determinations were made)

[d] The molar ratio of monosaccharide composition were estimated by HPLC

polysaccharides, consisted primarily of galactose and rhamnose, respectively. However, there is still insufficient evidence to establish any systematic relationship between structure, including monosaccharide composition, and algal order.

Molecular weight of sulfated polysaccharides The molecular weight of the sulfated polysaccharides was determined by gel permeation chromatography on Sephadex G-100. Sulfated polysaccharides of CCB-F0.3, CCB-F0.5, CCB-F1.0, and CCB-F2.0 had apparent molecular weights of 155, 130, 155, and 170 kDa, respectively (Table 1).

Polysaccharide analysis by agarose gel electrophoresis Electrophoretic mobility of sulfated polysaccharides on agarose gel, using diaminopropane/acetate buffer, is shown in Fig. 2. The staining pattern of polysaccharides on the agarose gel electrophoresis with toluidine blue revealed that all fractions consisted of sulfated polysaccharides. In addition, *C. cupressoides* mainly synthesized three types of sulfated polysaccharides, separated from each other through acetone precipitation, followed by gel permeation chromatography on Sephadex G-100. Polysaccharides with low electrophoretic mobility were found in CCB-F0.3 and CCB-F0.5 whereas CCB-F1.0 contained polysaccharides with higher mobility. Unfortunately, CCB-F2.0 was not used in this and other test since only a small amount of this polymer was obtained (Table 1). We were able to individualize the sulfated polysaccharides due to the property of 1,3-diaminopropane/acetate buffer. Diamine is able to complex with the *Caulerpa* polysaccharides through their sulfated groups as was previously observed for sulfated glycosaminoglycans (Dietrich and Dietrich 1976). This complexation is more dependent on the spacing of vicinal charges than the absolute charge of the compounds (Dietrich and Dietrich 1976). Thus, chondroitin sulfate and dermatan sulfate, which have the same charge/mass ratio, have different electrophoretic mobilities in diaminopropane/acetate buffer (Fig. 2b).

FT-IR analysis Infrared spectroscopy has proved to be a powerful tool in observing similarities between compounds. Characteristic absorptions of sulfate were identified in the FT-IR spectra of all polysaccharides: bands around 1,264–

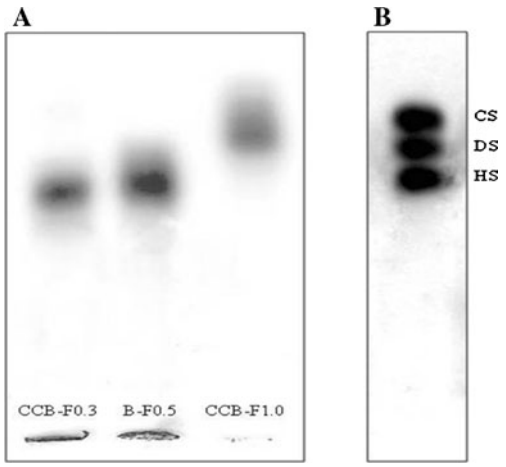

Fig. 2 Electrophoresis of sulfated polysaccharides from *C. cupressoides* (**a**) and electrophoresis of sulfated glycosaminoglycans (**b**). About 5 μl aliquots (50 μg) of the *Caulerpa* polysaccharides or (5 μg) of glycosaminoglycans were applied in agarose gel (107.5 cm, 0.2 cm thick) prepared in 0.05 M 1,3-diaminopropane–acetate buffer, pH 9.0, and subjected to electrophoresis at 110 V/cm for 60 min. The gels were then maintained in 0.1% cetyltrimethylammonium bromide for 2 h and dried, and the polysaccharides were stained with 0.1% toluidine blue in a solution containing 50% ethanol and 1% acid acetic in water for 15 min. The gels were then destained with the same solution lacking toluidine blue

1,259 cm^{-1} (CCB-F0.3, CCB-F0.5, and CCB-F1.0) for an asymmetric S=O stretching vibration (Silva et al. 2005); bands around 808 cm^{-1} (CCB-F0.3, CCB-F0.5, and CCB-F1.0) for C–O–S bond stretching (Cao and Ikeda 2009); the bands near 845 cm^{-1} (CCB F0.3), 830 cm^{-1} (CCB-F0.5 and CCB-F1.0) and 820 cm^{-1} (CCB-F0.3 and CCB-F0.5) were assigned to the 4-sulfate, 2-sulfate, and 6-sulfate of D-galactose units, respectively (Ghosh et al. 2004; Wang et al. 2010).

Additionally, at 3,000–3,400 cm^{-1} and around 2,920 cm^{-1} all polysaccharides showed signs from the stretching vibration of O–H and C–H, respectively (Wang et al. 2010). Bands around 1,638–1,654 cm^{-1} were due to the bound water.

Antioxidant activity Recently, several sulfated polysaccharides from marine algae have been described as having antioxidant activity (Rupérez et al. 2002; Souza et al. 2007; Wang et al. 2008; Lim and Ryu 2009). Antioxidant activity of sulfated polysaccharide from *C. cupressoides* was evaluated in different in vitro assays: total antioxidant capacity, ferrous chelating, and hydroxyl and superoxide radical scavenging.

In the total antioxidant activity assay, all sulfated polysaccharides were capable of reducing Mo(VI) to form a green phosphate/Mo(V) complex (Fig. 3). However, CCB F0.3 and CCB F0.5 were significantly ($p<0.05$) more potent than CCB F1.0. The purification process did not increase the total antioxidant capacity of polysaccharides compared to the total antioxidant capacity of sulfated polysaccharide-rich extract from *C. cupressoides* (Costa et al. 2010). In a previous study, Chandini et al. (2008), using extracts from seaweeds *Padina tetrastomatica* and *Turbinaria conoides*, found 9.79 and 9.65 ascorbic acid equivalent, respectively, which was considered an elevated total antioxidant capacity. More recently, Camara et al. (2011) showed heterofucans with high total antioxidant capacity of approximately 20 ascorbic acid equivalent.

We also found that sulfated polysaccharides with a greater sulfate/sugar ratio (CCB-F0.3 and CCB-F0.5) were more potent than CCB-F1.0. Several studies have demonstrated that other algal sulfated polysaccharides also increased antioxidant activity in accordance with the degree of polymer sulfation (Qi et al. 2005; Wang et al. 2008). The ability of sulfated polysaccharides from *C. cupressoides* seaweed to scavenge hydroxyl and superoxide radicals was evaluated as described in "Materials and methods." Sulfated polysaccharides did not show antioxidant activity by the mechanism of scavenging hydroxyl and superoxide radicals until a concentration of 2.0 mg mL^{-1}.

Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions. It is known to generate ROS through the Fenton and Haber-Weiss reaction. Metal ion-chelating activity of an antioxidant molecule prevents oxyradical generation and consequent oxidative damage (Kumar et al. 2008). The activity of CCB-0.3 was independent of concentration, which showed dose-dependent chelating up to a concentration of 1.5 mg $mL^{-1}$and declined at 2.0 mg mL^{-1}. The most active compound was CCB-F1.0 with 44% of ferrous chelating at 2.0 mg mL^{-1} (Fig. 4). In addition, data illustrated that all polysaccharides from *C. cupressoides* showed moderate chelating ability on ferrous ions. Moderate chelating ability also has been recorded for several sulfated polysaccharides from seaweed, such as those extracted from brown alga *Undaria pinnitafida* (Hu et al. 2010) and *Laminaria japonica* (Wang et al. 2008) and from the green alga *U. pertusa* (Qi et al. 2005). The metal-

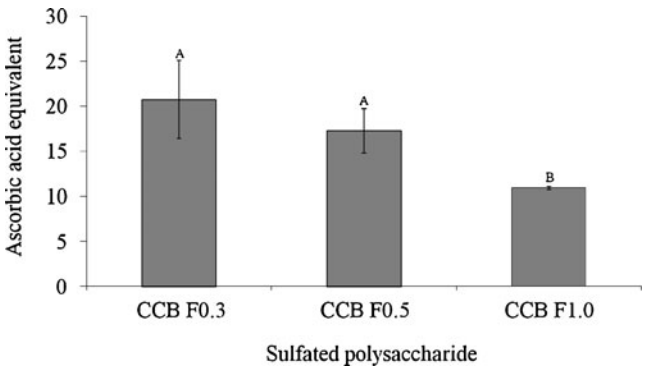

Fig. 3 Total antioxidant capacity of sulfated polysaccharides from *C. cupressoides*. The results are expressed as ascorbic acid equivalents (mg of ascorbic acid/g of polysaccharide). The data are the mean values of tree determinations ± standard deviation. **a**, **b** *Different letters* indicate a significant difference between sulfated polysaccharides ($p<0.05$)

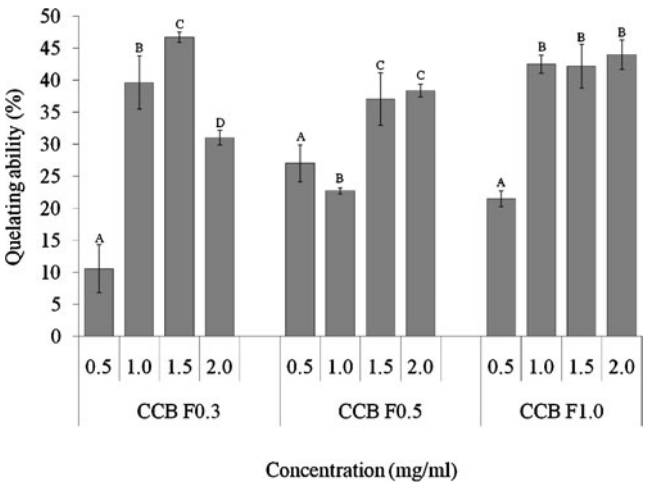

Fig. 4 Chelating effect of sulfated polysaccharides from *C. cupressoides* on ferrous ions. The data are the mean values of tree determinations ± SD. **a**, **b**, **c**, **d** *Different letters* indicate a significant difference between sulfated polysaccharides when compared at each concentration ($p<0.001$)

Table 2 Anticoagulant activity in aPTT test of sulfated polysaccharides isolated from *C. cupressoides*

Polysaccharide	aPTT (s)[a]			
	Concentration (μg/mL)			
	1.5	3.0	8.0	16.5
Clexane®[b]	$47.8\pm2.6_{AX}$	$65.2\pm1.7_{BX}$	$168.0\pm7.3_{CX}$	$>240_{DX}$
CCB F0.3	$66.7\pm0.6_{AX}$	$58.2\pm1.1_{BY}$	$160.9\pm1.9_{CX}$	$163.1\pm0.1_{DX}$
CCB F0.5	$56.8\pm3.3_{AX}$	$72.3\pm0.9_{BX}$	$206.7\pm6.1_{CY}$	$>240_{DX}$
CCB F1.0	$31.1\pm0.2_{AY}$	$31.0\pm0.6_{AY}$	$42.6\pm0.2_{BY}$	$79.5\pm2.1_{CY}$

The data are the mean values of tree determinations ± standard deviation. Different letters (A–D) indicate a significant difference between sulfated polysaccharides when compared at each concentration ($p<0.001$). Different letters (X, Y) indicate a significant difference between sulfated polysaccharides when compared with Clexane® at same concentration ($p<0.001$)

[a] *aPTT* activated partial thromboplastin time. For control sample without compound 30±3.3 s

[b] *Clexane*® low molecular weight heparin

chelating property of these polymers suggested that they might be applied in adsorption, metal ions separation or wastewater treatment, and antioxidant therapy. Furthermore, different from that observed for total antioxidant capacity, in the ferrous chelating test CCB-F1.0 (low sulfate/sugar ratio) was significantly ($p<0.001$) more potent than CCB-F0.5 (high sulfate/sugar ratio) at a concentration of 2.0 mg mL^{-1}. This indicated that ferrous chelating activity of sulfated polysaccharides from *C. cupressoides* depended on the spatial patterns of sulfate groups and is unlikely to be a mere charge density effect.

Anticoagulant activity Anticoagulant activity is a well-known effect of sulfated polysaccharides in general. The present study evaluated anticoagulant activity of sulfated polysaccharides from *C. cupressoides* by aPTT and PT tests, which assess the intrinsic and extrinsic pathways of coagulation, respectively.

Anticoagulant activity of the sulfated polysaccharides is depicted in Tables 2 and 3. In the aPTT test, all polysaccharides showed considerable dose-dependent activity (Table 2). A significant finding was the aPTT activity of polysaccharides CCB-F0.3 and CCB-F0.5, similar to that of Clexane®, a commercial low molecular weight heparin, at the same concentration (from 1.5 to 16.5 μg mL^{-1}). In the PT test, only CCB-F0.3 and CCB-F0.5 showed anticoagulant activity (Table 3). These data demonstrate that the anticoagulant activity sulfated polysaccharide-rich extract from *C. cupressoides* observed by Costa et al. (2010) was mainly due to the presence of CCB-F0.3 and CCB-F0.5 in that extract.

We found a positive relationship between the anticoagulant activity (aPTT and PT) and the sulfate/sugar ratio of sulfated polysaccharide from *C. cupressoides*. In the concentrations tested, anticoagulant activity decreased in the order of CCB-F0.5>CCB-F0.3>CCB-F1.0, the same order as the sulfate/sugar ratio in the samples (0.73, 0.63, and 0.23, respectively). Similar results were recorded with low molecular weight fucans (sulfated polysaccharides) from seaweed *Ascophyllum nodosum*, indicating that inhibitory fucan effects on coagulation were dependent on their sulfation degree (Haroun-Bouhedja et al. 2000).

In addition to the content of sulfate groups in polysaccharides, the positions of these groups in a monosaccharide residue also influenced anticoagulant activity (Mestechkina and Shcherbukhin 2010). Here, in contrast to CCB-F1.0, the more potent polysaccharides (CCB-F0.5 and CCB-F0.3) contained galactose-6-sulfate as observed in FT-IR, indicating this sulfated residue is important for anticoagulant activity of CCB-F0.5 and CCB-F0.3. In agreement with our results, Chaidedgumjorn et al. (2002)

Table 3 Anticoagulant activity in PT test of sulfated polysaccharides isolated from *C. cupressoides*

Polysaccharide	PT (s)[a]				
	Concentration (μg mL^{-1})				
	16.5	33.0	66.5	100.0	133.0
Heparin[b]	>120	>120	>120	>120	>120
CCB F0.3	$13.8\pm0.7_{A}$	$15.9\pm0.5_{A}$	$21.5\pm2.1_{B}$	$27.5\pm1.7_{C}$	$29.9\pm1.9_{C}$
CCB F0.5	$14.8\pm0.0_{A}$	$18.6\pm1.0_{B}$	$31.7\pm0.9_{C}$	$45.5\pm0.5_{D}$	$59.2\pm1.5_{E}$
CCB F1.0	$12.6\pm0.2_{A}$	$13.0\pm0.0_{A}$	$13.6\pm0.3_{A}$	$13.7\pm0.1_{A}$	$14.0\pm0.0_{A}$

The data are the mean values of tree determinations ± standard deviation. Different letters indicate a significant difference between sulfated polysaccharides when compared at each concentration ($p<0.001$)

[a] *PT* prothrombin time. For control sample without compound 13.2±0.2 s

[b] Heparin: unfractionated heparin

showed that polysaccharides specifically desulfated at position C6 lost their anticoagulant activity, whereas desulfation on C4 did not affect polysaccharide anticoagulant activity. This result suggests sulfated polysaccharides from *C. cupressoides* may be used in anticoagulant therapy.

Conclusions

We obtained four high molecular weight sulfated heteropolysaccharides from *C. cupressoides* composed mainly of galactose (except CCB-2.0). Sulfated polysaccharides CCB-F0.3, CCB-F0.5, and CCB-1.0 exhibited antioxidant activity (CAT test) and significant ferrous chelating ability. Moreover, CCB-F0.3 and CCB-F0.5 showed potent anticoagulant activity in PT and aPTT tests, directly proportional to the ratio of sulfate and sugar found in these polymers. With such strong concomitant antioxidant and anticoagulant activities, CCB-0.3 and CCB-0.5 were identified as potential multipotent drugs. Further work using NMR spectroscopy and MS-based methylation analysis will help us to understand of the complete structure of the polysaccharides, including configuration of glycosidic linkages, position of glycosidic linkages, and pattern of O-sulfation, which will certainly present a good opportunity to elucidate the biological roles of polysaccharides and develop potential anticoagulant and antioxidant drugs based on the three-dimensional structures.

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