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BIOMEDICAL SCIENCES

Partial characterization and anticoagulant activity of sulfated galactan from the green seaweed *Halimeda opuntia*

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Abstract: The number of deaths associated with cardiovascular diseases (CVD) increases every year, leading to an intense search for new compounds that may be employed as anticoagulants. One of the classes of bioprospected molecules comprises sulfated polysaccharides (SP) from seaweed, as heparin displays many adverse effects associated with its use. The present study aimed to characterize and evaluate the anticoagulant potential of SP extracted from the green algae *Halimeda opuntia*. Four PS-rich fractions, F23, F44, F60 and F75, were obtained by proteolytic digestion in papain followed by ethanol precipitation. The presence of SP was confirmed by agarose gel electrophoresis, revealing different populations in each fraction. The F44 fraction is noteworthy compared to the other fractions, presenting a 5% yield compared to the initial algae weight and anticoagulant activity revealed by the activated partial thromboplastin time (APTT) assay (intrinsic/common coagulation pathway). Surprisingly, F44 purification (SP peak P1F44) resulted in prothrombin time (PT) activity (extrinsic coagulation pathway) at a 160 µg/mL, in addition to enhanced APTT activity. The P1F44 anticoagulant activity mechanism was shown to be dependent on two coagulations factors, IIa and Xa, more potent via IIa. Future assessments will be performed to assess this fraction in the medical clinic.

Key words: Marine algae, Sulfated polysaccharides, Blood clotting, Heparin-like, Factor IIa.

INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases (CVD) are currently the leading cause of death worldwide. About 17.9 million people lose their lives each year due to CVD, representing 31% of all global deaths, with a third of these deaths befalling people under 70 years old (WHO 2017). CVDs include coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions generally associated with thromboembolic events due to the formation of thrombi in the circulatory system. Because of this, CVD diagnosis and treatment employing anticoagulant therapy is essential for people presenting CVD or at high cardiovascular risk (Mourão 2015, WHO 2017).

The use of anticoagulant agents began with the discovery of unfractionated heparin (UFH), a sulfated polysaccharide (SP) belonging to the glycosaminoglycan (GAGs) family. This polymer, composed mainly of L-iduronic-2-O-sulfated acid and D-glucosamine-N-sulfated, displays several advantages due to its specificity and the fact that is does not cause anaphylaxis, and is considered one of the most important advances for the development of cardiac

surgery (Olson et al. 1992, Sommers et al. 2011), followed by the discovery of hydroxycoumarin (warfarin). However, despite their effectiveness, both exhibit a series of limitations and adverse effects that restrict their clinical use (Alban 2005, White 2006, Pan et al. 2010, Mourão 2015). Because of this, low molecular weight heparin (LMWH) was developed in the 1980s, with the aim of reducing such limitations, and is still employed today. In fact, LMWH has been shown to be a safe and effective drug that does not require laboratory monitoring, with a longer half-life and predictable response. However, its administration, as well as that of UFH, remains parenteral, also presenting thrombocytopenia risks (Sattari & Lowelthal 2011, Van Der Wall et al. 2017).

The development of semi-synthetic compounds, such as LMWH and pentasaccharides (fondaparinux), in recent decades has triggered the search for the ideal anticoagulant. In this regard, the need for equally effective, easy to use oral anticoagulants displaying better safety profiles has led to the development of novel molecules with mechanisms of action based on direct thrombin or factor Xa inhibition, such as dabigratan, rixoxaban, prasugrel and ticagrelor (Flato et al. 2010, Yoshida et al. 2011, Lorga Filho et al. 2013). However, scientific data have reported adverse reactions in several patients, often leading to treatment discontinuation, as well as high costs (Sattari & Lowelthal 2011, Afonso et al. 2016, Van Der Wall et al. 2017). Currently, although heparin remains the ideal model, numerous substances are at different development stages, aimed at obtaining fully synthetic compounds from mimetic oligosaccharides and developing orally active heparin formulations (Correia-da-Silva et al. 2013, Afonso et al. 2016). In this regard, many research groups have begun prospecting and investigating biologically active molecules with anticoagulant potential that can function as substitutive or alternative therapies to heparin in certain clinical indications (Melo et al. 2012, Sujian et al. 2019). Thus, SP constitute a complex group of macromolecules that have aroused great interest in the scientific community, mainly due to the fact that these compounds naturally display a polyanionic character capable of interacting with important functional proteins (Pomin & Mourão 2014, Adrien et al. 2019), found in microorganisms (Kusaykin et al. 2006), animals (both vertebrates and invertebrates) (Nader et al. 2004), seaweed (Costa et al. 2010) and some higher aquatic plants (Aquino et al. 2005, Dantas-santos et al. 2012).

Marine algae are the main source of SP, which are often found in the extracellular matrix of these organisms, containing many active substances and metabolites (Silva & Neto 2009). SP are complex constituents, and their structure varies between different algae species, both concerning the type of constituent sugar and the position of the glycosidic bond and sulfation site, an important factor that determines their specific biological functions (Hu et al. 2012, Mourão 2015). Furthermore, seaweed use increases every year (Calumpong et al. 2017, Ferdouse et al. 2018), with the extraction of SP exhibiting numerous biological activities, i.e., anticoagulant (Anderson et al. 1976, Pereira et al. 1999, 2002, 2005, Mourão & Pereira 2000), antithrombotic (Mourão & Pereira 2000, Mourão 2004), antitumoral (Soeda et al. 1994), antiproliferative (Costa et al. 2010), antiviral (Damonte et al. 1994), antioxidant (Zhang et al. 2003), antiinflammatory (Berteau & Mulloy 2003) and antinociceptive (Vieira et al. 2004) properties.

In this context, bioprospecting is undoubtedly of paramount importance for the specific knowledge of the chemical and pharmacological potential of marine natural products, especially concerning green algae, as

only 1.3% of all compounds described from 1963 to 2013 belong to this phylum (Chlorophyta), against 7.3% extracted from Rodophyta (Blunt et al. 2015). In this regard, the green macroalgae *Halimeda opuntia* is noteworthy, as it is abundant throughout the Brazilian coast and easily cultivated, and scarce information is available concerning its anticoagulant activity. Although one study suggesting the structure of *H. opuntia* SP and anticoagulant activity has been recently published (Arata et al. 2015), the extraction technique differs from the one proposed herein. Additionally, selective precipitation and purification methods were not carried out, leading to different results.

Therefore, this study aimed to extract and characterize SP from the seaweed *H. opuntia* and evaluate its anticoagulant activity concerning the intrinsic and/or common pathway and the extrinsic pathway, through the activated partial thromboplastin time (APTT) and prothrombin time (PT) assays, respectively, as well as the specific coagulation system antithrombin-dependent pathway (anti-Xa and anti-IIa).

MATERIALS AND METHODS

SP extraction and precipitation

Halimeda opuntia specimens were obtained at Cumuxuratiba beach (17˚ 6` 57.841" S and 39˚ 10` 18.779" O), in the Prado municipality (BA, Brazil), separated from other species, washed with distilled water and dried at room temperature. Subsequently, the crushed seaweed (3.5 g) was immersed and maintained in acetone for 24 h for delipidation and depigmentation. The collected sediment was dried at 60°C, suspended in an extraction buffer (0.1 M sodium acetate, 5.0 mM ethylenediamine tetraacetic acid (EDTA) and 5.0 mM cysteine at pH 6.0 with the addition of papain) and incubated at 60 °C for 24 h under agitation (200 rpm). The incubation mixture was then centrifuged (2500 x g, 20 min) at room temperature and the supernatant separated. The residue was resuspended in the same extraction buffer until the absence of SP in the supernatant, verified by the metachromatic property in dimethylethylene blue (DMB - Sigma-Aldrich) at A_{525nm}. Positive supernatants were combined (1,2 L) and termed the crude extract (CE), which was then fractionated by precipitation in increasing ethanol concentrations (30, 80, 150 and 300%). Based on this methodology, final ethanol concentrations were considered as 23, 44, 60 and 75%, respectively. Subsequently, ethanol volumes were added to the solutions, which were maintained at 4 °C for 12 h. The precipitates were then separated from the solutions by centrifugation (10,000 x g, 10 min), dialyzed exhaustively against distilled, the SP were concentrated by freeze-drying, weighed and stored for further analysis. This procedure was repeated with increasing ethanol volumes up to the final concentration in a 75% solution. Each fraction obtained by precipitation was renamed using the number corresponding to the final applied ethanol concentration, namely F23 (25 mg), F44 (172 mg), F60 (566 mg) and F75 (302 mg). Yields were calculated compared to the initial seaweed weight (3.5 g).

Agarose gel electrophoresis

An initial SP analysis was performed by agarose gel electrophoresis. The obtained fractions were applied to a 0.5% agarose gel for 1 h at 110 V in 1,3-diaminopropane-acetate 0.05 M (pH 9.0). The SP were then fixed using a 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution. After 12 h, the gel was dehydrated and stained with 0.1% toluidine blue in 0.1:5:5 (v/v) acetic acid-ethanol water (Dietrich & Dietrich 1976). Chondroitin sulfate (Sigma-Aldrich) and bovine heparin (200.5 IU mg^{-1} - National Institute of Standards

and Biological Control/INPCB) were used as standards.

In vitro coagulation assays

To analyze the anticoagulant activity of each fraction, human plasma was collected in a 3.8% sodium citrate solution at a 9:1 ratio (Anderson et al. 1976) and analyzed by the activated partial thromboplastin time (APTT) and prothrombin time (PT) assays. Both tests were carried out using commercial kits under experimental conditions recommended by the manufacturer (Labtest, São Paulo, SP, Brazil). A curve was performed using 100 µl volume of plasma plus sample, varying the volumes of SP samples by 2, 4, 6, 8 and 10 µ l. Clotting times were determined using a microcoagulometer (Amelug, model KC4A). The concentration of SP was calculated considering the final volume of the entire assay, being 300 µl in aPTT and 200 µl in PT. All assays were performed in triplicate and the results were compared to an unfractionated heparin (UFH) control (Melo et al. 2008).

SP purification

The F44 fraction (172 mg) was purified by ion exchange chromatography employing a Diethylaminoethyl (DEAE)-cellulose (BioRad) column. The column was equilibrated and washed with an equilibration buffer (20mM Tris-hydrochloric acid (HCl), 50 mM EDTA pH 7.4) and the SP were eluted under a linear sodium chloride (NaCl) gradient from 0 to 4.0 M, solubilized in an equilibrium buffer, at a 1 mL/min flow, and 2 mL fractions were collected and monitored at A_{525nm} by means of the metachromatic property with DMB (Farndale et al. 1986) and at A_{490nm} to monitor the presence of hexoses by the phenol/sulfuric acid analysis. The obtained SP fractions were pooled, dialyzed exhaustively against distilled water and filtered through 3 kDa Amicon filters with Milli-Q water until complete absence of salts (monitored with $AgNO_3$). Finally, the SP were concentrated by freeze-drying, weighed (20 mg) and stored at -20°C. Yields were calculated compared to the initial fraction weight (Farndale et al. 1986).

Enzyme inhibition assays

Purified samples exhibiting anticoagulant potential detected by the APTT and TP assays were incubated in 96-well plates with 40 µL of a 20 mM Tris-HCl solution containing 150 mM NaCl and 0.1% polyethylene glycol (PEG) 8000, pH 7.5, AT (10 nM) and factor Xa (2 nM) or thrombin (2 nM) for 1 min at 37°C. Factor Xa and thrombin activities were determined by adding the chromogenic substrates S-2765 or S-2238 (100 µM), respectively. Kinetic test was performed and Substrate hydrolysis was detected (A_{405nm}), accompanied by 5 minutes, using a microplate reader and the absorbance change rates were proportional to the residual thrombin or factor Xa activity remaining in the incubation solution (Glauser et al. 2009).

Chemical characterization

Sulfate content was determined according to the gelatin-barium method (Dodgson & Price 1962) employing a standard sodium sulfate (1 µg/µL). The Bradford method (1976) was performed for total protein determination using bovine albumin as standard (1 µg/µL). Uronic acid content was quantified according to Dische (1947) in the presence of H_2SO_4 and borate, using glucuronolactone (0.1 µg/µL) as the standard. In all dosages, calibration curves were used with determined volumes of the standards.

Structural assessment

Infra-red analyses

About 0.1 mg of the purified and freeze-dried samples were added to 1 mg of potassium

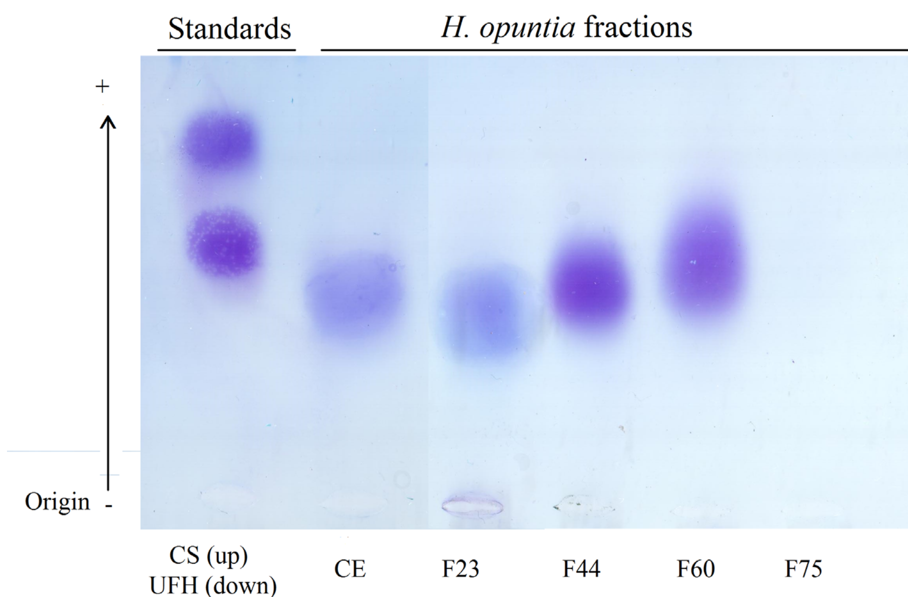

Figure 1. **Agarose gel electrophoresis of ethanol-precipitated SP fractions (F23, F44, F60 and F75) obtained from *H. opuntia*. UFH – Unfractionated Heparin; CS – Chondroitin sulfate; CE – Crude extract.**

bromide and crushed until obtaining a homogeneous mixture. Next, pellets were prepared applying a force of 15 tons using a hydraulic press, which were then submitted to infrared analyses. Infrared spectra were recorded employing a Perkin Elmer Spectrum 65 FT-IR spectrophotometer between 400 and 4000 cm^{-1}. Measurements were performed under a 4 cm^{-1} scanning resolution and normal environmental conditions (Chopin & Whalen 1993).

Monosaccharide composition analyses

Initially, 5 mg of purified SP were hydrolyzed in the presence of trifluoroacetic acid at a final concentration of 5 M for 4 hours at 100 °C. After cleavage, the sugars were reduced with sodium borohydride and the produced alditols acetylated with acetic anhydride:pyridine (1:1, v/v). The acetylated alditols were then dissolved in chloroform and analyzed by gas chromatography (GC MS-QP2010 Shimadzu, Japan) using a Restek RTX-5MS column. The initial run temperature was 110 °C and the final one, 250 °C, at an increasing rate of 2 °C/min (Kircher 1960).

Statistical analyses

Results were expressed as the means ± standard error of the means (SEM) of an indicated number of experiments. Results were analyzed by the Student t test using the OriginPro 8 software. Statistical significance was set as $p<0.05$ and only curves with $R > 0.98$ were considered for the calculation purposes at the chemical dosages mentioned above.

RESULTS AND DISCUSSION

H. opuntia SP extraction and characterization

SP were extracted by enzymatic treatment and the obtained extracts following proteolysis were grouped (crude extract - CE) and precipitated with increasing and cumulative ethanol concentrations (23%, 44%, 60% and 75%). Sufficient amounts of SP were not detected in the fraction precipitated with 9%. Thus, only four fractions were considered, F23, F44, F60 and F75.

After the initial procedures, the seaweed material was freeze-dried and weighed. The dry weight obtained for each fraction was compared to the initial seaweed weight, allowing for SP

yield calculations for each fraction. Fraction F60 exhibited the highest yield, at 16% of the total extracted mass, while the F23 fraction yield was less than 1%. Fractions F44 and F75 presented 5 and 8.5% yields, respectively. Several SP extraction methodologies are available, such as the use of hot and/or cold aqueous solutions (Adrien et al. 2017) under controlled acidic conditions and enzymatic extraction employing different proteolytic enzymes displaying nonspecific action, in addition to the papain used herein (Farias et al. 2000, 2008, Melo et al. 2012). Each experimental protocol is capable of extracting compounds displaying different properties, chemical compositions, and yields. For example, Arata and collaborators extracted SP from *H. opuntia* using two different methods: aqueous solutions at room temperature and controlled acidic conditions. Total SP yields were low for both procedures, at 0.04% and 0.22%, respectively (Arata et al. 2015). On the other hand, the yield obtained by proteolytic digestion employing papain was more efficient, at 30.5%.

Concerning the initial analysis of the sulfated constituents, each fraction was subjected to agarose gel electrophoresis and later stained with toluidine blue, allowing for the identification of SP in F23, F44 and F60. On the other hand, the F75 fraction, even when applied in large amounts, did not present the characteristic SP violet band in this electrophoresis system. The SP fractions extracted from *H. opuntia* presented different electrophoretic mobility patterns, although all bands were homogeneous and co-migrated close or slower than the heparin standard. The agarose gel electrophoresis initially suggested that SP with higher negative charges (high sulfation content) tend to display greater mobility, although the diaminopropane buffer present in the gel can interact with the sulfate groups of these molecules, resulting in negative charge neutralization, conformationally altering sulfate structure and exposure and, consequently, reducing molecule mobility. Therefore, this technique has been widely used in the study of carbohydrates, as it is capable of show marked differences in charge density between isolated SP fractions, which is why it is used in the identification of these molecules, allowing the preliminary analysis of extracted SP, indicating that different electrophoretic mobilities are associated to different structures. This would also explain why the F75 fraction is not visualized in this type of electrophoresis (Dietrich & Dietrich 1976, Rodrigues et al. 2011, Melo et al. 2012, Barbosa et al. 2019).

The anticoagulant activities of the four extracted fractions were tested in the activated partial thromboplastin time (APTT) and prothrombin time (PT) *in vitro* coagulation assays. These assays are used in clinical laboratory settings to determine the absence and/or functioning of coagulation factors present in the intrinsic and extrinsic (and/or common) pathways in the classic blood coagulation system model, respectively. Fractions F60 and F75 did not exhibit significant anticoagulant activity at any assessed concentration in the APTT assay (Figure 2a), while fractions F23 and F44, on the other hand, displayed similar anticoagulant effects, prolonging plasma clotting time by 300 seconds, at about 133 µg/mL using 1 µg/mL of unfractionated heparin (UFH) as a positive control. None of the precipitated fractions obtained from *H. opuntia* exhibited activity in the PT assay (Figure 2b).

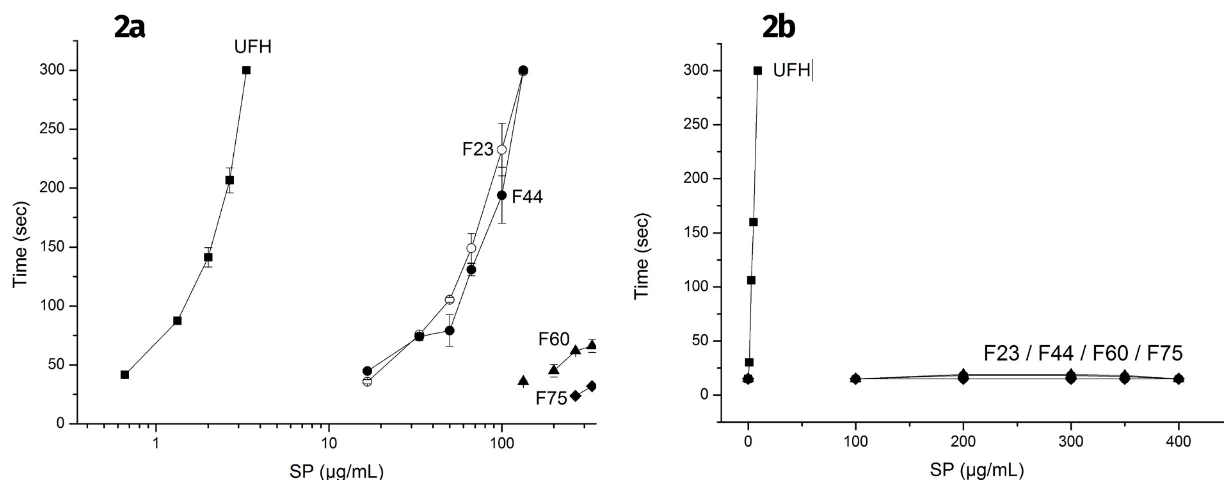

Figure 2. Anticoagulant activity of the precipitated SP fractions (F23, F44, F60 and F75) obtained from *H. opuntia* revealed by the APTT (2a) and PT (2b) assays. UFH – Unfractionated Heparin.

Purification of the F44 fraction obtained from H. opuntia and *in vitro* anticoagulant activity

SP purification of the precipitated F44 fraction and electrophoretic analysis

Although two fractions (F23 and F44) exhibited anticoagulant activity in the APTT assay, the F23 fraction resulted in a very low yield, as mentioned previously. Thus, only the F44 fraction was further investigated. The fraction was first subjected to ion exchange chromatography purification employing DEAE-cellulose under a linear NaCl gradient (0 to 4 M). The F44 fraction eluted in a single metachromatic peak (A_{525nm}), termed P1F44 (Figure 3a). Furthermore, the presence of a neutral polysaccharide was observed by the exclusively positive reading noted at A_{490nm}, by phenol/sulfuric acid analysis for total sugar, where a first major peak (LF44) was observed prior the start of the NaCl gradient, and a second peak was noted coinciding with the metachromatic peak, associated to SP (P1F44). The LF44 was discarded and the P1F44 fraction was subsequently dialyzed exhaustively against distilled water, filtered with Milli-Q water, freeze-dried and weighed, resulting in a 12% yield. This fraction was then submitted to agarose gel electrophoresis (Figure 3b), where an electrophoretic mobility pattern identical to the parent fraction was observed.

In vitro anticoagulant activity in the APTT, PT and enzyme inhibition assays

The *in vitro* anticoagulant activity of the purified P1F44 fraction was reassessed by the APTT and PT assays. This fraction prolonged the clotting time in both *in vitro* assays (Figure 4). The P1F44 fraction doubled its activity as revealed by the APTT assay when compared to the precipitated fraction (F44), prolonging clotting time by 300 seconds at about 66 µg/mL of material, with an activity only 20-fold lower than the UFH. This behavior was expected, as the purification step is able to remove contaminants that do not display anticoagulant activity and, consequently, concentrate sample SP, increasing specific activity. It is important to highlight that sulfate presence and molecule position are extremely important for their anticoagulant activity. Furthermore, several studies concerning SP have demonstrated that their activities are also associated to their structural variability, given the wide diversification of their monosaccharide constitutions and types of glycosidic bonds,

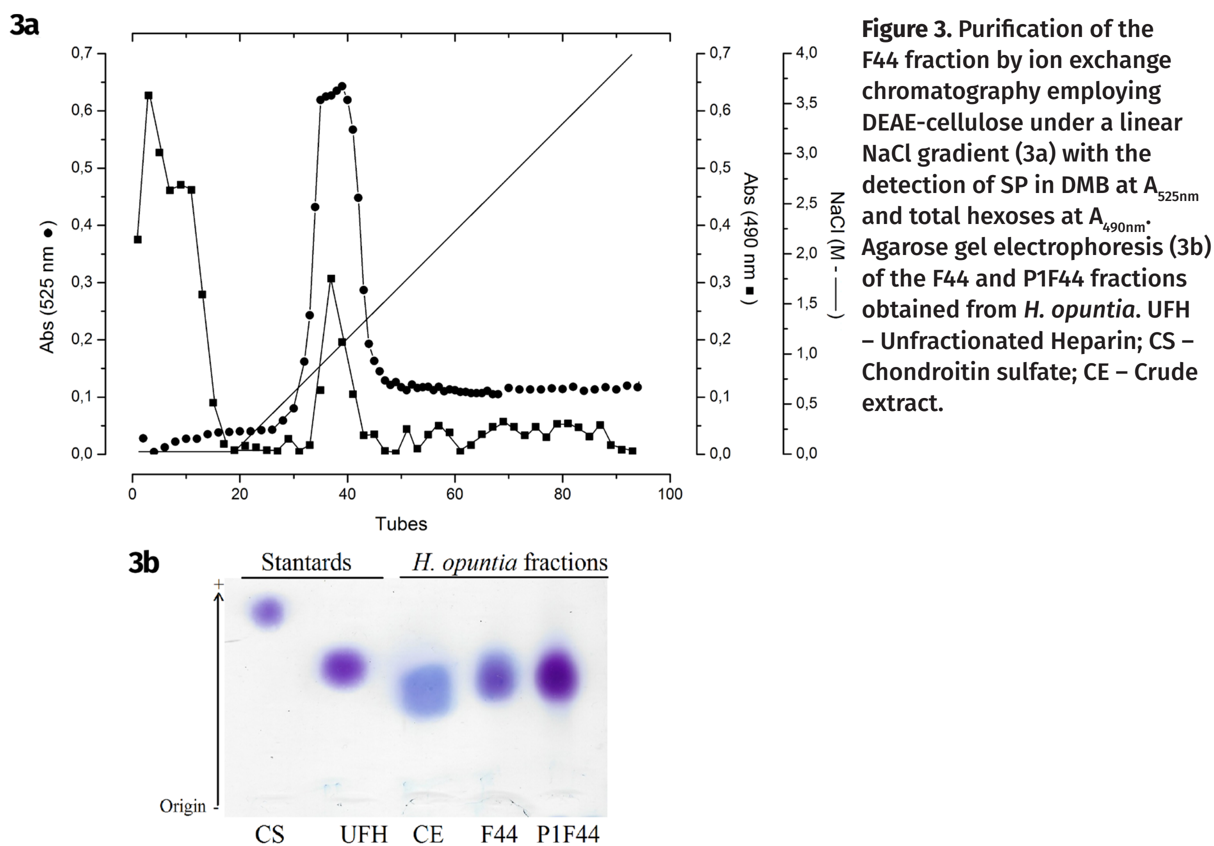

Figure 3. Purification of the F44 fraction by ion exchange chromatography employing DEAE-cellulose under a linear NaCl gradient (3a) with the detection of SP in DMB at A_{525nm} and total hexoses at A_{490nm}. Agarose gel electrophoresis (3b) of the F44 and P1F44 fractions obtained from *H. opuntia*. UFH – Unfractionated Heparin; CS – Chondroitin sulfate; CE – Crude extract.

resulting in unique structural SP conformations with different patterns and anticoagulant potentials (Mendes et al. 2009, Fonseca et al. 2010).

In addition, this fraction was also able to prolong the clotting time by 300 seconds at a concentration of 400 μg/mL via the PT assay, which is not common, as PS usually exhibit activity in only one pathway, intrinsic or extrinsic (Yang et al. 2002, Yoon et al. 2002, 2007, Ronghua et al. 2003, Zhang et al. 2008, Medeiros et al. 2008, Silva et al. 2010, Dore et al. 2013).

Figure 4 indicates the anticoagulant activities of several green algae SP as revealed by the APTT and PT assays. In general, the P1F44 fraction exhibits promising activity, since, in addition to activity detected via the APTT assay when compared to other algae, it also exhibits activity via the PT assay, while dual activity was noted only for *Caulerpa cupressoides*. It is important to note that the *H. opuntia* fraction assessed herein displayed better results than those reported by Arata et al. (2015) as, in addition to being able to double the APTT assay results with an amount 6.5-fold lower than *H. opuntia*, it was also able to double the PT assay results, which was not reported by Arata et al. (Table I). This is probably due to the differences regarding the SP selective alcoholic extraction and precipitation processes applied herein. The algae *Monostroma nitidum*, *Monostroma latissinum* and *Codium cylindricum* exhibited better activity via the APTT assay, but no activity was observed via the PT assay at the concentrations evaluated, while it was possible to observe activity at the concentration of 400 μg/mL of the P1F44 fraction.

If, on the one hand, activity via the APTT assay is common, on the other hand, activity via the PT assay is unusual and is rarely reported in the literature. The double positive activity

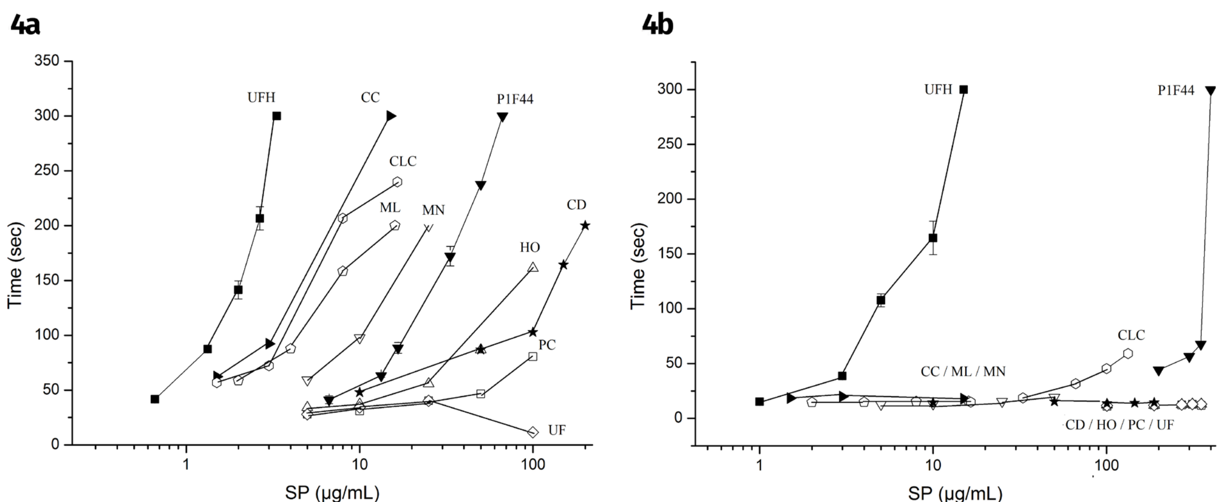

Figure 4. **Anticoagulant activity revealed by the APTT (4a) and PT (4b) assays for the purified SP fraction (P1F44) from *H. opuntia* compared to the fractions obtained by Arata et al. (2015) (HO, PC, UF) and other authors. Caption: UFH – Unfractionated Heparin; HO – *Halimeda opuntia*; PC – *Penicillus capitatus*; UF – *Udotea flabellum;* MN – *Monostroma nitidum*; ML – *Monostroma latissinum*; CC – *Codium cylindricum*; CLC – *Caulerpa cupressoides*; CD – *Codium divaricatum*.**

Table I. **Comparison between the activities of different SP in the APTT and PT assays.**

Algae	APTT*	PT*	Reference
***Halimeda opuntia* (P1F44)**	15 mg/mL	160 mg/mL	This study
***Halimeda opuntia* (HO)**	100 mg/mL	N.A.	Arata et al. 2015
***Penicillus capitatus* (PC)**	50 mg/mL	N.A.	
***Udotea flabellum* (UF)**	S.A.	N.A.	
***Monostroma nitidum* (MN)**	8 mg/mL	N.A.	Cao et al. 2019
***Monostroma latissinum* (ML)**	3.5 mg/mL	N.A.	Li et al. 2011
***Codium cylindricum* (CC)**	2.5 mg/mL	N.A.	Matsubara et al. 2001
***Caulerpa cupressoides* (CLC)**	3 mg/mL	66,5 mg/mL	Rocha et al. 2012
***Codium divaricatum* (CD)**	45 mg/mL	N.A.	Li et al. 2015

***Mean SP concentration required to double the control times in the ATPP and PT assays considering a mean control time of 35 seconds and 15 seconds, respectively. Caption: N.A. – no activity.**

of the investigated fraction (aPTT and PT) is, thus, extraordinary, although some reports have indicated that SP extracted from the brown algae *Ecklonia kurome* (Nishino et al. 1991), the green algae *Codium dwarkense* (Siddhanta et al. 1999) and the fucoidan obtained from the brown seaweed *Fucus vesiculosus* (Azevedo et al. 2009) exhibit dual activities.

To explore the anticoagulant mechanism of action, P1F44 was tested alongside factors isolated from the blood coagulation system. The results indicate that the P1F44 fraction interfered with the coagulation system by inhibiting thrombin (IIa) (Figure 5a) and factor Xa (Figure 5b) activities, both mediated by antithrombin (AT).

Table II displays the mean inhibitory concentration (EC_{50}) of SP extracted from the red algae *Botryocladia occidentalis* and *Gellidium crinale* and from the sea urchins *Strongylocentrotus purpuratus* I, *Strongylocentrotus purpuratus* II and *Arbacia lixula* compared to the P1F44 fraction from *H. opuntia* with the reference drug (heparin) in relation to factors IIa and Xa in the presence of AT. All the listed SP exhibited lower EC_{50} in tests in which their anticoagulant potential via IIa/AT was compared to Xa/AT assays.

There is growing scientific evidence that suggests different interactions between SP and specific coagulation system proteins based on their different structural and sulfation patterns, forming a particular complex between the plasma inhibitor and the target protease. Some SP from marine organisms are capable of anticoagulant activity independent of interactions with coagulation proteins, as in the case of the sulfated galactan present in the seaweed *Botryocladia occidenalis*, which display activity through serpin-dependent and independent mechanisms. This SP exhibited excellent results in the *in vitro* APTT and PT coagulation assays and in enzymatic assays performed with purified proteases and serpins, displaying anti-factor

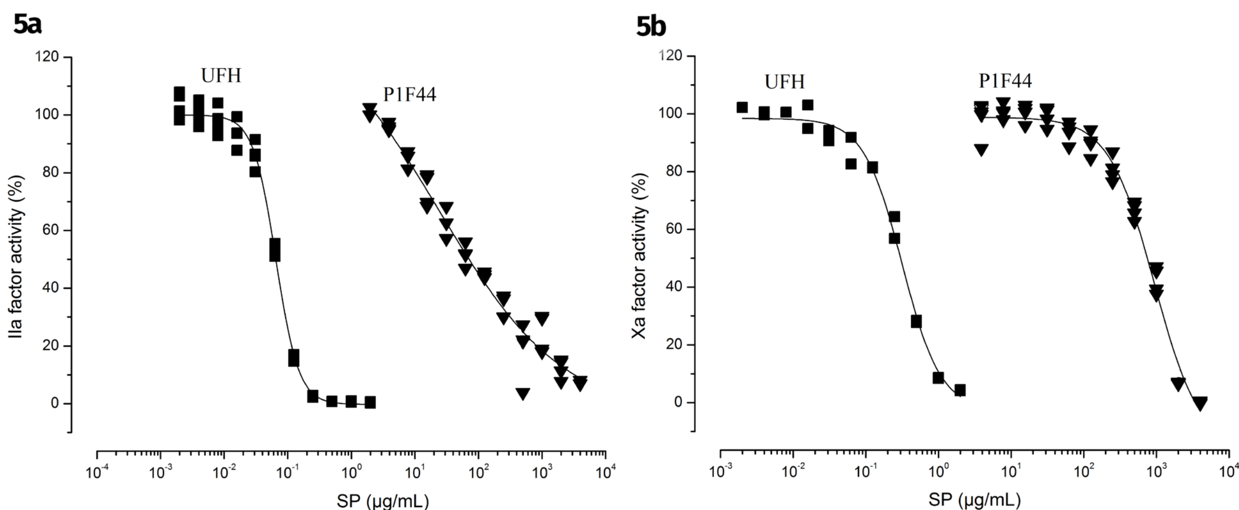

Figure 5. Inhibition of coagulation factors IIa (a) and Xa (b) by the P1F44 fraction obtained from *H. opuntia* in the presence of antithrombin. UFH – Unfractionated Heparin.

Table II. Inhibitory concentration of the P1F44 fraction obtained from *H. opuntia* compared to other polysaccharides of marine origin and to the heparin standard.

Polysaccharides	EC_{50} (µg/mL)	
	IIa/AT	Xa/AT
P1F44	67.6	>500
SG from *Botryocladia occidentalis*	0.02	2.5
SG from *Gelidium crinale*	0.02	1.5
SF from *Strongylocentrotus purpuratus I*	0.3	2
SF from *Strongylocentrotus purpuratus II*	0.9	N.D.
SF from *Arbacia lixula*	150	>500
UFH	0.061	0.31

SG – Sulphated galactan; SF – Sulphated Fucan; UFH – Unfractioned heparin. Adapted from Pomin & Mourão (2014).

IIa and anti-factor Xa activities mediated by AT. In addition to the examples previously cited in Table I, one SP isolated from the green alga *Codium cylindricum* by Matsubara et al. (2001) also exhibited anticoagulant activity with a direct thrombin inhibitory mechanism, independent of the presence of AT and heparin cofactor II (Matsubara et al. 2001, Rodrigues et al. 2013). This possible multiplicity of anticoagulant action mechanisms should be better investigated to further understand the biotechnological potential of this class of molecule.

Chemical characterization of SP obtained from H. opuntia

The chemical characterizations of the F44 and P1F44 fractions as presented in Table III. The sulfate percentages in each fraction were 8.9% and 26%, respectively. The purification process may have removed sample interferences, generating better composition determinations. Sulfate contents, on the other hand, were higher than in the fraction reported by Arata et al. (2015) of 21.7%. This difference corroborates the data obtained in the coagulation tests and shows the importance of the presence of sulfates for the anticoagulant activity of SP, hence the difference also observed for *in vitro* activity (ATTP and PT) between the SP obtained herein and by Arata et al. (2015). The uronic acid content detected herein (1,6%) was low when compared to other green algae, such as *Ulva rigid* (30%) and *Caulerpa cupressoides* (7.18%) (Rodrigues et al. 2013, Adrien et al. 2019). No protein content was detected, indicating the efficiency of the applied extraction method.

The monosaccharide composition analysis of the P1F44 fraction revealed the presence of mostly galactose (77.5%), followed by mannose (21.1%) and glucose (1.4%). Arata et al. (2015) found the same constituent monosaccharides, with similar galactose contents (77.2%) and different mannose (9.8%) and glucose (6.1%) contents, also detecting the presence of ramnose (1%), fucose (1.3%), arabinose (2.3%) and xylose (2.1%). The results reported herein corroborate literature data, demonstrating that green algae exhibit more heterogeneous SP, usually rich in galactose, mannose, xylose, arabinose and glucose and/or uronic acids (Chevolot et al. 2001).

Finally, an infrared (IR) spectroscopy assay was performed to analyze the primary structural characteristics of the SP present in the P1F44 fraction (Figure 6). The findings indicate characteristic carbohydrate bands, such as at 1250 cm^{-1}, confirming the bond between monomers due to typical C—O—C bond stretching. The most prominent band was observed between 3500 - 3300 cm^{-1}, corresponding to O—H bond stretching characteristic of monosaccharides.

Characteristic signs of sulfate grouping were also observed, such as S=O bond stretching between 1050 - 950 cm^{-1}, C-O-S bond stretching at 850 cm^{-1} and C-O bond stretching at 600 cm^{-1}, as exhibited in Table IV (Abreu 1997, Silverstein et al. 2006, Wu et al. 2013). Bands referring to C—H bond stretching around 2900 cm^{-1} and COO- or OH around 1650 cm^{-1} were also observed, the latter associated with the carboxylic acid group of uronic acid, confirming the data obtained in the chemical uronic acid analysis reported in Table I (Abreu 1997, Silverstein et al. 2006, Wu et al. 2013). The small band present at 1400 cm^{-1} corresponds to the carboxyl group belonging to pyruvic acid (Estevez et al. 2009), thus confirming the data reported by Arata et al (2015), who describe the composition of SP present in *H. opuntia* as a sulphated pyruvated galactan.

Table III. Chemical characterization of SP from obtained from *H.opuntia.*

Fraction	Uronic acid (%)	Sulfate (%)	Proteins (%)	Monosaccharide composition (%)		
				Glu	Man	Gal
F44	1.6	8.9	ND	NT	NT	NT
P1F44	<1	26	ND	1.4	21.1	77.5
H.O.	NT	21.7	NT	6.1	9.8	77.2

H.O – *Halimeda opuntia* obtained by Arata et al. (2015); ND – Not detected; NT – Not tested; Glu – Glucose; Man - Mannose; Gal – Galactose.

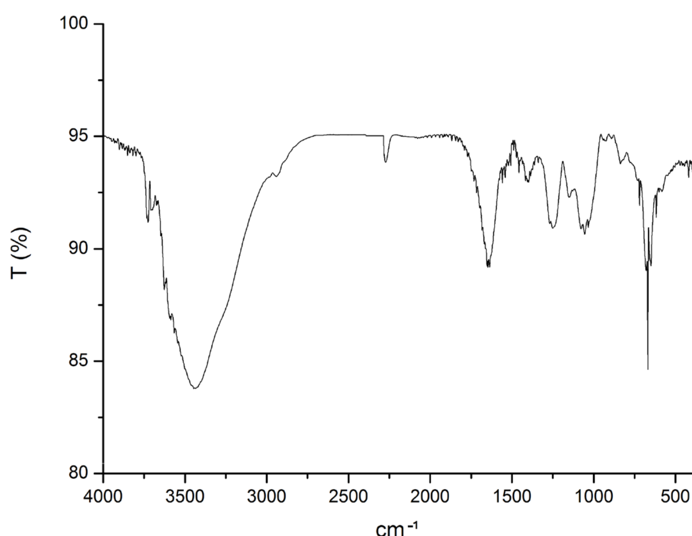

Figure 6. Infrared spectrum of the P1F44 fraction extracted from *H. opuntia.*

Table IV. Infrared spectrum attributions of the SP obtained from *H. opuntia.*

Bands	Wavelength (cm^{-1})	Attributions
1	3500 – 3300	OH bond stretching
2	2900	CH bond stretching
3	1650	COO- or OH stretching in uronic acid
4	1400	C=O stretching in pyruvic acid
5	1250	C—O—C bond stretching
6	1050 - 950	S=O stretching
7	850	C—O—S bond stretching
8	600	C-S bond stretching

CONCLUSIONS

A proteolytic extraction methodology followed by selective alcohol precipitation was effective in the extraction of four distinct and SP-rich fractions obtained from the green algae *H. opuntia*, two of which (F23 and F44) were active in the APTT (intrinsic coagulation system pathway) assay. F44 purification (P1F44) increased anticoagulant activity compared to the precipitated fraction (F44), doubling its intrinsic pathway action and also indicating extrinsic pathway activity (PT). The anticoagulant activity mechanism presented by P1F44 was shown to be dependent on both IIa and Xa factors, and more potent via IIa mediated by AT.

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BBC and JLDG contributed running the laboratory work, analysis of the data and drafted the paper. TAM and BFG contributed to biological studies. ARS contributed in collecting algae sample, in identification and herbarium confection. CDN, CMB and PASM contributed to critical reading of the manuscript. LPC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.