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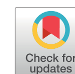
Influence of sulfated polysaccharides from *Ulva lactuca* L. upon Xa and IIa coagulation factors and on venous blood clot formation



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A R T I C L E I N F O

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A B S T R A C T

The interest in bioactive compounds from natural sources, such as marine organisms, has increased considerably in recent years. Among these compounds, sulfated polysaccharides from seaweed exhibit a broad spectrum of biological activities. Sulfated polysaccharides from green algae are still poorly investigated. For this reason, in this study, using unusual methodologies, such as extraction conditions, FACE, and RAMAN, we investigated the structural features of F50UL and F70UL sulfated polysaccharides from *Ulva lactuca* L. and their distinct in vitro anticoagulant and in vivo antithrombotic activities. Sulfated polysaccharides of *U. lactuca* were obtained by enzymatic proteolysis with ALCALASE® and fractionated by acetone precipitation. F50Ul and F70Ul sulfated polysaccharides with higher yield were partially chemically characterized by Fluorophore-assisted carbohydrate electrophoresis (FACE) and RAMAN spectroscopy analysis and submitted to an in vitro screening by APTT, PT, TT, and anti-factor Xa and IIa tests. The venae cavae ligature experimental model for the analysis of in vivo antithrombotic activity of F50Ul and F70Ul sulfated polysaccharides were also performed. The *U. lactuca* L. sulfated polysaccharides characterization by FACE and RAMAN showed a typical ulvans structure that contains as principal component rhamnose, but other monosaccharides (uronic acid, glucose, and galactose) are present. F50Ul (0.1–1.0 μg/μl) showed anticoagulant activity in vitro. However, F70Ul that has a similar composition did not present these effects. Also, only F50Ul sulfated polysaccharides (≥5 μg/g) showed a great in vivo antithrombotic concentration-dependent and time-dependent activity. In summary, we demonstrate the use of unusual extraction and characterization analysis procedures for *U. lactuca* L. sulfated polysaccharides and the ability of F50Ul to reduce the weight of thrombus in rats probably by the association with factors Xa and IIa inhibition. These results provide strong evidence of the anticoagulant potential of these sulfated polysaccharides isolated from *Ulva lactuca* L.

1. Introduction

According to the World Health Organization, cardiovascular diseases are the major cause of death worldwide. An approximate calculation has shown that over 23.6 million people will die due to cardiovascular diseases until 2030. It is associated with heart and blood vessels disorders and includes coronary heart disease, hypertension, stroke (cerebrovascular), rheumatic heart and peripheral vascular diseases [1]. Arterial thrombosis is one of the most common causes of death in the world due to either instability or rupture of an atherosclerotic plaque resulting in localized clot formation and blockage of blood flow with subsequent myocardial infarction (heart attack) or stroke [2].

The physiologic action of the hemostatic system removes fibrin deposits from vessels and maintains the circulating blood in a fluidic state. In cases of vessel injury, the tissue factor on adventitial cells, such as adventitial fibroblasts and smooth muscle cells activates the clotting system with a complex series of enzymatic reactions involving coagulation proteins, culminating in the activation of thrombin, which converts fibrinogen to fibrin. Thus, it forms a stable clot and prevents excessive blood loss. While hemostatic clots are localized to the vessel wall and do not impair blood flow, the thrombotic clots result in

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impairment of blood flow and even complete occlusion of the vessel [2]. In this case, anticoagulation therapy is required for inhibition of blood clotting using appropriate drugs, i.e., warfarin or heparin [3].

Heparin is a sulfated glycosaminoglycan of mammalian origin, and it is worldwide used as a therapeutic agent (as anticoagulant and antithrombotic) for many years. The activity of heparin is dependent on the ternary complex formation with antithrombin III and coagulation serine proteases, such as thrombin. Also, heparin may increase heparan sulfate proteoglycans release from the endothelial cells with antithrombotic activity [4,5]. However, heparin may be associated with a risk of hemorrhagic complications, thrombocytopenia cases [4,6,7], changes in lipid metabolism and osteoporosis [8].

Based on these facts, there is a need for the investigation of new sources of anticoagulants. Researchers and the pharmaceutical industry are looking for an ideal anticoagulant substance that presents the best cost-benefit-risk, with the maximum activity and the minimum of hemorrhagic risk and lowest side effects than the drugs currently in use [9].

Brazil has an extensive coastline and a rich flora of marine macroalgae, which represents potential sources of biotechnological interest due to the production of a vast diversity of compounds with a broad spectrum of biological activities that offer opportunities for bioprospecting unexplored substances [10,11]. Marine macroalgae with high biotechnology interest belong to the Phaeophyta (brown algae), Rhodophyta (red algae) and Chlorophyta (green algae) phylum [12,13].

Marine algae polysaccharides are the most abundant organic components which present great molecular biodiversity. Sulfated and non-sulfated polysaccharides have attracted considerable attention due to their broad therapeutic action, i.e., the anticoagulant and antithrombotic activities [14].

Green algae are known to synthesize a large number of sulfated polysaccharides and have recently attracted the interest because of the many benefits they can bring to health. There are a variety of sulfated polysaccharides obtained from green algae, such as rhamnan obtained from the *Monostroma* species, arabinogalactan from the *Codium* species, galactan from the *Caulerpa* species [15] and the ulvan, which are isolated from the *Ulva* and *Enteromorpha* genera.

Ulva lactuca L. polysaccharides are heterogenous sulfated compounds known as ulvans that are constituted by residues of rhamnose, uronic acids, galactose, mannose, xylose, arabinose, and glucose. Some activities have been described for these polysaccharides such as anti-atherogenic action and protective of oxidative stress [17,18].

Based on these facts, it is essential to realize investigations with natural marine compounds for the development of new products with innovative applications in pharmacology and therapeutics. The principal objective of this work is to carry out the partial chemical characterization and the evaluation of the anticoagulant and antithrombotic activities of the sulfated polysaccharides obtained from the green algae *U. lactuca* L.

2. Materials and methods

2.1. Materials

Agarose (Standard Low-MR) was obtained from BioRad Laboratories (Richmond, CA, USA). Glucuronic acid, galacturonic acid, rhamnose, xylose, fucose, galactose, glucose monosaccharides, chondroitin sulfate, dermatan sulfate, heparan sulfate, bovine serum albumin, AMAC, DMSO, sodium cyanoborohydride, toluidine blue were purchased from Sigma Chemical Company, (St. Louis, MO, USA). Activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) commercial kits were purchased from Labtest (Labtest, Lagoa Santa, MG, Brazil). ACTICHROME® Heparin (Anti-FIIa) and ACTICHROME® Heparin (Anti-FXa) purchased from Sekisui Diagnostics, LLC (Stamford, CT, USA). Citrated plasma was purchased from Bionutrientes do Brasil, Ltda (Barueri, SP, Brazil). Ketamine 10% and xylazine 2% was purchased from Vetnil (Louveira, SP, Brazil). Propylenediamine (1,3-diaminopropane) was purchased from Aldrich, Milwaukee, WI, USA. Acetic acid, ethanol, chloric acid, and sulfuric acid were obtained from Merck (Darmstadt, Germany). All other solvents and chemical products were of analytical grade.

2.2. Extraction of sulfated polysaccharides from green algae Ulva lactuca L.

The green algae *U. lactuca* L. was collected in the seashore of Nísia Floresta, RN, Brazil, during the low tide period and it was used as starting material for the isolation of sulfated polysaccharides. After collection, the algae were washed with water to remove contaminants (epiphytes and calcareous inclusions), and it was dried at 40 °C (FANEM, Model 315 SE). Immediately after being dried, the algae were powdered and submitted to an extraction method based on the procedure previously described [17]. Algae powder (35 g) was incubated with acetone to remove lipids and pigments for 24 h, at room temperature, three times. The de-lipidated powder was dried and resuspended in 280 mL of 0.25 M NaCl, pH 8.0. Alkalase™ was added to the mixture for proteolytic digestion. After 24 h of incubation at 60 °C, the mixture was submitted to an enzymatic inactivation (100 °C, 5 min). After cooling the material was submitted to centrifugation (3000*g*, 30 min), filtered through cheesecloth and named crude extract.

2.3. Fractionation of sulfated polysaccharides crude extract from Ulva lactuca L.

Sulfated polysaccharides present in the crude extract were 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 (3000*g*, 30 min) and dried under vacuum, weighted, resuspended in distilled water, and analyzed. The operation was repeated sequentially with the supernatant by adding 0.5, 0.7 and 1.0 volumes of acetone according to the current solution volume [41]. The sulfated polysaccharides were named according to the acetone volume used for its precipitation as follow, F30Ul, F50Ul, F70Ul, and F70Ul.

2.4. Chemical analysis

The total sugar content was determined by the phenol-sulfuric acid method [18]. Protein was quantified by the Bradford method [19]. Total inorganic sulfate was measured by the barium chloride-gelatin method after acid hydrolysis (8 M HCl for 6 h at 100 °C), as previously described [20]. The uronic acid content was determined by the modified carbazole reaction [21]. Standard samples of known concentrations were used to build a standard curve for comparison.

2.5. Agarose gel electrophoresis

Agarose gel electrophoresis of the sulfated polysaccharides was performed in 0.5% agarose gels (7.5 × 5.0 × 0,2 cm thick) prepared in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0 [22]. Aliquots of the carbohydrates (about 50–250 μg) in the volume of 5 μL were applied to the gel and subjected to electrophoresis (100 V, 4 °C). As standard, a mixture of chondroitin sulfate, dermatan sulfate and heparan sulfate (5 μg of each) was used. After running, sulfated polysaccharides were precipitated in the gel with 0.1% cetyltrimethylammonium bromide solution for 2 h, dried under continuous ventilation, and stained for 20 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol. After, the gel was destained in 1% acetic acid in 50% ethanol and analyzed.

2.6. Estimation of the molecular weight

The molecular weight estimation was realized by Bhilocha method

with modifications [23]. The 1.5% agarose gel (7.5 × 5.0 × 0,2 cm thick) was prepared in 100 mM Tris buffer, pH 8.3, with 100 mM boric acid and 1 mM EDTA. Aliquots of the carbohydrates (100 μg) were dried under vacuum and resuspended with 40 mM Tris·HCl buffer containing 20 mM NaCl, 2 mM EDTA, 40% glycerol and 0.05% cresol red dye. The samples prepared were then applied to the gel and subjected to electrophoresis (20 V, 4 °C, 30 min; 40 V, 4 °C, 4 h). After the run, the gel was stained for 30 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol and subjected to molecular weight analyses with TotalLab TL120 1D v2009 software, Nonlinear Dynamics Ltd.

2.7. Monosaccharide composition analysis by Fluorophore-assisted carbohydrate electrophoresis – FACE

Monosaccharide analysis was realized with sulfated polysaccharides after hydrolysis (6 M HCl, 6 h, 100 °C). Derivatization has done by the method described by Calabro [24], with some modifications. Dried samples (2–80 μg) were derivatized by addition of 5 μL of 50 mM AMAC (250 nmol) in 85% DMSO/15% acetic acid. Samples were incubated at room temperature for 15 min, then 5 μL of a newly prepared 1 M sodium cyanoborohydride solution was added. Samples were incubated for 16 h at 37 °C, and then 5 μL of 60% glycerol was added. Electrophoresis was carried out in the Mini-Protean Tetra Cell System (BioRad Laboratories, Richmond, CA, USA) in a borate-containing buffer system [25,26]. Polyacrylamide separating gels (25%) were prepared as described by [26], with some modifications. The electrode buffer used for the run was 0.5 M glycine, 0.6 M Tris base, and 0.5 M boric acid, final pH 8.3. The run (100–220 V, 70 min) was followed with the aid of an UV-lamp (320–400 nm). After electrophoresis, gels images were obtained with a UV transilluminator (365 nm) (Scientific grade CCD Gel Cam 310) and photographed (GelDoc-It Imaging System, UVP). The images were analyzed with TotalLab TL120 1D v2009 software, Nonlinear Dynamics Ltd.

2.8. Raman spectroscopy analyses

Fourier-transform Raman spectra were recorded using a Bruker RFS 100 spectrometer and an Nd:YAG laser operating at 1064 nm, equipped with a Ge detector cooled with liquid nitrogen. A spectral resolution of 4 cm^{-1} was used, and good signal-to-noise ratios were obtained with 1000 interferogram scans accumulated, using laser power of 100 mW at the sample.

2.9. Anticoagulant activity

Activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) coagulation assays were performed with Labtest kits (Labtest, Lagoa Santa, MG, Brazil), in a Cascade-M coagulometer (Helena Laboratories Company) and measured using citrate-treated plasma. The results were expressed as the ratio, which was determined as follows: control time/sample time. The anti-factor-Xa and anti-factor-IIa activities were determined with ACTICHROME® Heparin (Anti-FIIa) and ACTICHROME® Heparin (Anti-FXa) (Sekisui Diagnostics, LLC, Stamford, CT, USA). Heparin was used as standard and activity was measured using citrate-treated plasma.

2.10. In vivo antithrombotic activity

The in vivo antithrombotic activity was evaluated using the inhibition of venous thrombosis produced after venae cavae ligature according to previous works [27–30]. Wistar rats weighing 180–200 g were granted by the Biology and Reproduction Animals Center of Federal University of Juiz de Fora and divided into groups (n = 5). The sulfated polysaccharides were injected intravenously into the caudal vein of animals in a volume of 0.2 ml of saline in different times (1, 3, 5, 7 and 23 h) before the surgical procedures. The method consisted of exposing 1 cm of the inferior venae cavae of rats (below the left renal vein) and performing a ligature with Nylon thread (number 4) at specific times after intravenous injection of the test substance. The abdominal cavity was then closed. After 1 h, the cavity was reopened, and the eventual thrombi formed were removed from the vein, washed, blotted with filter paper, dried under vacuum for 24 h, and weighed. Heparin was used as a control. Animals were anesthetized by intramuscular injection with a solution of ketamine 10%/xylazine 2% (90 mg/kg/10 mg/kg) (Vetnil, Louveira, SP, Brazil) before surgical procedures. The animal assays were approved by the Ethical Animal Research Committee of the Federal University of Juiz de Fora (CEP 037/2014).

3. Results and discussion

3.1. Chemical analysis and monosaccharides composition of sulfated polysaccharides

Sulfated polysaccharides present in the *U. lactuca* L. crude extract were obtained by precipitation with different volumes of acetone and named F30Ul, F50Ul, F70Ul, and F100Ul which correspond of 1.07%, 11.17%, 8.73%, and 1.37% of the starting material (35 g of dry algae powder), respectively. These samples were analyzed by agarose gel electrophoresis in PDA buffer (Fig. 1). All polysaccharides showed metachromatic shift bands after staining with toluidine blue. The metachromatic shift bands are attributed to the aggregation of the toluidine blue at the sulfate groups in the sulfated polysaccharides. However, only intense bands were visualized in F50Ul and F70Ul. According to the extraction yield and the appearance of the metachromatic bands, F50Ul and F70Ul were selected for the continuation of this work.

Previous studies demonstrated that *U. lactuca* L sulfated polysaccharides could be extracted by hot water [16,31–33], ethanol 85% [32] or NaOH 2% [34]. It is important to note that there are no reports about the extraction of sulfated polysaccharides from *U. lactuca* L. with enzymatic proteolysis followed by acetone precipitation. The yields of a percent of dry mass obtained by these different methods were around 20% [33], 10.32–14.52% [32] and 9.33–17.57% [34]. These values are close to the yield obtained in the present work (22.34%).

F50Ul and F70Ul presented the total carbohydrate content of 68.02% and 20.14%, respectively (Table 1). Other extraction procedures of *U. lactuca* L. polysaccharides using hot water extraction followed by ethanol precipitation obtained different levels of total carbohydrate content, for example, 65,4% [31] and 79% [35], 21–36% [33] and 37–51% [32].

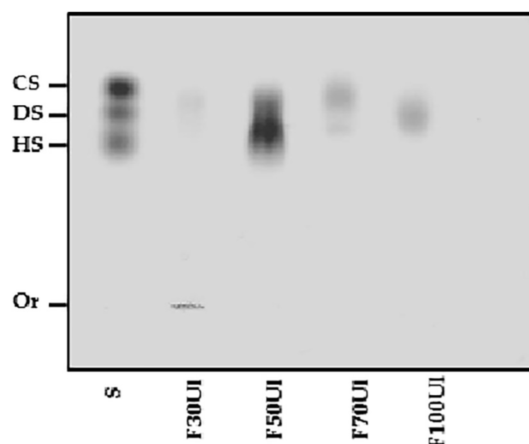


Fig. 1. Agarose gel electrophoresis of *U. lactuca* L. polysaccharides. Aliquots of F30Ul (250 μg), F50Ul (50 μg), F70Ul (50 μg), F100Ul (150 μg) and standards (5 μg) were applied. Standards (S); origin (Or); chondroitin sulfate (CS); dermatan sulfate (DS); heparan sulfate (HS).

Table 1
The chemical composition of *U. lactuca* L. sulfated polysaccharides.

Sulfated polysaccharides	Yield (%)	Total sugar (%)	Uronic acid (%)	Sulfate (%)	Ratio sulfate/sugar[b]	Protein (%)	Molecular weight (kDa)
F50Ul	11.17	68.02 ± 0.04	32.71 ± 0.05	19.97 ± 0.00	0.29	n.d.[a]	185.28
F70Ul	8.73	29.14 ± 0.05	4.32 ± 0.00	52.81 ± 0.01	1.81	n.d.[a]	163.44

[a] nd - not detected.
[b] Sulfate/sugar: the ratio between sulfate content and total sugars content.

Proteins were not detected in F50Ul and F70Ul after enzymatic proteolysis indicating a low contaminant's concentration. Concerning the uronic acid content, F50Ul showed 32.14%, similar to those already found in the literature 17.2% [31], 30% [35], 20.37–23.60% [36], while for F70Ul (4.32%) the uronic acid content was lower. On the other hand, sulfate content was higher in F70Ul (52.81%) than in F50Ul (19.97%). These sulfate content values were higher than those reported in earlier works where *U. lactuca* L. polysaccharides were extracted by hot water method, 11–14% [33], 17.4% [31], 8.52–12.45% [32].

Polyacrylamide gel electrophoresis analysis in TBE (Tris-Borate-EDTA) buffer showed polysaccharides with high molecular weight, F50Ul (185.28KDa) and F70Ul (163.44KDa) (Table 1). According to different works [33,37] *U. lactuca* L. high molecular weights polysaccharides can be obtained with less drastic pH and temperature conditions.

The monosaccharide composition of these polysaccharides and the images of Fluorophore-Assisted Carbohydrate Electrophoresis (*FACE*) analysis are presented in Fig. 2.

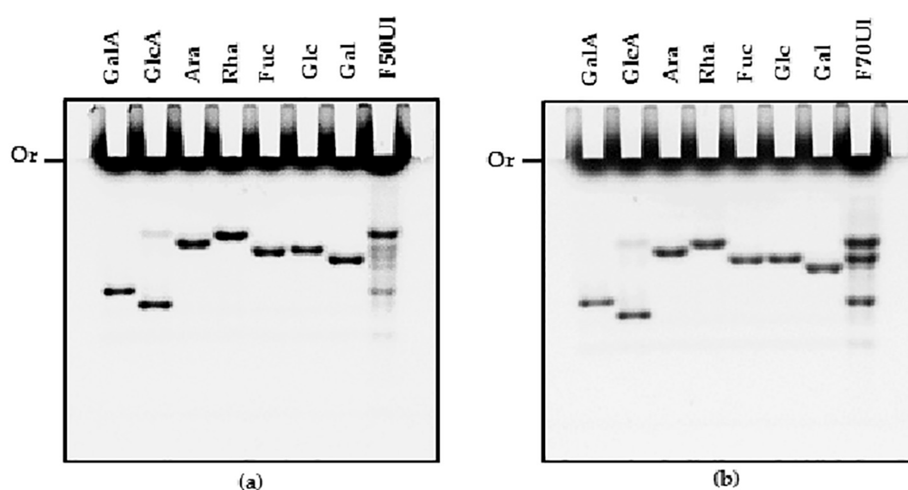
The principal monosaccharide component of sulfated polysaccharides from *U. lactuca* L (F50Ul and F70Ul) is rhamnose. These compounds also contain other monosaccharides, such as uronic acid, glucose, and galactose. These data are in agreement with other works in which *U. lactuca* L. polysaccharides were obtained by different methods of extraction. Hot water sulfated polysaccharide extraction procedures resulted in rhamnose content of 22.4%, glucuronic acid 22.5%, xylose 3.7%, iduronic acid 3.1% and glucose 1.0% [38]. Polysaccharides obtained by hot acid water extraction showed rhamnose content of 13.35–15.59%, glucose 2.90–10.79% and xylose 2.36–2.73% [36] and, polysaccharides obtained by hot basic water extraction followed by deproteination, rhamnose 51.2; 60.8%, uronic acid 16.4; 16.8% and glucose 8.2–20.1% [34].

It is important to emphasize that these difference in extraction methodologies may result in changes in polysaccharides yield, chemical composition and molecular features [37].

3.2. Spectroscopy analyses of F50Ul and F70Ul sulfated polysaccharides

Raman analyses of F50Ul and F70Ul have shown characteristic bands of carbohydrates in the spectral region of 1200–800 cm^{-1} assigned to stretching vibrations of the C—O/C—C groups such as bending modes of the C—O—H group observed at 1074 cm^{-1} (F50) and 1091 cm^{-1} (F70). The spectra have also revealed a C—O—C— skeletal mode of β-anomer linkage at 896 cm^{-1} and deformational modes of CCO groups in the range of 800–200 cm^{-1} (Table 2) [39]. Bands observed at 1380 cm^{-1} have been attributed to deformation mode of a methyl group from rhamnose and a weak band at 1419 cm^{-1} that could be assigned to stretching mode of COO^- group. The presence of sulfate groups was confirmed due to stretching vibrations of OSO_3^- at ca. 1060 and C—O—S at 848 cm^{-1} [40,41]. The absence of Raman bands associated with amide I (1600–1690 cm^{-1}) and amide II (1480–1580 cm^{-1}) modes from proteins confirmed the efficiency of the enzymatic proteolysis extraction method (Fig. 3).

The structure suggested to ulvanobioronic acid, which is the principal unit of ulvans, is formed by an glucuronic acid residue linked β (1 → 4) to a 3-sulfated rhamnose residue (Type A), or by an iduronic acid molecule linked α(1 → 4) to a molecule of 3-sulfated rhamnose (Type B) [38]. Raman signals registered from F50Ul and F70Ul could be correlated with ulvan class of compound since the main functional groups typical of ulvanobioronic acid such as OSO_3^-, C—O—S, COO^-, C—O—H, C—O—C, and methyl groups have been observed (Table 2). A band at 1008 cm^{-1} could be assigned to stretching vibrations of C—O—C and C—O—S in equatorial position according to Matsuhiro and



Fraction	Rhamnose	Uronic acid	Glucose	Galactose
F50Ul	1	0.333	0.081	0.052
F70Ul	1	0.666	0.835	0,009

(c)

Fig. 2. Fluorophore-assisted carbohydrate electrophoresis (FACE) of the hydrolysis product of *U. lactuca* L. polysaccharides in Tris-borate buffer. FACE of F50Ul polysaccharides (a); FACE of F70Ul polysaccharides (b); monosaccharide composition of *U. lactuca* L. polysaccharides (c). Arabinose (Ara); fucose (Fuc); galactose (Gal); galacturonic acid (GalA); glucose (Glc); glucuronic acid (GlcA); rhamnose (Rha); origin (Or).

Table 2
Tentative assignments of main Raman bands observed for F50Ul and F70Ul sulfated polysaccharides.

F50Ul	F70Ul	Tentative assignment
1454 m	1456 w	δCH_2
1419 m	1419 w	νCOO^-; δCH_2
1380 m	1379 w	δCH_3
1340 m	1340 w	δCOH; δCH
1137 m	1139 m	νCC; νCO, δCOH
1091 sh	1093 m	δCOH
1089 sh	–	δCOH
1074 s	–	δCOH; CCO
1060 s	1059 m	νSO_3; CO
1008 s	1007 s	νCOC, $OSO_{3(equatorial\ position)}^-$
985 s	985 s	CC_{ring}
896 m	898 w	$\nu COC_{\beta\text{-glycosidic linkage}}$
848 m	850 w	νCOS; anomeric C_1H
792 w	790 w	νCO_{ring}; δCOC
621 vw	621 m	δCCC; δCCO
478 vw	472 sh	δCCO
457 w	460 m	δCCC
416 m	416 vw	δCCC

s: strong; m: media; w: weak; vw: very weak; sh: shoulder.

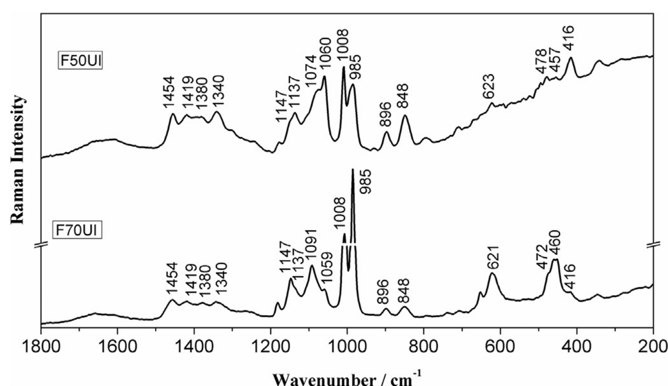


Fig. 3. FT-Raman spectra of F50Ul and F70Ul sulfated polysaccharides.

coworkers [42].

Raman spectral analyses have shown subtle differences between F50Ul and F70UI, they share Raman bands with similar wavenumber position, however, with inverse intensity mainly in the region of 1100–416 cm^{-1}. The higher amount of galactose in F50Ul (0.052) compared to F70Ul (0.009) shown in Fig. 2, could be associated with the broad band centered at 1704 cm^{-1} and the band at 416 cm^{-1}. The correlation has been suggested due to comparison with Raman spectrum of the monosaccharide galactose which presents bands at ca. 1070/1054 cm^{-1} attributed to the coupling of CO/CC stretches with the bend of δCOH band at 417 cm^{-1} (δ skeletal mode) and some other minor bands [39]. These results have demonstrated that Raman spectral profiles corroborate the variation of sugar content revealed by FACE.

3.3. Clotting assays

The anticoagulant activity of F50Ul and F70Ul sulfated polysaccharides initially was assessed by evaluating their potential to inhibit different coagulation cascade pathways: the intrinsic (activated partial thromboplastin time: APTT), extrinsic (prothrombin time: PT), and common (thrombin time: TT) anticoagulant pathways. General clotting assays were performed with citrate-treated plasma, which was incubated with the samples in different concentrations and with commercial heparin (0.025 μg/mL) as a positive control reference.

A dose-dependent relationship in the APTT assay was observed for the F50Ul sulfated polysaccharide (0.2–1.0 μg/mL), and it was able to extend the plasma coagulation time (0.5 and 1.0 μg/mL) about two times in comparison to the negative control (Fig. 4(a)). F50Ul (1.0 μg/mL) increased three times the plasma coagulation time in prothrombin time (PT) assay, within the concentration range used in the APTT test (Fig. 4(b)). The thrombin time (TT) was measured with different concentrations of F50Ul but only in 1.0 μg/mL concentration a significantly prolonged plasma coagulation time was observed when compared to the negative control (Fig. 4(c)).

On the other hand, the F70Ul sulfated polysaccharide (1.0–2.0 μg/mL), did not significantly prolonged any plasma coagulation time assay at any of the concentrations evaluated (Figs. 4(a, b, c)).

Heparin (0.025 μg/mL) was used as positive control, and as expected, prolonged the plasma coagulation time in the intrinsic pathway about six times (Fig. 4(a)), prolonged the plasma coagulation time in the extrinsic pathway about two times (Fig. 4(b)) and on thrombin-induced clot formation making the plasma uncoagulable.

Only a few works have reported anticoagulant activities of sulfated polysaccharides from the *U. lactuca* L. algae. Some authors have studied the anticoagulant activity of two different extracts of *U. lactuca* L., one obtained with hot water extraction and ethanol precipitation, while the other obtained with 85% ethanol extraction and ethanol precipitation. Both extracts (100 μg/mL) prolonged the plasma coagulation time in the presence of thrombin [32]. Meanwhile, Adrien and coworkers [3] investigated the anticoagulant activity of a hot water crude extract of *U. lactuca* L. and showed that this extract did not have activity on any of the coagulation pathways (APTT, PT, and TT). These authors indicated that one of the reasons for the absence of the activity could be due to the low sulfate content of the extract obtained (about 13%) [3].

It is interesting to note that the F50Ul evaluated in the present work, extracted by enzymatic digestion and acetone precipitation, interfered in the coagulation process. These results convinced us to investigate the possible coagulation cascade proteins that are involved in this activity.

The effect of the sulfated polysaccharides of *U. lactuca* L. on the antithrombin-mediated inhibition of factors Xa and IIa (thrombin) was investigated. As can be observed in Fig. 4(d), F50Ul showed significant anti-Xa activity with strong inhibition of factor Xa, close to 96%, and a reduction in factor IIa activity about 45%. For the F70Ul (1.0 μg/μL) the anti-Xa activity was close to 80% and the reduction in factor IIa activity is close to 60,18% (Fig. 4(d)). Heparin (0.4 μg/μL) completely inhibited the factor Xa activity (100%) and about 50% the factor IIa activity.

In previous work, the authors [3] showed that a water crude extract of *U. lactuca* L. was devoid of any anticoagulant activity upon Xa factor and also did not present any antithrombin-mediated inhibition of the IIa factor, regardless of the concentration tested.

It is important to emphasize that the differences between the activities found are related to the differences in extractions methods. Anticoagulant activity depends on the presence of sulfate groups, their position in the polysaccharide molecule, as well as the structural conformation of the molecule [43]. In the case of green algae, another interesting note is that the presence of sulfate groups at position 3 in the rhamnose residue was directly associated with anticoagulant activity [44,45].

3.4. "In vivo" antithrombotic activity

The inhibition of venous thrombus formation after venae cavae ligature by *U. lactuca* L sulfated polysaccharides intravenously injected into the caudal vein of rats was evaluated after 24 h. As can be observed in Fig. 5(a) the F50Ul (20 μg/g of rat weight) reduced in 56% ($p < 0,05$) the weight of the thrombus formed after the ligature, while F70Ul did not show significant effects. Heparin (1.5 μg/g of rat weight) was used as positive control and after 2 h of intravenously injected reduced the thrombus weight in 92% ($p < 0,05$), as observed in a previous work [30].

To determine the F50Ul antithrombotic activity, a dose-response curve was constructed, and the results are present in Fig. 5(b). The F50Ul significantly reduced ($p < 0,05$) the thrombus weight after 24 h

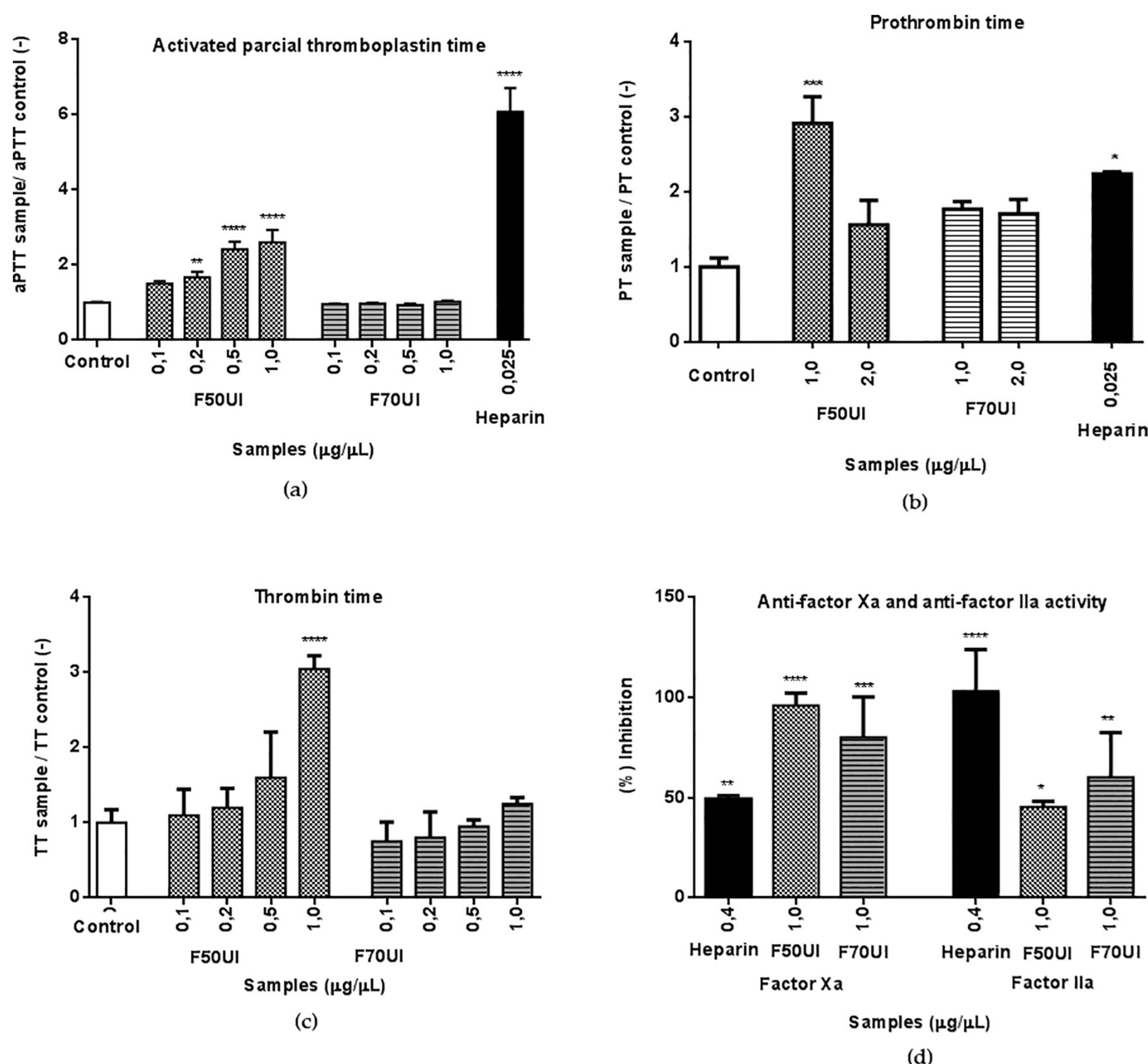


Fig. 4. Anticoagulant activity of F50Ul and F70Ul *U. lactuca* L sulfated polysaccharides. Activated partial thromboplastin time assay (aPTT) for *U. lactuca* L sulfated polysaccharides (a); prothrombin time assay (PT) for *U. lactuca* L sulfated polysaccharides (b); thrombin time (TT) assay for *U. lactuca* L sulfated polysaccharides (c). These results were expressed as the ratio of sample clotting time for negative control clotting time. Citrated plasma without samples was used as negative control and heparin (0,025 µg/µL) as a positive control. Anti-factor IIa and anti-factor Xa activity are shown in (d). Results of this assay were expressed as percent inhibition relative to the background of each of the factors. *U. lactuca* L. Original aPTT and PT for control were 27.0 s and 10.7 s, respectively, and for Heparin were 150 s and 25.5 s, respectively. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

of the polysaccharide injection in different concentrations (2, 5, 10, 15 and 20 µg/g of rat weight). As can be observed in Fig. 5(b) there is a dose-dependent response, and from the concentration (5 µg/g) no thrombus was formed. Moreover, a time-response curve was obtained with a concentration of 10 µg/g of rat weight. Fig. 5(c) shows that this effect is also time-dependent ($p < 0,05$), reaching the maximum of the antithrombotic effect after 12 h after injection of the sulfated polysaccharide.

The significant “in vivo” antithrombotic effect of F50Ul can be correlated with the “in vitro” results obtained in APTT, PT, TT assays as well as the inhibition of Xa and IIa factors (thrombin) trials. The reduction in thrombus formation can be associated with the effects of F50Ul sulfated polysaccharides on Factors Xa and IIa of the common anticoagulant pathway intermediated by antithrombin III. Factor Xa is a common enzyme to both coagulation pathways (extrinsic and intrinsic) and is associated with Factor V to form prothrombinase (Xa-Va) which converts prothrombin to thrombin that cleaves fibrinogen to form fibrin, thereby initiating the formation of the clot [2]. Then, when there are some effects upon the Xa factor, the prothrombinase complex is destabilized, and thrombin is not correctly activated, interfering with clot formation.

The antithrombotic activity as observed for heparin could be associated to the enzymatic inhibition (Xa and IIa) but also with the increase of heparan sulfate and nitric oxide synthesized by endothelial cells [5,46–48]. The relation between “in vivo” antithrombotic activity of sulfated polysaccharides from algae and heparan sulfate synthesis has been reported earlier. The polysaccharide of Spatoglossum schröederi algae (20 µg/g), for example, reduced the thrombi weight after 24 h of action and these polysaccharides showed “in vitro” antithrombotic activity too. It increased the heparan sulfate synthesis by endothelial cells culture [29,30].

4. Conclusions

In the present work, four sulfated polysaccharides from the green algae *Ulva lactuca* L. were obtained by acetone precipitation and named F30U1, F50U1, F70U1, and F100U1. Among them, two (F50U1 and F70U1) showed carbohydrate content higher than 8% and an intense metachromatic band indicating the presence of sulfate groups. The sulfated polysaccharides exhibited molecular weights of 185.28 kDa,

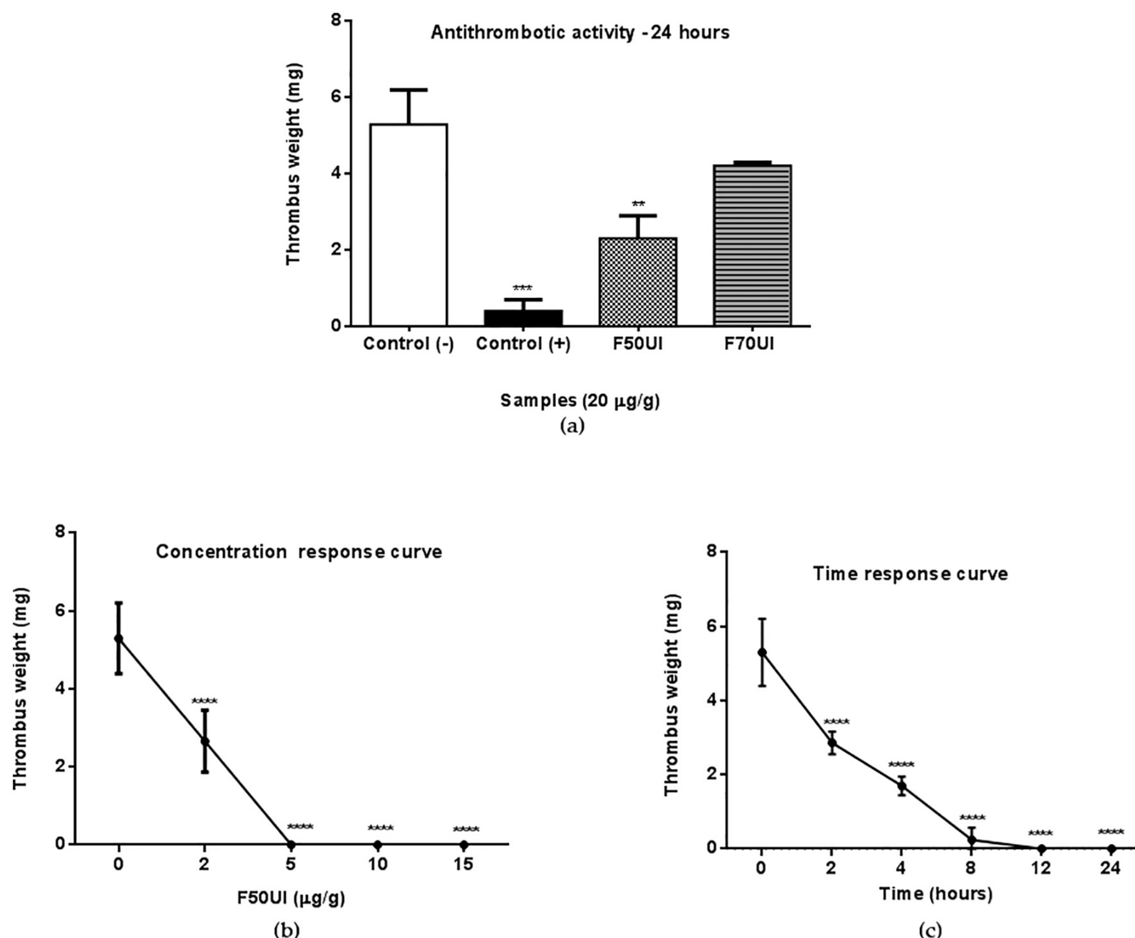


Fig. 5. Antithrombotic activity of *U. lactuca* L. sulfated polysaccharides. *U. lactuca* L. sulfated polysaccharides (20 µg/g) after 24 h of action is shown in (a); dose-response curve of *U. lactuca* L. F50Ul is shown in (b); time response curve of *U. lactuca* L F50Ul is shown in (c). *U. lactuca* L F50Ul; *U. lactuca* L F70Ul; ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$.

and 163,44 kDa, respectively, for F50U1 and F70U1. Monosaccharide analysis by FACE exhibited a majority presence of rhamnose, and also the presence of uronic acid, glucose, and galactose. The Raman signals from both sulfated polysaccharides are characteristic of the presence of the main functional groups, the ulvanobioronic acid, of the ulvans. F50U1 was able to inhibit all the coagulation pathways, the common, intrinsic and extrinsic pathways. This activity was higher than that of F70U1 sulfated polysaccharides. This fact is mainly due to the inhibitory action on factor Xa of the coagulation cascade. About antithrombotic activity, F50U1 was able to inhibit in vivo thrombus formation in a dose- and time-dependent fashion. Although F70Ul exhibits an in vivo antithrombotic activity, it was lower than observed for F50U1. Taken together, these results provide strong evidence of the anticoagulant potential of these sulfated polysaccharides isolated from *Ulva lactuca* L, making an exciting option for future investigation of the mechanisms involved in this action.

Author contributions

S.E.R., J.A.K.A., and V.P.M. conceived and designed the experiments; V.P.M. performed the collection of seaweed *Ulva lactuca* L.; S.E.R., and R.G.C.A. performed the chemical analyses; L.F.M., and L.F.C.O. performed the RAMAN spectroscopy analyses; S.E.R., C.M.A., and H.B.N. performed the anticoagulant tests; S.E.R., and V.P.M. performed the antithrombotic analyses; H.B.N., J.A.K.A., and V.P.M. analyzed the data; S.E.R., H.B.N., J.A.K.A., and V.P.M. wrote the paper. J.A.K.A and V.P.M. funded and revised the paper.

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Declaration of competing interest

The authors declare no conflict of interest.

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