Structural and physical-chemical analyses of sulfated polysaccharides from the sea lettuce *Ulva lactuca* and their effects on thrombin generation

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ABSTRACT. *Ulva lactuca* (Chlorophyceae) has biotechnologically-important sulfated-polysaccharides (Ul-SPs), but their potentials on thrombin generation (TG) are unknown. This study analyzed the structural and physical-chemical features of the Ul-SPs as modulators of TG. Proteolytic digestion yielded (13.13%) extract containing sulfate (20.43%) and total sugars (65.72%), besides ulvan consisting of rhamnose, xylose, glucose, glucuronic acid and α-/β-types glycosidic linkages as characterized by one-/two-dimensions nuclear magnetic resonance (NMR) experiments. Fractionation of the Ul-SPs by DEAE-cellulose chromatography yielded Ul-SP1 and Ul-SP2 (0.50 and 0.75 M NaCl, respectively) showing sulfation (15.72-18.04%) and total sugars (59.73-60.58%) consistent with the charge density pattern by combination of agarose/polyacrylamide gel electrophoresis using sequential staining with toluidine blue and stains-all, although with slight differences in their sizes (40 and >100 kDa, respectively). By both activated partial thromboplastin time (APTT) and prothrombin time (PT) tests, anticoagulation of the fractions was virtually detected by APTT (0.39 and 0.43 IU, respectively) against heparin (193 IU). Fractions acted differently on both intrinsic/extrinsic pathways in TG using 60-fold diluted human plasma, with 50% efficacies up to 8.3 μg, whereas at high concentrations suggested intrinsic hypercoagulability since heparin abolished both systems at low amounts. Ul-SPs block TG, but predicting thrombosis in increasing doses.

Keywords: Ulvales; complex glycans; chemical analysis; thrombosis.

Análises estrutural e físico-química de polissacarídeos sulfatados da alface do mar *Ulva lactuca* e seus efeitos sobre geração de trombina

RESUMO. Clorofíceas *Ulva lactuca* possuem polissacarídeos sulfatados (Ul-PSs) importantes biotecnologicamente, porém são desconhecidos seus potenciais sobre geração de trombina (GT). Analisaram-se as características estruturais e físico-químicas dos Ul-PSs como moduladores de GT. Digestão proteolítica rendeu (13,13%) extrato contendo sulfato (20,43%) e açúcares totais (65,72%), além de ulvan, como caracterizada por experimentos de ressonância magnética nuclear uni-/bi-dimensionais, consistindo de rhamnose, xilose, glucose, ácido glucurônico e ligações glicosídicas tipos-α/-β. Fracionamento dos Ul-PSs por cromatografia de DEAE-cellulose rendeu Ul-PS1 e Ul-PS2 (0,50 e 0,75 M de NaCl, respectivamente) mostrando sulfatação (15,72-18,04%) e açúcares totais (59,73-60,58%) consistentes com o grau de densidade de carga por combinação de eletroforese em gel de agarose/poliacrilamida usando coramento sequencial com azul de toluidina e "stains-all", embora com diferenças quanto aos seus tamanhos (40 e >100 kDa, respectivamente). Por ambos os testes do tempo de tromboplastina parcial ativada (TTPA) e do tempo de protrombina, anticogulação das frações foi detectada virtualmente pelo TTPA (0,39 e 0,43 UI, respectivamente) frente heparina (193 UI). Frações atuaram diferentemente sobre ambas as vias intrínseca/extrínseca na GT usando plasma humano diluído 60 vezes, com eficácia de 50% até 8,3 μg, enquanto em concentrações maiores sugeririam hipercoagulabilidade intrínseca visto que heparina aboliria ambos os sistemas em quantidades baixas. Ul-PSs bloqueiam GT, porém prevendo trombose em doses crescentes.

Palavras-chave: Ulvales; glicanos complexos; análises químicas; trombose.

Introduction

Since ancient times in Asia seaweeds have been recognized as nutritional and functional food sources because of their health benefits (Cardozo et al., 2007; Wang, Wang, Wu & Liu, 2014). They have intrinsic components without toxicity and with multifunctionality in many commercially-important
food and pharmaceutical products, taking advantage of clinically-used drugs that have systemic complications (Cardozo et al., 2007; Pomin & Mourão, 2008; Mourão, 2015). One particularly interesting feature of seaweeds is their alternative as anticoagulants, a property proved by their mucilaginous matrix sulfated polysaccharides (SPs), which can be extracted by different protocols (Athukorala, Jung, Vasanthan, & Jeon, 2006; Fidelis et al., 2014; Imjongjairak et al., 2016; Rodrigues, Torres, Alencar, Sampaio, & Farias, 2009; Rodrigues et al., 2016a).

Cell-wall SPs vary among algal species and polymeric identity (Cardozo et al., 2007; Thomas & Kim, 2013), having strongly charged nature with certain structural features conserved among phyla (Pomin & Mourão, 2008). Structurally, these polyanionics are classified into three major chemical classes: 1) sulfated galactans (mainly carrageenans and agarans) are the hydrocolloids expressed in Rhodophyceae (Cardozo et al., 2007; Mourão, 2015; Rodrigues et al., 2009; Rodrigues et al., 2016a); 2) fucans or fucoidans are synthesized in Phaeophyceae, owing to the presence of sulfated fucose (Pomin & Mourão, 2008); and 3) sulfated heteropolysaccharides naturally occur in Chlorophyceae as dominant polymers, but little are known about their highly complex structural and physical-chemical features and biotechnological properties (Rodrigues et al., 2014; Wang et al., 2014; Yaich et al., 2014). SPs from seaweeds have high molecular weights (usually > 100 kDa) and variable sulfation (-SO₄²⁻) on their chains making them as modulators on coagulation (Rodrigues et al., 2009; Pomin, 2012; Fidelis et al., 2014) and inflammation (Rodrigues et al., 2012; Pomin, 2012; Thomas & Kim, 2013) by interacting with health/disease-associated proteins.

Although being the initial treatment in cardiovascular or thromboembolic diseases, unfractionated heparin (UHEP) systemic therapy can induce bleeding complications and thrombocytopenia. Its anticoagulant mechanism is proved by antithrombin (AT)-binding specific pentasaccharide sequence, which it is not found in other natural sources (Pomin, 2012; Mourão, 2015). The main targets of the algal anticoagulants are the thrombin (Rodrigues et al., 2013; Mourão, 2015), the final enzyme that converts fibrinogen into fibrin clot (Castoldi & Rosing, 2011), and the factor Xa (Athukorala et al., 2006; Glauser et al., 2009; Mourão, 2015), a coagulation protease that generates thrombin (Zavyalova & Kopylov, 2016), and/or by independent effect of serpins (Quinderé et al., 2014), preventing thrombosis without producing hemorrhage (Rodrigues et al., 2011; Quinderé et al., 2014; Mourão, 2015). However, at high dose, SPs from seaweeds may lead to thrombosis due to activation of factor XII in the intrinsic coagulation system (Rodrigues et al., 2011; Quinderé et al., 2014; Mourão, 2015).

The anticoagulant effects of the seaweeds SPs are routinely measured by two tests: 1) the activated partial thromboplastin time (APTT); and 2) the prothrombin time (PT), which distinguish the anticoagulant potential on both intrinsic/extrinsic coagulation pathways, respectively (Rodrigues et al., 2009; Mourão, 2015); but, they do not indicate the normal function status and thrombin generation (TG)-based coagulation assays have been introduced to develop more accurately antithrombotic drugs (Jung et al., 2014; Zavyalova & Kopylov, 2016) and for clinical prognostics (bleeding disorders and surgical procedures), and epidemiology of thrombosis (Castoldi & Rosing, 2011; Duarte et al., 2017).

Some species of tropical seaweeds have been revealed as promising sources of SPs displaying in vitro TG inhibition/activation. SPs isolated from the Phaeophyceae Ecklonia kurome (Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999) and from the Rhodophyceae Botryocladia occidentalis (Glauser et al., 2009), Acanthophora muscosoides (Rodrigues et al., 2016b) and Gracilaria birdiae (Rodrigues et al., 2017a) revealed as modulators of TG by blocking both blood coagulation pathways. Contrasting result was found for the SP isolated from the brown seaweed Fucus vesiculosus that acted as activator of TG by tissue factor pathway using calibrated automated thrombography (Zhang et al., 2014). However, studies on the biological role of the SPs isolated from the Chlorophyta species on in vitro TG inhibition assays are lacking in the literature (Rodrigues et al., 2017b; Rodrigues, Benevides, Tovar, & Mourão, 2017c; Rodrigues et al., 2018).

Species of the algal order Ulvales (Chlorophyta) usually overgrow as “green tides” in eutrophicated coastal waters, causing ecological and economical problems (Fletcher, 1996). Ulva lactuca Linnaeus (known as sea lettuce) represents an available biomass with very low added-value and opportunities to use it have emerged as an alternative source of nutritional components (Tabarsa, Rezaei, Ramezanpour, & Waaland, 2012) and ulvan-type SP consisting of rhamnose, xylose, glucose and uronic acid (Shanmugam, Ramavat, Mody, Oza, & Tewari, 2001; Siddhanta, Goswami, Ramavat, Mody, & Mairh, 2001; Wang et al., 2014; Yaich et al., 2014). This SP has shown as a natural protector against experimentally-induced renal toxicity in rats (Mahmoud & Hussein, 2014;
Sathivel, Balavinayagamani, Rao, & Devaki, 2014), besides of surfactant (Tian, Yin, Zeng, Zhu, & Chen, 2015) and analgesic/anti-inflammatory agent devoid of systemic damage in mice (Aratijo et al., 2016). On this ulvan polysaccharide opens a versatility of potential applications in different fields as a novel renewable biomaterial to be explored extensively regarding its chemical/functional nature (Lahaye & Robic, 2007; Li et al., 2018; Wang et al., 2014).

The scope of the present study was to analyze the structural features from SPs samples of U. lactuca collected on the coast of Ceará/Brazil using NMR technique and to characterize by agarose/polyacrylamide gels electrophoreses by sequential staining employing toluidine blue and stains-all regarding their physical-chemical properties; also, in vitro assessments were conducted with respect to its SPs on classical coagulation tests (APTT and PT) and TG assay in 60-fold diluted human plasma using a continuous detection system.

Material and methods

Ulva lactuca samples and physical-chemical analyses of their SPs

Specimens of the Chlorophyceae U. lactuva were collected in natural bed from the Northwestern coastline of Brazil (Flecheiras beach, Trairi-Ceará). All the samples were placed in plastic bags and conducted to the Carbohydrates and Lectins laboratory (CarboLec), Universidade Federal do Ceará. After collection, the material was cleaned and removed from macroscopic epiphytes, followed by washing with distilled water and stored at -20°C until further use (Aratijo et al., 2016). A voucher specimen (#4978) was deposited in the Herbarium Prisco Bezerra of the Department of Biological Sciences, Universidade Federal do Ceará, Brazil. The experimental analyses of the U. lactuca SPs were performed at the Connective Tissue laboratory, Universidade Federal do Rio de Janeiro (UFRJ), Brazil.

A five grams sample of dehydrated algal tissue was cut into small pieces and subjected to papain digestion (60°C, 24 hours) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM) (all Sigma Aldrich). Percent yield (%) was determined as based on the dehydrated weight of the algae obtained for papain extraction (Aratijo et al., 2016). Crude SP extract (20 mg) was dissolved in 10 mL of 50 mM sodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose column (1.2 × 12 cm) equilibrated with the same buffer (all Sigma Aldrich). Fractionation was conducted using a stepwise of NaCl from 0 to 1.5 M NaCl in the same buffer, with intervals of 0.25 M between each concentration. Fractions of 2.5 mL were collected and analyzed for SPs using the metachromasy assay ($A_{525}$ nm) containing dimethymethylene blue (Sigma Aldrich) with an Amersham Bioscience Ultraspec 3100 spectrophotometer at 525 nm (Farndale, Buttle, & Barrett, 1986). Extract and fractions U1-SP1 and U1-SP2 (eluted with 0.5 and 0.75 M NaCl, respectively) were examined for their contents of sulfate (Dodgson & Price, 1962), total sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and proteins (Bradford, 1976) according to the respective quantitative assays. SPs present in extract and fractions were checked by 0.5% agarose gel electrophoresis procedure (Dietrich & Dietrich, 1976). All these experiments were also conducted as described (Aratijo et al., 2016). Next, polyacrylamide gel electrophoresis (6%) was carried out to estimate the molecular size of SPs by comparison with the electrophoretic mobility of standard compounds dextran sulfate (8 kDa), chondroitin-4-sulfate (40 kDa) and chondroitin-6-sulfate (60 kDa) (all Sigma Aldrich) (Rodrigues et al., 2013; Rodrigues et al., 2017a). After electrophoretic experiments, both gels were subjected to sequential staining with toluidine blue and stains-all (both Sigma Aldrich) to reveal nonSPs from the polymer samples (Volpi & Maccari, 2002; Rodrigues et al., 2017a; Rodrigues et al., 2017b; Rodrigues et al., 2017c).

Structural analysis by NMR spectroscopy

$^1$H and $^{13}$C, one-dimensional and two-dimensional spectra, of the crude SP extract (U. lactuca) was recorded using a Bruker DRX 600 MHz apparatus with a triple resonance probe. About 5 mg sample was dissolved in 0.6 mL of 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA). All spectra were recorded at 25°C with HOD suppression by presaturation. 1D $^1$H-NMR spectrum was recorded with 32768 points and 8 scans and inter-scan delay set to 1 s (Tian et al., 2015; Rodrigues et al., 2014). Regarding the two-dimension NMR experiment, the $^1$H/$^{13}$C edited-HSQC spectrum (64 scans, 1024 × 512 points) was run and globally optimized alternating phase rectangular pulses (GARP) for decoupling (Quinderé et al., 2014). Chemical shifts are displayed relative to external trimethylsilylethionic acid at 0 ppm for $^1$H and relative to methanol for $^{13}$C. All spectra were processed using the SpinWorks 3.1.8 software package (Quinderé et al., 2014; Rodrigues et al., 2016a).
**In vitro experimental coagulation models**

**Blood samples**

Coagulation analyses were conducted using venous blood samples collected in citrated vacutainer tubes containing 3.2% sodium citrate from 10 different donors (University Hospital Clementino Fraga Filho, UFRJ), followed by centrifugation (2000 × g, 15 min.) prior to tests. Normal citrated human plasma aliquots of 1 mL were frozen and stored at -70°C until use (Rodrigues et al., 2017a).

**APTT and PT assays**

Fractions were assessed by both in vitro APTT and PT tests based on manufacturers’ specifications, for measuring anti-coagulant effect in a coagulometer Amelung KC4A before the in vitro TG assay. For APTT assay, a mixture of 100 μL of plasma and concentration of SPs (1 mg mL⁻¹) was incubated with 100 μL of APTT reagent (kaolin bovine phospholipid reagent) (Sigma Aldrich). After 2 min of incubation at 37°C, 100 μL of 25 mM CaCl₂ was added to the mixtures, and the clotting time was recorded. Regarding the PT assay, a mixture of 100 μL plasma and concentration of SPs (1 mg mL⁻¹) was incubated at 37°C for 1 min. After that, 100 μL of PT reagent (Sigma Aldrich) was added to the mixtures, and the clotting time was recorded using the same coagulation equipment. UHEP from the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK (193 international units per mg (IU mg⁻¹) of polysaccharide) was used as the standard in both tests. Data were expressed as mean ± S.E.M. (n = 3).

**TG assay in 60-fold diluted human plasma**

This assay was performed in a microplate format as described (Rodrigues et al., 2016b). For this, microplate containing: 10 μL cephalin (contact-activator system) or thromboplastin (830 μg well-plate⁻¹, factor tissue-activator system) + 30 μL 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 μL SPs (U. lactuca fractions: 0, 4.1, 8.3, 41.6 or 83.3 μg well-plate⁻¹ or UHEP: 2 or 4 μg well-plate⁻¹) + 60 μL 20 mM CaCl₂ and 0.33 mM chromogenic substrate S2238 (10:50 ratio, v/v), was used. The in vitro reaction was triggered at 37°C by addition of diluted plasma (10 μL), and the absorbance (405 nm) was continually recorded for 60 min. (Molecular Devices, Menlo Park, CA, USA). The inhibitory response of TG by SPs was determined by lag phase, peak thrombin (PTh) and/or time to peak (TPeak). Graphics were processed using Statistical Analysis Software version 8.0 Origin Program.

**Results and discussion**

Wall matrix SPs from the green seaweed *Ulva lactuca* (popularly known as sea lettuce) were extracted with papain to examine their structure, and then fractionated by DEAE-cellulose anion-exchange chromatography to analyze their physical-chemical features and in vitro effects on the coagulation.

The yield of crude SP (13.13%) was about 5.95-fold higher than that of the overall extract (2.21%) from the same algal species, applying the papain method (Araújo et al., 2016). This comparative examination of the total yield with a previous investigation also reflected highest levels of 20.43 and 65.72% for sulfate and total sugars, respectively, from the analytical assays of the samples, and could be the result of the drying (60°C, 24 hours) for obtaining crude SP extracted from the dehydrated raw biomass in comparison with the lyophilization procedure employed by Araújo et al. (2016), who previously examined the *U. lactuca* crude SP extract composition (15.10% sulfate and 54.72% total sugars).

Studies on the algal order Ulvales demonstrated variability for sulfate (12-26.8%) and yield (3.04-29%) using different protocols (Siddhanta et al., 2011; Yaich et al., 2014; Wang et al., 2014; Tian et al., 2015). Such variations could also be determined by algae collected from different regions and ecophysiological growth conditions (Cardozo et al., 2007; Wang et al., 2014; Rodrigues et al., 2016a and 2017a), as already reported for SPs from *Ulva* species (Siddhanta et al., 2001).

Carbohydrates-bound matrix proteins (like glycoproteins) (Wang et al., 2014) were removed by protease treatment (Rodrigues et al., 2011; Araújo et al., 2016) as an essential step to commercially-important biomaterial production (Cardozo et al., 2007). This was important because species of the genus *Ulva* Linnaeus contain high protein levels (17-33%) (Siddhanta et al., 2001; Tabarsa et al., 2012; Wang et al., 2014).

**NMR analyses reveal characteristics of ulvan**

The structural nature of the *U. lactuca* crude SPs extract was further investigated by NMR (both 1D ¹H and 2D ¹H-¹³C edited-HSQC experiments) (Quinderé et al., 2014) and the spectra of raw sample were recorded in D₂O solution at 25°C (Figure 1) (Tian et al., 2015) to offer information on the native molecule with other studies (Cardozo et al., 2007; Rodrigues et al., 2014; Wang et al., 2014). ¹H NMR spectrum revealed some unresolved resonance.
peaks at spectral window of expansion $\delta_H$ 5.4-1.3 ppm (Quinderé et al., 2014; Rodrigues et al., 2014) since heterogeneous composition (Figure 1A) is typical for SPs from seaweeds (Pomin & Mourão, 2008; Mourão, 2015; Rodrigues et al., 2016a).

Signals of anomeric H-1 suggested both $\alpha$-type and $\beta$-type glycosidic bonds as also recorded in the spectrum for a crude SP from *U. lactuca*, when obtained by ultrasonic-assisted extraction (Tian et al., 2015). Low-field signals ($\delta_H > 5$) in the $^1H$ NMR spectrum of the glycan confirmed the presence of $\alpha$-pyranoses (Tian et al., 2015) of the protons directly linked to carbons involved in glycosidic linkages (Pomin & Mourão, 2008; Rodrigues et al., 2014; Wang et al., 2014). The chemical shift at $\delta_H$ 5.23 and 5.05 ppm revealed minor amounts of $\alpha$-rhamnopyranosil units and possibly starch (Li et al., 2012), and at $\delta_H$ 4.92 ppm indicated C-1 of $\alpha$-L-rhamnose ($\alpha$-L-Rha) (Tian et al., 2015). Notably, $\beta$-D-xylose ($\beta$-D-Xyl) (at $\delta_H$ 4.42-4.55 ppm) linked to C-1/C-2 and glucose (Gluc) (at $\delta_H$ 4.66 ppm) appeared as considerably highest identities than those spectral values observed previously for these sugar residues co-extracted with native polymer (Tian et al., 2015).

Our observations could support the high content of total sugars prior measured these respective residues making part of the same molecule (Rodrigues et al., 2014) since enzymes help to obtain highest sugar yield and contents (Athukorala et al., 2006). Siddhanta et al. (2001) examined crude SPs extracts from *Ulva* species, when obtained by cold and hot water procedures. Those extracted with cold water showed to be enriched with high levels of hexose sugars, whereas the hot water extracts were rich in Rha, Xyl and Gluc as parts of the structural polysaccharide. By contrast, this question deserve to be better analyzed because the Dubois, Gilles, Hamilton, Rebers, & Smith (1956)' method measures small chains of polysaccharides.

Anomeric signals located at the high-field region ranging from $\delta_H$ 3.35 to 4.26 ppm could correspond to protons of the C-2-C-5 of sugar residues; of uronic acid at $\delta_H$ 3.70 (COO$^-$); and peaks assigned to $\delta_H$ 4.5 to 4.8 ppm would be of sulfated sugar residues and/or of uronic acid in polymeric blocks (Rodrigues et al., 2014; Wang et al., 2014). The spectral datum attributable to methyl of L-Rha was recorded at $\delta_H$ 1.33 ppm (Rodrigues et al., 2014; Tian et al., 2015).

Analysis by 2D $^1H$-$^13C$ edited-HSQC of the *U. lactuca* crude SP allowed to assign spin systems (Table 1C) 5.4-1.3/106-16 ppm (Quinderé et al., 2014; Rodrigues et al., 2016a) and some rhamnose, xylose, glucose and glucuronic acid residues were tentatively identified (Figure 1B), as also previously demonstrated from the $^1H$ NMR analysis (Figure 1A). The spectrum revealed at $\delta_{1H}$ 4.66/101.0 and 5.12/94.2 ppm resonances to the C-1 of D-glucuronic acid (GlcA) and $\alpha$-(1→4)-L-Rha, respectively (Tian et al., 2015). $\beta$-Gluc residue could also occur on GlcA signal; therefore, in the amorphous cellulose co-extracted with polymer (Wang et al., 2014). GlcA (at $\delta_{1C}$ 3.69/77.6 and 3.38/72.6 ppm assigned to the C-4 and C-2, respectively) and Rha/Xyl (at $\delta_{1C}$ 3.78/76.0, 4.4/66/68.0 and 4.3-4.7/73-77.0 ppm) were also identified, except for Xyl to C-5 that did not appear in the complex spectrum contrasting with the study of Tian et al. (2015), but characteristic values of C-6 of Rha (Li et al., 2012, Rodrigues et al., 2014) and CH$_3$-Rha (Li et al., 2012) were recorded (at $\delta_{1C}$ 1.33/15.60 and 3.6/56.1 ppm, respectively). Sugar residues not assigned here were derived from Xyl and Gluc (Tian et al., 2015). Ulvan is formed of aldobiouronic acid and 4-O-$\alpha$-D-glucuronosyl-L-Rha (Lahaye & Robic, 2007; Wang et al., 2014).
Collectively, our findings supported ulvan as characterized by infrared analysis from a more recent study (Araújo et al., 2016). On the basis of these comparative structural analyses, the current investigation suggested that the use of papain to obtain polysaccharides from the Brazilian samples of *U. lactuca* could also be useful as a bioprospecting strategy during comparison with others studies (Rodrigues et al., 2014; Rodrigues et al., 2016a).

**Electrophoretic analyses reveal physical-chemical homogeneity of complex glycans**

The profile of DEAE-cellulose anion-exchange chromatography of the crude SP confirmed the separation into two SPs fractions (UL-SP1 and UL-SP2) at 0.50 and 0.75 M NaCl, respectively (Figure 2A), as documented by Araújo et al. (2016), who formerly reported *U. lactuca* cell wall SPs isolation.

However, as observed in Figure 1A, DEAE-cellulose column-bound SPs were eluted with increasing molarity of NaCl and produced similar metachromasy between fractions, accounting the material recovered from the column almost equal between them, as also supported by their respective integrated metachromatic areas (IMAs) estimated from the chromatogram (Table 1). Siddhanta et al. (2001) extracted SPs from *U. lactuca* by cold and hot water and noted an important difference in the number of fractions eluted with NaCl on DEAE-cellulose column.

Total fraction yield was about 6.8-fold greater than that obtained for this same algal species (Araújo et al., 2016). Similar tendency of the fractions in this study was noted for their high contents of both sulfate (15.72 and 18.04%) and total sugars (60.58 and 59.73%) and no protein contamination from the analyzed polymer samples. This compositional profile also showed to be distinct than that found by Araújo et al. (2016), who revealed 0.75 M NaCl fraction (UL-SP2) with levels of 9.73- and 1.75-fold higher for sulfate and total sugars, respectively, than that obtained for this same algal species (Araújo et al., 2016). On the basis of their structural conformation and total sugars/sulfate ratio (Table 1) based on their interactions with the diamine (Dietrich & Dietrich, 1976) that showed similar eletrophoretic mobilities on gel, after toluidine blue treatment (Volpi & Maccari, 2002; Fidelis et al., 2014; Rodrigues et al., 2014a; Salles et al., 2017). This presumed certain regularity on their chains of ordered helical conformation of the *U. lactuca* SPs as commonly isolated from seaweeds (Athukorala et al., 2006; Pomin & Mourão, 2008; Wang et al., 2014) and/or when different methods are used, when using various algal species (Siddhanta et al., 2001).

**Figure 2.** (A) Separation of the crude SP from *Ulva lactuca* by DEAE-cellulose. Fractions were collected and checked by metachromasy using 1,9-dimethylmethylene blue (■). Electrophoreses in agarse gel (B) and polyacrylamide gel (C) of the SPs isolated from *U. lactuca*. Extract (E) and fractions (UL-SP1 and UL-SP2) and standard glycosaminoglycans chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa), dextran sulfate (DexS, 8 kDa) and/or uninfractionated heparin (UHEP, 14 kDa) present on gels were stained with 0.1% toluidine blue (a) and stains-all (b).

The physical-chemical characteristics of the SPs from *U. lactuca* were further analyzed by two electrophoretic techniques. For agarose gel analysis (Figure 2Ba), extract and fractions exhibited single, homogeneous and coincident metachromasy bands co-migrating as CS, but not as UHEP, suggesting SPs with same structural conformation and total sugars/sulfate ratio (Table 1) based on their interactions with the diamine (Dietrich & Dietrich, 1976) that showed similar eletrophoretic mobilities on gel, after toluidine blue treatment (Volpi & Maccari, 2002; Fidelis et al., 2014; Rodrigues et al., 2016a; Salles et al., 2017). This presumed certain regularity on their chains of ordered helical conformation of the *U. lactuca* SPs as described, but more refined studies are required (Wang et al., 2014; Yaich et al., 2014). Previous report from Araújo et al. (2016) described polydispersive SPs using the same procedure. On this view, results postulated that *U. lactuca* could perhaps biochemically change its matrix polysaccharide composition based on collection period (Cardozo et al., 2007; Wang et al., 2014; Rodrigues et al., 2016a).

**Table 1.** Yield and composition of SPs fractions obtained by DEAE-cellulose ion-exchange chromatography from the green seaweed *Ulva lactuca*.

| Fraction | NaCl (M) | IMA | Yield | Sulfate | Sugars | Sugar/sulfate | Proteins |
|----------|----------|-----|-------|---------|--------|--------------|---------|
| UL-SP1   | 0.50     | 52.65 | 36.88 | 15.72   | 60.58  | 3.85         | *       |
| UL-SP2   | 0.75     | 47.35 | 36.25 | 18.04   | 59.73  | 3.31         | *       |

* a – NaCl concentration; b – Integrated metachromatic area (IMA) determined as percentage from the Statistical Analysis Software version 8.0 Origin Program; c – Yield calculated as the percentage from a sample of extract applied on DEAE-cellulose column; d – Chemical dosages were correlated as previously reported by Araújo et al. (2016); e – total sugars/sulfate ratio; * Not detected.
The molecular distribution of the SPs from *U. lactuca* was clearly determined by stepwise of NaCl (DEAE-cellulose). The characterization by polyacrylamide gel analysis evidenced 0.5 M NaCl fraction (UI-SP1) of low molecular weights SPs as C-4-S (ca. 40 kDa) (Figure 2Ca) and similar to a SPs subfraction obtained from *Caulerpa cupressoides* (Chlorophyta) (Rodrigues et al., 2013), while a wide dispersion in the molecular masses (> 100 kDa) was noted for extract and fraction UI-SP2 (0.75 M NaCl) as usually described for SPs from seaweeds (Pomin, 2012; Fidelis et al., 2014; Rodrigues et al., 2016a and 2017a). Ulvaless contains two major SPs populations (500-800 kDa and 150-200 kDa) (Wang et al., 2014). However, our observations did not support Yaich et al. (2014), who previously reported that enzymatic extraction does not induce a decrease in the molecular size of the SPs from *U. lactuca* in comparison with the acid extraction (HCl, pH 1.5, 90°C); therefore, without impacting on the structure of the algal SPs. More refined studies using chromatographic methods are needed (Yaich et al., 2014; Mourão, 2015), since similar polyanionic characters between fractions were visualized (Figure 2Ba).

Although SPs were detected in extract and fractions by treatment with toluidine blue (Figure 2Ba), our investigations were also conducted to more precisely examine all the SPs preparations, when associated with the use of stains-all, to improve the sensitivity of the complex glycans (Figure 2Bb). This combined strategy resulted not only in a strong increase of the staining for standards and samples (especially fraction UI-SP1) similar to sulfated glycosaminoglycans from animal tissues (Volpi & Maccari, 2002), but also suggesting the presence of non-sulfated sugar residues from the examined material (Rodrigues et al., 2017a, b and c), reinforcing the solution NMR experiments which revealed acidic sugars residues, such as GlcA (Figure 1), a highly conserved structural identity in Ulvaceae (Wang et al., 2014; Tian et al., 2015). Rodrigues et al. (2016) isolated and compared three crude SPs extracts obtained from the red seaweed *Acanthophora musicoidea*. Procedures of agarose gel electrophoresis and NMR revealed charge density and structural homogeneities, respectively, of sulfated glycans extracted along the algal extracellular matrix.

In this study, such interpretations were also extended to the SPs stained with toluidine blue and stains-all from the polyacrylamide gel analysis (Figure 2Cb) (Rodrigues et al., 2017), as observed for glycosaminoglycans (Andrade, Oliveira, Tovar, Mourão, & Vilanova, 2017). On this basis, it was also hypothesized that the elimination of minerals (like cadmium and calcium ions) associated with the algal matrix by means of protease extraction could perhaps affect the molecular weight of the *U. lactuca* SPs or only deducing their molecular nature, when obtained by papain protocol, as reported in Ulvales (Wang et al., 2014; Yaich et al., 2014). Further studies should be conducted to clarify this supposition (Siddhanta et al., 2001; Wang et al., 2014).

Overall, these approaches would allow to analyze the course of extraction of matrix polysaccharides for the quality control (Volpi & Maccari, 2002; Salles et al., 2017), at least on initial level (Andrade, Oliveira, Tovar, Mourão, & Vilanova, 2017), of complex carbohydrates-based products (Cardozo et al., 2007; Wang et al., 2014) in the detection of polymeric contaminants, as already reported for sulfated glycans from animals (Volpi & Maccari, 2002; Salles et al., 2017).

### Table 2. Analysis of the SPs fractions, obtained by DEAE-cellulose chromatography, from the green seaweed *Ulva lactuca* on the coagulation *in vitro* using UHEP as a reference.

| Fractions | NaCl (M) | APTT (s) * | PT (s) ** | T IT & | IU mg -1 # |
|----------|---------|-----------|----------|-------|-----------|
| Ul-SP1   | 0.50    | 34.49 ± 0.09 | 11.20 ± 0.01 | 1.01 | 0.39 |
| Ul-SP2   | 0.75    | 38.31 ± 0.11 | 10.20 ± 0.02 | 1.10 | 0.43 |

**NaCl** - Sodium chloride; *Activated partial thromboplastin time (APTT)*; **Prothrombin time (PT)**; #SPs concentration to prolong the APTT or PT in seconds; &Ration for prolong the APTT test; *Effect expressed in international units (IU) per mg of SPs (IU mg -1); UHEP (193.00 IU mg -1): APTT [2.5 μg mL -1]: 42.15 ± 0.6 s; PT [100 μg mL -1]: 20.13 ± 0.6 s; Controls: 33.5 ± 0.08 s and 10.02 ± 0.01 s for APTT and PT tests, respectively (n = 3, p > 0.05 vs. control).
These preliminary results on the coagulation were consistent with those found by Shanmugam et al. (2001), who noticed uronic acid-rich low molecular masses SPs (U. lactuca) devoid of anticoagulation according to both APTT and PT models. In fact, the presence of acid sugar residues revealed by NMR analysis in the U. lactuca SPs composition (Figure 1) had no impact on the routine clotting assays, although soluble SPs extracted with papain and highly charged fractions (Table 1 and Figure 2A). Athukorala et al. (2006) obtained an enzymatic hydrolysate containing SPs from the brown seaweed Ecklonia cava and found strong anticoagulation dependently of their high degree of sulfation in detriment to low uronic acid content. Structural variability of the seaweeds SPs may reveal different anticoagulant mechanisms making analogies with that of UHEP (Pomin, 2012; Fidelis et al., 2014; Quinderé et al., 2014), which has thrombin inactivation by AT (Pomin & Mourão, 2008; Pomin, 2012; Mourão, 2015).

The limited values proved by classical coagulation models on the amount of thrombin generated (Castoldi & Rosing, 2011; Duarte et al., 2017) to measure plasma anticoagulants (Table 2) (Jung et al., 2014; Rodrigues et al., 2017a; Rodrigues et al., 2017c) led us to further evaluate possible effects of SPs from U. lactuca on an in vitro TG system. In this assay, different concentrations (4.1, 8.3, 41.6 and 83.3 μg well-plate⁻¹) (Rodrigues et al., 2016b; Rodrigues et al., 2017a; Rodrigues et al., 2017b; Rodrigues et al., 2017c; Rodrigues et al., 2018) of both fractions Ul-SP1 and Ul-SP2 added to 60-fold diluted human plasma attenuated TG in contact-activated system (intrinsic pathway) and thromboplastin-activated system (extrinsic pathway) (Nishino et al., 1999; Glauser et al., 2009), when the amidolytic activity of thrombin was continually measured at 37°C for 60 min., using in parallel UHEP as standard anticoagulant. No activator response of TG in plasma in the absence of cephalin or thromboplastin (negative controls) was observed in vitro for 60 min. (Rodrigues et al., 2016b; Salles et al., 2017; Rodrigues et al., 2018) (Figure 3).

Interestingly, under the conditions of our study, increasing concentrations of the SPs fractions samples of U. lactuca decreased the in vitro anticoagulant response on TG induced by cephalin vs. control without SPs (Figures 3A and B). Ul-SP1 and Ul-SP2 at 4.1 and 8.3 μg well-plate⁻¹ more drastically attenuated TG as analyzed by the PTh and TPeak parameters, inducing more than 50% inhibition of PTh (51.02 → 54.33%) compared with the positive control (TPeak: 20 and 24 min.), whereas the lag phase time (14 → 17 min.) remained almost unchanged. The required amount of the algal SPs was at least 4.15-fold greater than UHEP, which abolished TG at 2 μg well-plate⁻¹ (Rodrigues et al., 2016b) as a result of its AT-dependent specific mechanism absence to others SPs-rich marine organisms (Glauser et al., 2009; Pomin, 2012; Mourão, 2015).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effect of different concentrations of SPs fractions (Ul-SP1 and Ul-SP2), obtained by DEAE-cellulose, from the green seaweed Ulva lactuca on cephalin (A and B)- or thromboplastin (C and D)-triggered TG in 60-fold diluted human plasma using chromogenic method by a continuous detection system (37°C, 60 min.).
TG in diluted plasma treated with 41.6 and 83.3 µg well-plate\(^{-1}\) of the samples only inhibited the intrinsic system by 18.36→45.08% (PTh) (Figures 3A and B). This also indicated that the \(U.\) lactuca SPs increased the PTh as a suggestion of plasma hypercoagulability \textit{in vitro} (Zhang et al., 2014) as demonstrated by direct thrombin inhibitors (Furufohri & Morishima, 2015) and similar to the \textit{in vivo} experiments of venous thrombosis, in which algae SPs stimulated prothrombotic actions in rats (Rodrigues et al., 2011; Pomin, 2012; Quinderé et al., 2014) due to activation of factor XII involved in physiological formation of bradykinin, a vasoactive amine (Mourão, 2015). These observations could be connected with the analgesic and anti-inflammatory profiles on bradykinin pathway of UI-SP2 (Aratijo et al., 2016) and structure-function relationships deserve to be further examined (Pomin, 2012). Herein, SPs from \(U.\) lactuca also prolonged lag phase time (26→37 min.) from the controls, possibly predicting risk of bleeding on a prognostic view (Castoldi & Rosing, 2011), but animal studies must be conducted since seaweeds SPs do not manifest extensive bleeding effects based on those effects of UHEP inducing hemorrhage in its administration in rats (Rodrigues et al., 2011; Quinderé et al., 2014; Mourão, 2015).

TG in extrinsic pathway-activated plasma in the presence of the fractions UI-SP1 and UI-SP2 to evaluate their anticoagulant potential was also evaluated (Figures 3C and D). The capacity of TG was markedly reduced in plasma treated with low concentrations of the algal SPs (4.1 and 8.3 µg well-plate\(^{-1}\)) as verified by the PTh (50.90→58.18% inhibition) and TPeak (30→58 min.) parameters; but, with the lag phase time longer in UI-SP2 (39 min.) (Figure 3D) (Rodrigues et al., 2017c). No hypercoagulant stimulus at high doses of SPs was recorded for 60 min. based on total inhibition achieved at 83.3 µg well-plate\(^{-1}\).

In fact, the similar effects of the SPs fractions on the TG response were supported by their respective total sugar and sulfate contents (Table 1), being almost equal with the increasing molarity of the salt eluents, but these combined results were not expected (Aratijo et al., 2016). Furthermore, the SPs fractions samples of \(U.\) lactuca added in water did not produce viscosity in the presence of chloride calcium to display the coagulation \textit{in vitro} (Table 2 and Figure 3) since that the gel formation is described as a function of pH, association between borate and free hydroxyl groups of the polysaccharide, as well as the chelation of calcium ion with borate, and sulfation pattern (Wang et al., 2014). The papain would play an important role in the solubility of these polymers in water to experimentation (Aratijo et al., 2016).

Seaweeds have various chemical components (Cardozo et al., 2007; Thomas & Kim, 2013), including the possible existence of phenolic compounds as suggested for water extracts containing SPs obtained from the red seaweed \textit{Gracilaria} fisheri. These findings revealed a positive correlation between the \textit{in vitro} antioxidant effect and the phenolic compound content (Imjongjairak et al., 2016). However, to the best our knowledge, phenolic compounds have been widely described in higher plant showing antimalarial effect (Gadetskaya et al., 2015).

Although some phenolic compounds may influence the thrombin response (Bijak et al., 2014), it is still very premature to infer effects of these compounds derived from seaweeds on the TG system because no studies have also been done. Furthermore, the use of the papain method as mentioned above results in purity and enhanced yield of biologically active SPs for biotechnology (Athukorala et al., 2006; Mourão, 2015; Aratijo et al., 2016) displaying \textit{in vitro} TG inhibition (Glauser et al., 2009; Rodrigues et al., 2016b; Rodrigues et al., 2017a; Rodrigues et al., 2017b; Rodrigues et al., 2017c). The structural features revealed by NMR analysis supported the presence of ulvan-type polysaccharide in the crude SP extract (Figure 1) (Lahaye & Robic, 2007; Wang et al., 2014).

Therefore, it was observed in the present study that the \(U.\) lactuca SPs displayed their inhibitory effects more in the extrinsic pathway than in the intrinsic one, as an opposite behavior compared with a fucoidan isolated from the brown seaweed \textit{Ecklonia kurome} (Nishino et al., 1999); and for the SPs isolated from the Rhodophyta \textit{Gracilaria birdiae} (Rodrigues et al., 2017a); and Chlorophyta \textit{Caulerpa racemosa} (Rodrigues et al., 2017c); besides of skin of Nile tilapia \textit{Oreochromis niloticus} (Salles et al., 2017) on the basis of TG parameters and intrinsic inhibitory efficacy by UHEP at 2-fold higher. This peculiar role of the \(U.\) lactuca SPs fractions corroborated as activators of the intrinsic coagulation at high concentrations because UHEP only acted as a potent inhibitor of the coagulation \textit{in vitro} (Figures 3A and B) (Mourão, 2015). Collectively, our results seemed to be of practical use because \textit{in vivo} models of thrombosis in experimental animals are always a laborious assay (Pomin & Mourão, 2008; Mourão, 2015), although TG \textit{in vitro} no reflecting the physiological environment (Pomin & Mourão, 2008).

Overall, SPs from \(U.\) lactuca had no actions on the classical coagulation models (Table 2), but were clearly apparent using \textit{in vitro} TG assay (Figure 3) (Castoldi & Rosing, 2011; Zhang et al., 2014).
2014; Rodrigues et al., 2016b; Salles et al., 2017).

The degree of inactivation on TG by algal SPs
ecludes a positive correlation of sulfation and
molecular size (Figure 2) on these biological
processes, and seemed by dependence on their
stereospecific features, as also suggested for other
SPs from seaweeds (Rodrigues et al., 2016b;
Rodrigues et al., 2017a). SPs from seaweeds have
various bioactivities (Cardozo et al., 2007;
Rodrigues et al., 2012; Wang et al., 2014) and have
traditionally been used in food products as
thickening and gelling agents for hydrocolloid
industry worldwide (Cardozo et al., 2007; Wang
et al., 2018), and recently in diets for hens (Li
et al., 2018). Excessive consumption of
U. lactuca as raw or semi-processed material could perhaps lead to
physiological reactions associated with thrombosis and bioavailability studies regarding dietary fibers
have yet been scarce (Wang et al., 2014).

Regarding U. lactuca food safety, elucidation of
molecular mechanisms behind the biological effects
on the coagulation in vitro (Pomin, 2012; Mourão,
2015) is still needed as a critical step to
antithrombotic drug formulation (Zavyalova &
Kopylov, 2016).

Conclusion

The green seaweed Ulva lactuca contains ulvan-
type polysulfated polysaccharides with low
molecular sizes (ca. 40 and > 100 kDa) and non-
sulfated glycans with experimental thrombosis
inhibition by both intrinsic and extrinsic pathways
independently of charge and molecular size, when in
60-fold diluted human plasma using continuous
method of thrombin generation in vitro, but with
lower efficacies than heparin. Low amounts reveal as
potent inhibitors of contact-activated thrombin
generation, whereas a tendency of plasma
hypercoagulability is manifested with increasing
concentrations, arousing the attention of their
physiological risks linked to thrombosis.

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