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ANALYSIS OF *Botryocladia occidentalis* (RHODOPHYTA) SULFATED GALACTANS AS MODULATORS OF THROMBIN GENERATION

ANÁLISE DE GALACTANAS SULFATADAS DE *Botryocladia occidentalis* (RHODOPHYTA) COMO MODULADORAS DE GERAÇÃO DE TROMBINA

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Abstract The cell-wall of *Botryocladia occidentalis* (Rhodophyta) contains sulfated galactans (BoSGs) with anti- and procoagulant properties, but their effects on thrombin generation (TG) were fewer explored. This study evaluated BoSGs by different extraction times (3, 6, 16 or 24 h) on yield, physical-chemical features and *in vitro* effects on cephalin-induced TG in 60-fold diluted human plasma using continuous system. Yield of papain extraction from ground matter increased ($p<0.05$) with time from 5.81±0.71% (BoSGs-3) to 10.03±0.74% (BoSGs-24), with a difference reaching 4.49%. Agarose/polyacrylamide gels electrophoreses revealed profiles of discrete charge density patterns and molecular masses >100kDa among all the wood color-visualized BoSGs after independent staining with toluidin blue or Stains-All *vs.* glycosaminoglycans standards. Infrared spectroscopy characterized a system of agaran/carrageenan-unrelated BoSGs structure. Regarting TG assay, BoSGs-24 abolished TG at concentration >4.1 µg competing with thrombin, but induced TG in cepahlin-free system. Heparin also inhibited TG, but not stimulated it. Therefore, the technique optimized yield and selected BoSGs with *in vitro* TG modulation.

Key words: ultrastructure; anionic polymers; chemical aspects; clot.

Resumo A parede celular de *Botryocladia occidentalis* (Rhodophyta) contém galactanas sulfatadas (BoGSs) com propriedades anti- e procoagulante, porém foram pouco explorados seus efeitos sobre geração de trombina (GT). Este estudo avaliou BoGSs por tempos de extração diferentes (3; 6; 16 ou 24 h) sobre rendimento, características físico-químicas e efeitos *in vitro* sobre GT induzida por cefalina em plasma humano diluído 60 vezes usando sistema contínuo. O rendimento da extração com papaína, da matéria moída, aumentou ($p<0.05$) com o tempo de 5,81±0,71% (BoSGs-3) para 10,03±0,74% (BoSGs-24), alcançando diferença de 4,49%. Eletroforeses em géis de agarose/poliacrilamida revelaram perfis de graus de densidade de cargas discretos e massas moleculares >100kDa entre todas as BoGSs visualizadas em cor "madeira" após coramento independente com azul de toluidina ou "Stains-All" *vs.* padrões de glicosaminoglicanos. Espectrocopia de infravermelho caracterizou um sistema de BoGSs de estrutura não relaciona a agarana/carragenana. A respeito do essaio de GT, BoGs-24 aboliu GT em concentração >4.1 µg competindo com trombina, porém induziu GT em sistema sem cefalina. Heparina inibiu também GT, mas não estimulou ela. Portanto, a técnica otimizou rendimento e selecionou BoGSs com modulação de GT *in vitro*.

Palavras-Chave: ultraestrutura; polímeros aniônicos; aspectos químicos; coágulo.

Introduction

Seaweeds are sources rich in sulfated polysaccharides (SPs) playing important roles in structure and algal physiology to environmental responses (Cardozo et al., 2007). Their cell-wall architectures, in which these polymers occur abundantly (Rodrigues et al., 2009, 2016a), are diverses and varing the pattern of cell arrangement/organization (like cell shape, size and/or number) of the assembled structures. In these fibrilar compartments are found in the amorphous matrix SPs linked each other by proteins making part of the matrix comprexity (Pomin & Mourão, 2008). It has been believed that the study of the SPs morphology would offer important data for the undertading of the cell-wall anatomy in the seaweeds (Rodrigues et al., 2016a). Structurally, SPs are highly charged macromolecules due to the presence of sulfate ester (S=O) and, by phylum, red seaweeds are rich sources in sulfated galactans (SGs) (carrageenan and agaran) (Cardozo et al., 2007), while fucans or fuicodans are present in brown seaweeds (Pomin & Mourão, 2008); and sulfated heteropolysaccharides biosynthetized by green seaweeds (Rodrigues et al., 2019). The yield and composition of SPs vary depending on seaweed species, when also obtained by *e.g.*, different extraction protocols, period/region of collect, and post-collect treatment (Cardozo et al., 2007; Farias et al., 2000; Pomin & Mourão, 2008; Rodrigues et al., 2017).

SPs from seaweeds have been reported, for exemple, as antinociceptive, anti-inflammatory (Rodrigues et al., 2012), anticoagulant (Rodrigues et al., 2009) and antithrombotic (Mourão, 2015). Anticoagulant action of SPs has been the most studied, particularly those from the red seaweeds (Mourão, 2015; Pomin & Mourão, 2008; Rodrigues et al., 2009). SPs derivated-anticoagulant property is traditionally analyzed by the activated partial thromboplastin time and the prothrombin time tests (Rodrigues et al., 2009, 2017, 2021); however, their uses show limited response on the thrombin generated in these systems, reflecting only traces of this protein formed in the plasma (Castoldi & Rosing, 2011). Thrombin generation (TG)-based coagulation assays have been developed by some companies or alternatively by different scientific groups in order to examine plasma coagulability (Castoldi & Rosing, 2011) and anticoagulants (Glauser et al., 2009; Rodrigues et al., 2016b, 2017, 2021; Zhang et al., 2014). The search by new methods for analysis of alternative sources of anticoagulants is currently necessary because the clot complications are originated by disorders of the cardiovascular system and the risks associated to unfractionated heparin (UHEP) (*e.g.*, thrombocytopenia, pathogenic particles, and hemorrhagic effect) used in clinical practice, whose drug is obtained from pig intestine or bovine lungs where it is commercially extracted, although in low concentrations (Mourão, 2015).

Studies on the *Botryocladia occidentalis* (Børgesen) Kylin, 1931 (Rhodymeniaceae, Rhodymeniales) red seaweed, a widespread species in many regions of the world, are still scarce, not only on its population structure (Venera-Pontón et al., 2019), but also its cell-wall SPs (Sampaio et al., 2020). This arborescent species has dark red/rose coloring of an anatomy of cell-walls that make up clusters of round pneumatic vesicles in which SPs fill the amorphous matrix (Figure 1A) (Sampaio et al., 2020). Farias et al. (2000) isolated a D-SG from Brazilian samples collected from reef ecosystem at Pacheco beach, Ceará. It is formed by a homogeneous backbone, consisted of the disaccharide repeating unit [→4)-α-D-Gal-(1→3)-β-D-Gal-(1→], but revealing heterogeneous based on its sulfation pattern. Nuclear magnetic resonance (NMR)-based studies showed that this SP is constituted by 2,3-disulfated (~33%) or 2-sulfated (33%) in α-D-galactose and, in β-D-galactose units, by 2-sulfated (Figure 1B). The addition of two sulfate esters to a single α-galactose residue displays a potent anticoagulant effect, similar that of UHEP. The *B. occidentalis* SGs (BoSGs) have also been investigated as antithrombotic (Farias et al., 2001; Fonseca et al., 2008) and its mechanisms of pro- and anticoagulant action (Farias et al., 2001; Fonseca et al., 2008; Glauser et al., 2009), as well as reducing mortality in shrimp post-larvae (Barroso et al., 2007), antimalarial, antiophidic (Sampaio et al., 2020) and antiviral (Kim et al., 2022) properties. Maurya et al. (2023) reexamined its structure and observed that the occurence of 4-linked α-3,6-

anhydrogalactose units displayed both anti-SARS-CoV-2 and anticoagulant properties. Nevertheless, an extensive study on its matrix SGs and *in vitro* TG potential using a continuous system remain unexplored.

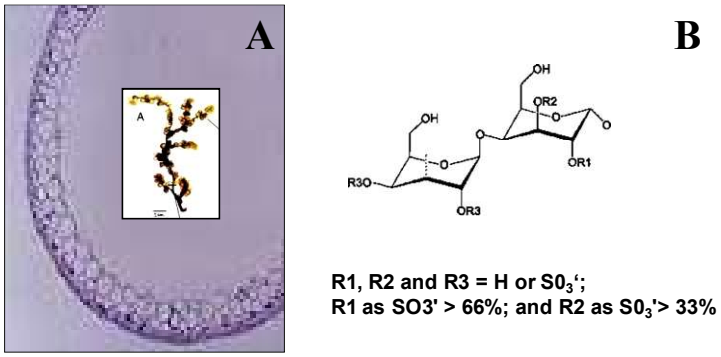

Figure 1. Anatomic structure of the cell-wall (A) in which sulfated D-galactans (in purple red) (B) occur embedded in the amorphous matrix of the red seaweed *Botryocladia occidentalis* (Børgesen) Kylin (Farias et al., 2000; Sampaio et al., 2020).

The scope of this study was to expand our understanding of the BoSGs on two directions: 1) applying different times to extract SGs on an effective approach to identify and biochemically characterize its cell-wall architecture; and 2) by this technique, it could be used as an auxiliary model to select modulators of TG *in vitro* using diluted human plasma.

Material and Methods

Seaweed collection and enzimatic extraction of BoSGs

Brazilian samples of *B. occidentalis* red seaweed were collected by hand during low tide from a natural bed located at Pacheco beach (Caucaia, Ceara) throughout a research expedition carried out by our group. The specimens were pre-selected in the environment and then placed in plastic bags for transport to the Marine Biochemistry laboratory of the Center of Biotechnology Applied to Aquaculture, Department of Fishing Engineering, Federal University of Ceará for SGs extraction. They were manually cleaned from epiphytes, washed with distillated water to remove residues and salt, and further frozen at – 20°C (Farias et al., 2000). As archive, a sample of this specimen was reposted in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil, being part of the biological structure used in this investigation. The collection was also authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

B. occidentalis biomass was dehydrated in the sunlight for 24 h, cut into small pieces and then ground before the extraction process. BoSGs were obtained according to the Farias et al. (2000)' method. Based on a brief scheme (Figure 2), 5 g ground samples were suspended in 100 mL of 100 mM sodium acetate buffer (pH 5.0) containing 510 mg of crude papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 60°C for different times (3, 6, 16 or 24 h). The incubation mixtures were then filtered using a nylon screen and the supernatants were saved. BoSGs that were present in solution were precipitated with 16 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature for 24 h. The mixtures were then centrifuged at 9.560 × *g*, for 20 min. The *pellet*s containing the BoSGs were washed with 100 mL of 0.05% CPC solution, dissolved under agitation (for 20 min) in 100 mL of a 2 M NaCl: ethanol (100:15 ratio, v:v) solution, and then re-precipitated for 24 h at 4°C with addition of 100 mL of ice-cold commercial ethanol. Separately, the precipitates

obtained were centrifugated (9.560 × *g* for 20 min), washed twice with 100 mL of 80% ethanol, and once with the same volume of commercial ethanol. The BoSGs were finally dried using an air drying oven (60°C, 6 h) and the yields were expressed as the percentage (%, n = 3) of the dehydrated matter. The crude extracts obtained in each period were named as BoSGs-3, BoSGs-6 BoSGs-16 and BoSGs-24, respectively.

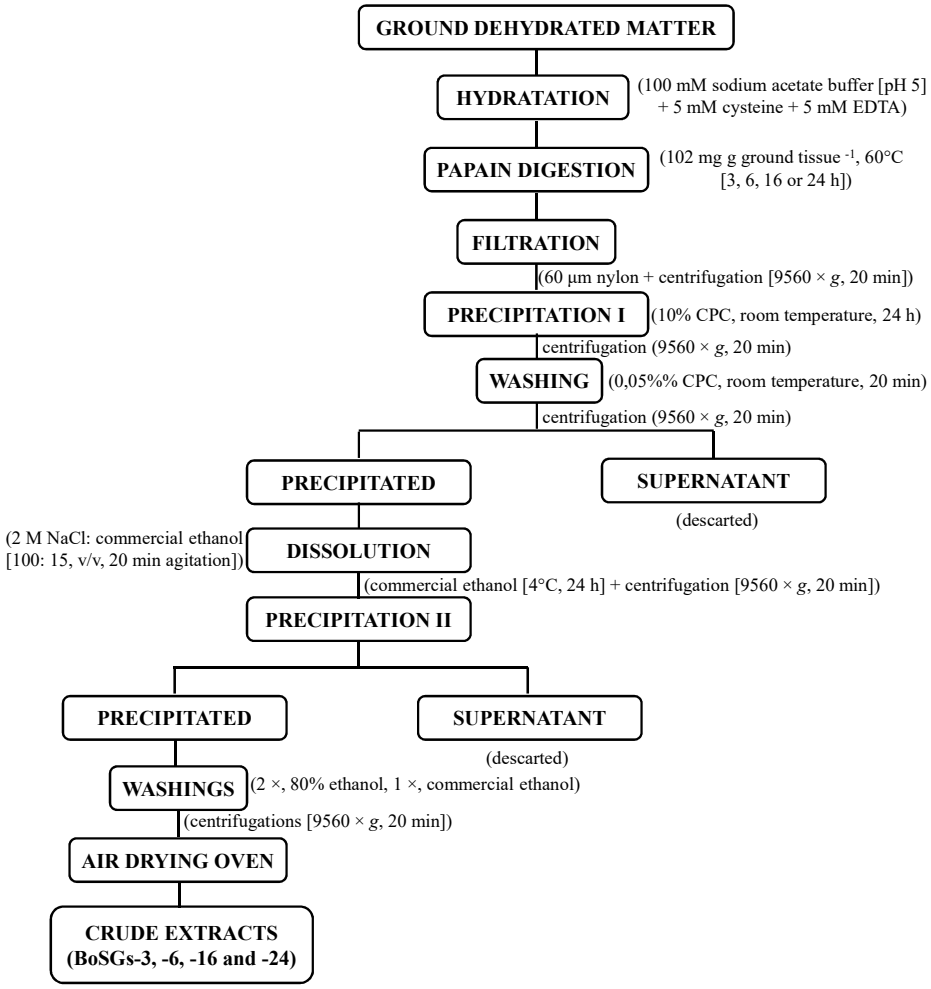

Figure 2. Scheme of obtaining of SGs from the red seaweed *B. occidentalis*.

Physical-chemical characterization by electrophoreses

The BoSGs were analyzed by two electrophoretic techniques: 1) in agarose gel, regarding pattern and charge density, and 2) in polyacrylamide gel, regarding molecular mass distribution. In the first technique, the BoSGs-3, -6, -16 or -24 samples were applied to a 0.5% agarose gel prepared with 0.05 M 1,3-acetate diaminopropane buffer (pH 9.0) and the run was carried out at constant voltage (100 V, 1 h). After the run, the BoSGs present in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for 24 h and then dehydrated (Dietrich & Dietrich, 1976). In the second procedure, the same samples were applied to a 6% polyacrylamide gel using 0.02 M Tris/HCl buffer (pH 8.6) and the run was performed at 500 mA for 1 h (Rodrigues et al., 2016a). The BoSGs present in both gels were revealed with 0.1% toluidine blue or Stains-All and, subsequently, the gels were decolorized with a solution containing absolute ethanol, distilled water and acetic acid or using distilled water only. Chondroitin-6-sulfate (C-6-S,~60 kDa), chondroitin-4-

sulfate (C-4-S,~ 40 kDa), sulfated dextran (DexS,~ 8 kDa), dermatan sulfate (DS,~ 40 kDa) and/or UHEP (~15 kDa) were used as standards (Andrade et al., 2017; Volpi & Maccari, 2002).

Characterization of BOSGs by Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR spectra were generated using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, 10 mg of BOSGs samples were pressed in KBr *pellets*. The measurements were performed at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at 500-4000 cm^{-1}.

Human plasma preparation for *in vitro* clotting analysis

A total of 10 different healthy donors (University Hospital Clementino Fraga Filho, Rio de Janeiro, Brazil) were used for TG study. A quantity of 8 mL venous blood was drawn into 4.5 mL Vacutainer polypropylene tubes containing 3.2% sodium citrate. After collection, platelet-poor plasma was separated by centrifugation (2000 × *g*, 15 min.) and the plasma supernatant aliquoted (1 mL) into Eppendorf vials and frozen at - 70°C until use (Rodrigues et al., 2021). The biological study of the BoSGs were performed at Connective Tissue laboratory, Federal University of Rio de Janeiro, Brazil, and the approved by the Ethical Committee of the same institution.

Effects of BoSGs-24 on TG *in vitro*

This assay was based on Rodrigues et al. (2016b and 2021) in microplate format with 10 µL rabbit brain cephalin (contact-activator system) + 30 µL 0.02 M Tris HCl/PEG buffer, pH 7.4 + 10 µL polysaccharides (BoSGs-24: 0, 4.1, 8.3, 41.6 or 83.3 µg well-$plate^{-1}$ or UHEP: 2 well-$plate^{-1}$) + 60 µL 20 mM CaCl2 and 0.33 mM chromogenic substrate S-2238 (10:50 ratio, v:v). The reaction was triggered at 37°C by addition of 60-fold diluted normal human plasma (10 µL), and the absorbance (405 nm) was read for 60 min. using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). Inhibitory response of TG by polysaccharides after the addition of the diluted plasma was determined by lag time (related to the initiation phase of coagulation), peak thrombin and time to peak (that reflect the amplification phase of coagulation).

Examination of the use of BoSGs-24 on substrate using purified protease system

The interaction of BoSGs-24 on the substrate was also continuously analyzed using a purified thrombin system (Rodrigues et al., 2021). Different concentrations of BoSGs-24 (0, 4.1, 8.3, 41.6 or 83.3 µg well-$plate^{-1}$) or UHEP (2 µg well-$plate^{-1}$) (10 µL) were used with 1.66 nM thrombin (10 µL) in 0.02 M Tris HCl/PEG buffer, pH 7.4. The reaction mixture was triggered in final volume of 120 µL by adding 60 µL 20 mM CaCl2 and 0.33 mM chromogenic substrate S-2238 (10:50 ratio, v:v), and the absorbance (405 nm) was read at 37°C for 80 min using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA).

Effect of BoSGs-24 on TG in plasma devoid of cephalin

This study was carried out using a microplate format, without cephalin. The test was conducted as follow: 40 µL 0.02 M Tris-HCl/PEG buffer (pH 7.4) + 10 µL BoSGs-24 (0, 4.1, 41.6 or 83.3 µg well $plate^{-1}$; UHEP: 2 µg.well-$plate^{-1}$) + 60 µL 20 mM CaCl2/0.33 mM chromogenic substrate S-2238 (10:50 ratio, v:v). The *in vitro* reaction was triggered at 37°C by the addition of plasma (diluted 60-fold well-$plate^{-1}$, 10 µL), and the substrate hydrolysis was detected at 405 nm every 1 min (120 min., 37°C) using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The stimulatory response of TG by BoSGs-3 was analyzed by absorbance of the assay (Rodrigues et al., 2021).

Statistical analyses

For extraction yield analysis, values by time (mean ± standard deviation, n = 3) were subjected to one-way ANOVA, followed by Tukey' test, applying $p < 0.05$ as significant. For *in vitro* TG study, experimental data (mean ± standard deviation, n = 3), calculated from the control curves (%), were analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. The graphical representations of TG were constructed using the Origin software version 8.0 as the Statistical Analysis Software (USA). All the experimental data were calculated from the controls (%) and the statistical analyses performed applying GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com).

Results and Discussion

Extraction yield of the *B. occidentalis* extracelular matrix SGs

The ground tissue of the dehydrated *B. occidentalis* biomass was sujected to nonspecific proteolytic digestion using papain, followed by both CPC and ethanol precipitations, and showed to be an effective approach to recover BoSGs, when different times (for 3, 6, 16 or 24 h, at 60°C) were separately applied. The yields of BoSGs were also compared with other studies (Table 1).

Table 1. Yields of SPs extracted from red seaweeds collected from coastal zones of Brazil/USA.

Seaweeds	SPs	Protocol / time	Tissue	Yield	Reference
B. occidentalis (Pacheco beach)	BoSGs-3	papain/3 h	ground	5.81±0.71%[a]	This study
	BoSGs-6	papain/6 h		9.11±1.37%[b]	
	BoSGs-16	papain/16 h		10.75±0.42%[b]	
	BoSGs-24	papain/24 h		10.03±0.74%[b]	
B. occidentalis (Pacheco beach)	BoSGs	papain/24 h	tritured	4.00%	Farias et al. (2000)
B. occidentalis (Gulf coast)	BoSGs	papain/24 h	tritured	6.00%	Maurya et al. (2023)
Halymenia pseudofloresia (Flecheiras beach)	SGs	papain/24 h	tritured	40.50% 4.90% 1.74%	Rodrigues et al. (2009)
Acanthophora muscoides (Pacheco beach)	SGs	papain/24 h	tritured	17.00% 4.65% 1.82%	Rodrigues et al. (2016a)

***** Yield was calculated as percentage (%) with basis of the dehydrated algal tissue; Different letters among the lines indicate significative diference at level of 5% (ANOVA, Tukey' test, $p < 0.05$).

BoSGs obtained after 3 h presented the lowest extraction yield (5.81 ± 0.71%, $p < 0.05$) compared with 6 (9.11 ± 1.37%), 16 (10.75 ± 0.42%) or 24 h (10.03 ± 0.74%), respectively, whose yields were almost equal among them ($p > 0.05$), as also shown in table 1. Therefore, the prolonged extraction period was decisive to obtain larger amounts of BoSGs, since the enzymes are useful to isolate and characterize biotecnologically-important cell-wall SPs (Barroso et al., 2007; Rodrigues et al., 2009, Kim et al., 2022; Maurya et al. (2023).

Another observation of this study was to an extraction yield level of BoSGs, from ground tissue, ranging, of at least, from 1.45 (BoSGs-3) to 2.68- (BoSGs-16) fold higher than those found by Farias et al. (2000) and Maurya et al. (2023), using tritured tissues of this same algal species, but a smaller source in SPs than other Rhodophyta species (Table 1). In fact, the ground *B. occidentalis*

biomass would allow greater action of the papain on the tissue, resulting in the greater yield of extracted SGs. It could also be explained based on its cell-wall anatomy (Figure 1A), in which the use of proteolysis would lead to a disorganization of the matrix structure by the cleavage between SGs and proteins (Rodrigues et al., 2016a); initially, releasing minor amounts of these components (BoSGs-3) from the surface texture and intercelular spaces of bolth stalks due to the existence of numerous peripheral cells (Sampaio et al., 2020). Extending digestion time (from 6 to 24 h), SGs would be extracted in greater amounts from the space of the pneumatic vesicles, in which the molecules will be more concentrated in the *B. occidentalis* tissue (Sampaio et al., 2020).

This methodology may be valuable to characterize and recover SGs of the red seaweed cell-wall ultrastructure compared to other protocols (Rodrigues et al., 2009; 2016a), as well as by production of the polymers by seaweed when it is affected by ecophysiological factors and antropogenic activity of the litoral region (Cardozo et al., 2007; Rodrigues et al., 2017).

Agarose/polyacrylamide gels electrophoreses

Further step of this study was to identify and characterize BoSGs from its extracelular matrix (Figure 1) by two electrophoretic procedures (agarose/polyacrylamide gels) (Figure 3). In agarose analysis, all the BoSGs extracts exhibited metachromasia and polydispersion visible after staining with toluidine blue alone, especially for BoSGs-24 extraction that showed a strongest band (charge density) among all the analyzed samples (Figure 3A), as typical for seaweeds-derived SPs (Farias et al., 2000; Rodrigues et al., 2009, 2017).

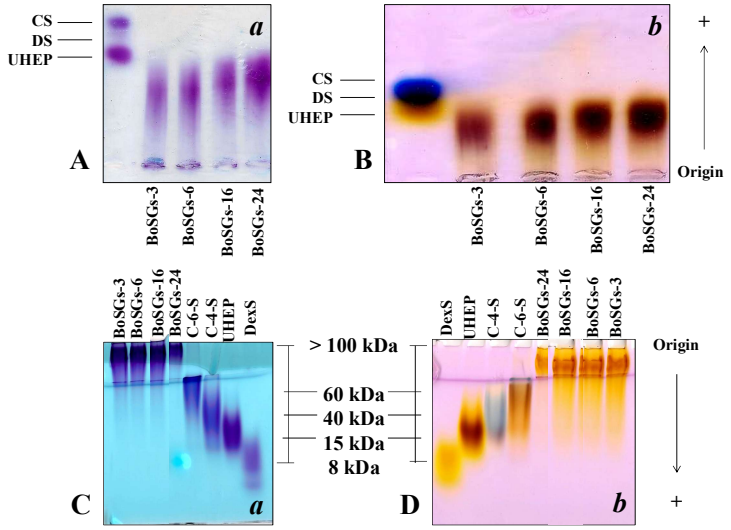

Figure 3. Agarose (A, B) / polyacrylamide (C, D) gels electrophoreses of *B. occidentalis* SGs (BoSGs-3, -6, -16, or -24), and standards chondroitin-6-sulfate (C-6-S,~60 kDa), chondroitin-4-sulfate (C-4-S,~40 kDa), dextran sulfate (DexS,~8 kDa), dermatan sulfate (DS,~ 40 kDa) and unfractionated heparin (UHEP,~15 kDa) present on gels were stained with 0.1% toluidine blue (a) or Stains-All (b).

The different preparations of BoSGs applied on gel presented a discrete mobility among them and close to UHEP, suggesting that these BoSGs would have a difference in their spatial configurations (structures) when isolated from different regions of the algal tissue (Rodrigues et al., 2009, 2017) or revealing distinct sulfate contents making part of the highly complex structure (Farias et al., 2000; Sampaio et al., 2020). These interpretations are reasonable due to fact that the diamine of the buffer system form a complex with SPs based on those from animals (Dietrich & Dietrich, 1976).

This highly heterogenous composition of BoSGs was expected for specimens from natural habitat, since no here analyzed sample was purified to yield a non-polydisperse blot comigrating as

DS standard (Sampaio et al., 2020). While staining with Stains-All alone (Figure 1B), as another cationic reagent employed in order to increase the capacity of detection, revealed an electrophoretic profile of BoSGs extracts with an intense colorating of bands in the agarose gel as visualized in a specific color-code for wood; and standards showing colors: UHEP in yellow and mixture of CS/DS in blue as preponderant in this system; therefore, it was possible to distinguish the SPs based on contrasting colors present in all analyzed samples *vs.* standards, at least on initial level, in the course of the extraction processes, than when compared those detected by sequential staining (Rodrigues et al., 2017, 2021; Volpi & Maccari, 2002).

Electrophoretic procedure using polyacrylamide gel was able to reveal SPs in purble/pink after toluidin blue treatment (Figure C) considerating other authors (Andrade et al., 2017; Maurya et al., 2023; Kim et al., 2022; Rodrigues et al., 2016a, 2017). Clearly, each SP was also identified with basis on its molecular weight where were easily distinguished by their distinct mobility on gel. In the case of the BoSGs, they did not show any migration and the blots stayed concentrated in the origin, which were characterized as high molecular weight SGs (> 100 kDa), as previosly described by Maurya et al. (2023) and Sampaio et al. (2020). Even with Stains-All application alone (Figure D), it was observed a coincidence between BoSGs and UHEP in terms of color to recognize themselves on gel. It is probably due to their higher anionic/composition charge compared with C-6-S (reddish-brown color), C-4-S (reddish-blue color) and DexS (light yellow) which have lower-relatively anionic charge and carboxylated groups of uronic acid yielding distinct blots (Andrade et al., 2017).

Overall, both gels revealed physical-chemical properties of SPs to use by glycobiolists, although their chemical interations be versatiles in nature, opening frontiers for new studies (Rodrigues et al., 2017).

Structural analysis of BoSGs by FT-IR

To understand what BoSGs accessibility in the cell-wall after each period of extraction, the SGs-rich extracts obtained by protease (BoSGs-3 or -24 h) were further characterized and compared by FT-IR. The BoSGs extracted not only by 3 h, but also after 24 h comparatively showed signals almost identical from the two analyzed samples (Figure 4).

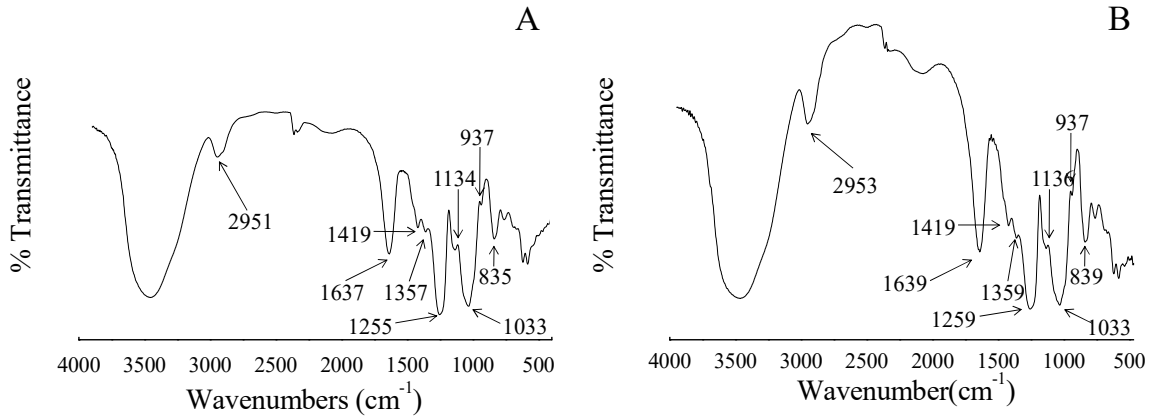

Figure 4. FT-IR spectra of the *B. occidentalis* SGs (BoSGs-3 [A] and BoSGs-24 [B]) at 500-4000 cm^{-1}.

Among the main absorption bands in the examined range (500-4000 cm^{-1}), it was found typical signals of ester sulfate groups at 1357-1359 cm^{-1} and S=O (at 1255-1259 cm^{-1}), whose intensities of these functional groups were also similar between them. The FT-IR spectra of both Bo-SGs-3 and -

24 h also displayed bands at 937 cm^{-1} corresponding to 3,6-anhydrogalactose, at 1033 cm^{-1} (galactose) and at 835-839 cm^{-1} (galactose-4-sulfate); as well as the presence at 2951-2953 cm^{-1} of C-H content. Uronic acid was also speculated at 1637-1639 cm^{-1} and 1419 cm^{-1}; and at 1134-1136 cm^{-1} of vibrating piranosic ring in native polysaccharide.

Collectively, these values of FT-IR partially suggested as a complex galactan structure (Rodrigues et al., 2021), since the initial study was performed by Farias et al. (2000) using NMR analysis that offered more detailed spectral data. The structure is composed by a simple repeating disaccharide of alternating 4-linked α-galactopyranose and 3-linked β-galactopyranose units with variable sulfation pattern, and one-third of 2,3-disulfation and one-third of 2-monosulfation.

Current investigation revealed the absence of galactose-4-sulfate at 845 cm^{-1}, galactose-6-sulfate at 820 cm^{-1} and 3,6-anhydrogalactose-2-sulfate at 805 cm^{-1}, which are related to agaran and/or carrageenan structures in red seaweeds (Cardozo et al., 2007; Rodrigues et al., 2021). In fact, Sampaio et al. (2020) reported to an agaran/carrageenan-unrelated BoSGs structure, but revealing important physical-chemical and biological properties. Maurya et al. (2023) carried out a more refined analysis regarding on the BoSGs structure and discovered that it contains a more complex sulfation pattern due to the presence of 4-linked α-3,6-anhydro-galactopyranose units. Anhydrosugars-rich SGs display gelling properties for industrial use (*e.g.*, cosmetic and pharmaceutical) (Cardozo et al., 2007). On an algal basis, the occurence this type of complex SGs with variable sulfation pattern and anhydrogalactose residues making part of its extracelular matrix could exercise a crucial role in the face of environmental disturbances (Cardozo et al., 2007; Rodrigues et al., 2017).

Therefore, FT-IR spectroscopy suggested structural regularity of the BoSGs extracted from cell-wall. This procedure combined with the use of different periods of extraction and analysis by conventional biochemical techniques could represent as an additional supplement to identify, at initial level, molecular basis to assist with criteria that also aim at the taxonomy of these marine organisms (Joly, 1965).

Based on the physico-chemical and structural results (Figures 3 and 4), BoSGs-24 was chosen for *in vitro* TG studies.

Analysis of BoSGs-24 on a TG system

The *in vitro* anti-TG potency of BoSGs-24 was assessed by an alternative method developed by Rodrigues et al. (2016b). This protocol is characterized by a 60-80 min. period of continuous measurement of thrombin formation in 60-fold diluted plasma well-plate^{-1} using chromogenic substrate and UHEP as anticoagulant drug (Rodrigues et al., 2017, 2021).

In this study, BoSGs-24 samples of concentrations from 4.1 to 83.3 µg well-plate^{-1} were capable of inibiting the TG response in diluted human plasma when stimulated by cephalin-activated intrisic coagulation pathway (Figure 5A). BoSGs-treated diluted plasma modified the TG parameters (peak thrombin (%), time to peak, and *lag* time) and reduced by 58.67 ± 0,25% at 48 ± 1.00 min. and with *lag* time at 35 ± 1.00 min., respectively, when at the lowest concentration used ($p < 0.005$), while at higher concentrations (> 4.1 µg well-plate^{-1}) abolished TG *in vitro* (100% peak thrombin inhibition) *vs.* control curve (at 40 min.).

The *in vitro* effects of BoSGs-24 were less effectives than UHEP, obtained from porcine, which completely supressed TG at 2 µg well-plate^{-1}; therefore, ~2-fold lower concentration than BoSGs-24. As espected, no inhibition of TG was detected in the absence of cephalin (negative control) based on Rodrigues et al. (2016b, 2017, 2021).

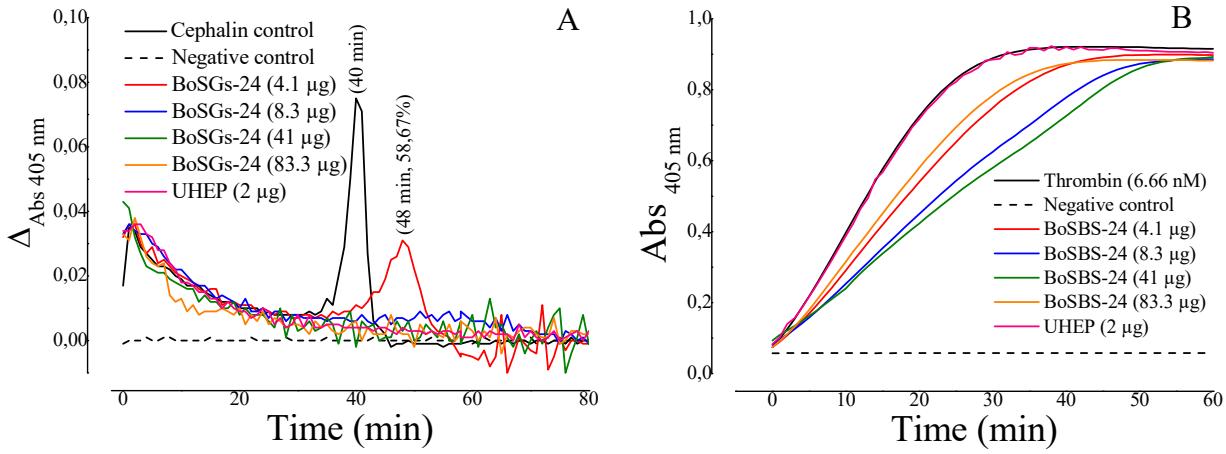

Figure 5. Effect of different concentrations of BoSGs-24 on cephalin-induced TG using 60-fold diluted human plasma (A) and purified Factor II system (B) by continuous detection method (37°C, 60-80 min.).

TG assays are global tools to more refined detection the clot formation (Castoldi & Rosing, 2011) and verify the dynamic of anticogulant SPs (Glauser et al., 2009; Rodrigues et al., 2016b, 2017, 2021; Zhang et al., 2014). The potent anticoagulant action of BoSGs was initially described by the activated partial thromboplastin time test *in pool* plasma of healthy donors and specific proteases assays that revealed with effects similar to those of UHEP, although these SPs exhibing distinct structures and origins (Farias et al., 2000; Mourão et al., 2015; Sampaio et al., 2020; Maurya et al., 2023). The nature of these interations in plasma are complex, not as mere consequence from their sulfation level, but also sterospecific features (Pomin & mourão, 2008; Mourão, 2015).

By electrophoreses it was show that BoSGs-24 had highest charge density that BoSGs-3-16 and high molecular masses (> 100 kDa) (Figure 3). BoSGs-24 at concentrations of > 4.1 µg well-plate^{-1} had higher electrostatic specifity of its sites de action with the inhibitors of the coagulation system for abolish TG (Figure 5A). UHEP has a particular anticoagulant mechanism not found in seaweeds SPs; it is formed by an antithrombin-binding pentasaccharide displying anticoagulation (Mourão, 2015). These modulatory actions by SPs are also important tools to the understanding of the anticlotting process, including *in vitro* and *in vivo* assays (Farias et al., 2001; Fonseca et al., 2008; Glauser et al., 2009).

In purified system, BoSGs-24 at concentrations of > 4.1 µg well-plate^{-1}, there were difference marked among the analyzed polymer samples (Figure 5B). As expected, UHEP no interacted with thrombin substrate in the concentration used; however, a different profile of BoSGs-24 was noted, especially at intermediate concentrations than those extremes tested. Therefore, BoSGs-24 acted as a competitor for thrombin in contrast with SGs isolated from the Rhodophyta *Acanthophora muscoides* that no modified the control assay profile (Rodrigues et al., 2021). These combinated observations would be determinate by molecular heterogeneities of the seaweeds SPs to differently modulate the coagulation (Pomin & Mourão, 2008; Glauser et al., 2009).

BoSGs were also reported as inductors of coagulation (thrombus formation), but *in vivo* experimental models using venous procedure require at high doses of sample (> 0.5 mg kg body weight) (Farias et al., 2001; Fonseca et al., 2008); therefore, are laborious and associated to molecular structure with coagulation cofactors (Mourão, 2015; Pomin & Mourão, 2008).

In order to partially investigate the *in vitro* TG-inducing seaweed SPs (Zhang et al., 2014), it was developed a practical approach using a cephalin-free TG model (Rodrigues et al., 2019). BoSGs-24

induced the clot formation in 60-fold diluted human plasma during 2 h, with only at concentration of 83.3 µg well-plate^{-1} (~ 60% induction); while, as espected, UHEP did not produce such effect (data not shown) (Rodrigues et al., 2019, 2021). This initial action of BoSGs-24 in plasma would be perhaps related to FXII activation as a procoagulant response derivated from its high molecular mass as already mentionated (Figures 3C, D) and observed for other seaweeds SPs (Rodrigues et al., 2019, 2021). In previous studies, BoSGs did not produce bleeding effect (hemorrhagic risk) (Farias et al., 2001) and toxicity in shimps (Barroso et al., 2007) and HEK-293T-hACE2 cells (Maurya et al., 2023).

In summary, *B. occidentalis* biomass pretreatment before extraction process using its ground tissue was an important step to select appropriate and effective conditions that optimized yield, physical-chemical aspects and anti- and pro-TG properties of its SGs. Due to the increasing use of seaweeds as raw or semi-processed material and the lack of studies on the physiological reactions of these dietary fibers (Carneiro et al., 2014), as well as the use them for the development of various products by the industry (Smit, 2004; Cardozo et al., 2007), their chemical and biological analyses are extremely important to determine the health-related potential risks (*e.g.*, thrombosis-related effects).

Conclusion

This study contributed as an effective technique to optimize yield and select *Botryocladia occidentalis* (Rhodophyta) agaran/carrageenan-unrelated sulfated polysaccharides as modulators of thrombin generation *in vitro*, when extracted from its ground biomass.

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