



***In vitro* effects of an *Acanthophora muscoides* (Ceramiales, Rhodophyta) native and modified sulfated polysaccharide fraction on thrombin generation**

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ABSTRACT. The structural complexity of the agaran type-sulfated polysaccharides (SPs) found in *Acanthophora muscoides* limits its investigation as anticoagulant alternative to heparin which induces clot complications. This study was extended to evaluate the properties of a SPs fraction and its alkali/desulfated derivatives on an intrinsic pathway-induced thrombin generation (TG) continuous model using 60-fold diluted normal or serpins-depleted human plasma. 0.75 M NaCl-eluted SPs fraction by DEAE-cellulose chromatography containing sulfate (35.20%), total sugars (55.97%) and no proteins showed charge homogeneity and heterogeneous molecular weight by agarose/polyacrylamide gel electrophoresis, respectively, using sequential staining with toluidine blue and Stains-All. Fourier Transform Infrared spectroscopy confirmed agaran-structure. Intact fraction poorly acted on the activated partial thromboplastin time (3.10 IU) than heparin (193 IU), but there was a preponderance of the serpin-independent effect than serpin-dependent one in TG assay comparing both systems was continually recorded. Heparin abolished plasma TG, but was inactive in depleted human plasma. While desulfated derivative of the respective fraction anticipated and induced thrombin formation vs. untreated plasma. The results suggested that sulfated sugars residues in the saccharide units of the polymer appear to be important to attenuate TG *in vitro*.

Keywords: anionic polymers; chemical modification; plasma systems; thrombosis.

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Introduction

The blood coagulation system is a complex sequence of enzymatic reactions orchestrated by coagulation factors. The end stage of this event is the generation of thrombin resulting in a fibrin clot. There are two pathways to its formation, the tissue factor-dependent extrinsic pathway and the contact factor-dependent intrinsic pathway, which are regulated by blood endogenous components, such as antithrombin, the most important physiological inhibitor of the coagulation (Rau, Beaulieu, Huntington, & Church, 2007). Conventionally, these pathways are explored by the activated partial thromboplastin time (APTT) and the prothrombin time tests, respectively, but they are of limited value and reflect only the first traces of the amount of thrombin formed. These thrombin generation (TG)-based coagulation assays aforementioned have been used as global tools for the study of plasma coagulability (Castoldi & Rosing, 2011; Duarte, Ferreira, Rios, Reis, & Carvalho, 2017) and analysis of anticoagulant agents (Chahed et al., 2020; Furugohri & Morishima, 2015; Furugohri, Sugiyama, Morishima, & Shibano, 2011; Wu et al., 2011; Mourão et al., 2001; Nishiro, Fukuda, Nagumo, Fujihara, & Kaji, 1999; Salles et al., 2017; Zhang et al., 2018).

The chemical and structural diversity of sulfated polysaccharides (SPs) varies according to the marine environment in which the analyzed organisms are found (Aquino, Landeira-Fernandez, Valente, Andrade, & Mourão, 2005; Cardozo et al., 2007; Mayer, Rodriguez, Berlinck, & Hamann, 2007). Diverse SPs displaying anticoagulation from innumerable origins (Chahed et al., 2020; Cardozo et al., 2007; Dantas-Santos et al., 2012; Mourão et al., 2001; Pereira et al., 2005; Rodrigues et al., 2011), particularly from macroalgae (Athukorala, Lee, Kim, & Jeon, 2007; Fonseca et al., 2008), have already been isolated and studied in glycomics (Pomin, 2012). These marine glycans have highly heterogeneous structures varying with algal species,

accounting for these glycans distinct modes of action (Glauser et al., 2009; Mourão, 2015; Pomin, 2012). These complex polyanionic polymers with high sulfate content bind to a number of plasma proteins and their stereospecific interactions result in differential levels of anticoagulant responses (Mourão, 2015; Pomin, 2012). Furthermore, SPs widely found in red seaweeds, in which are known as agaran and carrageenan (Campo, Kawano, Silva Junior, & Carvalho, 2009; Cardozo et al., 2007), do not have the antithrombin-binding pentasaccharide required for anticoagulation in unfractionated heparin (UHEP), which leads to high rates of bleeding, heparin-induced thrombocytopenia and other undesirable consequences (Mourão, 2015; Mourão & Pereira, 1999).

A particular SP present in the Rhodophyta *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vicent has been isolated by Quinderé et al. (2013). The polysaccharide acted as a candidate agent to control nociception and inflammation devoid of systemic toxicity *in vivo*. Subsequently, structure studies of the intact SP and its chemically-modified (i.e. desulfated and anhydro-enriched) derivatives revealed a high degree of structural heterogeneity due to the occurrence of anhydrogalactose units, methyl ethers, variable sulfation, and pyruvate (Figure 1) (Quinderé et al., 2014) along of its system of mucilaginous matrix agarocolloid structures (Rodrigues et al., 2016a) as found in the *Acanthophora* genus (Duarte et al., 2004).

This highly complex nature of the SPs from *A. muscoides* may also be reduced by mild acid hydrolysis producing sulfated oligosaccharides that act as blockers of TG *in vitro* (Rodrigues et al., 2016b). Besides, the unmodified SPs from *A. muscoides* presented mostly serpin-independent anticoagulation mechanism and drastically reduced arterial thrombosis *in vivo* (Quinderé et al., 2014). This same molecule also reduced macrophage chemotaxis in atherosclerotic mice (Quinderé et al., 2015), viral infections (Vanderlei et al., 2016) and, recently, its addition to cryodiluent media revealed to be a safe tool for fish semen (Pereira et al., 2020).

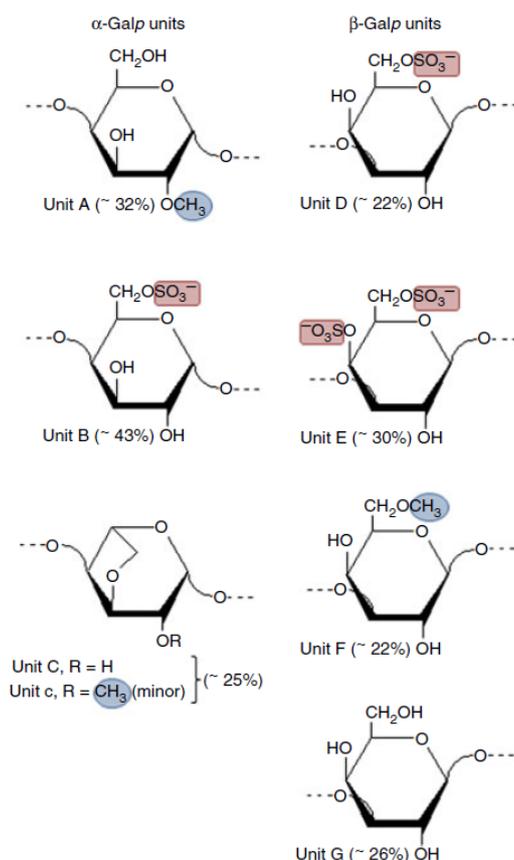


Figure 1. Presumed structure by nuclear magnetic resonance of the SP from *A. muscoides*. This glycan is composed of alternating 4-linked α -galactose and 3-linked β -galactose units. Its complexity arises from substitutions with methyl ethers (blue-shaded ellipses) and sulfate esters (red-shaded rectangles). Some α -units also occur as anhydro-galactose residues. Units are indicated as percentages in the panel of the total α - or β -residues. Letters represent the structural assignments per unit (Quinderé et al., 2014).

The current study investigated whether a SPs fraction isolated from *A. muscoides* and its chemically-modified derivatives inactive TG in 60-fold diluted depleted and/or normal human plasma by a continuous *in vitro* method. The structural identity of the SPs was also examined by Fourier Transform Infrared (FT-IR) spectroscopy.

Material and methods

Brazilian samples of *A. muscoides* (Linnaeus) Bory de Saint-Vincent (Ceramiales, Rhodophyta) were manually collected in September 2012 at Pacheco beach (Caucaia, Ceará State) at low tide at mesolittoral zone, and then conducted in plastic bags to the Carbohydrates and Lectins Laboratory, Federal University of Ceará, Brazil. After collection, salt, macroscopic epiphytes and sand were removed from the algal samples with tap water, and then carefully rinsed with distilled water. The cleaned algal samples were stored at 20 °C until use as described (Quinderé et al., 2013, 2014). A voucher specimen (no. 46093) was deposited in the Herbarium Prisco Bezerra, Department of Biology, Federal University of Ceará. The experimental analyses were carried out at the Connective Tissue Laboratory, Federal University of Rio de Janeiro, Brazil.

SPs were extracted from a dehydrated *A. muscoides* tissue sample (5 grams) by papain digestion at 60 °C for 6 h in 100 mM sodium acetate buffer, pH 5.0, containing EDTA and cysteine (both 5 mM) and fractionated by anion-exchange chromatography on a DEAE-cellulose column using a stepwise of NaCl from 0 to 1.25 M, with intervals of 0.25 M, in 50 mM sodium acetate buffer, pH 5.0 (Quinderé et al., 2013). Fractions (F I → F IV) of 2.5 mL were collected and analyzed for SPs using the metachromasy assay with dimethylmethylene blue (Farndale, Buttle, & Barrett, 1986) and for total sugars by the phenol-sulfuric acid method in microplate format according to Masuko et al. (2005). Integrated metachromatic area of the fractions was also calculated (%) from the chromatogram as an additional parameter for sulfate using a Statistical Analysis Software version 8.0 Origin Program.

Chemical tests were conducted to characterize the lyophilized fractions for their total sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), sulfate (Dodgson & Price, 1962) and proteins (Bradford, 1976) contents using galactose, Na₂SO₄ and bovine serum albumin as standard curves, respectively. The purity and molecular mass distribution were verified by agarose (Dietrich & Dietrich, 1976) and polyacrylamide gel (Rodrigues et al., 2016a) electrophoresis, respectively, using sequential staining with toluidine blue and Stains-all to reveal complex polysaccharides (Volpi & Maccari, 2002) by comparison with the electrophoretic mobility of standard glycosaminoglycans dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa), chondroitin-6-sulfate (~ 60 kDa), heparan sulfate and/or dermatan sulfate (Salles et al., 2017). Desulfation of the pyridinium salt from a fraction with dimethylsulfoxide was carried out at 80 °C for 4h (Nagasawa, Inoue, & Kamata, 1977) and the alkali-treatment using NaOH, as previously reported (Quinderé et al., 2014). FT-IR spectroscopy of the fractions was performed using KBr in the wavenumber range of 500-4000 cm⁻¹. The anti-clotting analyses were performed with the fraction that had the highest yield, named F II.

Coagulation experiments were conducted using human blood samples collected in citrated vacutainer tubes containing 3.2% sodium citrate from 10 different healthy donors (University Hospital Clementino Fraga Filho, Rio de Janeiro, Brazil), 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. This study was approved by the Ethical Committee of the Federal University of Rio de Janeiro, Brazil (protocol 01200.001568/2013-87). Fractions were assessed by conventional APTT coagulation assay based on manufacturers' kit specifications, to measure their anti-clotting effect in a coagulometer (Amelung KC4A) before the *in vitro* TG assays. A mixture of 100 µL plasma and SPs concentrations varying from 0 to 1 mg mL⁻¹ were incubated with 100 µL APTT reagent (kaolin bovine phospholipid reagent). After 2 min of incubation at 37 °C, 100 µL 25 mM CaCl₂ was added to the mixtures, and the clotting time was recorded. The values were expressed as ratios of clotting time in the presence (T1) or absence (T0) of the polysaccharide. UHEP (193 IU mg⁻¹) was used as the standard anticoagulant.

Screening on the inhibitory effect of TG by SPs fractions in diluted normal human plasma was performed according to Salles et al. (2017) and Rodrigues et al. (2016b) in microplate format employing: 10 µL rabbit brain cephalin (contact-activator system) + 30 µL 0.02 M Tris HCl/PEG buffer, pH 7.4, + 10 µL polysaccharides or chemically-generated derivatives (SPs fractions (F I → F III): 0, 4.1, 8.3, 41.6 or 83.3 µg well-plate-1 or UHEP: 2 µg well-plate-1) + 60 µL 20 mM CaCl₂ and 0.33 mM S-2238 chromogenic substrate for displays TG (10:50 ratio, v:v). The reaction was triggered at 37 °C by addition of 60-fold diluted plasma (10 µL), and the absorbance (405 nm) was recorded for 60 min using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The inhibitory response of TG by polysaccharides/derivatives after the addition of the diluted serpin-free and normal human plasmas 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) based on Mansour et al. (2017) and Chahed et al. (2020).

In order to analyze the interaction of the intact F II on the substrate by the purified system, different concentrations of F II (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 addition of 60 μL of 20 mM CaCl_2 and 0.33 mM S-2238 chromogenic substrate (10:50 ratio, v:v), and the absorbance (405 nm) was recorded for 80 min at 37°C using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA).

The graphical representations were constructed using Origin Program version 8.0 as the Statistical Analysis Software. Data between the concentrations expressed as mean \pm standard deviation (SD) ($n = 3$) were analyzed by one-way ANOVA, followed by Tukey' test for unpaired data. Differences were considered statistically significant at a level of $p < 0.05$. All statistical analyses were performed applying GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com).

Results

Analyses suggest acidic sugar residues in *A. muscoides*

The profile of DEAE-cellulose chromatography of a sample of the total extract is shown in Figure 2A. Four fractions (F I, F II, F III, and F IV) were eluted at 0.5, 0.75, 1, and 1.25 M NaCl, respectively. The main peaks, F I and F II, showed higher metachromasy than F III and F IV, which were in conformity to their yields and sulfate contents (Table 1).

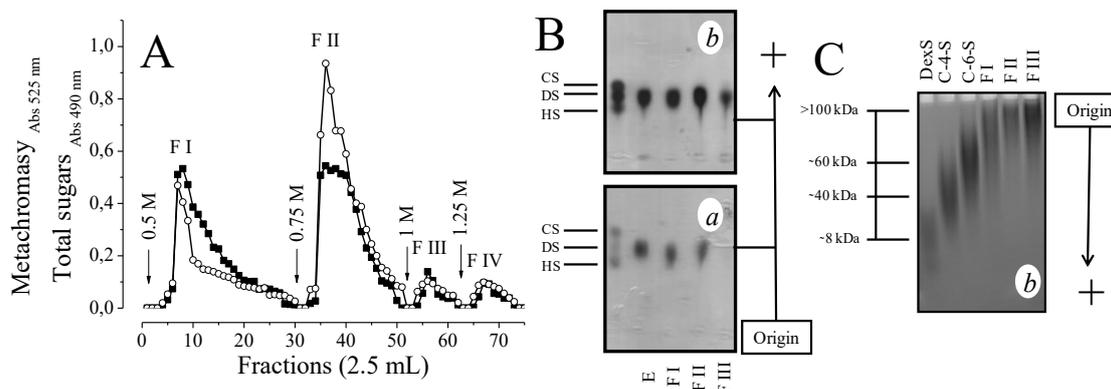


Figure 2. (A) Fractionation of the SPs from *A. muscoides* by DEAE-cellulose. Fractions were collected and detected by metachromasy using 1,9-dimethylmethylene blue (■—■) and phenol- H_2SO_4 (○—○). Arrows represent the NaCl concentration (↓). Agarose (B) and polyacrylamide (C) gel electrophoresis of extract (E) and/or fractions and standard glycosaminoglycans chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa), dextran sulfate (DexS, 8 kDa), dermatan sulfate (DS) and heparan sulfate (HS) were stained with 0.1% toluidine blue (a) and Stains-All (b).

F II presented the highest total sugars level, with an IMA of approximately 1.26-fold higher than F I (total sugars/metachromasy), while fraction F IV showed scarce material for analysis. Additionally, proteins were not detected in any sample (Table 1). The physical-chemical characterization by agarose analysis revealed that the extract and the fractions F I and F II migrated as single and coincident metachromatic components in comparison to standard glycosaminoglycan dermatan sulfate, while fraction F III was not visualized on the gel using staining with toluidine blue due to its relatively lower charge density. F III was only detected by sequential toluidine blue and Stains-All staining (Figure 2B).

Table 1. Yield, integrated metachromatic area, chemical analysis and molecular weight of the fractions obtained by anion-exchange chromatography (DEAE-cellulose) from *A. muscoides*.

Fractions	NaCl (M) ^a	Yield (%) ^b	IMA (%) ^c	Chemical analyses (%)		
				Sulfate ^d	Protein ^e	Total sugars ^f
F I	0.5	26.67	43.94	19.93	-	46.72
F II	0.75	52.67	48.07	30.20	-	52.97
F III	1	5.33	4.27	3.27	-	18.12
F IV	1.25	0.67	3.72	*	*	*

^aNaCl concentration; ^bYield calculated as the percentage from a sample of total extract applied on a DEAE-cellulose column; ^cIntegrated metachromatic area (IMA); ^dDosage by Dodgson and Price' (1962) method using NaSO_3 as standard; ^eDosage by Bradford' (1976) method using bovine serum albumin (-not detected); ^fDosage by Dubois et al.' (1956) method using D-galactose as standard. * Scarce material for analysis.

All the fractions exhibited a molecular mass of more than 100 kDa according to the polyacrylamide analysis because they remained close to the origin of the gel, except fraction F I that showed electrophoretic mobility similar to the chondroitin-6-sulfate. Staining of the standards and fractions associated with Stains-all also led to an improved visualization of the complex glycans, including fraction F III at low amount (Figure 2C). The FT-IR spectra of F I, F II and F III showed typical signals of agaran. The bands 1253-1253 (sulfate ester), 929-931 (3,6-anhydro- α -L-galactopyranose), 1074-1076 (structural form of galactan), 889-890 (sign of agar), 830-831, 819-823 (sulfation in the C-2 and C-6, respectively), 1631-1639 and 1415-1419 cm^{-1} (uronic acid) were common to all fractions. The elimination of sulfation and the reduced charge of the modified F II were also confirmed by FT-IR (spectra not shown). Because F II presented a higher SPs yield and sulfate content than F I, more detailed biological experiments were performed with it.

F II poorly modifies the APTT assay, but is effective inhibitor of TG, while its desulfated product stimulates the system

The effects of the fractions F I, F II, and F III on the APTT test in normal plasma confirmed that they are as poor inhibitors of the intrinsic coagulation pathway as estimated by T1 T0-1 ratio, where only F II extended in 2.19 times the clotting time at 1 mg mL⁻¹. The SPs fractions ($p < 0.05$) added to plasma had effects in the order of 1.80 ± 0.09 , 3.10 ± 0.05 , and 1.48 ± 0.07 IU mg⁻¹, respectively, vs. 193 IU standard UHEP, which still displayed anticoagulant action up to 2 $\mu\text{g mL}^{-1}$ (results not shown).

Figure 3A illustrates the effects of increasing concentrations of F II on the inactivation of TG by the intrinsic coagulation pathway in 60-fold diluted normal human plasma. Overall, F II concentration-dependently decreased ($p < 0.05$) the amount of thrombin generated by means of a continuous system using a specific chromogenic substrate. The inhibitory effects of F II ($53.70 \pm 0.25\%$ inhibition) on the activity of generated thrombin under the conditions used showed an association between the three TG parameters considered and the range of F II concentration from 4.1 to 83.3 $\mu\text{g well-plate}^{-1}$. The average values of the peak thrombin (%), time to peak, and lag time for each concentration tested are listed in Table 2.

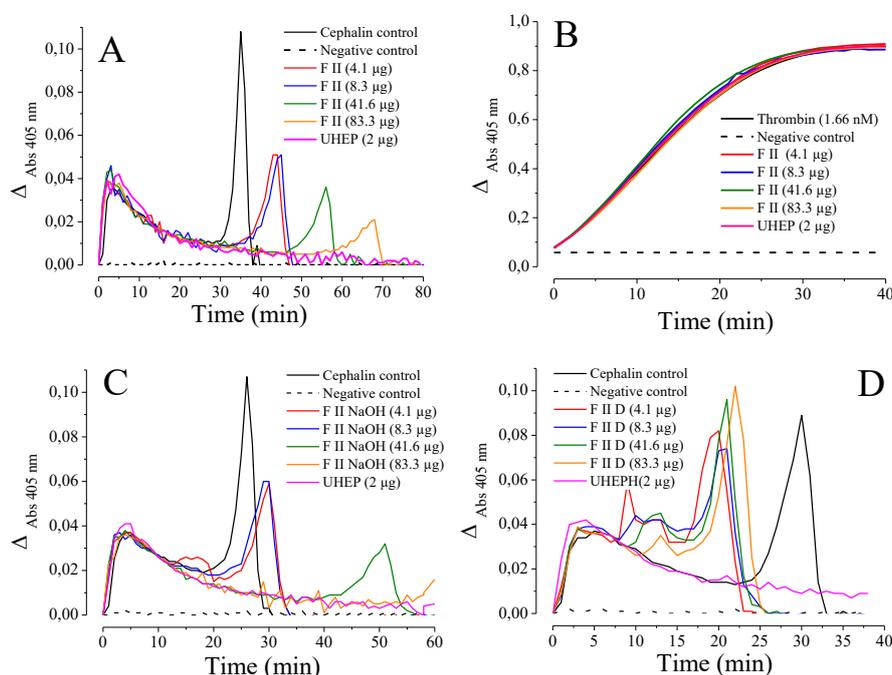


Figure 3. Effect of different concentrations of an intact SPs fraction (F II) (A), in a purified system (B) and its anhydro-enriched derivative (C) and desulfated derivative (D) from *A. muscoides* on the cephalin-activated TG in 60-fold diluted normal human plasma using chromogenic method by a continuous detection system (37° C, 40-80 min).

The concentration of F II required for 50% reduction of the amidolytic reaction of TG was of at least 4.1 $\mu\text{g well-plate}^{-1}$ as revealed by the peak thrombin, with time to peak reached at 43 min from the control curve (time to peak: 35 ± 1.00 min). The maximum inhibitory potential in this study was about 80% at 83.3 $\mu\text{g well-plate}^{-1}$ of F II. Overall, F II concentration-dependently prolonged the lag time (33-57 min) in diluted normal plasma. Similar inhibition profiles were also found for F I and F III modifying plasma TG *in vitro* (data not

shown). UHEP abolished TG by the intrinsic pathway in plasma treated with 2 $\mu\text{g well-plate}^{-1}$. Compared to UHEP, fractions were weaker inhibitors of the TG using this diluted plasma system. No inhibition of TG in plasma was detected in the absence of cephalin.

Accordingly, no interaction of intact F II and UHEP with the substrate was observed using purified thrombin since the concentrations of the SPs did not alter the control assay profile (Figure 3B). The residual TG inhibition of the anhydro-enriched derivative of F II in diluted normal human plasma revealed an almost similar profile than intact F II (Figure 3C).

Table 2. Average values of the TG parameters in diluted normal plasma treated with fraction F II and its chemically-modified derivatives from *A. muscoides* vs. UHEP.

Polysaccharide ($\mu\text{g well-plate}^{-1}$)	peak of thrombin (%)	time to peak (min)	lag time (min)
Intact F II			
F II: 4.1	52.77 \pm 0.120 ^a	43 \pm 1.00 ^a	33 \pm 1.00 ^a
F II: 8.3	53.70 \pm 0.257 ^a	45 \pm 1.00 ^a	34 \pm 1.00 ^a
F II: 41.6	66.67 \pm 0.200 ^b	56 \pm 1.10 ^b	45 \pm 1.00 ^b
F II: 83.3	80.56 \pm 1.502 ^c	68 \pm 1.20 ^c	57 \pm 1.00 ^c
UHEP: 2	100 \pm 0.00 ^d	–	–
Control (cephalin)	–	35 \pm 1.00	28 \pm 1.00
anhydro-enriched derivative			
F II: 4.1	44.85 \pm 1.32 ^a	30 \pm 1.00 ^a	22 \pm 1.00 ^a
F II: 8.3	43.93 \pm 1.49 ^a	29 \pm 1.00 ^a	20 \pm 1.00 ^a
F II: 41.6	70.01 \pm 1.08 ^c	51 \pm 1.00 ^b	39 \pm 1.00 ^b
F II: 83.3	100 \pm 0.87 ^d	–	–
UHEP: 2	100 \pm 0.00 ^d	–	–
Control (cephalin)	–	26 \pm 1.00	18 \pm 1.00
desulfated derivative			
F II: 4.1	17.98 \pm 1.68 ^a	20 \pm 1.00 ^a	8 \pm 1.00 ^a
F II: 8.3	7.87 \pm 1.57 ^b	20 \pm 1.00 ^a	8 \pm 1.00 ^a
F II: 41.6	7.87 \pm 1.34 (+) ^c	21 \pm 1.00 ^a	9 \pm 1.00 ^a
F II: 83.3	14.61 \pm 1.49 (+) ^d	22 \pm 1.00 ^a	10 \pm 1.00 ^a
UHEP: 2	100 \pm 0.00 ^e	–	–
Control (cephalin)	–	30 \pm 1.00	23 \pm 1.00

Data represent means \pm SEM (n = 3). Different letters among the polysaccharide concentrations indicate significant difference at $p < 0.05$ vs. control. Values were analyzed by one-way ANOVA followed by Tukey's test. – not determined; (+) algal polysaccharide-induced TG.

Regarding the desulfated F II (Figure 3D), the response of cephalin-induced TG was drastically altered in plasma treated with the range of concentrations from the control curve. Increasing amounts of the desulfated derivative of F II significantly modified the TG parameters, not only stimulating the system when at high concentrations of the sample (7.87 \pm 1.34 and 14.61 \pm 1.49% over inductions for 41.6 and 83.3 $\mu\text{g well-plate}^{-1}$, respectively), but also reducing the lag time almost equal among the concentrations assessed (Table 2). Collectively, these results suggested to an opposite stimulus on the blood coagulation under conditions where the absence of sulfation was important in this process.

The *in vitro* TG model stimulated by cephalin (intrinsic pathway) using 60-fold diluted serpin-free plasma detected continually at 37° for 60 min inhibitory actions of the intact F II and its anhydro-enriched derivative as shown in Figure 4A. The inhibitory capacity in the assays using the same F II concentrations was also markedly determined by a response of dependence of the amount of sample in plasma. Interestingly, in highest concentration assessed (83.3 $\mu\text{g well-plate}^{-1}$), TG was abolished as verified by peak thrombin, but requiring a relatively higher amount of F II (41.45-fold) than UHEP by intrinsic coagulation pathway. The time to peak and lag time also assumed the same tendency with the increasing concentration of anhydro-enriched product compared to intact F II in contact-activated plasma.

In contrast to thrombin generated in normal plasma, the control curves using depleted plasma were not expected to a better resolution process. The residual inhibition effect of the respective preparations showed serpin-independent ability by different concentrations of SPs tested. For intact F II, high concentrations (41.6 and 83.3 $\mu\text{g well-plate}^{-1}$, 100% inhibition) suppressed TG compared to 8.3 $\mu\text{g well-plate}^{-1}$ of SPs with basis on maximum absorbance of active thrombin formed without polysaccharides by contact-pathway and characterized by a lag time of about 37 min. When the plasma was treated with 4.1 $\mu\text{g well-plate}^{-1}$ of intact F II, no alteration on the TG was observed vs. control curve without SPs. As expected, any anticoagulant action and TG induction were found in depleted plasma in the presence of UHEP and absence of cephalin, respectively.

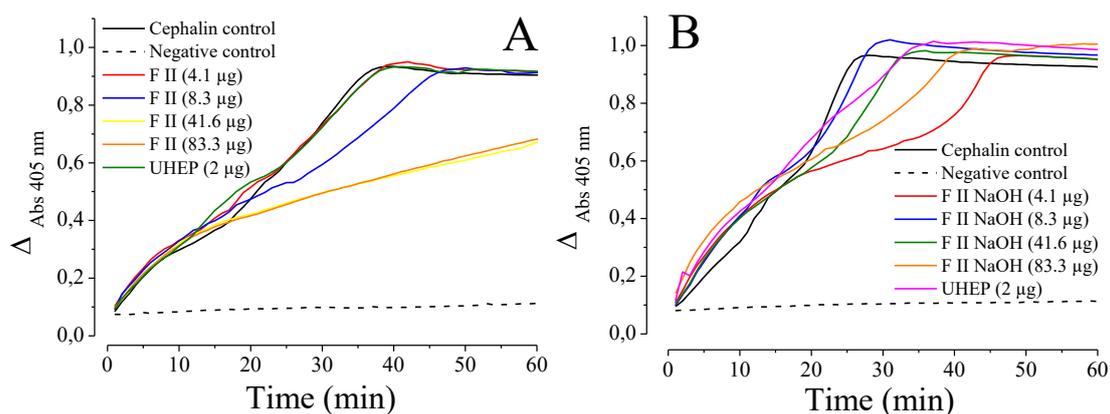


Figure 4. Effect of different concentrations of the intact SPs fraction (F II) (A) and its anhydro-enriched derivative (B) from *A. muscoides* on the cephalin-activated TG in 60-fold diluted serpin-free plasma using chromogenic method by a continuous detection system (37 °C, 60 min).

In terms of effects of the anhydro-enriched derivative, the serpin-independent thrombin inhibitor F II also altered the intrinsic pathway-induced thrombin formation (Figure 4B). However, this peculiar role occurred as maximum reduction of the TG at dose of 4.1 $\mu\text{g well-plate}^{-1}$ of alkali-modified F II vs. other concentrations displaying less pronounced TG inhibitions from the control plasma. Again, UHEP and negative control did not induce anticoagulation and TG response, respectively.

Discussion

Diverse chemical classes of complex SPs naturally occurring have been described in a great variety of living organisms (Aquino et al., 2005; Athukorala et al., 2007; Campo et al., 2009; Cardozo et al., 2007; Mayer et al., 2007; Rodrigues et al., 2012). In this current study, employing anion-exchange (DEAE-cellulose) column for charged variously-fractions separation and electrophoresis in agarose or polyacrylamide gel for respective physical-chemical characterization confirmed that the abundance and the compositional profile of the SPs produced by *A. muscoides* would change according to the collection period (Quinderé et al., 2013; Rodrigues et al., 2016a). The homogeneous charge pattern and the heterogeneous molecular weight characterized the SPs after combined staining with toluidine blue and Stains-All as glycosaminoglycans (Volpi & Maccari, 2002; Salles et al., 2017). When associated with the use of Stains-All, more intense bands were revealed by gels (Salles et al., 2017), especially fraction F III eluted at 1 M NaCl vs. F I and F II (at 0.5 and 0.75 M NaCl, respectively), suggesting distinct levels of uronic acids as analyzed by FT-IR (Vanderlei et al., 2016) and pyruvylated-galactose residues (Rodrigues et al., 2016a), but with structural regularity.

The chemical identity by mean of FT-IR of the fractions confirmed agaran-structures as also demonstrated by Vanderlei et al. (2016). *A. muscoides* sulfated galactans are composed of alternating 4-linked- α -galactopyranosyl units and 3-linked- β -galactopyranosyl units revealing variable sulfation, CH_3 substitutions and 3,6-anhydro- α -L-galactose units, besides of pyruvated-galactose residues, respectively (Quinderé et al., 2013; Rodrigues et al., 2016a), characteristics of the genus (Duarte et al., 2004). The complex nature of the polymer after chemical reactions (alkali and desulfation) resulted in a more homogeneous structure by conversion of 6-sulfated α -galactopyranosyl units into their 3,6-anhydro- α -galacto-derivatives and elimination of sulfation, respectively (Quinderé et al., 2014). These methods are traditionally applied in the hydrocolloid industry (Campo et al., 2009; Cardozo et al., 2007) and in scientific investigations (Dantas-Santos et al., 2012; Mourão, 2015; Pereira et al., 2005). Rodrigues et al. (2016a) suggested that studies on the ultrastructure of the SPs could contribute to better clarify the cell-wall matrix architecture in *A. muscoides*.

A. muscoides has SPs with peculiar effects on the coagulation, when estimated by *in vitro* and *in vivo* experimental assays (Quinderé et al., 2014; Rodrigues et al., 2016a, 2016b). The APTT standard plasma clotting assay is stimulated by a mixture of contact activators and phospholipids, in which mimic *in vitro* a negative surface of the contact-pathway. It is traditionally used to record the anticoagulant potential in clinic and in experimental studies (Duarte et al., 2017; Mourão & Pereira, 1999; Zhang et al., 2018), revealing anticoagulant SPs from different organisms (Athukorala et al., 2007; Campo et al., 2009; Dantas-Santos et al., 2012; Mansour et al., 2017; Mourão, 2015; Mourão & Pereira, 1999; Pomin, 2012; Rodrigues et al., 2011).

In this study, the effects of the low molecular mass SPs fractions, as revealed by polyacrylamide analysis

(Quinderé et al., 2013; Rodrigues et al., 2016a), on APTT test in normal human plasma treated at high concentration of SPs supported those modest APTT values vs. the molecular size distribution found by Rodrigues et al. (2016a) testing fractions from *A. muscoides* by revealing themselves lacking in the intrinsic and/or common coagulation pathway inhibition, as also previously observed for SPs from *A. spicifera* using APTT model (Duarte et al., 2004). As expected, UHEP importantly modified the APTT due to its known antithrombin-binding specific pentasaccharidic sequence absence in others SPs-expressing organisms (Mourão, 2015; Mourão & Pereira, 1999; Pomin, 2012). However, the conventional coagulometric measurement by APTT model is limited to the initiation phase of the coagulation process, detecting the formation of only 5% of total thrombin; therefore, the production of thrombin and fibrin would be still occurring in plasma (Castoldi & Rosing, 2011; Duarte et al., 2017). Thrombin exerts a relevant role in the coagulation system because it induces a wide range of physiological effects, as well as it is agonist for the formation of thrombin changed under pathological conditions, such as thrombosis disease and bleeding disorder (Duarte et al., 2017; Mourão & Pereira, 1999; Rau et al., 2007).

TG protocols have also been developed (Glaser et al., 2009; Mourão et al., 2001; Nishino et al., 1999; Wu et al., 2014) to analyze the total clot formation dynamic in a plasma sample, accurately reflecting the conditions of the hemostatic process (Castoldi & Rosing, 2011; Duarte et al., 2017), as well as anticoagulant tools examination (Glaser et al., 2009; Furugohri et al., 2011; Wu et al., 2011; Mourão et al., 2001; Nishino et al., 1999). However, there is no a currently available standard test to routine clinical use, although the calibrated automated thrombogram has been aforementioned (Castoldi & Rosing, 2011; Furugohri et al., 2011; Wu et al., 2011; Mansour et al., 2017; Zhang et al., 2014; Zhang et al., 2018) having great clinical potential (Duarte et al., 2017).

Herein, the effects of the SPs from *A. muscoides* were studied by means of a TG alternative protocol developed by our group (Rodrigues et al., 2016b). TG assay confirmed F II to act as inhibitor of the activity of thrombin formed by contact-activation using diluted normal plasma treated between concentrations of 4.1 - 83.3 $\mu\text{g well-plate}^{-1}$ by exhibiting dose-response inhibition curve in connectivity with the considered TG parameters vs. control plasma (Rodrigues et al., 2016b), and by interpreting with others intrinsic coagulation pathway-induced TG inhibitor glycans by whole or diluted plasma (Mourão et al., 2001; Nishino et al., 1999; Salles et al., 2017). Similarly, F I and F III, in a concentration-dependent manner, had an almost identical inhibitory profile as that of F II in the assay to alter the amidolytic activity of thrombin by diluted plasma, as verified by reduced thrombin peak and delaying lag time and time to peak compared to baseline level (Rodrigues et al., 2016b; Salles et al., 2017). The complete inhibition of the TG response by UHEP (Rodrigues et al., 2016b; Salles et al., 2017) reinforced our data because it has a preponderant effect by contact-activation derived of its molecular structure with high affinity by plasma antithrombin (Mourão & Pereira, 1999).

These combined results were also supported by the absence of response of F II and UHEP with the substrate in purified thrombin system. Our observations suggested that the agarocolloid-type polysaccharide structural composition did not influence the anticoagulant dynamic of the fractions in diluted normal human plasma along the detection process as already demonstrated by APTT test using whole plasma (Rodrigues et al., 2016a), since this classical clotting assay is relatively insensitive (Castoldi & Rosing, 2011; Duarte et al., 2017) to the anticoagulant effects of glycans (Glaser et al., 2009; Mourão et al., 2015; Mourão et al., 2001; Salles et al., 2017).

Results revealed that the SPs from the red seaweed *A. muscoides* prevented thrombosis *in vitro* (Rodrigues et al., 2016b), but independently of their degree of sulfation and molecular mass distribution as similar features to the dermatan sulfate-type glycosaminoglycans isolated from the skin of *Oreochromis niloticus* inhibiting TG *in vitro* by intrinsic coagulation pathway (Salles et al., 2017); it also already revealed *in vivo* (Quinderé et al., 2014), although the conditions *in vitro* do not reflect the *in vivo* physiological environment (Fonseca et al., 2008; Mourão, 2015; Pomin, 2012).

The use of chemically-modified glycans also allows develop novel materials revealing potential biomedical applications (Campo et al., 2009; Cardozo et al., 2007). In this study, F II was conducted using its chemically-modified products (Quinderé et al., 2014) to better clarify the effects in diluted depleted and normal plasmas on a structural basis. In diluted normal plasma system, the anhydro-enriched polysaccharides concentration-dependent attenuated the TG by blocking the intrinsic coagulation pathway at concentrations as those of native F II using the same analytical method.

Comparing native F II and alkali-modified F II, the significant rates of inactivation in diluted plasma by both test samples could involve the same chemical requirements to modulate the active coagulation system (Rau et al., 2007). This common inhibitory level of contribution on the intrinsic pathway seemed be related

to sulfated galactose residues in the D and E units of F II (Figure 1) (Quinderé et al., 2014), in which alkali-treatment would not generate anhydro sugars due to presence of sulfation on the carbon 6 of the β -Galp units (Campo et al., 2009), besides di-sulfated units in the E residue (Quinderé et al., 2014). It has been reported that the proportion and/or the distribution of di-sulfated units along the galactans could be a specific structural requirement for the anticoagulant action (Mourão, 2015; Pereira et al., 2005; Pomin, 2012). Moreover, the residual inhibitory action of the alkali-derivative persisted despite the decrease in molecular size postulated by alkali-treatment of the SPs (Cardozo et al., 2010), reinforcing their actions as previously correlated with the properties of the intact molecule due to lack of structural-function relationship data (Quinderé et al., 2014). Rodrigues et al. (2016b) reported that sulfated oligosaccharides from *A. muscoides*, obtained by mild-acid hydrolysis (0.04 HCl, 60 °C) from unmodified F II, displayed more potent *in vitro* effects on TG than the intact fraction, with efficacies similar to that of UHEP using same assay. Thus, alkali-treatment of F II had no influence in the evaluation by TG assay, although high levels of 3,6-anhydro sugars residues could increase the intrinsic ability of strength of gels formed by SPs (Cardozo et al., 2007).

Results were in line with others published studies, where chemically-modified polysaccharides were biologically active (Mourão, 2015). Thus, given the importance of the agarophytes species for hydrocolloid industry worldwide (Cardozo et al., 2007), the respective approach indicated new focus to gain insight into the study of Rhodomelaceae bioactive glycans modulating circulatory dysfunctions (Rodrigues et al., 2016b).

By contrast, desulfated F II anticipated and induced TG due to the elimination of the total charge of the *A. muscoides* SPs. This result confirmed the dependence of sulfate on the inhibitory potential by native SPs in plasma when also compared with different coagulation models already used (Duarte et al., 2004; Mansour et al., 2017; Zhang et al., 2014). Intact SPs from *A. muscoides* were shown to be less able to activate the contact-pathway (by factor FXIIa) due to its low molecular size (Quinderé et al., 2014) than known high molecular size SPs, whose effects on the respective pathway resulted in a prothrombotic action occurring at higher doses on model of venous thrombosis using experimental animals (Fonseca et al., 2008; Rodrigues et al., 2011; Mourão et al., 2015). UHEP, an antithrombin-dependent anticoagulant, not induced coagulation as accordingly reported (Fonseca et al., 2008; Quinderé et al., 2014; Barcellos et al., 2018).

Zhang et al. (2014) showed higher procoagulant effect of the desulfated fucoidan than original fucoidan from the brown seaweed *Fucus vesiculosus*, when assessed by the calibrated automated thrombography method. Mansour et al. (2017) revealed that the oversulfated fucosylated chondroitin sulfate from the sea cucumber *Holothuria polii* body wall exhibited unusual procoagulant effect by means of calibrated automated thrombography. *Ulva lactuca* (Chlorophyta) predicted thrombosis in high doses of SPs ($> 8.3 \mu\text{g well-plate}^{-1}$) by contact-activation using our test (Barcellos et al., 2018). Procoagulant stimulus by seaweeds SPs could be a practical aspect as therapeutic treatment for haemophilic patients (Fonseca et al., 2008). Possibly, our results could help to analyze the stimulus on the balance between procoagulant and anticoagulant forces of the blood coagulation system (Duarte et al., 2017).

In view of the major serpin-independent anticoagulant/antithrombotic effects using APTT in serpin-free plasma and in animal model of experimental thrombosis, respectively (Quinderé et al., 2014), F II (native and anhydro-derivative) was further explored using our *in vitro* model of TG in 60-fold diluted serpin-free plasma. Both polysaccharidic preparations delayed TG in depleted plasma, but with different potency, showing intrinsic inhibitory effect by native F II more remarkable than that of modified F II. Clearly, the native polysaccharide at high concentrations (41.6 and $83.3 \mu\text{g well-plate}^{-1}$) was even more active on serpin-free plasma, entirely abolishing the formation of thrombin, with basis on study of Glauser et al. (2009) evaluating the actions of *Botryocladia occidentalis* SPs on contact pathway-induced TG using serpin-free plasma. However, an almost inhibition by anhydro-derivative on the TG was observed, because the maximum reduction was achieved with lowest amount compared to control. Therefore, there was the impact of reduced charge density on the alkali-derivative F II effect in depleted plasma vs. intact F II, since the anticoagulant effect of SPs could depend on their sulfation pattern and molecular size (Pereira et al., 2005; Fonseca et al., 2008; Pomin, 2012; Mourão, 2015).

The relative difference between these results confirmed the preponderance of the serpin-independent effect than serpin-dependent one of F II, supporting APTT' results using serpin-free plasma in another study (Quinderé et al., 2014). In addition, this serpin-unrelated inhibitory action combined with the lack of effects on the purified serpins and coagulation proteases would determine inhibition of the prothrombinase complex by F II (Glauser et al., 2009; Quinderé et al., 2014), since this system is critical for TG (Rau et al., 2007). At a low amount, UHEP did not induce inhibition due to antithrombin-free plasma, reinforcing those APTT results

in serpin-free plasma obtained by Quinderé et al. (2014), as well as supporting the *in vivo* antithrombotic action of F II as serpin-independent by the same authors.

The introduction of an alternative method to examine the inhibitory levels of TG on both diluted plasma systems by SPs led to a comparison of effects. Considering that thrombin is initiated by vessel-wall injury leading to exposes the blood to tissue factor in the subendothelium playing important roles in hemostasis, it is also involved in inflammatory reactions (Rau et al., 2007). The modulation of TG by algae polysaccharides could give information additional correlating with other bioactivities already published (Quinderé et al., 2013, 2015; Rodrigues et al., 2012), since their actions appear to be independent of their degree of sulfation and/or molecular size (Quinderé et al., 2014; Rodrigues et al., 2016a; Vanderlei et al., 2016), characterizing a peculiar structural level of the *A. muscoides* SPs for the development of an interesting anticoagulant/antithrombotic agent. Complementary studies of mechanistic characterization of these molecules by which they would modulate the TG inhibitory response in different plasma systems are still needed.

Conclusion

An agaran-revealing SPs fraction and its modified products from Rhodophyta *A. muscoides* have variably inhibitory effects on the intrinsic pathway-dependent thrombin generation in both 60-fold diluted depleted and normal human plasmas, besides desulfated derivative induces thrombin formation. It seems that the sulfated galactose residues in the saccharide units of the alkali-derivative would contribute for the preponderance of the serpin-independent effect than serpin-dependent one of the SP leading to an inhibition of the prothrombinase complex.

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