



Sulfated agaran with 4,6-pyruvated form from red seaweed *Acanthophora muscoides* attenuates thrombin formation: *in vitro* and *ex vivo* studies

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ABSTRACT. *In vitro* studies have described the sulfated agaran from *Acanthophora muscoides* as an intrinsic inhibitor of thrombin generation (TG), but not in *ex vivo* assay. This investigation partially characterized a pyruvate fraction with *in vitro* and *ex vivo* effects on an intrinsic/extrinsic pathway-induced thrombin generation (TG) continuous model using 36 or 60-fold diluted mice or defibrinated, normal human plasma. Fraction separated by DEAE-cellulose chromatography exhibited charge homogeneity and non-sulfated polysaccharides (<100 kDa) by agarose and polyacrylamide gel electrophoresis, respectively, using Stains-all alone. Fourier Transform Infrared and Nuclear Magnetic Resonance studies indicated a 4,6-pyruvated agaran-structure. The fraction and heparin had no effect on prothrombin time, but there was a preponderant intrinsic rather than extrinsic pathway inhibition in TG assay; themselves, acting on both free and fibrin bound thrombin activity without chromogenic substrate interaction. Both fractions, desulfated and native, anticipated and induced thrombin formation in activators-devoid or normal plasma. In addition, mice pretreated with fraction (20 mg kg⁻¹, intraperitoneally) reduced intrinsically plasma TG *ex vivo* after 2h. Heparin suppressed TG *in vitro*, but induced it *ex vivo*. Therefore, agaran from *A. muscoides* blocks TG on *in vitro* and *ex vivo* studies, suggesting to evaluate the blood coagulability status.

Keywords: sulfated polysaccharide; chemical analysis; alternative system; clot formation.

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Introduction

Blood coagulation is a complex reaction involving plasma factors that circulate in an inactive form until proteolytic activation by an upstream factor, and thrombin is the major effector for clot formation. Thrombin is generated by the tissue factor-dependent extrinsic pathway and the contact factor-dependent intrinsic pathway (Rau, Beaulieu, Huntington, & Church, 2007), which are analyzed by prothrombin time (PT) and activated partial thromboplastin time tests, respectively. However, these tests do not accurately prove the amplification and propagation steps of hemostasis. Because of these limitations, thrombin generation (TG) tests have been suggested since 1953 as global assays of plasma coagulability, including automated (Castoldi & Rosing, 2011; Duarte, Ferreira, Rios, Reis, & Carvalho, 2017) and alternative (Ofosu et al., 1984; Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999; Glauser et al., 2009; Rodrigues et al., 2016a) methods. The measurement of thrombin activity *via* fluorogenic or chromogenic thrombin substrates added to plasma evaluate the prognostic of bleeding or thrombosis (Castoldi & Rosing, 2011; Duarte et al., 2017; Lau, Berry, Mitchell, & Chan, 2007), and plasma anticoagulants (Ofosu et al., 1984; Glauser, Pereira, Monteiro, & Mourão, 2008; Perzborn, Heitmeier, Buethorn, & Laux, 2014; Wu et al., 2014; Furugohri & Morishima, 2015; Chahedet al., 2020).

Thrombin has pro- and anticoagulant roles connected to coagulation factors and inhibitors, such as antithrombin on protease regulation (factors Xa, IXa and FIIa - thrombin), an amino acid glycoprotein produced in the liver (Rau et al., 2007), which has unfractionated heparin (UHEP) as its cofactor due to the unique pentasaccharide sequence with high affinity binding to antithrombin displaying *in vitro* and *in vivo* effects (Nader et al., 2001). Although effective inhibitor of TG (Wu et al., 2014), UHEP induces thrombocytopenia and extensive bleeding complications, besides parental contamination (Gurbuz, Elliott, & Zia,

2005; Guerrini et al., 2008), sparking efforts to develop substitutes (Mourão & Pereira, 1999; Mestechkina & Shcherbukhin, 2010; Rodrigues et al., 2011; Xie, Chen, Li, Yang, & Huang, 2011; Pomin, 2012; Mourão, 2015).

Seaweed sulfated polysaccharides (SPs) are strongly anionic due to their high content of sulfate esters (S=O) originated from nucleophilic substitution of hydroxy groups, having these molecules large molecular distribution (>100 kDa) (Mestechkina & Shcherbukhin, 2010; Pomin, 2012), with their complex structures varying among species (Cardozo et al., 2007; Farias et al., 2008; Recalde et al., 2016; Mourão et al., 2015). They induce anti-clotting effects in mammalian plasma, not only by sulfated galactans (carrageenan and agaran-type structures) (*Rhodophyceae*) (Cardozo et al., 2007), but also by fucans or fucoidans (*Phaeophyceae*) (Rocha et al., 2005; Pomin, 2012; Mourão et al., 2015) and by ulvans (*Chlorophyceae*) (Farias et al., 2008; Arata et al., 2015; Rodrigues et al., 2019). Besides that, complexity occurs also by specific requirement degree for anticoagulation independently from sulfation level on their chains (Pomin, 2012; Mourão et al., 2015). Furthermore, they have great advantage as alternatives to industrial and pharmacological applications because of their minimum viral/toxic risks (Li, Zhang, & Song, 2005; Cardozo et al., 2007; Rodrigues et al., 2012 and 2013; Mourão et al., 2015).

TG studies have been applied to characterize SPs on their functionalities in plasma. Ofosu et al. (1984) found that heparan sulfate and dermatan sulfate had complementary modes for blocking the intrinsic pathway-induced TG in normal plasma. Fucoidan from the brown seaweed *Ecklonia kurome* inhibited TG in both coagulation pathways using normal or defibrinated plasmas (Nishino et al., 1999). Mourão et al. (2001) demonstrated that a fucosylated chondroitin sulfate from *Ludwigothurea grisea* (sea cucumber) interfered with TG in defibrinated plasma by both contact-activated and thromboplastin-activated systems. Glauser et al. (2009) revealed that SPs from Rhodophyta *Botryocladia occidentalis* inhibited the prothrombinase complex in TG using serpin-free plasma. Dermatan sulfate from the skin of the freshwater fish *Oreochromis niloticus* (Salles et al., 2017) and SPs isolated from the Rhodophyta *Gracilaria birdiae* (Rodrigues et al., 2017a) and Chlorophyta *Caulerpa cupressoides* (Rodrigues et al., 2017b; Rodrigues, Benevides, Tovar, & Mourão, 2019) continually attenuated TG in diluted human plasma by both intrinsic and extrinsic pathways. Conversely, Zhang et al. (2014) and Mansour et al. (2017) revealed that a fucoidan from *Fucus vesiculosus* (*Phaeophyceae*) and a fucosylated chondroitin sulfate from Mediterranean sea cucumber *Holothuria polii* body wall exhibited dual effects on TG in whole plasma by intrinsic coagulation pathway using the Calibrated Automated Thrombogram. Barcellos et al. (2018) predicted thrombosis *in vitro* in diluted human plasma, by contact pathway when in continuous system, evaluating increasing amounts of SPs fractions isolated from *Ulva lactuca* (Chlorophyta). Despite descriptive studies, sensitivity of continuous systems for TG detection using different plasmas treated with seaweeds SPs remain poorly investigated.

Acanthophora muscoides (Linnaeus) Bory de Saint-Vicent (*Rhodomelaceae*, *Ceramiales*) is a tropical red seaweed found along the Brazilian coastline. Quinderé et al. (2014) characterized a SPs fraction by Nuclear Magnetic Resonance (NMR) spectroscopy, which showed structural heterogeneity in terms of variable sulfate ester and/or methyl ether substitutions, 3,6-anhydro- α -galactosyl units, and pyruvate. Subsequently, it revealed agaran-structures along its matrix structure (Rodrigues et al., 2016b). There has already been described *in vitro* and/or *in vivo* with antinociceptive, anti-inflammatory (Quinderé et al., 2013), antithrombotic (Quinderé et al., 2014), antiatherogenesis (Quinderé et al., 2015), anti-TG (Rodrigues et al., 2016a) and antiviral (Vanderlei et al., 2016) effects devoid of toxicity *in vivo* (Quinderé et al., 2013); and as safe supplement to cryodiluent media for fish semen (Pereira et al., 2020). Recently, Rodrigues, Quinderé, and Benevides (2021) reported *in vitro* effects of a native and modified SPs fraction on intrinsic pathway-induced TG in diluted depleted or normal plasma and suggested that sulfated galactose residues in saccharide units of the alkali-derivative acted on the prothrombinase complex. Nevertheless, its effects on the extrinsic coagulation pathway and *ex vivo* anti-clotting potential remain unexplored.

The aim of this investigation was to expand our knowledge concerning *in vitro* inhibitory effects of a SPs fraction from *A. muscoides* on TG by extrinsic/intrinsic coagulation pathways and to continually measure its *ex vivo* action on TG using systemically-treated mice plasma.

Material and methods

Drugs and reagents

The unfractionated heparin was obtained from the Europharma Lab (São Paulo, Brazil). The standard glycosaminoglycans dextran sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, heparan sulfate and

dermatan sulfate were purchased from Sigma-Aldrich (St, Louis, MO, USA). Activated partial thromboplastin time and prothrombin time reagents and calcium chloride were from Wiener Lab (Rosario, Argentina). While chromogenic substrates for thrombin S-2238 or Factor Xa S-2222, and thrombin and Factor Xa were purchased from Chromogenix (Mölnådal, Sweden) and from Haematologic Technologies (Essex Junction, VT, USA), respectively. The snake venom enzyme Ancrod was obtained from Sigma-Aldrich (St, Louis, MO, USA). Other reagents were purchased from Brazilian companies or analytical grade.

Seaweed and SPs fraction preparation

Specimens of red seaweed *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 placed in plastic bags and sent to the Carbohydrates and Lectins Laboratory, Federal University of Ceará, Brazil, and treated as described (Rodrigues et al., 2021). A voucher specimen (#46093) was deposited in the Herbarium Prisco Bezerra, Department of Biology, Federal University of Ceará. Studies were carried out at the Connective Tissue Laboratory, Federal University of Rio de Janeiro, Brazil.

SPs were enzymatically extracted from dehydrated algal tissue by papain incubation (60°C, 6h) in 100 mM sodium acetate buffer, pH 5.0, containing EDTA and cysteine (both 5 mM), and then 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, yielding four fractions, as recently described (Rodrigues et al., 2021). Fractions of 2.5 mL were collected and analyzed for SPs using the metachromasy assay (A_{525nm}) with dimethylmethyleneblue (Farndale, Buttle, & Barrett, 1986). Biological analyses were performed with the fraction showing the highest lyophilized yield, named SP-Am.

Desulfation of the pyridinium salt of the SP-Am with dimethylsulfoxide was carried out at 80°C for 4h (Nagasawa, Inoue, & Kamata, 1977). Its purity and molecular mass distribution was checked by electrophoresis on agarose (Dietrich & Dietrich, 1976) and polyacrylamide gels (Rodrigues et al., 2016b), respectively, associated with toluidine blue or Stains-all to reveal complex polysaccharides (Volpi & Maccari, 2002; Andrade, Oliveira, Tovar, Mourão, & Vilanova, 2017; Rodrigues et al., 2019) by comparison with the mobility of standard glycosaminoglycans dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa), chondroitin-6-sulfate (~ 60 kDa), heparan sulfate and/or dermatan sulfate (Rodrigues et al., 2021). The identity of the polymer (5 mg) was verified by Fourier Transform Infrared (FT-IR) spectroscopy in KBr using a Shimadzu IR spectrophotometer to characterize agar polysaccharide (Vanderlei et al., 2016; Rodrigues et al., 2021) and NMR spectroscopy ($^1H/^{13}C$ HMBC) in 0.6 mL 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA) using Bruker DRX 800 MHz (1024 × 256 points, with a 60 ms delay for evolution of long-range couplings and set with no decoupling during acquisition time) with trimethylsilyl propionic acid as a standard to characterize the SP pyruvate form (Quinderé et al., 2014), respectively, and spectrum processed using the SpinWorks 3.1.8 software package (USA).

In vitro clotting assay

Blood collection and animal/human plasma sample preparation

A total of 10 different healthy donors (University Hospital Clementino Fraga Filho, Rio de Janeiro, Brazil) were used for coagulation studies. 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). Human plasma was defibrinated by incubation with 0.1 unit mL⁻¹ Ancrod (snake venom enzyme) at 37°C for 30 min. Then, the clot formed was removed, and the plasma centrifuged (2,700 × g, 10 min) was used for TG analysis (Glauser et al., 2009). Mice peripheral blood was collected, centrifuged (500 × g, 15 min) and plasma samples containing 3.2% sodium citrate were stored at 70°C prior to use. This study was approved by the Ethical Committee of the Federal University of Rio de Janeiro (protocol 01200.001568/2013-87).

Traditional PT test

SP-Am was assessed by coagulation assay based on manufacturer kit specifications, to measure its effect in a coagulometer (Amelung KC4A) before the *in vitro* TG assays. A mixture of 100 µL plasma and SP-Am concentration of 1 mg mL⁻¹ was incubated at 37°C for 1 min. After incubation, 100 µL PT reagent was added

to the mixtures, and the clotting time was automatically recorded. Values were expressed as ratios of clotting time in the presence (T_1) or absence (T_0) of the polysaccharide (Rodrigues et al., 2016b).

Effects of SP-Am on TG *in vitro*

This assay was based on Salles et al. (2017) and Rodrigues et al. (2016a and 2021) in microplate format with 10 μ L rabbit brain cephalin (contact-activator system) or PT reagent (830 μ g well-plate, factor tissue-activator system) + 30 μ L 0.02 M Tris HCl/PEG buffer, pH 7.4 + 10 μ L polysaccharides (SP-Am or desulfated product: 0, 4.1, 8.3, 41.6 or 83.3 μ g well-plate⁻¹ or UHEP: 2 or 4 well-plate⁻¹) + 60 μ L 20 mM CaCl_2 and 0.33 mM chromogenic substrate S-2238 (10:50 ratio, v:v). The reaction was triggered at 37°C by addition of 36 or 60-fold diluted defibrinated or normal human plasma (10 μ L), and the absorbance (405 nm) was read for 60 min. using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). Inhibitory response of thrombin generation 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) (Mansour et al., 2017; Rodrigues et al., 2021).

Analysis of the use of SP-Am on substrates using two purified protease systems

The interaction of SP-Am on the substrate by the purified systems (thrombin or Factor X) was further analyzed continuously. Different concentrations of SP-Am (0, 4.1, 8.3, 41.6 or 83.3 μ g well-plate⁻¹) or UHEP (4 μ g well-plate⁻¹) (10 μ L) were used with 1.66 nM thrombin (10 μ L) in 0.02 M Tris HCl/PEG buffer, pH 7.4. The reaction mixture was triggered in final volume of 120 μ L by adding 60 μ L 20 mM CaCl_2 and 0.33 mM chromogenic substrate S-2238 or S-2222 (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 SP-Am on TG in plasma devoid of cephalin

This study was based on Rodrigues et al. (2019) using a microplate format, without cephalin. The test was conducted as follow: 40 μ L 0.02 M Tris-HCl/PEG buffer (pH 7.4) + 10 μ L SP-Am (0, 4.1, 41.6 or 83.3 μ g well-plate⁻¹; UHEP: 2 μ g.well-plate⁻¹) + 60 μ L 20 mM CaCl_2 /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 μ L), 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 SP-Am was analyzed by absorbance of the assay.

Ex vivo effect of SP-Am on TG in mice plasma

Male Swiss mice (28-32 g) from the Animal Care Unit of the Federal University of Rio de Janeiro, Brazil, were kept in a temperature-controlled room (20-22°C) with free access to water and food on a 12/12h light/dark cycle and all care was taken to avoid environmental disturbances that might influence animal responses. For systemic treatment, groups of five animals were segregated and handled separately, and then they received SP-Am (20 mg kg⁻¹ body weight, ip.) (Quinderé et al., 2013), UHEP (5 mg kg⁻¹ body weight, ip.) (Gurbuz et al., 2005) or saline (0.9% NaCl w/v, ip) for 1 or 2h. After that, mice were anesthetized (100 mg kg⁻¹ body weight ketamine and 16 mg kg⁻¹ body weight xylazine, i.m.) and citrated plasma samples used for TG analysis.

Statistical analysis

The graphical representations were constructed using the Origin software version 8.0 as the Statistical Analysis Software (USA). For *in vitro* TG study, experimental data (mean $\pm$ standard deviation, n = 3), calculated from the control curves (%), were analyzed by one-way ANOVA, followed by Tukey' test for unpaired data, with differences considered statistically significant at p < 0.05. For *ex vivo* TG analysis, data per hour (mean $\pm$ standard deviation, n = 3) were also subjected to Student *t*-test for unpaired values, applying p < 0.05 as significant. All the experimental data were also 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) (Rodrigues et al., 2021).

Results

Sulfated galactan from *A. muscoides* reveals 4,6-pyruvate

Separation of the crude SP in *A. muscoides* by DEAE-cellulose chromatography showed a prominent peak of metachromasy at 0.75 M NaCl-eluted fraction (SP-Am) accounting for ~50% total polysaccharide (w/w%). By specific staining with toluidine blue, the physical-chemical analysis by agarose gel electrophoresis revealed SP-Am as a single band exhibiting strong metachromasy migrating to dermatan sulfate (Figure 1Aa), whereas the polyacrylamide analysis indicated a distinct profile from standard glycosaminoglycans on gel, which was because the high molecular size SP-Am (> 100 kDa) revealed concentrated in the origin (Figure 1Ba).

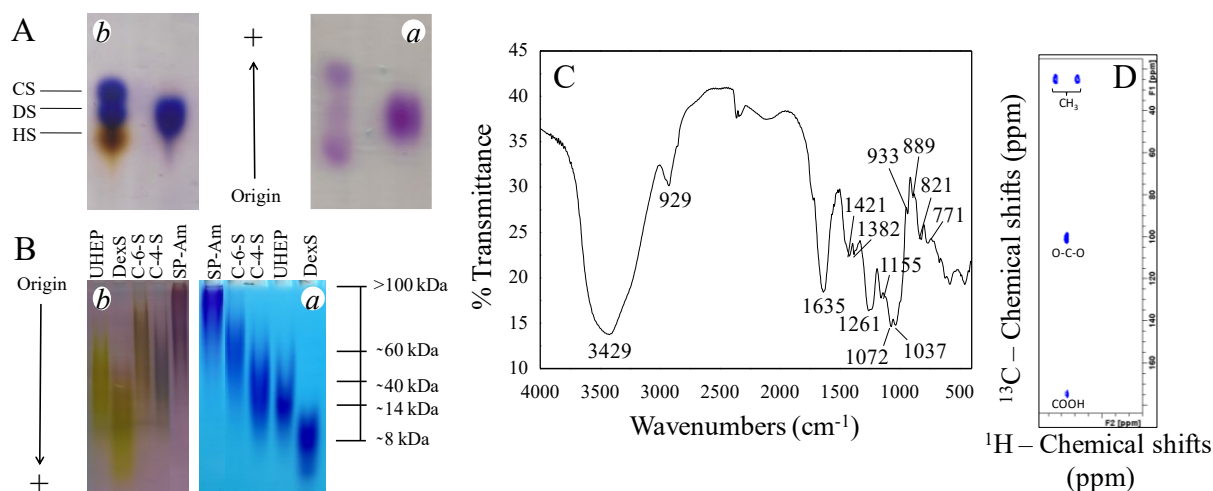


Figure 1. Agarose (A) and polyacrylamide (B) gel electrophoresis of SP-Am and standard glycosaminoglycans chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa), dextran sulfate (DexS, 8 kDa), dermatan sulfate (DS) and heparan sulfate (HS) were stained with 0.1% toluidine blue (a) or Stains-all (b). FT-IR spectrum at 500–4000 cm^{-1} (C) in KBr and 2D $^1\text{H}/^{13}\text{C}$ HMBC spectral region (D) of SP-Am characterize an agarocolloid of 4,6-pyruvylated form.

Regarding the electrophoresis gels stained with cationic dye Stains-all, polysaccharides assumed their identities by distinct color and more sensitivity of the single polysaccharide species than toluidine blue alone (Figures 1Ab and Bb). More relevant was a brown polydisperse blot of SP-Am migrating like chondroitin-6-sulfate until the central portion of the gel, therefore, it was recognized due to its contrasting color vs. standards (Figure 1Bb).

FT-IR showed characteristic absorption bands of agar-polysaccharide in SP-Am sample (Figure 1C). Among the main signals, it was attributed to the presence of sulfate ester bond (at 1382 cm^{-1}), S=O (at 1261 cm^{-1}), C-O-C corresponding to 3,6-anhydro- α -L-galactopyranose (at 933 cm^{-1}), galactan (at 1072 cm^{-1}), sign of an agar (at 889 cm^{-1}) and galactose structure at C-6 position (at 821 cm^{-1}), which were chemical signals for agaran-structures. In addition, at 1635–1421 and at 1155 cm^{-1} were related to the occurrence of uronic acid and C-O, and C-C of vibrating piranosic ring, respectively, in native polysaccharide. The desulfated product of SP-Am had no important chemical vibration for the sulfated signals described above, indicating the efficacy of the process in eliminating these charged groups attached to saccharide chains (not shown).

The HMBC analysis of SP-Am showed spectral signals at $\delta_{\text{H/C}}$ 1.51–26, 1.51–101.2 and 1.51–175.6 ppm corresponding to the groups CH_3 , O-C-O and COOH of pyruvate, respectively (Figure 1D). These data led to the identification of a six-membered cyclic ketal, including O-4 and O-6 positions (4,6-O-(1'carboxy)-ethylidene cyclic ketals) of galactoses located at non-reducing ends of the polysaccharide.

SP-Am has no effect on PT test, but inhibits TG *in vitro* and induces it by desulfation

Analysis by means of PT test revealed that the SP-Am was not an inhibitor *in vitro* of the extrinsic coagulation pathway based on $T_1 T_0^{-1}$ ratio (1.01 ± 0.03), when normal human plasma was treated with a high polysaccharide concentration (1 mg mL^{-1}). Its clotting time ($11.23 \pm 0.01 \text{ s}$) remained similar to plasma control ($11.01 \pm 0.02 \text{ s}$); likewise, UHEP ($12.37 \pm 0.04 \text{ s}$, 1 mg mL^{-1}), $p > 0.05$.

By contrast, TG study evidenced that the SP-Am modulated the factor tissue-activator system when in diluted human plasma (36 or 60-fold), with different resolution pattern as observed by thrombin activity

(Figure 2). SP-Am altered *in vitro* ($p < 0.05$) the TG parameters in the range of concentration tested (4.1 – $83.3 \mu\text{g well-plate}^{-1}$). These extrinsic inhibitory effects by SP-Am were more remarkable in 60-fold diluted plasma than the 36-fold one. Table 1 lists the values of peak thrombin (%), time to peak, and *lag* time for each concentration used in the assays. It inactivation on TG in 36-fold diluted plasma led to relatively parental dose-response curves, showing *lag* time at 11 min and maximum inhibitory potential with $41.6 \mu\text{g well-plate}^{-1}$ ($67.79 \pm 0.60\%$ peak thrombin inhibition) at 33 ± 1.10 min. vs. control (time to peak: 17 ± 1.00 min.), whereas at $83.3 \mu\text{g well-plate}^{-1}$ of SP-Am the inhibitory response ($62.92 \pm 0.39\%$) was anticipated for 27 ± 1.00 min. in plasma (Figure 2A). In the presence of UHEP, total inactivation of TG was achieved at $4 \mu\text{g well-plate}^{-1}$.

The profile of TG inhibition in 60-fold diluted plasma by SP-Am was concentration-dependent and highly sensitive ($p < 0.05$) than the 36-fold one (Figure 2). When $83.3 \mu\text{g well-plate}^{-1}$ of SP-Am was added to thromboplastin-activated plasma, the TG curve was abolished. The dose-effect with SP-Am (4.1 – $41.6 \mu\text{g well-plate}^{-1}$) was observed ($p < 0.05$), yielding average inhibition rates of 35.35 ± 0.25 , 40.71 ± 0.24 and $53.92 \pm 0.28\%$ for peak of thrombin in times of 22 ± 1.00 , 26 ± 1.00 and 43 ± 1.00 min; and *lag* times at 11 ± 1.00 , 14 ± 1.00 and 33 ± 1.00 min, respectively, vs. control curve. While at highest dose ($83.3 \mu\text{g well-plate}^{-1}$), it required an amount 20.82-fold of SP-Am higher than UHEP to reach complete inhibition of TG by extrinsic pathway (Figure 2B). Considering both plasma preparations, no inhibition of TG was found in the absence of thromboplastin.

Table 1. Average values of TG parameters in diluted plasma treated with SP-Am and its desulfated product using UHEP as a reference.

Polysaccharide ($\mu\text{g well-plate}^{-1}$)	peak of thrombin (%)	time to peak (min)	<i>lag</i> time (min)
thromboplastin-activated TG in 36-fold diluted plasma			
SP-Am: 4.1	56.17 ± 0.20 ^{aA}	22 ± 1.00 ^{aA}	10 ± 1.00 ^{aA}
SP-Am: 8.3	56.92 ± 0.23 ^{aA}	23 ± 1.00 ^{aA}	10 ± 1.00 ^{aA}
SP-Am: 41.6	67.79 ± 0.60 ^{bA}	33 ± 1.00 ^{bA}	13 ± 1.00 ^{bA}
SP-Am: 83.3	62.92 ± 0.39 ^{cA}	27 ± 1.00 ^{cA}	11 ± 1.00 ^{cA}
UHEP: 2	58.42 ± 0.27 ^{ad}	20 ± 1.00 ^{ad}	02 ± 1.00 ^d
UHEP: 4	100 ± 0.00 ^{eA}	-	-
Control	-	17 ± 1.00	06 ± 1.00
thromboplastin-activated TG in 60-fold diluted plasma			
SP-Am: 4.1	35.35 ± 0.25 ^{aB}	22 ± 1.00 ^{aA}	11 ± 1.00 ^{aA}
SP-Am: 8.3	40.71 ± 0.24 ^{bB}	26 ± 1.00 ^{bB}	14 ± 1.00 ^{bB}
SP-Am: 41.6	53.92 ± 0.28 ^{cB}	43 ± 1.00 ^{cB}	33 ± 1.00 ^{cB}
SP-Am: 83.3	100 ± 0.37 ^{dB}	- ^B	- ^B
UHEP: 4	100 ± 0.00 ^{dB}	-	-
Control	-	18 ± 1.00	8 ± 1.00
tissue factor pathway-inducing desulfated product in 60-fold diluted plasma			
SP-Am: 4.1	18.07 ± 0.98 (+) ^a	28 ± 1.00 ^a	15 ± 1.00 ^a
SP-Am: 8.3	18.07 ± 0.99 (+) ^a	30 ± 1.00 ^b	17 ± 1.00 ^b
SP-Am: 41.6	6.62 ± 1.22 (+) ^b	28 ± 1.00 ^{abc}	15 ± 1.00 ^{abc}
SP-Am: 83.3	3.61 ± 1.13 (+) ^c	31 ± 1.00 ^{abcd}	17 ± 1.00 ^{abcd}
UHEP: 4	100 ± 0.00 ^e	-	-
Control	-	37 ± 1.00	24 ± 1.00

Data represent means $\pm$ SEM ($n = 3$). Letters among the polysaccharide concentrations indicate significant difference at $p < 0.05$ vs. control. Letters between polysaccharide concentration reveal significant difference at $p < 0.05$, considering the diluted plasmas. Values were analyzed by one-way ANOVA followed by Tukey's test or *t*-Student; - not determined; (+) algal polysaccharide-induced TG.

Accordingly, SP-Am and UHEP did not interact with thrombin substrate in purified system since the different concentrations of the polysaccharides did not modify the control assay profile (not shown). When evaluated in purified Factor Xa system, only UHEP had a distinct profile in the presence of the substrate compared with the normal dynamic of the assay (Figure 3C).

SP-Am inhibited intrinsic pathway-triggered TG in 60-fold diluted plasma (Table 2), but when devoid of cephalin, it increased the clot formation dose-dependently, although with modest role vs. UHEP which had no effect on it (Figure 2D). By contrast, desulfated SP-Am importantly stimulated thromboplastin-induced TG in 60-fold diluted plasma treated in the range of concentrations vs. control (Figure 2E). The desulfated product led to a hypercoagulation response in connectively with the TG parameters by active extrinsic pathway, not only inducing the system, but also reducing the *lag* time compared with the control ($p < 0.05$). These effects were preponderant in low doses, up to $8.3 \mu\text{g well-plate}^{-1}$ (Table 1). The absence of sulfation had an opposite effect on blood coagulation based on total inhibition of UHEP.

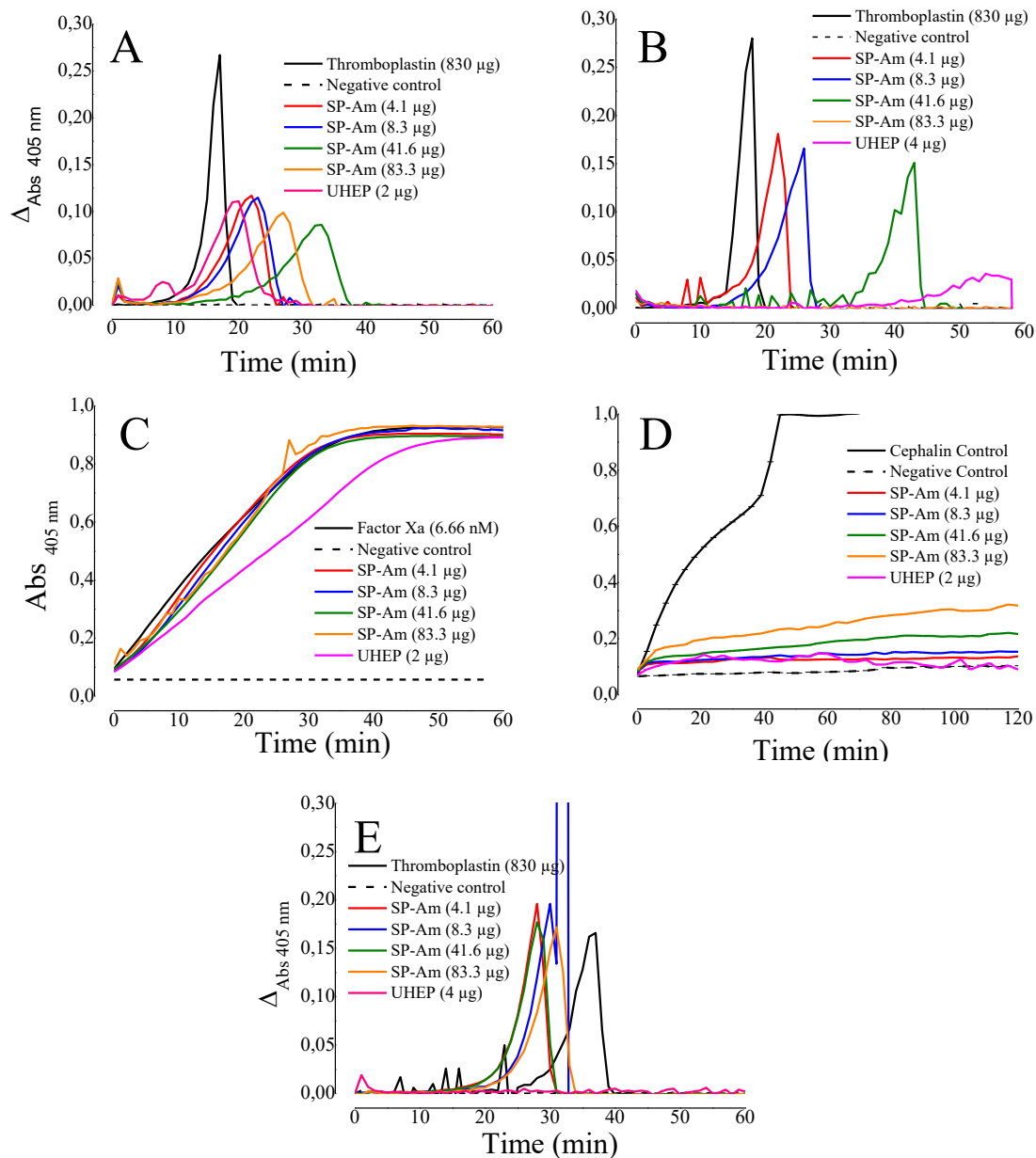


Figure 2. Effect of different concentrations of SP-Am from *A.muscoides* on thromboplastin or cephalin-induced TG using 36 (A) or 60 (B)-fold diluted human plasma, purified Factor Xa system (C), without contact-activator (D) and desulfated-derivative (E) by continuous detection method (37°C, 60-120 min.).

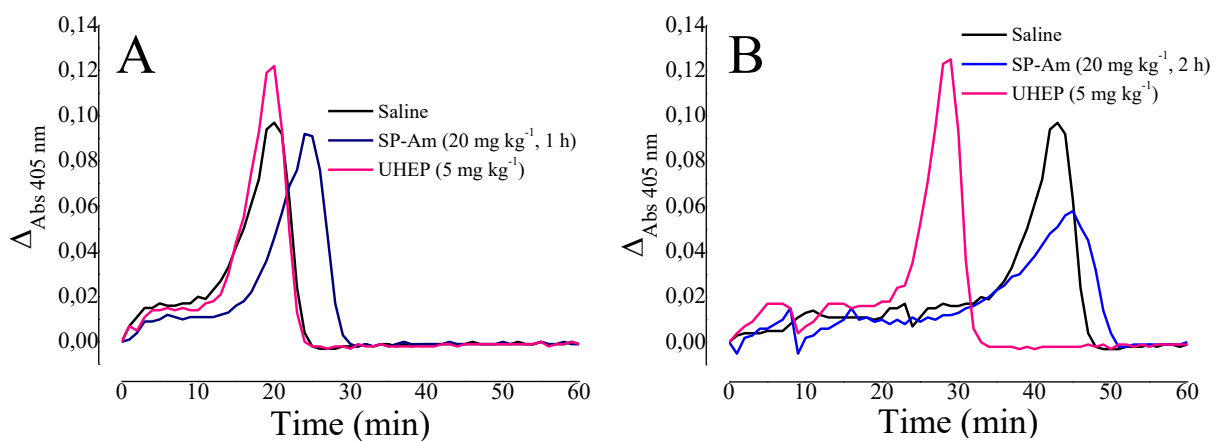


Figure 3. Effect of SP-Am on TG by intrinsic pathway in mice plasma. Mice received SP-Am (20 mg kg^{-1} body weight, ip.), UHEP (5 mg kg^{-1} body weight, ip.) or saline (0.9% NaCl wv⁻¹, ip.). After 1 (A) or 2 (B) h treatment, peripheral blood was collected and plasma analyzed ex vivo for TG.

SP-Am intrinsically modulates TG using normal or defibrinated plasma

Table 2 lists that normal plasma treated with SP-Am entirely inhibited TG at high doses (100% inhibition). In other way, the effect of SP-Am reduced cephalin-induced TG using defibrinated plasma (Table 2). It was an anti-TG agent concentration-dependent effective ($p < 0.05$), although inhibiting by $72.97 \pm 0.35\%$ in highest dose ($83.3 \mu\text{g well-plate}^{-1}$) the peak of thrombin recorded at $18 \pm 1.00 \text{ min.}$, and *lag* time of $14 \pm 1.00 \text{ min.}$ For UHEP, at concentration 41-fold lower, it completely suppressed TG. Even with thromboplastin, SP-Am had no significant effect in this system (data not shown).

Table 2. Average values of TG parameters in cephalin-triggered diluted plasma system using SP-Am and UHEP as a reference.

Polysaccharide ($\mu\text{g well-plate}^{-1}$)	peak of thrombin (%)	time to peak (min)	<i>lag</i> time (min)
cephalin-activated TG in 60-fold diluted plasma			
SP-Am: 4.1	58.25 ± 0.22^a	40 ± 1.00^a	29 ± 1.00^a
SP-Am: 8.3	58.25 ± 0.25^a	42 ± 1.00^a	37 ± 1.00^b
SP-Am: 41.6	100.00 ± 0.00^b	52 ± 1.00^b	43 ± 1.00^c
SP-Am: 83.3	100.00 ± 0.00^b	53 ± 1.00^c	44 ± 1.00^d
UHEP: 2	100 ± 0.00^c	-	-
Control	-	34 ± 1.00	28 ± 1.00
cephalin-activated TG in 60-fold diluted defibrinated plasma			
SP-Am: 4.1	41.44 ± 0.61^a	17 ± 1.00^a	11 ± 1.00^a
SP-Am: 8.3	41.44 ± 0.47^a	16 ± 1.00^a	08 ± 1.00^b
SP-Am: 41.6	68.46 ± 0.27^b	17 ± 1.00^a	10 ± 1.00^{ac}
SP-Am: 83.3	72.97 ± 0.35^c	18 ± 1.00^a	14 ± 1.00^{ad}
UHEP: 4	100 ± 0.00^d	-	-
Control	-	15 ± 1.00	09 ± 1.00

Data represent means $\pm$ SEM ($n = 3$). 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.

Treatment with SP-Am reduces TG *ex vivo* in mice plasma

Systemic administration of SP-Am (20 mg mL^{-1} , *ip.*) to mice with a single injection produced *ex vivo* TG inhibition. From the collected plasma, no effect on the intrinsic coagulation pathway by polysaccharide was noticed after 1 hour treatment from the saline group ($p > 0.05$), except for a discrete delay in *lag* time (Figure 3A).

As a reference, UHEP induced TG in analyzed plasma sample, similar to time of peak generated by saline group ($20 \pm 1.02 \text{ min.}$) in the assay (Table 3). Extended treatment of the animals for 2 hours allowed to clearly detect an inhibitory effect of TG at 25 min. (Figure 3B), with $40.20 \pm 1.22\%$ reduction ($p < 0.05$) of the response generated by cephalin (Table 3). UHEP potentially increased the hypercoagulation in plasma vs. saline group, as well as anticipating the *lag* time for $19 \pm 1.00 \text{ min.}$ in the same experiment. Significant effects of SP-Am by means of the thromboplastin-stimulated TG were not detected (not shown).

Table 3. Average values of TG parameters in cephalin-triggered diluted mice plasma using SP-Am and UHEP as a reference, after 1 or 2 hours.

Polysaccharide ($\mu\text{g well-plate}^{-1}$)	peak of thrombin (%)	time to peak (min)	<i>lag</i> time (min)
1 hour			
SP-Am (20 mg kg^{-1} , <i>ip.</i>)	06.18 ± 1.51^a	25 ± 1.05^a	12 ± 1.00^a
UHEP (5 mg kg^{-1} , <i>ip.</i>)	$25.77 \pm 1.35^{(+)^b}$	20 ± 1.12^a	10 ± 1.00^b
Saline ($0.9\% \text{ NaCl}$, <i>ip.</i>)	-	20 ± 1.02	09 ± 1.00
2 hours			
SP-Am (20 mg kg^{-1} , <i>ip.</i>)	40.20 ± 1.22^a	45 ± 1.00^a	25 ± 1.00^a
UHEP (5 mg kg^{-1} , <i>ip.</i>)	$28.86 \pm 1.26^{(+)^b}$	29 ± 1.00^a	19 ± 1.00^b
Saline ($0.9\% \text{ NaCl}$, <i>ip.</i>)	-	43 ± 1.00	26 ± 1.00

Data represent means $\pm$ SEM ($n = 3$). 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; (+) polysaccharide-induced TG.

Discussion

In this study, SP-Am was physically, chemically and structurally checked, and further evaluated *in vitro* and *ex vivo* on TG using human and mice-collected plasmas, respectively. SP-Am also contained nonSPs of low molecular mass (Rodrigues et al., 2021) as previously confirmed by Vanderlei et al. (2016) using gel permeation chromatography, which showed a heterogeneous system formed by a polysaccharide chain with

low molecular distribution. This result appeared on gel electrophoresis, after the use of Stains-all alone, in brown color, suggesting uronic acid-containing glycans in *A. muscoides* and purity of the agarocolloid confirmed by its structure as identified on FT-IR and NMR analyses (Quinderé et al., 2014; Vanderlei et al., 2016; Rodrigues et al., 2021) and by comparisons with other authors who found agar polysaccharide in *Acanthophora* (Duarte et al., 2004) and other seaweed species (Ferreira et al., 2012; Recalde et al., 2016). Similar to our study, polysaccharides isolated from animal (Volpi & Maccari, 2002; Andrade et al., 2017), plant (Souza et al., 2015) and seaweed (Rodrigues et al., 2017b and 2019) were also characterized by their specific colors, reinforcing the use of Stains-all alone as a more precise strategy to also reveal acidic polysaccharides in red seaweeds than in combination with toluidine blue (Rodrigues et al., 2021). Additionally, HMBC analysis showed a doublet signal due to nondecoupling of the pulse sequence relatives to a sulfated galactan pyruvate form of six-membered cyclic ketal, including O-4 and O-6 positions (4,6-O-(1'carboxy)-ethylidene cyclic ketals), since that this structural feature has not been frequently found in agarophytes (Cardozo et al., 2007; Ferreira et al., 2012; Mourão, 2015). Other authors reported a possible coexistence in sulfated galactan pyruvate forms of five-membered/six-membered cyclic ketals, as in green seaweeds (Farias et al., 2008; Arata et al., 2015).

Collectively, our investigation led us to, for the first time, report a peculiar pyruvylated galactan for *Acanthophora*, but typical for the order Ceramiales, whose members show high structural complexity of agarophyte forms (Ferreira et al., 2012). Notably, with low molecular weight agarans independent of other galactans, like carrageenans (Recalde et al., 2016).

TG has a vital role in hemostatic balance (Rau et al., 2007), when it is reduced antithrombotic and hemorrhagic events increase, while stimulates thrombosis (Castoldi & Rosing, 2011; Duarte et al., 2017). Studies on the extrinsic pathway, which is a physiological trigger for hemostasis or thrombus production (Rau et al., 2007), have been little explored using SPs (Nishino et al., 1999; Wu et al., 2014; Rodrigues et al., 2017a; Barcellos et al., 2018; Salles et al., 2017). SP-Am modified TG parameters at dose-dependent effects, in 36- or 60-fold diluted human plasmas, on the tissue-factor pathway compared to control curves, respectively. On the contrary, the used doses of SP-Am were relatively lower than in the PT test (Barcellos et al., 2018), by which none allowed to detect slight differences due to its low lack, since it has fast kinetic to reveal thrombin formed (Arata et al., 2015), while TG assay had more precision to measure total thrombin (Castoldi & Rosing, 2011; Duarte et al., 2017), thus showing the anticoagulant dynamic of SP-Am compared with other studies (Nishino et al., 1999; Wu et al., 2014; Rodrigues et al., 2017a and 2019; Salles et al., 2017). UHEP abolished TG at an amount 2-fold higher than that required to intrinsically inactivate TG (Rodrigues et al., 2019; Salles et al., 2017), in which UHEP had affinity for plasma antithrombin (Rodrigues et al., 2021); whereas in the PT assay, it did not alter the normal values, as reported by Rodrigues et al. (2016b). The intrinsic inhibitory effect from all the polysaccharides was more important than the extrinsic one, similarly to brown seaweed *E. kurome* (Nishino et al., 1999) and freshwater fish *O. niloticus* (Salles et al., 2017) SPs. In this way, SP-Am could interact with factors (V, VII/Va, IX and X) and/or other mechanisms involved in the extrinsically TG inhibition (Rau et al., 2007).

No difference at the level of absorbance between the thrombin generated in control plasmas was noted as by intrinsic pathway, confirming that SP-Am and UHEP did not interact with the substrate for thrombin (Rodrigues et al., 2021). Similarly, SP-Am did not interfere with purified FXa system, which is critical for TG (Rau et al., 2007), contrasting UHEP that directly interacted with the slow-reacting substrate in the continuous assay; therefore, acting as a competitor for thrombin. Thus, our method was able to monitor SPs with affinities or not when substrate was involved during its cleavage for thrombin activity (Lau et al., 2007).

Anti-clotting effects by SPs are due to sulfate groups attached to polymer, but not when desulfated (Quinderé et al., 2014; Mourão, 2015). SP-Am displayed serpin-independent TG inhibition by contact-activation related to its chemical structure (Rodrigues et al., 2021). In this study, desulfated SP-Am was devoid of anti-TG action in 60-fold diluted human plasma by extrinsic pathway, but, interestingly, potentially anticipated and induced TG, as also previously observed by the intrinsic one, while UHEP did not induce TG as expected (Furugohri & Morishima, 2015; Rodrigues et al., 2021). The expression of both free thrombin activity and thrombin-A2M with unmodified or desulfated SP-Am could be hard to interpret due to unknown interactions with slow-acting substrate along of the reactions (Lau et al., 2007). Taking with literature data, our results were in line with the procoagulant effect of the desulfated fucoidan from the brown seaweed *Fucus vesiculosus*, when examined by the calibrated automated thrombogram method (Zhang et al., 2014). Direct thrombin inhibitors, as melagatran, induced coagulation *in vitro* initiated by tissue factor based on its serpin-independent anticoagulant mechanism (Perzborn et al., 2014).

Cephalin or thromboplastin-devoid TG allowed to examine whether SP-Am would stimulate 60-fold diluted plasma, and suggested that, in the concentration range tested, the modestly TG-induced algal polysaccharide as FXII-speculated procoagulant effect previously related to low molecular mass accordingly, while UHEP did not activate FXII (Quinderé et al., 2014). This was also postulated for a SPs fraction from the green seaweed *Caulerpa cupressoides*, but 100% TG inducing at highest dose (Rodrigues et al., 2019). 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. As report, induced TG by melagatran, but not UHEP, was recorded by intrinsic pathway in human plasma (Furugohri & Morishima, 2015). Thus, desulfated and native SP-Am require future comparison on TG based on balance between pro- and anticoagulant effects, as already studied *in vivo* using SPs (Rodrigues et al., 2011; Quinderé et al., 2014; Mourão, 2015).

Ancrod is a non-thrombin inhibitor that acts by cleaving fibrinopeptide in plasma (Gurbuz et al., 2005). Herein, assay with SP-Am-treated 60-fold diluted defibrinated human plasma stimulated by intrinsic pathway dose-dependently reduced TG, and was suppressed by UHEP from the control, as revealed by TG parameters. Glauser, Pereira, Monteiro, and Mourão (2008) also detected actions of sea cucumber *L. grisea* SPs on the contact pathway using defibrinated plasma. Comparison between the assays using normal and defibrinated plasmas suggested that the free thrombin activity predominated over that in *in vitro* normal plasma system. It could be convenient because substrate cleavage would feed normal plasma TG, contrasting to plasma treated with Ancrod prior to assay, in which the activity of free and fibrin bound thrombin reduced clot formation (Lau et al., 2007). In the presence of SP-Am in the same plasma, when induced by thromboplastin, no important inactivation was achieved in the absence of fibrin, demonstrating its effects as preponderant in intrinsic coagulation. UHEP abolished TG as expected (Glauser et al., 2008). Therefore, SP-Am acted on the presence or absence of fibrin in diluted human plasma, similar to fibrin polymerization inhibitors (Lau et al., 2007).

Finally, mice treated with SP-Am for 1 or 2h changed TG response in 60-fold diluted peripheral blood plasma, in terms of lag time and peak of thrombin activity, by reducing thrombosis risk, since similar levels of active thrombin were generated by intrinsic pathway in the saline controls. These effects were coherent with those *in vivo*, which are involved in antithrombotic action devoid of hemorrhage in SP-Am-treated rats (Quinderé et al., 2014). Rats orally administered fucoidan (900 and 2500 mg kg⁻¹) from the brown seaweed *Laminaria japonica* presented anti-clotting *in vivo* as an adverse consequence considering renal patients (Li et al., 2005). Rocha et al. (2005) discovered that a SP from the brown seaweed *Spatoglossum schroederi* had time-dependent antithrombotic effect in rats, displaying an important effect up to 8h after intravenous administration. Rodrigues et al. (2013) discovered that SPs from the green seaweed *Caulerpa cupressoides* administered (9 mg kg⁻¹, ip.) to mice along fourteen consecutive days altered the platelet count in peripheral blood. Its SPs had *in vivo* important pharmacological effects from other studies (Rodrigues et al., 2011, 2012). Early, SP-Am did not induce any systemic damage in mice (Quinderé et al., 2013). Conversely, heparinized animals showed high risk of thrombocytopenia by a possible interaction with the circulating platelets (Gurbuz et al., 2005), since the prolonged use of UHEP can induce clot complications (Mourão & Pereira, 1999), based on cephalin free and platelet *poor* plasma assays that showed no hypercoagulable state as already mentioned. Another result was the lack of *ex vivo* anti-TG effect by SP-Am-received mice plasma activated by thromboplastin, postulating, under our conditions, that its systemic action did not involve the extrinsic clotting pathway (Xie et al., 2011).

In summary, the application of a TG model using SP-Am allowed to compare continually *in vitro* and *ex vivo* effects on both coagulation pathways. Further correlation between these effects could help in the development of an antithrombotic agent to better understand its persistence in plasma, as well as bioavailability and prognostics of circulatory disorders (Mourão & Pereira, 1999) associated with inflammation (Quinderé et al., 2015) and infections (Vanderlei et al., 2016), using different routes in future studies, to direct investigations on its pharmacological actions (Rodrigues et al., 2017b).

Conclusion

The Rhodophyta *Acanthophora muscoides* presents a 4,6-pyruvylated agaran galactan fraction, containing acid uronic residues of low molecular mass, revealing peculiar structural characteristics distinct from other agarophyte species. This polysaccharide exerts intrinsic inhibitory effects on thrombin generation more remarkable than extrinsic effects in 60-fold diluted mice and human plasmas, whereas desulfated and native fractions stimulate coagulation with or without activator related to *in vitro* procoagulant events, respectively.

It also acts on both free and fibrin bound thrombin activity by contact *via* similar to fibrin polymerization inhibitors, besides in the *ex vivo* assay using heparin or fraction-treated mice plasma prove to be useful to evaluate the systemic coagulability status, as a pre-clinical prognostic. Overall, our study contributes with biological basis to the understanding of the anticoagulant dynamics of the algal polysaccharide to the regulation of clot formation *in vitro* and *ex vivo*.

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