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In vitro inhibition of thrombin generation by sulfated polysaccharides from the marine alga *Solieria filiformis* (Kützing) Gabrielson (Solieriaceae, Rhodophyta)

Inibição *in vitro* de geração de trombina por polissacarídeos sulfatados da alga marinha *Solieria filiformis* (Kützing) Gabrielson (Solieriaceae; Rhodophyta)

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Abstract *Solieria filiformis* is a rich source in carrageenans-type sulfated polysaccharides (SPs) containing two fractions (F I and F II), of which F I presented *in vivo* antinociceptive and inflammatory actions. However, the effects of its SPs on thrombin generation (TG) assays have not been investigated. This study characterized the physicochemical properties of SPs isolated from *Solieria filiformis* cultured in the coast of Ceará, Brazil and analyzed *in vitro* inhibitory effects on a TG system using a chromogenic method by continuous detection. Crude extract obtained with papain and fractionated by anion-exchange chromatography on a DEAE-cellulose column contained two fractions (F I and F II). Electrophoresis in agarose gel revealed an difference in terms of charge density between extract and fractions obtained from the DEAE-cellulose chromatography, suggesting the presence of both *kappa*- and *iota*-carrageenans, confirming a previous study using the respective *S. filiformis* fractions. Anticoagulant assays using normal human plasma and unfractionated heparin (UHEP) (193 IU mg^{-1}) by activated partial thromboplastin time test showed virtual effects in order of 1.34 (extract), 0.86 (F I) and 0.98 (F II) IU mg^{-1} less potents than UHEP; additionally, extract and fractions had no actions on the prothrombin time. 60-fold diluted human plasma treated with extract and fractions inhibited dependently of concentration TG stimulated by cephalin (intrinsic pathway) and thromboplastin (extrinsic pathway), with charge density-related TG inhibition pattern of the algal SPs. UHEP totally abolished TG at 4.15-fold lower concentration. Therefore, SPs from *S. filiformis* prevent thrombosis, *in vitro*, by interfering with the TG system, as a promising plasma alternative to modulate blood coagulation.

Keywords: sulfated glycans, physicochemical analysis, clot formation.

Resumo *Solieria filiformis* é uma fonte rica em polissacarídeos sulfatados (PSs) tipo carragenanas contendo duas frações (F I e F II), das quais F I apresentou ações antinociceptiva e inflamatória *in vivo*. Entretanto, não têm sido investigados os efeitos de seus PSs sobre ensaios de geração de trombina (GT). Este estudo caracterizou as propriedades físico-químicas de PSs isolados de *Solieria filiformis* cultivada na costa do Ceará, Brasil e analisada efeitos inibitórios *in vitro* sobre um sistema de GT usando um método cromogênico por detecção contínua. O extrato bruto obtido com papaína e fracionado por cromatografia de troca-iônica em uma coluna de DEAE-celulose conteve duas frações (F I e F II). Eletrophorese em gel de agarose revelou diferença de densidade de cargas entre extrato e frações obtidas da cromatografia em DEAE-celulose, sugerindo a presença de ambas carragenanas-*kappa* e -*iota*, respectivamente, e confirmando, ainda, um estudo prévio usando as frações de *S. filiformis*. Ensaios anticoagulantes usando plasma humano e heparina não-fracionada (HEPNF) (193 UI mg^{-1}) pelo teste do tempo de tromboplastina parcial ativada mostraram efeitos virtuais na ordem de 1,34 (extrato), 0,86 (F I) e 0,98 (F II) UI mg^{-1} potentes menos que HEPNF. Ademais, extrato e frações não possuíram ações sobre o time de protrombina. Plasma humano diluído 60 vezes tratado com extrato e frações inibiu GT estimulada por cefalina (via intrínsica) e tromboplastina (via extrínsica), com grau de inibição relacionada à densidade de carga dos PSs da alga. HEPNF aboliu totalmente GT em concentração 4,15 vezes menor. Os PSs de *S. filiformis* previnem trombose *in vitro* por interferir com o sistema de GT, alternativa promissora no plasma para modular a coagulação sanguínea.

Palavras-chave: glicanos sulfatados, análise físico-química, formação de coágulo

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Introduction

The marine habitat arises as a natural stock of living renewable resources at prospecting of potentially useful metabolites for health-related applications in many countries (Smit, 2004; Cardozo et al., 2007; Cheung, Ng, Wong, Chen & Chan, 2016). Among all coastal organisms, seaweeds (marine macroalgae) have a wide spatial and temporal distribution over both tropical and subtropical areas of the world (Ale et al., 2016) and have been widely explored by scientists and food and pharmaceutical firms as promising sources of biomaterials for human use (Smit, 2004; Cardozo et al., 2007; Silva et al., 2010; Prajapati, Maheriya, Jani & Solanki, 2014). However, the excessive exploration of the natural beds has generated disturbances on the marine aquatic communities worldwide, especially affecting the assemblages of marine benthic macroalgae in terms of diversity and distribution pattern along the several coastal systems (Pellizzari & Reis, 2011; Pereira & Costa-Lotufo, 2012). Mariculture of commercially-important seaweeds arises as a sustainable alternative for phycocolloid production in large scale and for the protection of the seaweeds natural beds (Rodrigues et al., 2011; Araújo et al., 2012).

Macroalgal resources are well-distributed along the Brazilian coast (Pellizzari & Reis, 2011; Pereira & Costa-Lotufo, 2012), where Ceará littoral is characterized by favorable tropical waters for the development of a diverse flora of seaweeds expressing structurally sulfated polysaccharides (SPs) with potential pharmacological values, such as anticancer (Costa-Lotufo et al., 2006), anticoagulant (Rodrigues et al., 2009; Rodrigues et al., 2013), anti-inflammatory (Pomin, 2012; Rodrigues et al., 2012a) and antithrombotic (Quinderé et al., 2014; Mourão, 2015) effects, and other high added-value products (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014; Paula et al., 2015). These marine sulfated glycans are biopolymers of highly anionic character (S=O) usually having large molecular sizes (> 100 kDa) (Pomin, 2012), occurring abundantly in the algae extracellular matrices as the main structural constituintes and vulnerable to changes in response to environment conditions (Pereira & Costa-Lotufo, 2012). On a basis of chemical classification, sulfated galactans (main carrageenan and agaran) are the most common molecular group found in Rhodophyceae (Prajapati, Maheriya, Jani & Solanki, 2014; Silva et al., 2010; Quinderé et al., 2014; Mourão, 2015). SPs are not only systhetized from algae (macro and microalgae) (Cardozo et al., 2007), but also in vertebrates (Rodrigues et al., 2012b), invertebrates (Mourão, 2015), freshwater plants (Dantas-Santos et al., 2012) and mushrom (Chang, Lur, Lu & Cheng, 2013); therefore, they have a wide phylogenetic distribution in nature (Pomin & Mourão, 2008). Their intrinsic functionatilies as texturing agents have also gained attention by hydrocolloid industry in the last decades because seaweeds SPs can be employed in various food and medical applications (Smit, 2004; Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014).

Reports have shown SPs from seaweeds endemic to the Ceará coast as plasma anternative anticoagulants to unfractionated heparin (UHEP) (Rodrigues et al., 2009; Rodrigues et al., 2013; Quinderé et al., 2014; Mourão, 2015) which it is an anticoagulant drug primarily required in clinic practice (eg., cardiovascular surgery and hemodialysis) to treat circulatory dysfunctions (thrombosis) inducing extensive bleeding effects and thrombocytopenia (Nader et al., 2001). For the analysis of anticoagulants, two classical coagulation assays are employed: 1) the activated partial thromboplastin time (APTT); and 2) the prothrombin time (PT), which explore the intrinsic and extrinsic blood coagulation pathways, respectively. Although conventionally used, both tests do not reflect the normal hemostatic function in comparison with the thrombin generation (TG)-based models which could better predict the overrall coagulation phases because they have more sensitivity to detect the coagulation process from a plasma sample (Castoldi & Rosing, 2011). Thus, TG assays would provide a more refined examination of different compounds regarding their anticoagulant dynamics, including marine SPs-type molecules (Nishino, Fukuda, Nagumo, Fujihara & Kaji, 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014; Rodrigues et al., 2016; Rodrigues et al., 2017).

Members of the family Solieriaceae belonging to the order Gigartinales have high global distribution pattern (Murano, Toffanin, Cecere, Rizzo & Knutsen, 1997; Chiovitti, Bacic, Kraft, Craik & Liao, 1999; Goulard, Diouris, Quere, G., Deslandes & Floc, 2001) and would correspond to a macroalgal biomass available along the Ceará coast, Brazil, for a versatility of applications, such as antimicrobial (Holanda et al., 2005), anti-inflammatory (Araújo et al., 2012) and in nutrition (Carneiro, Rodrigues, Teles, Cavalcante & Benevides, 2014). Within the genus *Solieria*, some SPs have been structurally characterized as carrageenan-type polysaccharide molecules (Murano, Toffanin, Cecere, Rizzo & Knutsen, 1997; Chiovitti, Bacic, Kraft, Craik & Liao, 1999; Goulard, Diouris, Quere, G., Deslandes & Floc, 2001), revealing composition dependent on the salinity changes (Goulard, Diouris, Quere, G., Deslandes & Floc, 2001), as well as

exhibiting biactivities (Stephanie, Eric, Sophie, Chistian & Yu, 2010). Araújo et al. (2011) identified two major fractions (F I and F II) derived from *S. filiformis* (Kützing) Gabrielson SPs, which revealed peculiar characteristics of *kappa* and *iota*-carrageenans, respectively, according to the infrared-structural analysis. Treatment with F I attenuated nociception in mice, but produced inflammation in rats. Subsequently, Araújo et al. (2012) reported that an extract containing *iota*-carrageenans reduced chemically-induced inflammation in rats, without modifying the *in vitro* normal coagulation time when measured by conventional APTT assay. More recently, Paula et al. (2015) examined the development of edible films from mixtures of carrageenans, including *S. filiformis iota*-carrageenans.

In this study, we evaluated the physicochemical properties of the *S. filiformis* SPs by agarose gel electrophoresis and also analyzed SPs capability of modulating an *in vitro* TG system in 60-fold diluted normal human plasma using the chromogenic method by a continuous measurement system in order to explore their anticoagulant dynamics.

Material and Methods

Solieria filiformis COLLECTION AND PREPARATION OF SPS FOR THE PHYSICOCHEMICAL ANALYSES

Samples of *S. filiformis* (Rhodophyta) were collected in open waters from *long line* structures located at 200 m of the Brazilian coast (03°13'06"S, 39°16'47"W) (Flecheiras beach, Trairí, Ceará State) as previously described by Araújo et al. (2012). These materials were taken in plastic bags for transport from field to the Carbohydrates and Lectins Laboratory (CarboLec), Federal University of Ceará. A voucher specimen (no. 40781) was deposited in the Herbarium Prisco Bezerra of the Department of Biological Sciences, Federal University of Ceará, Brazil. Seaweed samples were prepared based on Rodrigues et al. (2011) and Araújo et al. (2012), eliminating macroscopic epiphytes and small marine animals, and washing with distillated water to remove seawater and sediments prior to dehydration at room temperature. The analyses of the *S. filiformis* SPs were performed at Connective Tissue laboratory, Federal University of Rio de Janeiro (FURJ), Brazil.

In order to obtain SPs extract, dehydrated algal tissue (2 g) were cut into small pieces and subjected to papain digestion procedure (60°C, 24 h) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM), as previously published elsewhere (Araújo et al., 2012). A sample of the extract (20 mg) was dissolved in 10 mL of 50 mM sodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose (1.2 × 12 cm) column equilibrated with the same buffer. Elution of the SPs was conducted with a stepwise of NaCl (from 0 to 1 M, at 0.25 M intervals in the same buffer) and the fractions (2.5 mL) collected and analyzed for SPs using the metachromatic assays containing dimethymethylene blue with an Amersham Bioscience Ultrospec 3100 spectrosphotometer at 525 nm, according to Fandarle, Buttle & Barrett (1986). The metachromatic fractions were further dialyzed and freeze-dried. Detection of SPs on agarose gel by sequential staining with toluidine blue was also performed by comparison with the electrophoretic mobility of heparan sulfate (HS), dermatan sulfate (DS) and UHEP (Dietrich & Dietrich, 1976; Araújo et al., 2011; Araújo et al., 2012).

IN VITRO COAGULATION METHODS

BLOOD SAMPLES

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

ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) AND PROTHROMBIN (PT) TESTS

Extract and fractions were assessed by both *in vitro* APTT and PT tests according to the manufacturers' kit specifications, for measure anti-clotting effect in a coagulometer Amelung KC4A before of the *in vitro* TG assay. For APTT assay, a mixture of 100 µL of plasma and concentration of SPs (1 mg mL^{-1}) was incubated with 100 µL of APTT reagent (kaolin bovine phospholipid reagent). After 2 min of incubation at 37°C, 100 µL of 25 mM $CaCl_2$ was added to the mixtures, and the clotting time was recorded. Regarding the PT assay, a mixture of 100 µL of plasma and concentration of SPs (1 mg mL^{-1}) was incubated for 1 min at 37°C. After that, 100 µL of PT reagent was added to the mixtures, and the clotting time was recorded using same coagulation equipment. UHEP with 193 international units (IU mg^{-1}) of polysaccharide was used as the standard on both tests. All the tests were done in triplicate and the data were expressed as mean ± S.E.M.

TG ASSAY

This assay was performed in a microplate format as described by Rodrigues et al. (2016). The microplate contained 10 µL of cephalin (contact-activator system) or thromboplastin (1083 µg well-plate^{-1}, factor tissue-activator system) + 30 µL of 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 µL of SPs (*S. filiformis* extract and fractions: 0, 4.1, 41.6 or 83.3 µg.well-plate^{-1} or UHEP: 2 or 4 µg.well-plate^{-1}) + 60 µL of 20 mM $CaCl_2$ and 0.33 mM chromogenic substrate S2238 (10:50 ratio, v/v). The *in vitro* reaction was triggered at 37°C by addition of plasma (diluted 60-fold well-plate^{-1}) (10 µL), and the absorbance (405 nm) was continually recorded for 60 min (Plate reader Thermo-max, America Devices). The inhibitory response of TG by SPs was determined by lag phase, peak thrombin (PTh) and/or time to peak (TPeak).

Results and Discussion

PHYSICAL AND CHEMICAL CHARACTERIZATION OF THE *S. filiformis* CELL-WALL SPS

The figure 1 represents the electrophoretic analysis on the agarose gel of the *S. filfiormis* SPs. The DEAE-cellulose colunm-bound SPs eluted at 0.5 and 0.75 M NaCl (F I and F II, respectively) (Araújo et al., 2011), as monitored by metachromasy assay due to complex-binding properties of the isolated SPs by use of dimethylmethylene blue (Fandarle, Buttle & Barrett, 1986), revealed physical and chemical characteristics distincts between them by comparison with CS, DS and UHEP.

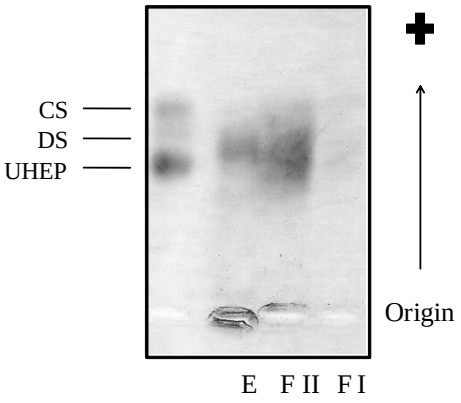

Figure 1 Agarose gel electrophoresis of the *Solieria filiformis* SPs. Extract (E), fractions (F I and F II) and standards glycosaminoglycans chondroitin sulfate (CS), dermatan sulfate (DS) and unfractionated heparin (UHEP) were stained with 0.1% toluidin blue.

All the samples contained high-density SPs after staining with toluidine blue, as visualized by the purple aspect (Dietrich & Dietrich, 1976; Rodrigues et al., 2009; Rodrigues et al., 2011; Dantas-Santos et al., 2012).Fraction F II, which had SPs retained in the column eluted with 0.75 M NaCl, showed strong homogeneous band due to its higher presence of sulfated groups from the examined polymer sample than fraction F I that did not apear on agarose gel as a consequence of its relatively lower charge density (Rodrigues et al., 2011; Dantas-Santos et al., 2012). These combined results supported the presence of *kappa* and *iota*-carrageenan-structures in the respective fractions as previously published results by Araújo et al. (2011), who prior examined the *S. filiformis* SPs by both agarose and infrared analyses. On this basis, carrageenans comprise a chemical class of polyanionics that have diferrential levels of charge density as a result of their primary differences regarding the number and position of ester sulfate groups, as well as the content of 3,6-anhydro-galactopyranose on their chains (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014). These structural differences could reflect in the degree of sulfation from the agarose gel (Figure 1) as expected (Araújo et al., 2011). The quality and content of SPs depend on environmental conditions, growth and age of the algae (Goulard, Diouris, Quere, G., Deslandes & Floc, 2001; Cardozo et al., 2007) to develop a natural product in industrial scale (Pereira & Costa-Lotufo, 2012). Moreover, extract and fraction F II also showed similar mobilities, sugesting same structural conformation of the SPs (Dietrich & Dietrich, 1976) co-migrating close to DS (Figure 1).

On the basis of these previous observations, this research was further designed to analyze the anticoagulant effects of the SPs from the red seaweed *S. filiformis*, when added to normal human plasma, on two classical coagulation tests (APTT and PT) before of the *in vitro* TG assay.

EFFECTS ON *IN VITRO* APTT AND PT ASSAYS AND TG RESPONSE

Table 1 shows the results of the normal human plasma treated with extract and fractions (F I and F II) obtained from the red seaweed *S. filiformis* and UHEP (193 IU mg^{-1}) used as a reference, when sujected to both *in vitro* APTT and PT assays. SPs from *S. filiformis* showed modest effects on the APTT assay and only reflected their roles in the intrinsic coagulation pathway by 1.34 (59.07 ± 0.62 s), 0.98 (38.10 ± 0.05 s) and 0.86 IU mg^{-1} (41.67 ± 0.20 s) for extract ($p < 0.05$) and fractions F I and F II, respectively. On a comparative

basis, these effects were in concordance with those APTT' results found by Araújo et al. (2012), using this same algal species. Therefore, the anticoagulant response of the *S. filiformis* SPs did not double the normal APTT in a high concentration of polysaccharide (1 mg ml^{-1}), with extract altering in only 1.59-fold the normal coagulation time in comparison with the control plasma without SPs (37.13 ± 0.21 s). No extrinsic pathway inhibition of these molecules on the PT method was dectected vs. control time (09.43 ± 0.32 s) (Mourão, 2015). Regarding the UHEP effects, it still showed, at concentrations of 5 and 100 µg.mL^{-1}, respectively, *in vitro* anti-clotting effects on the APTT (41.40 ± 0.6 s) and PT (23.40 ± 0.05 s) tests vs. control times (APTT: 37.13 ± 0.21 s; PT: 09.32 ± 0.32 s) (Nader et al., 2001).

Table 1 Evaluation of extract and fractions, obtained by anion-exchange chromatography (DEAE-cellulose), from the red seaweed *Solieria filiformis* on classical coagulation tests.

Polysaccharides	NaCl (M)	APTT (s)*	PT (s)**		
		1 mg.mL^{-1}***		$T_1.T_0^{-1}$&	$IU.mg^{-1}$#
Extract	-	59.07 ± 0.62[b]	10.73 ± 0.31	1.59	1.34
F I	0.50	38.10 ± 0.05[a]	09.83 ± 0.37	1.00	0.86
F II	0.75	41.67 ± 0.20[a]	09.52 ± 0.26	1.12	0.98

NaCl - Sodium chloride; *Activated partial thromboplastin time (APTT); **Prothrombin time (PT); ***SPs concentration to prolong the APTT or PT in seconds; &Ration for prolong the APTT; #Anticoagulant effect expressed in international units (IU) per mg of SPs ($IU.mg^{-1}$); UHEP (193.00 $IU.mg^{-1}$: 5 and 100 µg.mL^{-1} for APTT (41.40 ± 0.6 s[a]) and PT (23.40 ± 0.05 s), respectively); Controls: 37.13 ± 0.21 s and 09.43 ± 0.32 s for APTT and PT tests, respectively (n = 3, Anova, Tukey's test, $p < 0.05$ vs. control).

Anticoagulation of the SPs from seaweeds (Rodrigues et al., 2009; Silva et al., 2010; Rodrigues et al., 2011) is related to some molecular requirements, including sugar type, sulfation pattern, anomeric configuration, glycosidic linkage and molecular mass (Pomin, 2012; Rodrigues et al., 2013). Specific bindings of the SPs with plasma proteins lead to molecular interactions in the haemostatic system displaying coagulation inhibitory effects. Consequently, the structure-function relationships of these SPs remain to be difficult due to the fact that seaweeds SPs have heterogeneous structures (Prajapati, Maheriya, Jani & Solanki, 2014; Mourão, 2015). A research performed by Rocha et al. (2005) revealed that a sulfated galactofucan from the brown seaweed *Spatoglossum shroederi* had no anticoagulant action on several *in vitro* assays (due to the presence of non-sulfate xylose units at the non-reducing terminal ends of the branches), but stimuled the synthesis of antithrombotic heparan sulfate by endothelial cells. More recently, Quinderé et al. (2014) reported that a SPs fraction isolated from the red seaweed *Acanthophora muscoides* presented independent effect of serpin *in vitro*, demonstrating new focus to gain insight in the study of antithrombotic sulfated glycans.

This investigation indicated the higher sensitivity of the APTT test than the PT assay, although modestly inhibiting the APTT by algal SPs (Table 1). On the basis this comparation, it is speculated that these classical coagulation methods would not reflect the normal hemostatic function in order to more precisaly evaluate the anticoagulant potential of the SPs since limited values were primarily measured (Castoldi & Rosing, 2011). Thus, an *in vitro* TG model stimulated by cephalin (contact pathway) or thromboplastin (tissue factor pathway), in which 60-fold diluted normal human plasma was treated with *S. filiformis* SPs (0, 4.1, 41.6 or 83.3 µg.well-$plate^{-1}$) to measure continually at 37°C for 60 min their anticoagulant dynamics compared with that of UHEP (2 or 4 µg.well-$plate^{-1}$) (Rodrigues et al., 2016), as illustrated in Figure 2.

Extract and fractions (F I and F II) added to diluted human plasma had a differential level of *in vitro* inhibitory response on TG after activation by both contact-activated and thromboplastin-activated systems, with basis on control absorbance of the amydolytic activity of thrombin that reduced rapidly until a plateu was reached from 25 to 33 min (Mourão et al., 2001; Rodrigues et al., 2016; Rodrigues et al., 2017). As the inhibitory effects of the extract and fractions on the TG in diluted plasma were observed to be concentration-dependent on both coagulation pathways as also evaluated by PTh in other studies (Nishino, Fukuda, Nagumo, Fujihara & Kaji, 1999; Rodrigues et al., 2016; Rodrigues et al., 2017), our findings revealed percentage of inhibition of 43.33% (41 min) at 4.1 µg well-$plate^{-1}$ and a complete inhibition achieving when an extract concentration of 4.15-fold higher than UHEP was used (Figure 2A). This dynamic was similar to decrease the TG in the thromboplastin-activated system, presenting more 50% inactivation of TG by extract in terms of PTh (68.88 and 81.65% inhibitions at 4.1 and 8.3 µg well-$plate^{-1}$, respectively) (Figure 2B).

The inhibitory potential of the fraction F I on TG in the intrinsic pathway was dependently of concentration of the SPs, except at 4.1 µg well-plate^{-1} that had no efficacy (29 min) to inhibits the active coagulation factors converted by thrombin in plasma (Rau, Beaulieu, Huntington & Church, 2007). Any total inhibition of TG was also observed in all the F I concentrations as evaluated in the intrinsic pathway (Figure 2C). While complete suppressions of the fraction F II on the TG using concentrations of 41.6 and 83.3 µg well-plate^{-1} were evidenced in the intrinsic pathway as illustrated in Figure 2E. Considerating the time parameter, SPs from *S. filiformis* were also capable of prolonging lag time with increasing concentrations, especially in F I (Figure 2C). On the basis of the dose-response curves of the *S. filiformis* SPs, a higher level of contribution on the extrinsic pathway can be suggested (Figure 2). Nishino, Fukuda, Nagumo, Fujihara & Kaji (1999) discovered that a fucoidan isolated from the brown seaweed *Ecklonia kurome* was more potent inhibitor of the TG in the intrinsic pathway. No activator response of TG in 60-fold diluted human plasma in the absence of cephalin or thromboplastin (negative control) was also observed *in vitro* during 60 min, as expected based on the findings obtained by Rodrigues et al. (2016).

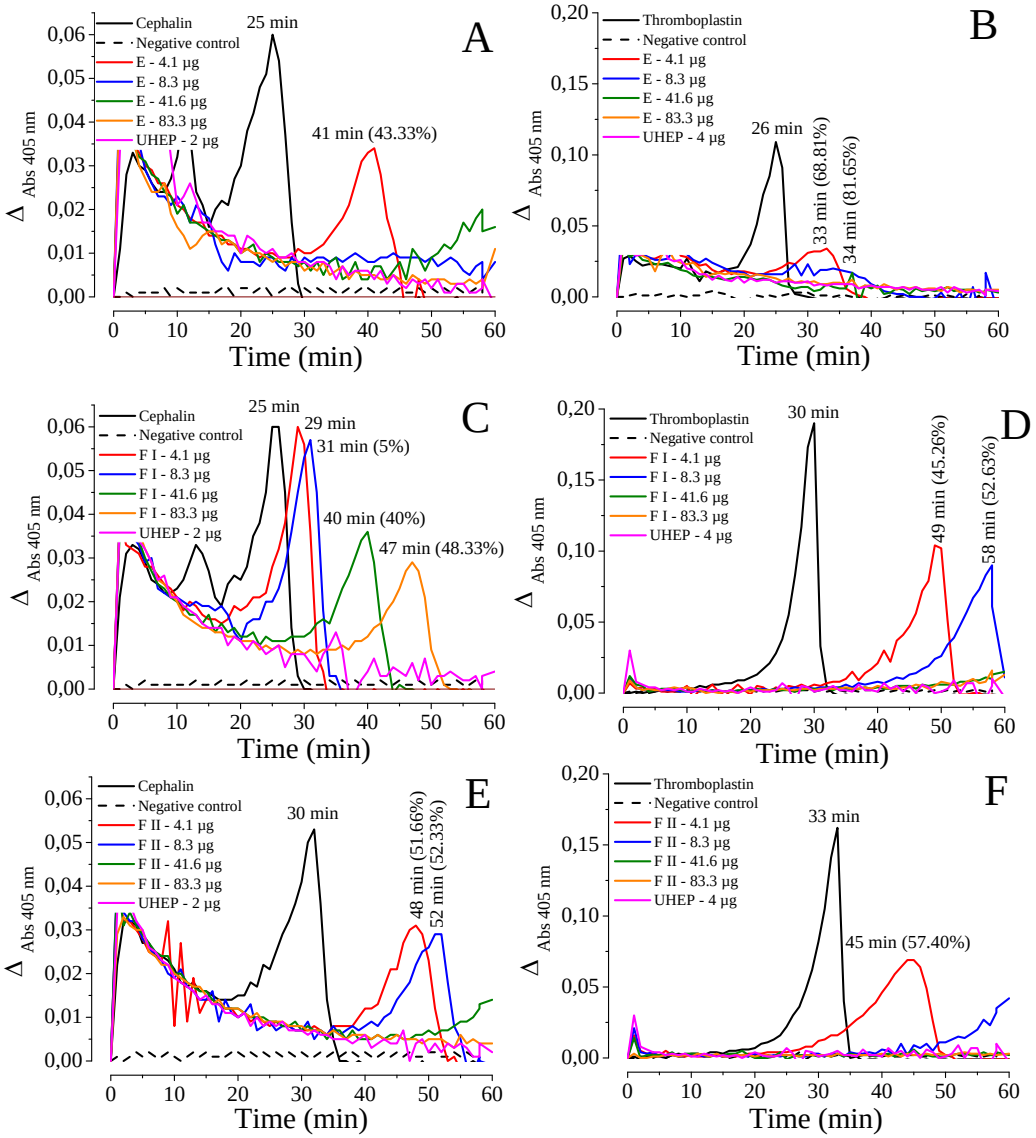

Figure 2 Effect of different concentrations of extract (A, B) and fractions F I (C, D) and F II (E, F), obtained by DEAE-cellulose, from the red seaweed *Solieria filiformis* on cephalin- or thromboplastin-triggered TG in 60-fold diluted human plasma using chromogenic method by a continuous detection system (60 min, 37°C).

These combined results led us to mention that the inhibition pattern depended on the degree of charge density of the glycans (Figure 1), supporting a higher sensitivity of our TG system than traditional APTT and PT assays (Castoldi & Rosing, 2011) for the analysis of anticoagulants (Nishino, Fukuda, Nagumo, Fujihara

& Kaji, 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014; Rodrigues et al., 2016; Rodrigues et al., 2017). UHEP required concentration 2-fold higher in the extrinsinc pathway compared with the intrinsic one using our *in vitro* TG model (Rodrigues et al., 2016) because it has mechanism of thrombin inhibition dependent of antithrombin-binding pentasaccharidic sequence (Nader et al., 2001) which is not found in other SPs-rich living sources (Mourão, 2015). Results here reported were in conformity to the fact that carrageenans-type polysaccharides display thrombin inhibition as an anticoagulant action mechanism (Silva et al., 2010; Prajapati, Maheriya, Jani & Solanki, 2014), as well as galactose-rich polysaccharides (Glauser et al., 2009; Mourão, 2015; Rodrigues et al., 2016).

The differential inhibition of the *S. filiformis* SPs on the TG (Figure 2) could be justified with basis on class of co-polymer previously demonstrated by agarose analysis (Figure 1) in which suggested *kappa*-/-*iota*-carrageenan, *kappa*-carrageenan and *iota*-carrageenan for extract and fractions F I and F II, respectively (Araújo et al., 2011). These SPs differ at an important level of sulfation and content of 3,6-anhydro-galactopyranose, with *kappa*-carrageenan showing lower levels of ester sulfate content than *iota*-carrageenan (Prajapati, Maheriya, Jani & Solanki, 2014) producing differential effects on the coagulation (Silva et al., 2010), as was also demonstrated in the current study (Figure 2). Another remark from this study was that the addition of calcium chloride to plasma to trigger the coagulation system, did not influence the evaluation by APTT, PT and TG assays since *kappa*- and *iota*-carrageenans form gels in the presence of calcium ions (Prajapati, Maheriya, Jani & Solanki, 2014). The algal protein digestion to obtain SPs resulted in enhanced biological effect of the water soluble SPs preparations (Rodrigues et al., 2011; Araújo et al., 2012).

As the inhibition pattern of the *S. filiformis* SPs on TG correlated with the TG parameters (PTh and TPeak) (Figure 2) (Rodrigues et al., 2016; Rodrigues et al., 2017), they could infer important implications to antithrombotic drug formulation. Given the ability of its SPs to control/stimulate certain reactions physiological, decreased TG in plasma treated with these sulfated glycans could also interplay on the antinociceptice/inflammatory/anti-inflammatory actions (Araújo et al., 2011; Araújo et al., 2012) since the concentration of SPs was about 244-fold-lower in the anticoagulant response, predicting a potential applicability of this TG model for further complementary studies on the interface between coagulation and inflammation due to lack of data concerning their structure-function relationships (Pomin, 2012). It is widely recognized that thrombin induces neutrophil adhesion during the inflammatory response initiated when injury to a vessel wall exposes the blood to tissue factor in the subendothelium (Rau, Beaulieu, Huntington & Church, 2007). Therefore, a more refined analysis on the mechanisms underlying involved in their anticoagulant effects on TG in both intrinsic and extrinsic pathways is still required for the understanding of the mode of action by which the algal SPs would modulate the circulatory system disorders.

Conclusion

The red seaweed *Solieria filiformis* experimentally cultured in open waters of the coast of Ceará State, Brazil (Flecheiras beach) contains sulfated polysaccharides with virtual anticoagulant effects using classical coagulation methods, but capable of modulating a thrombin generation system in both intrinsic and extrinsic coagulation pathways, reducing the thrombosis *in vitro*, although less potent than unfractionated heparin, when in 60-fold diluted human plasma. Therefore, sulfated polysaccharides from *S. filiformis* interfere with the thrombin generation parameters, as a promising plasma alternative to modulate blood coagulation.

References

Ale, M.T., Barret, K., Addico, G.N.D., Rhein-Knudsen, N., Graft-Johnsn, A.A. & Meyer, A.S. (2016). DNA-based identification and chemical characteristics of *Hypnea musciformis* from coastal sites in Ghana. *Diversity*, 8(14): 1-14.

Araújo, I.W.F., Vanderlei, E.S.O., Rodrigues, J.A.G., Coura, C.O., Quinderé, A.L.G., Fontes, B.P., Queiroz, I.N.L., Jorge, R.J.B., Bezerra, M.M., Silva, A.A.R., Chaves, H.V., Monteiro, H.S.A., Paula, R.C.M. & Benevides, N.M.B. (2011). Effects of a sulfated polysaccharide isolated from the red seaweed *Solieria filiformis* on models of nociception and inflammation. *Carbohydrate Polymers*, 86(3): 1207-1215.

Araújo, I.W.F., Rodrigues, J.A.G., Vanderlei, E.S.O., Paula, G.A., Lima, T.B. & Benevides, N.M.B. (2012). *Iota*-carrageenans from *Solieria filiformis* (Rhodophyta) and their effects in the inflammation and

coagulation. *Acta Scientiarum Technology*, 34(2): 127-135.

Cardozo, K.H.M., Guaratini, T., Barros, M.P., Falcão, V.R., Tonon, A.P., Lopes, N.P., Campos, S., Torres, M.A., Souza, A.O. & Pinto, E. (2007). Metabolites from algae with economical impact. *Comparative Biochemistry and Physiology* Part C: *Toxicology* &*Pharmacology*,146(102): 60-78.

Carneiro, J.G., Rodrigues, J.A.G., Teles, F.B., Cavalcante, A.B.D. & Benevides, N.M.B. (2014). Analysis of some chemical nutrients in four Brazilian tropical seaweeds. *Acta Scientiarum. Biological Sciences*, 32(2): 137-145.

Castoldi, E. & Rosing J. (2011). Thrombin generation tests. *Throm. Res.*, 127(suppl.3): S21-S25.

Chang, C.W., Lur, H.S., Lu, M. K. & Cheng, J.J. (2013). Sulfated polysaccharides of *Amillariella mellea* and their anti-inflammatory activities via NF-κB suppression. *Food Res. Inter.*, 54(1): 239-245.

Cheung, R.C.F., Ng, T.B., Wong, J.H., Chen, Y. & Chan, W.Y. (2016). Marine natural products with anti-inflammatory activity. *Appl. Microbiol. Biotechnol*., 100(4): 1645-1666.

Chiovitti, A., Bacic, A., Kraft, G.T., Craik, D.J. & Liao, M.L. (1999). Pyruvated carrageenans from *Solieria robusta* and its adelphoparasite *Tikvahiella candida*. *Hydrobiol*., 398/399(0): 401-409.

Costa-Lotufo, L.V., Pessoa, C.U., Moraes, M.E.A., Almeida, A.M.P., Moraes, M.O. & Lotufo, T.M.C. (2006). Marine organisms from Brazil as source of potential anticancer agents. *Adv. Phytom.*, 2(1): 181-196.

Dantas-Santos, N., Gomes, D.L., Costa, L.S., Cordeiro, S.L., Costa, M.S.S.P., Trindade, E.S., Chavichiolo, C.R., Scortecci, K.C., Leite, E.L. & Rocha, H.A.O. (2012). Freschwater plants synthesize sulfated polysaccharides: Heterogalactans from water hycinth (*Eicchornia crassipes*). *Inter. J. Mol. Sci.*, 13(1): 961-976.

Dietrich, C.P.; & Dietrich, S.M.C. (1976). Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Anal. Biochem.*, 70(2): 645-647.

Farndale, R.W., Buttle, D.J. & Barrett, A.J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta*, 883(2): 173-177.

Glauser, B.F., Rezende, R.M., Melo, F.R., Pereira, M.S., Fransischetti, I.M. B., Monteiro, R.Q., Rezaie, A.R. & Mourão, P.A.S. (2009). Anticoagulant activity of a sulfated: Serpin-independent effect and specific interaction with factor Xa. *Throm. Haemos.*, 102(6): 1183-1193.

Goulard, F., Diouris, M., Quere, G., Deslandes, E. & Floc, J.Y. (2001). Salinity effects on NDP-sugars, floridoside, starch, and carrageenan yield, and UDP-glucose-pyrophosphorylase and -epimerase activities of cultivated *Solieria chordalis*. *J. Plant Physiol*., 158:1387-1394.

Holanda, M.L., Melo, V.M M., Silva, L.M.C.M., Amorim, R.C.N., Pereira, M.G. & Benevides, N.M.B. (2005). Differential activity of a lectin from *Solieria filiformis* against human pathogenic bacteria. *Braz. J. Med. Biol. Res.*, 38(12): 1769-1773.

Mourão, P.A.S., Boisson-Vidal, C., Tapon-Bretaudière, J., Drouet, B., Bros, A. & Fischer, A.M. (2001). Inactivation of thrombin by a fucosylated chondroitin sulfate from Echinoderm. *Throm. Res.*, 102(2): 167-176.

Mourão, P.A.S. (2015). Perspective on the use of sulfated polysaccharides from marine organisms as a source of new antithrombotic drugs. *Mar. Drugs*, 1(5): 2770-2784.

Murano, E., Toffanin, R., Cecere, E., Rizzo, R. & Knutsen, S.H. (1997). Investigation of the carrageenans extracted from *Solieria filiformis* and *Agardhiella subulata* from Mar Piccolo, Taranto. *Mar. Chem.*, 58(3-4): 319-325.

Nader, H.B., Pinhal, M.A.S., Baú, E.C., Castro, R.A.B., Medeiros, G.F., Chavante, S.F., Leite, E.L., Trindade, E.S., Shinjol, S.K., Rocha, H.A.O., Tersariol, I.L.S., Mendes, A. & Dietrich, C.P. (2001). Development of new heparin-like compounds and other antithrombotic drugs and their interactions with vascular endothelial cells. *Braz. J. Med. Biol. Res.*, 34(6): 699-709.

Nishino, T., Fukuda, A., Nagumo, T., Fujihara, M. & Kaji, E. (1999). Inhibition of the generation of thrombin and factor Xa by a fucoidan from the brown seaweed *Ecklonia kurome*. *Throm. Res.*, 96(1): 37-49.

Paula, G.A., Benevides, N.M.B., Cunha, A.P., Oliveira, A.V., Pinto, A.M.B., Morais, J.P.S., & Azeredo, H.M.C. (2015). Development and characterization of edible films from mixtures of κ-carrageenan, ι-carrageenan, and alginate. *Food Hydrocoll.*, 47(1): 140-145.

Pellizzari, F. & Reis, R.P. (2011). Seaweed cultivation on the Southern and Southeastern Brazilian Coast. *Braz. J. Pharmacog.*, 21(2): 305-312.

Pereira, R.C. & Costa-Lotufo, L.V. (2012). Bioprospecting for bioactives from seaweeds: potential, obstacles and alternatives. *Braz. J. Pharmacog.*, 22(4): 894-905.

Pomin, V.H. & Mourão, P.A.S. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology.*, 18(12): 1016-1027.

Pomin, V.H. (2012). Fucanomis and galactanomics: Current status in drug discovery, mechanisms of action and role of the well-defined structures. *Biochim. Biophys. Acta*, 1820(12): 1971-1979.

Prajapati, V.D., Maheriya, P.M., Jani, G.K. & Solanki, H.K. (2014). Carrageenan: A natural seaweed polysaccharide and its applications. *Carbohydrate Polymers*, 105(25): 97-112.

Quinderé, A.L.G., Santos, G.R.C., Oliveira, N.M. C.G., Glauser, B.F.; Fontes, B.P., Queiroz, I.N. L., Benevides, N.M.B., Pomin, V.H. & Mourão, P.A.S. (2014). Is the antithrombotic effect of sulfated galactans independent of serpins? *J. Throm. Haemos.*, 12(1), 43-53.

Rau, J.C., Beaulieu, L.M., Huntington, J.A. & Church, F.C. (2007). Serpins in thrombosis, hemostasis and fibrinlysis. *J. Throm. Haemos.*, 5(suppl.1): 102-115.

Rocha, H.A.O., Moraes, F.A., Trindade, E.S., Franco, C.R.C., Torquato, R.J.S., Veiga, S.S., Valente, A.P., Mourão, P.A.S., Leite, E.L., Nader, H.B. & Dietrich, C.P. (2005). Structural and hemostatic activities of a sulfated galactofucan from the brown alga Spatoglossum schroederi. An ideal antithrombotic agent? *J. Biol. Chem.*, 280(5): 41278-41288.

Rodrigues, J.A.G., Torres, V.M., Alencar, D.B., Sampaio, A.H. & Farias, W.R.L. (2009). Extração e atividade anticoagulante dos polissacarídeos sulfatados da alga marinha vermelha *Halymenia pseudofloresia*. *Rev. Ciên. Agron.*, 40(2): 224-231.

Rodrigues, J.A.G., Araújo, I.W.F., Paula, G.A., Lima, T.B., Bessa, E.F. & Benevides, N.M.B. (2011). Carragenana da epífita *Hypnea musciformis* obtida do cultivo experimental de *Solieria filiformis* em Flecheiras, Estado do Ceará, Brasil. *Acta Scientiarum Technology*, 33(2): 137-144.

Rodrigues, J.A.G., Vanderlei, E.S.O., Silva, L.M.C.M., Araújo, I.W.F., Queiroz, I.N.L., Paula, G.A., Abreu, T.M., Ribeiro, N.A., Bezerra, M.M., Chaves, H.V., Lima, V., Jorge, R.J.B., Monteiro, H.S.A., Leite, E.L. & Benevides, N.M.B. (2012a). Antinociceptive and anti-inflammatory activities of a sulfated polysaccharide isolated from the green seaweed *Caulerpa cupressoides*. *Pharmacol. Rep.*, 64(2): 282-292.

Rodrigues, J.A.G., Vanderlei, E.S.O., Quinderé, A.L.G., Fontes, B.P., Queiroz, I.N.L., Benevides, N.N. B.(2012b). Glicosaminoglicanos isolados da pele de palombeta (*Chloroscombrus chrysurus*) e guaiúba (*Ocyurus chrysurus*): características e implicações biológicas. *Acta Scientiarum. Biological Sciences.*, 32(2): 141-148.

Rodrigues, J.A.G., Moura-Neto, E., Teixeira, L.A.C., Paula, R.C.M., Mourão, P.A.S. & Benevides, N.M.B. (2013). Structural features and inactivation of coagulation proteases of a sulfated polysaccharidic fraction from *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae, Chlorophyta). *Acta Scientiarum Technology*, 35(4): 611-619.

Rodrigues, J.A.G., Queiroz, I.N.L., Quinderé, A.L.G., Benevides, N.M.B., Tovar, A.M.F. & Mourão, P.A.S. (2016). Mild-acid hydrolysis of a native polysulfated fraction from *Acanthophora muscoides* generates sulfated oligosaccharides displaying *in vitro* thrombin generation inhibition. *Acta Scientiarum. Biological*

Sciences., 38(1): 7-15.

Rodrigues, J.A.G., Barcellos, P.G., Salles, T.C., Benevides, N.M.B., Tovar, A.M.F. & Mourão, P.A.S. (2017). *In vitro* inhibition of thrombin generation by sulfated polysaccharides from the tropical red seaweed *Gracilaria birdiae* Plastino & Oliveira. *Acta Fish. Aquat. Res.*, 5(1): 22-32.

Smit, A. (2004). Medicinal and pharmaceutical uses of seaweed natural products: A review. *J. Appl. Phycol.*, 16(4): 245-262.

Silva, F.R.F., Dore, C.M.P.G., Marques, C.T., Nascimento, M.S., Benevides, N.M.B., Rocha, H.A.O.; Chavante, S.F. & Leite, E.L. (2010). Anticoagulant activity, paw edema and pleurisy induced carrageenan: Action of major types of commercial carrageenans. *Carbohydrate Polymers*, 79(1): 29-33, 2010.

Stephanie, B., Eric, D., Sophie, F.M., Chistian, B. & Yu, G. (2010). Carrageenan from *Solieria chordalis* (Gigartinales): Structural analysis and immunological activities of the low molecular weight fractions. *Carbohydrate Polymers*, 81(2): 448-460.

Zhang, Z., Till, S., Jiang, C., Knappe, S., Reutterer, S., Scheiflinger, F., Szabo, C.M. & Dockai, M. (2014). Structure-activity relationship of the pro- and anticoagulant effects of *Fucus vesiculosus* fucoidan. *Throm. and Haemos.*, 111(3): 429-437.