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In vitro inactivation of thrombin generation by polysulfated fractions isolated from the tropical coenocytic green seaweed *Caulerpa racemosa* (Caulerpaceae, Bryopsidales)

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ABSTRACT. Pharmacological efficacy of *Caulerpa racemosa* (Chlorophyta) sulfated polysaccharidic (SPs) fractions (F I→III) on models of coagulation and inflammation has been demonstrated, but not their effects on thrombin generation (TG). This study examined fractions for composition and physical-chemical characteristics and *in vitro* inactivation of TG by F I and F II in 60-fold diluted human plasma using continuous method. Papain-extraction yield of 0.7% revealed F I→III by DEAE-cellulose chromatography, with differences among the relative proportions of sulfate (17.37-24.00%), total sugars (30.03-48.34%) and absence of proteins. Charge density patterns and molecular sizes > 100 kDa of the fractions were verified by both agarose/polyacrylamide analyses, respectively. These electrophoreses combined with toluidine blue/Stains-All also indicated nonSPs. Anticoagulant effects of 4.76 (F I), 12.00 (F II) and 2.32 (F II) IU mg^{-1} by activated partial thromboplastin time test were recorded against heparin (193 IU mg^{-1}), without changes in prothrombin time. Diluted plasma treated with F I and F II reduced concentration-dependent and sulfation pattern TG by both intrinsic and extrinsic pathways, with 50% inactivation by intrinsic pathway of F II even at 4.1 μg. Heparin abolished TG at least 4-fold lower. Therefore, *C. racemosa* produces SPs with TG inhibition.

Keywords: Chlorophyta, complex glycans, sulfation pattern, clot.

Inativação *in vitro* da geração de trombina por frações polissulfatadas isoladas da macroalga verde cenocítica tropical *Caulerpa racemosa* (Caulerpaceae; Bryopsidales)

RESUMO. Eficácia farmacológica de frações (F I→III) polissacarídicas sulfatadas (PSs) da Chlorophyta *Caulerpa racemosa* sobre modelos de coagulação e inflamação tem sido demonstrada, exceto seus efeitos sobre geração de trombina (GT). Examinaram-se frações quanto à composição, características físico-químicas e inativação *in vitro* de GT por F I e F II, em plasma humano diluído 60 vezes usando método contínuo. Rendimento de extração-papaína (0,7%) revelou, por cromatografia de DEAE-celulose, F I→III com diferenças entre as proporções relativas de sulfato (17,37-24,00%), açúcares totais (30,03-48,34%) e ausência de proteínas. Foram verificados, por ambas as análises agarose/poliacrilamida, graus de densidade de carga e tamanhos moleculares > 100 kDa das frações, respectivamente. Também essas eletroforeses, combinadas com azul de toluidina/Stains-All, indicaram polissacarídeos não sulfatados. Foram registrados, pelo teste do tempo de tromboplastina parcial ativada, efeitos anticoagulantes de 4,76 (F I), 12,00 (F II) e 2,32 (F II) UI mg^{-1} contra heparina (193 UI mg^{-1}), porém não modificando tempo de protrombina. Plasma diluído tratado com F I e F II reduziu GT por ambas as vias intrinsíca/extrínsica, dependente de concentração e grau de sulfatação, com F II em 4,1 μg apresentando eficácia de 50% pela via intrínsica. Heparina, quatro vezes menos, aboliu GT. Portanto, *C. racemosa* produz PSs com inibição de GT.

Palavras-chave: Chlorophyta, glicanos complexos, grau de sulfatação, coágulo.

Introduction

The coagulation system comprises a series of complex proteolytic reactions that lead to the formation of thrombin, which then form a fibrin clot. Two pathways are recognized: 1) intrinsic (initiated by contact activation with a negatively charged surface); and 2) extrinsic (initiated by exposure of tissue factor to blood components at the site of injury), converging them to a common pathway to display the activation of factor X. They are classically analyzed using the activated partial thromboplastin time (APTT) and the prothrombin

time (PT), respectively, although emphasizing that these two *in vitro* tests do not reflect the hemostasis (Ferreira, Sousa, Dusse, & Carvalho, 2010). Given these limitations and the complexity of the coagulation process, thrombin generation (TG)-based assays would offer a better understanding of the *in vivo* event (Wu et al., 2014) to measure TG in a blood plasma sample (Castoldi & Rosing, 2011; Xin, Chang, & Ovanesov, 2016) and analyze anticoagulants (Wu et al., 2014; Zavyalova & Kopylov, 2016).

Circulatory dysfunctions are usually associated with the sedentary lifestyle, poor eating habits and stress that potentially lead to venous thromboembolism, heart attack and pulmonary embolism as the most prevalent cardiovascular diseases worldwide, leading causing to patient death or else partial or total disability. Routinely, unfractionated heparin (UHEP) as anticoagulant drug has been primarily employed in clotting disorders, which inhibits thrombin and activated factor X mediated by antithrombin (AT) due to its specific pentasaccharide sequence (Mourão et al., 2015). Although effective, UHEP develops thrombocytopenia and bleeding being necessary to monitor its anticoagulation using the APTT test (Mourão et al., 2015) since UHEP significantly decreases TG (Wu et al., 2014). This therapeutic agent is mainly extracted from pig and bovine intestine and its potential risks of chemical contamination with other glycosaminoglycans for use in cardiac surgeries also justify the current efforts to develop alternatives to UHEP (Mourão & Pereira, 1999; Pomin, 2012; Rodrigues et al., 2016).

Seaweeds have structurally diverse unique chemicals (e.g., proteins, carbohydrates and lipids) (Carneiro, Rodrigues, Teles, Calvacante, & Benevides, 2014) with industrial relevance and benefits to human and animal health (Costa-Lotufo et al., 2006) as potentially useful ingredients in cosmetics (Cardozo et al., 2007), aquaculture (Rodrigues, Júnior, Lourenço, Lima & Farias, 2009), nutrition (Carneiro et al., 2014) and biomedicine (Pomin, 2012; Mourão, 2015). Of all algae products, extracellular matrix sulfated polysaccharides (SPs) comprise a diverse group of biomaterials exhibiting high degree of sulfation (S=O, sulfate radicals) and molecular distribution of more than 100 kDa, which are capable of displaying anticoagulant, antithrombotic and anti-inflammatory effects (Pomin, 2012; Mourão, 2015; Rodrigues et al., 2012a; Rodrigues et al., 2016). Their functionalities as gelling and stabilizing agents make them also economically attractive for hydrocolloid industry (Cardozo et al., 2007). Depending on the chemical class, sulfated galactans (Rhodophyta) (Kloareg & Quatrano, 1988; Mourão, 2015), fucans or fucoidan (Ochrophyta) (Athukorala, Jung, Vasanthan, & Jeon, 2006) and sulfated heteropolysaccharides (Chlorophyta) (Wang, Wang, Wu & Liu, 2014) are the main polymeric identities of the SPs playing regulatory roles of the algae (Kloareg & Quatrano, 1988). On a basis of abundance, seaweeds produce larger amounts of SPs than other natural sources (Aquino, Landeira-Fernandez, Valente, Andrade, & Mourão, 2005; Mourão & Pereira, 1999; Dantas-Santos et al., 2012; Chang, Lur, Lu, & Cheng, 2013).

There are very few reports concerning the effects of SPs isolated from aquatic organisms on *in vitro* TG assays. For example, SPs derived from the sea cucumber *Ludwigothurea grisea* (Mourão et al., 2001) and from the seaweeds *Ecklonia kurome* (Ochrophyta) (Nishiro, Fukuda, Nagumo, Fujihara & Kaji, 1999) and *Botryocladia occidentalis* (Rhodophyta) (Glauser et al., 2009) inactivated TG after induction by both contact-activated and thromboplastin-activated systems and/or by the prothrombinase complex. Zhang et al. (2014) demonstrated that the fucoidan derived from the brown seaweed *Fucus vesiculosus* showed both pro- and anticoagulant effects using calibrated automated thrombography. Studies by Rodrigues et al. (2016) revealed that a native SPs fraction and its various chemically (HCl treated)-modified products from the red seaweed *Acanthophora muscoides* attenuated coagulation status activated by the use of cephalin in an *in vitro* TG continuous system. Recently, SPs isolated from Brazilian samples of *Gracilaria birdiae* (Gracilariales, Rhodophyceae) exerted *in vitro* inhibitory potential on a chromogenic TG assay in diluted human plasma (Rodrigues et al., 2017a). The skin of Nile tilapia (*Oreochromis niloticus*) also contained SPs (dermatan sulfate-type glycosaminoglycans) with *in vitro* TG inhibition (Salles et al., 2017). To the best of our knowledge, there is only one report concerning the *in vitro* inhibitory effects of SPs isolated from Chlorophyta species on TG tests (Rodrigues et al., 2017b).

Studies on the order Bryopsidales of green seaweeds of the *Caulerpa* genus Lamouroux (1809), which is distributed in tropical areas to warm-temperate zones, revealed compositional complexity (galactose, glucose, arabinose, sulfate, xylose and uronic acid and traces of fucose residues) and high structural heterogeneity of their SPs exhibiting bioactivities (Hayakawa et al., 2000; Ji, Shao, Zhang, Hong & Xiong, 2008; Maeda, Ida, Ihara & Sakamoto, 2012; Rodrigues, Vanderlei, Quinderé, Fontes, & Benevides, 2010; Rodrigues et al., 2012a; Wang et al., 2014). Focusing on the *C. racemosa*

(Forsskal) J. Agardh species, a crude SP was shown to be anticoagulant and antiviral *in vitro* (Ghosh et al., 2004; Ghosh et al., 2009). Subsequently, three SPs fractions (F I, F II and F III) from this algal species have been isolated (Rodrigues et al., 2010), of which F II had anticoagulant (Rodrigues et al., 2012b), antinociceptive and anti-inflammatory effects devoid of toxicity in mice (Ribeiro et al., 2014). Fractions F I and F II modestly altered the normal APTT method compared with UHEP (Rodrigues et al., 2010; Rodrigues et al., 2012b).

Considering the above and the invasive biology of the *Caulerpa* species (Verlaque, Durand, Huisman, Boudouresque, & Parco, 2003), SPs from *C. racemosa* were examined for their physical-chemical characteristics using a combination of agarose/polyacrylamide gel electrophoresis and sequential staining with toluidine blue/Stains-All. Analyses of the fractions F I and F II on an *in vitro* TG system in 60-fold diluted human plasma using chromogenic method in a continuous measurement system were also conducted to explore their anticoagulant dynamics.

Material and methods

Marine alga and analysis of SPs

The *C. racemosa* (Forsskal) J. Agardh coenocytic seaweed was manually collected in August 2011 from the rocky shore located at Pacheco beach (Caucaia, Ceará state, Brazil). Algal samples were placed in plastic bags and then transported to the Carbohydrates and Lectins laboratory (CarboLec), *Universidade Federal do Ceará*, Brazil, where macroscopic epiphytes attached to material were removed, the material was washed with distillated water to eliminate salt and sand, followed by dehydration of the algal tissue at room temperature prior to crude SP extraction. A voucher specimen (# 52418) was deposited in the Herbarium Prisco Bezerra of the Department of Biological Sciences, *Universidade Federal do Ceará*, Brazil. The analyses of the *C. racemosa* SPs were conducted at Connective Tissue laboratory, *Universidade Federal do Rio de Janeiro* (UFRJ), Brazil.

Essentially, two grams of dehydrated algal tissue cut in small pieces were subjected to papain digestion (60°C, 24 hours) as previously described (Rodrigues et al., 2010), followed by fractionation of a sample of extract (20 mg) dissolved in 10 mL of 50 mM sodium acetate buffer (pH 5.0) by anion-exchange chromatography (DEAE-cellulose), where the column (1.2 × 12 cm)-bound SPs were eluted with NaCl (from 0 to 2 M, with 0.25 M of intervals) added to buffer of equilibrium and the collected fractions (2.5 mL) checked for SPs using metachromatic assay containing dimethymethylene blue in an Amersham Bioscience Ultrospec 3100 spectrosphotometer at 525 nm (Farndale, Buttle, & Barrett, 1986). The metachromatic fractions (F I, F II and F III) were further freeze-dried and examined for their contents of sulfate (Dogdson & Price, 1976), total sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and proteins (Bradford, 1976), while their purity and molecular mass were revealed by agarose (Dietrich & Dietrich, 1976) and polyacrylamide (Rodrigues et al., 2016) analyses, respectively, using toluidine blue/Stains-All as staining reagents (Volpi & Maccari, 2002) by comparison with the electrophoretic mobility of standards dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa) and chondroitin-6-sulfate (~ 60 kDa) (Rodrigues et al., 2016; Salles et al., 2017). Since F I and F II were available in higher quantities, more refined *in vitro* coagulation experiments focused only on these fractions.

In vitro coagulation experiments

Blood samples

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

APTT and PT tests

Fractions were assessed by both *in vitro* APTT and PT tests according to the manufacturers' specifications, for measure anti-clotting effect in a coagulometer Amelung KC4A before the *in vitro* TG assay. For APTT assay, a mixture of 100 µL plasma and concentrations of SPs (0-1 mg mL^{-1}) was incubated with 100 µL APTT reagent (kaolin bovine phospholipid reagent). After 2 min incubation at 37°C, 100 µL 25 mM $CaCl_2$ was added to the mixtures, and the clotting time was recorded. Regarding the PT assay, a mixture of 100 µL plasma and concentration of SPs (1 mg mL^{-1}) was incubated for 1 min at 37°C. After that, 100 µL PT reagent was added to the mixtures, and the clotting time was recorded using the same coagulation equipment. UHEP with 193 international units per mg (IU mg^{-1}) of polysaccharide was used as the standard in both tests. All the tests were done in triplicate and the data were expressed as mean ± S.E.M.

TG assay

This assay was based on Rodrigues et al. (2016) in a microplate format, containing: 10 μL cephalin (contact-activator system) or thromboplastin (830 μg well-plate^{-1}, factor tissue-activator system) + 30 μL 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 µL SPs (*C. racemosa* fractions (F I and F II): 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 $CaCl_2$:0.33 mM specific 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 recorded for 60 min (Plate reader Thermo-max, America Devices). The *in vitro* inhibitory response of TG by SPs was determined by peak thrombin (PTh) and time to peak (TPeak).

Results and discussion

In this study, 24 h after papain-assisted extraction, a total yield only of 0.7% crude SP from the dehydrated raw matter was achieved against 2.2 and 4% obtained by Rodrigues et al. (2010) and by Ribeiro et al. (2014), respectively, who formerly analyzed the *C. racemosa* crude SP extraction yield using protease treatment. This lowest yield result was consistent with those of other studied *Caulerpa* species that revealed total yields varying from 0.6 to 20% (Ghosh et al., 2004; Ji et al., 2008; Rodrigues et al., 2012b; Wang et al., 2014). Although presenting a yield of about 5.7-fold lower than those previously published, the DEAE-cellulose column-bound coenocytic tissue structure SPs were separated into three SPs fractions (F I, F II and F III) at 0.5, 0.75 and 1 M NaCl, respectively, as assessed by metachromasy due to the complex-binding capacity of SPs (Farndale et al., 1986). On the basis of these results, the total amount of material (76.5%) recovered from the column (Table 1) was also as expected (Rodrigues et al., 2010). Thus, regarding the chemical analyses, the 0.75 M NaCl fraction (F II) confirmed its highest levels of sulfate (24%), total sugars (48.34%) and absence of SPs-bound proteins among the fractions (Table 1), as accordingly published on the heterogeneous composition of the *C. racemosa* SPs from the total sugars/sulfate ratio (Ribeiro et al., 2014) by using the DEAE-cellulose algal crude SP separation (Rodrigues et al., 2010).

Since the chemical profiles of the *C. racemosa* SPs were similar to those reported, our studies were further extended to characterize complex glycans-containing fractions by a combination of electrophoreses (agarose/polyacrylamide) and sequential toluidine blue/Stains-All staining. Furthermore, the anticoagulant dynamics of the fractions F I and F II (Table 1) on an *in vitro* TG system by continuous measuring (60 min, 37°C) of the amidolytic activity of thrombin using the specific chromogenic substrate in 60-fold diluted normal human plasma samples was also examined in this current investigation.

Table 1. Yield and composition of the SPs fractions obtained by ion-exchange chromatography (DEAE-cellulose) from the green seaweed *Caulerpa recemosa*.

Fraction	NaCl (M)[a]	Yield (%)[b]	Chemical analyses (%)			
			Sulfate[c]	Sugars[d]	Sugars/sulfate[e]	CPs[f]
F I	0.50	19.50	19.61	37.35	1.90	⋆
F II	0.75	50.30	24.00	48.34	2.01	⋆
F III	1.00	07.00	17.37	30.03	1.74	⋆

a - NaCl concentration; b - Yield calculated as the percentage from a sample of extract applied on DEAE-cellulose column; c - Dosage by Dodgson and Price (1976) method using $NaSO_3$ as standard; d - Dosage by Dubois et al. (1956) method using D-galactose as standard; e - Sulfate/hexose ratio; f - Dosage by Bradford (1976) method using bovine serum albumin (- not detected); ⋆ Non-detected.

Agarose/polyacrylamide analyses combined with toluidine blue/Stains-All reveal *Caulerpa racemosa* nonSPs in complex mixtures

To evaluate the physical-chemical characteristics of the *C. racemosa* SPs (extract/fractions) and standards (glycosaminoglycans chondrotin sulfate, UHEP or dextran sulfate) in complex mixtures, agarose/polyacrylamide techniques associated with sequential toluidine blue/Stains-All staining were carried out as a function of charge density and molecular size, respectively, as already reported for glycosaminoglycans extracted from animal tissues (Volpi & Maccari, 2002; Salles et al., 2017) (Figure 1).

Conventional treatment with the cationic dye toluidine blue confirmed SPs in extract and fractions exhibiting polydispersion (Rodrigues et al., 2012b; Ribeiro et al., 2014) co-migrating close to UHEP on agarose gel (Figure 1Aa). Additionally, F I and F III had lowest metachromasy between extract and F II as supported the sulfate analysis (Table 1) due to a relatively lower degree of sulfation (ester sulfate groups, S=O) of the SPs (Rodrigues et al., 2010). F I had highest mobility on agarose gel electrophoresis, suggesting a different structural conformation and charge/mass ratio of the *C. racemosa* polysaccharides during comparisons with other studies (Dietrich & Dietrich, 1976; Fidelis et al., 2014). Polyacrylamide gel electrophoresis (Figure 1Ba) revealed large molecular sizes SPs (> 100 kDa) since they remained close to the origin of the gel, typical for algal SPs (Pomin, 2012; Fidelis et al., 2014; Mourão, 2015; Rodrigues et al., 2017a).

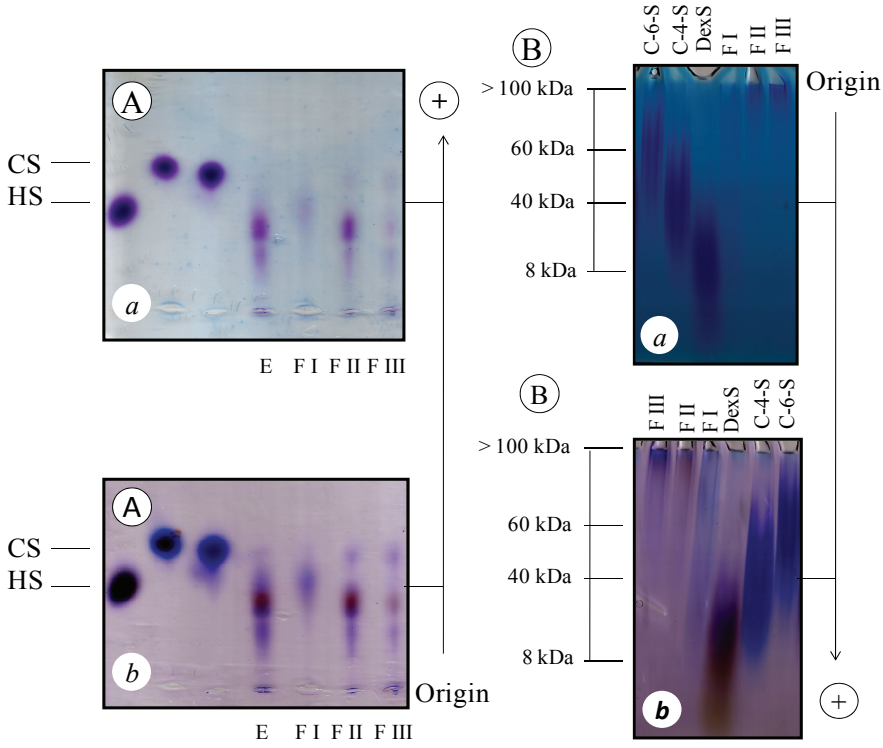

Figure 1. Electrophoreses in agarose gel (A) and polyacrylamide (B) gel of the SPs isolated from the green seaweed *Caulerpa racemosa*. Extract (E) and fractions (F I, F II and F III) and standard glycosaminoglycan chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa), dextran sulfate (DexS, 8 kDa) and/or unfractionated heparin (UHEP, 14 kDa) present on gels stained with 0.1% toluidine blue (*a*) or Stains-All (*b*).

Such observations contrasted with those found for two SPs fractions from the green seaweed *C. cupressoides* var. *lycopodium*, where average molecular masses varying from 8 to > 100 kDa were observed by polyacrylamide gel electrophoresis (Rodrigues et al., 2013; Rodrigues et al., 2017b). Ghosh et al. (2004) obtained a hot water crude SP from the red seaweed *C. cupressoides* and found apparent molecular weights of two major peaks (F 1 and F 2) to be 120 and 70 kDa, respectively, when a size exclusion chromatography (Sephacryl S-100 column) was used. For the SPs from *Caulerpa lentillifera* by treating with water, Maeda et al. (2012) obtained a xylogalactan with a molecular size of more than 100 kDa using a Superdex 200 HR 10/30 column. Therefore, the revealed molecular characteristics by *Caulerpa* SPs can also vary according to algal species and the analytical procedure used (Wang et al., 2014).

Staining of the *C. racemosa* SPs after separation by agarose gel electrophoresis procedure with toluidine blue/Stains-All is illustrated in figure 1Ab. Extract and fractions became more visible on gel compared with the toluidine blue alone (Figure 1Aa) (Rodrigues et al., 2017a) as previously demonstrated for glycosaminoglycans (Volpi & Maccari, 2002; Salles et al., 2017), postulating non negatively charged polysaccharides in preparations since the fractions F II and F III appeared an additional second band with higher mobility as CS, which is composed of alternate disaccharide sequences of differently sulfated residues of D-glucuronic acid and of *N*-acetyl-D-galactosamine (Volpi & Maccari, 2002).

The degree of staining among the fractions compared with extract and standards was also individualized with the stepwise of NaCl, where 0.75 M NaCl-eluted SPs (F II) showed staining pattern similar to that of the extract; therefore, suggesting compositional variability of the *C. racemosa* polysaccharides of high molecular sizes (Figure 1Bb) (Rodrigues et al., 2017a) based on the proportions of its sugar residues (Ghosh et al., 2004; Ji et al., 2008). The combined method of electrophoreses and toluidine blue/Stains-All staining to reveal *Caulerpa* nonSPs was also important for distinguishing other glycans present in the fractions, when the polysaccharides were co-eluted from the DEAE-cellulose column (Rodrigues et al., 2017a, b), given the difficult in the determination of sugar composition after chemically-modified polysaccharides (Rodrigues et al., 2016).

Collectively, the use of this methodology could also be useful for the co-detection of acidic components naturally occurring in coenocytic structures of green seaweeds, as already reported for SPs isolated from other natural sources (Volpi & Maccari, 2002; Rodrigues et al., 2017a, b). Further studies on the sensitivity to heavy metal–acid polysaccharide complexes are also needed since the concentrations of glycans are regulated in response to environmental stresses (Cardozo et al., 2007; Wang et al., 2014).

Higher levels of anticoagulation by *Caulerpa racemosa* SPs are measured on a TG system than in traditional APTT and PT tests

Anticoagulant effects of the *C. racemosa* SPs were initially checked by routine coagulation screening tests (APTT and PT) using a 193 IU mg^{-1} UHEP standard (Table 2). Algal SPs modified the normal intrinsic APTT values (31.80 ± 0.1 s) in the orders of 4.76 (250 μg mL^{-1}, 38.15 ± 0.35 s), 12.00 (100 μg mL^{-1}, 38.60 ± 0.30 s), and 2.32 IU mg^{-1} (500 μg mL^{-1}, 37.35 ± 0.35 s) for the fractions F I, F II and F III, respectively. Fraction F II had effect on APTT at least 2.52 IU mg^{-1}-fold higher than F I and F III and doubling the APTT at 500 μg mL^{-1} of the test samples

This anticoagulant profile of the fractions was positively correlated with the sulfation pattern of the complex SPs (Table 1 and Figure 1) to prolong the APTT (Rodrigues et al., 2010; Pomin, 2012; Mourão, 2015). UHEP prolonged the intrinsic pathway by APTT model (Mourão et al., 2015) up to 2.5 μg mL^{-1} (42.15 ± 0.60 s) (Rodrigues et al., 2017a). SPs from *C. racemosa* had no effect on PT (1 mg mL^{-1}) compared with UHEP (100 μg mL^{-1}, 20.30 ± 0.70 s) vs. control without SPs (11.70 ± 0.50 s), reflecting the disability of the algal SPs on the extrinsic pathway factors inhibition based on other published studies (Rodrigues et al., 2010; Dantas-Santos et al., 2012; Rodrigues et al., 2017a).

SPs from the *Caulerpa* genus act on the coagulation by blocking the production of thrombin (which degrade fibrinogen into fibrin) depending on their sulfate content and charge density (Ghosh et al., 2004; Rodrigues et al., 2010; Rodrigues et al., 2012b; Wang et al., 2014), as also confirmed in the present study (Figure 1 and Table 2). The action of these compounds would be mediated by serpins (antithrombin and heparin II cofactor) (Rodrigues et al., 2013; Wang et al., 2014) because galactose-rich polysaccharides reveal as inhibitors of thrombin (Hayakawa et al., 2000; Ghosh et al., 2004), but the relationship between structure and anticoagulation still lacks in-depth details since the existence of complexity and heterogeneity of the algae glycans make comparison difficult with UHEP at molecular level and bioactivity (Athukorala et al., 2006; Pomin, 2012; Fidelis et al., 2014; Mourão, 2015; Rodrigues et al., 2016). TG assays may measure several feedback reactions to evaluate diverse classes of SPs regarding their anticoagulant dynamics (Nishino et al., 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014; Rodrigues et al., 2017b; Salles et al., 2017).

The current study demonstrated that 60-fold diluted human plasma treated with concentrations ranging from 4.1 to 83.3 μg well-$plate^{-1}$ of the *C. racemosa* SPs (F I and F II) reduced dependently TG induced by cephalin or thromboplastin (data not shown). This was in conformity to the PTh and TPeak parameters (Nishino et al., 1999; Rodrigues et al., 2016; Rodrigues et al., 2017a; Rodrigues et al., 2017b; Salles et al., 2017), based on the amidolytic activity of thrombin that decayed immediately until a plateau was reached at 28 and the negative control without activators min (Mourão et al., 2001; Salles et al., 2017). The anticoagulant dynamic of the fractions was monitored for 60 min at 37°C in parallel with the control curve of UHEP used (Figure 2) (Rodrigues et al., 2017a; Rodrigues et al., 2017b; Salles et al., 2017).

As the effects of the fractions F I and F II on TG examined in diluted plasma were of concentration-dependent manner and sulfation, the *in vitro* inhibitory reactions of the *C. racemosa* SPs also reflected as coherent responses with those of classical APTT assays (Table 2), but not with PT results (data not shown) (Mourão et al., 2001; Rodrigues et al., 2016; Rodrigues et al., 2017a; Salles et al., 2017).

Table 2. Anticoagulant effect of fractions obtained by anion-exchange chromatography (DEAE-cellulose) from the green seaweed *Caulerpa racemosa* compared to UHEP.

Fractions	NaCl (M)	APTT test (s)*					$T_1T_0^{-1}$***	IU&
		100** μg mL^{-1}	250** μg mL^{-1}	500** μg mL^{-1}	750** μg mL^{-1}	1000** μg mL^{-1}		
F I	0.50	-	38.15±0.35s	42.10±0.10s	52.9±0.10s	55.50±0.2s	1.74	4.76
F II	0.75	38.60±0.30s	50.75±0.75s	77.35±0.305s	105.2±0.105s	158.1±1.05s	2.43	12.00
F III	1.00	-	-	37.35±0.35s	42.07±0.50s	47.85±0.05s	1.50	2.32

NaCl – Sodium chloride; *Activated partial thromboplastin time (APTT); **SPs concentration to prolong the APTT in seconds; ***Ratio for double the APTT; &Anticoagulant effect expressed in international units (IU) per mg of SPs (IU mg^{-1}); UHEP (193.00 IU mg^{-1}: 2.5 μg mL^{-1} for APTT: 42.15 ± 0.60 s); Plasma control: 31.80 ± 0.10 s (n = 3)

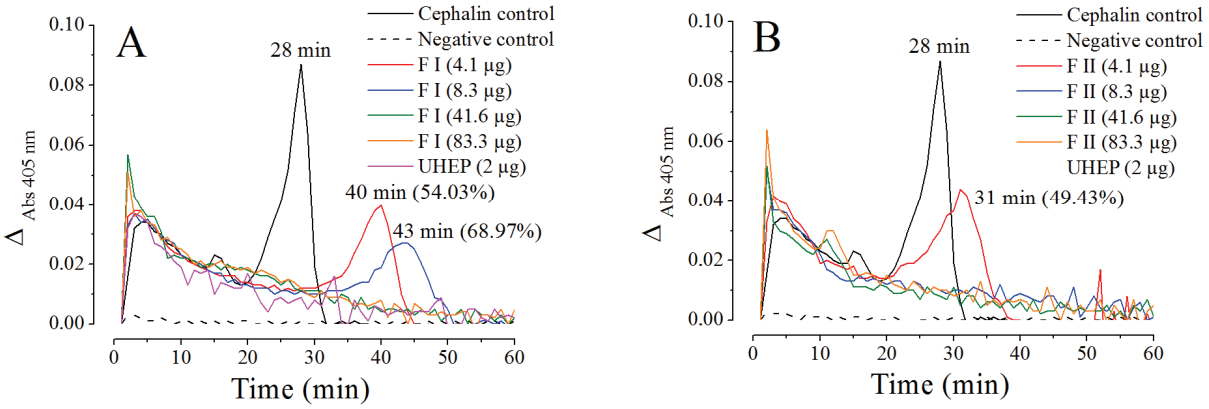

Figure 2. Effect of different concentrations of F I (A) and F II (B) from the red green seaweed *Caulerpa racemosa* on cephalin-triggered TG in 60-fold diluted human plasma using chromogenic method in a continuous detection system after 60 min at 37°C.

Our TG system had higher more accuracy at a level of concentration 24.4 and 243.9-fold lower than respective APTT and PT assays, and 50% inhibition of PTh by intrinsic pathway was observed even at 4.1 μg well-plate^{-1}, given the limited results proved by classical tests (Castoldi & Rosing, 2011; Rodrigues et al., 2017a; Salles et al., 2017).

Complete suppression of TG in intrinsic pathway-activated diluted human plasma was achieved in all the F II concentrations, except at 4.1 μg well-plate^{-1} that still had an inactivation at 31 min (49.34% PTh inhibition) (Figure 2B) (Rodrigues et al., 2017b) compared with those of F I, which abolished TG at 41.6 and 83.3 μg well-plate^{-1} (Figure 2A). These combined observations led to an intrinsic inhibition level of 10-fold higher than F II, reinforcing F I to be less potent in preventing *in vitro* clot formation due to its comparatively lower sulfate composition (Table 1) as previously confirmed the APTT assay (Table 2).

This distinct inhibitory profile of the *C. racemosa* fractions on TG by the coagulation reaction of the intrinsic pathway stimulated by cephalin (contact-activation) could perhaps be revealed as serpins-dependent thrombin inhibitors (Rodrigues et al., 2013; Wang et al., 2014) at differential inactivation patterns to modulate thrombosis *in vitro* (Nishino et al., 1999; Mourão et al., 2001) because antithrombin-dependent anticoagulants do not enhance TG in plasma and have ability to attenuate thrombin activity (Furugohri, Sugiyama, Morishima & Shibano, 2011). On the contrary, the impact of sulfation on the extrinsic-pathway induced TG did not appear using both fractions F I and F II, showing that the sulfation level was not relevant in this process because the algal SPs had only a discrete inhibitory action from the positive control (thromboplastine); therefore, a significant response was not found in plasma treated with the polymers (data not shown).

Overall, these data indicated that the SPs from *C. racemosa* do not have important action in the inhibition of thrombus by extrinsic pathway. Considering this question, it was postulated that the SPs from this algal species revealed with their most inhibitory effects to be on intrinsic pathway-activated coagulation than extrinsic one. Similar to the SPs isolated from the red seaweed *G. birdiae* (Rodrigues et al., 2017a) and from the skin of Nile tilapia (*O. niloticus*) (Salles et al., 2017) that displayed *in vitro* TG inhibition.

Although no correlation with the molecular masses of the SPs fractions (*C. racemosa*) was attributed on the TG inactivation because the polysaccharide chains were not affected after protease treatment as visualized by polyacrylamide gel electrophoresis (Figure 1) (Athukorala et al., 2006; Rodrigues et al., 2013; Fidelis et al., 2014), our study clearly evidenced SPs as blockers of TG dependently of charge, contrasting with the SPs from the red seaweed *G. birdiae* that suggested stereospecific mechanisms (Rodrigues et al., 2017a) to modulate the active coagulation factors converted by thrombin in plasma (Rau et al., 2007).

In these connections, decrease in TG by *C. racemosa* SPs reflected a different mode of action from the other classes of polysulfated that have diverse structures and mechanisms of thrombin inhibition (Nishino et al., 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014; Rodrigues et al., 2016) distinct from that of UHEP, which abolished TG at 2 (intrinsic pathway) (Figure 2) (Rodrigues et al., 2016; Rodrigues et al., 2017b) or 4 (extrinsic pathway) (data not shown) μg well-plate^{-1} (Rodrigues et al., 2017a; Salles et al., 2017) because of its specific pentasaccharide sequence with high

antithrombin affinity displaying thrombin inhibition (Mourão, 2015). These results allowed us to postulate that the modulation of TG by intrinsic pathway in the presence of UHEP were in concordance with the automated method (Jun et al., 2014), but occurred at concentration 5-fold lower than that of Glauser et al. (2009), who reported no inhibition of TG by UHEP up to 10 μg.

As our TG system would be sensitive to analyze anticoagulants (Rodrigues et al., 2016; Rodrigues et al., 2017a; Salles et al., 2017), inactivation of TG by *C. racemosa* SPs could interfere with their anti-inflammatory actions (Ribeiro et al., 2014), because the concentration of SPs was about 2.43-fold lower in the anticoagulation. Increased TG induces thrombosis associated with neutrophil adhesion during the inflammatory response initiated when injury to a vessel wall exposes the blood to tissue factor in the subendothelium (Rau et al., 2007).

By contrast, TG inhibition by intrinsic pathway in plasma treated with *C. racemosa* SPs was correlated with the antiviral property based on another study (Ghosh et al., 2004). Observation suggested that our system could also constitute as an useful tool to guide complementary analyses for antivirally active algal SPs development (Cardozo et al., 2007) in parallel to prevention of thrombosis *in vitro* (Nishiro et al., 1999; Mourão et al., 2001; Rodrigues et al. 2016, 2017b) as support to predict the risk of bleeding or coagulopathy (Castoldi & Rosing, 2011; Zavyalova & Kopylov, 2016) on the focus of patients subjected to antiviral treatment (Ghosh et al., 2004; Ghosh et al., 2009).

In summary, *C. racemosa* features SPs have potential applicability as novel, biomaterial to prevent thrombosis *in vitro* as demonstrated in the TG assay and test parameters could indicate to studies of their underlying mechanisms (Glauser et al., 2009; Rodrigues et al., 2016) when an increase of plasma prothrombin's referential values increase the TG after activation by both intrinsic and extrinsic pathways (Rao et al., 2007; Castoldi & Rosing, 2011).

Conclusion

The green seaweed *Caulerpa racemosa* reveals large molecular sizes complex glycans displaying *in vitro* diluted human plasma thrombin generation inactivation, with their most inhibitory effects to be on intrinsic pathway-activated coagulation than extrinsic one dependent on the concentration and charge density, when in a continuous system, although less potent than unfractionated heparin.

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