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In vitro inhibition of thrombin generation by sulfated polysaccharides from the tropical red seaweed *Gracilaria birdiae* Plastino & Oliveira

Inibição *in vitro* da geração de trombina por polissacarídeos sulfatados da alga marinha vermelha tropical *Gracilaria birdiae* Plastino & Oliveira

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Abstract *Gracilaria birdiae* is a red macroalga found on the Brazilian coastline and experimentally cultivated to obtain industrially-important sulfated polysaccharides (SPs) for biotechnology, but their anticoagulant potentials on thrombin generation (TG) *in vitro* have not been examined. This study compared the *in vitro* inhibition of SPs isolated and physical-chemically characterized from samples of *G. birdiae* collected in natural-bed (Gbnb) and cultured (Gbc) on the coastal of Flecheiras beach on TG. Papain extraction yield difference reached 11.76%, and different DEAE-cellulose chromatograms were obtained, with fractions (Gbnb-I→II and Gbc-I→IV) revealing charge density patterns and molecular sizes > 100 kDa according to the both agarose and polyacrylamide electrophoreses, respectively. These procedures, associated with toluidine blue/Stains-All staining, also revealed nonSPs. By both activated partial thromboplastin time (APTT) and prothrombin time assays, fractions had no anticoagulant actions, except Gbc-I that discretely altered the APTT (0.51 IU mg mL^{-1}) compared with unfractionated heparin (UHEP, 193 IU mg mL^{-1}). Gbc-I inhibited concentration-dependent both intrinsic and extrinsic coagulation pathways in *in vitro* TG assay using 60-fold diluted human plasma, with 50% efficacy on thromboplastin-activated TG, similar to Gbnb-I, although less potent than UHEP which abolished it at a 41.65-fold lower concentration. The results suggest that *G. birdiae* change its matrix-polysaccharide content under field conditions and TG assay could guide complementary analyses on algal species anticoagulant potential for pharmacological use.

Keywords: Rhodophyta, marine environment, polysulfated, chemical analysis, thrombin.

Resumo *Gracilaria birdiae* é uma macroalga vermelha encontrada na costa brasileira e cultivada experimentalmente para obter polissacarídeos sulfatados (PSs). Seus potenciais anticoagulantes não têm sido examinados sobre geração de trombina *in vitro*. Comparou-se a inibição *in vitro* dos PSs isolados e caracterizados físico-quimicamente de *G. birdiae* coletados em banco natural (Gbbn) e cultivados (Gbc) na praia de Flecheiras sobre GT. A diferença nos rendimentos, na extração com papaína, foi 11,76%. Foram obtidos cromatogramas diferentes (DEAE-celulose), apresentando frações (Gbbn-I→II and Gbc-I→IV), revelando, por eletroforeses em agarose e poliacrilamida, diferenças na densidade de carga e tamanhos moleculares >100 kDa. Esses procedimentos, associados com coramento azul de toluidina/"Stains-All", revelaram também polissacarídeos não-sulfatados. Por ambos os testes do tempo de tromboplastina parcial ativada (TTPA) e do tempo de protrombina, as frações não apresentaram anticoagulação, exceto Gbc-I que alterou discretamente o TTPA (0,51 UI mg mL^{-1}) comparada ao da heparina não-fracionada (HEPNF, 193 UI mg mL^{-1}). Gbc-I inibiu, dependente de concentração, ambas as vias intrínseca e extrínseca no ensaio de GT *in vitro* usando plasma humano diluído 60 vezes e apresentando, semelhante a Gbnb-I, eficácia de 50% sobre GT estimulada por tromboplastina, embora potente menos a HEPNF que aboliu GT em uma concentração 41,65 vezes menor. Os resultados sugerem que *G. birdiae* muda, *in situ*, seu conteúdo de polissacarídeo de matrix e ensaio de GT poderia guiar análises sobre o potencial anticoagulante da algácea para uso farmacológico.

Palavras-chave: Rhodophyta, ambiente marinho, poli-sulfatado, análise química, trombina.

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Introduction

Seaweeds comprise well-adapted photosynthetic bioresources in marine ecosystem (Costa-Lotufo et al., 2006) expressing a variety of matrix sulfated polysaccharides (SPs) with economical impact worldwide due to their wide spectrum of intrinsic applications in various food preparations and in biotechnology (Pereira & Costa-Lotufo, 2012; Prajapati et al., 2014). These polyanionic glycans of high molecular weights have highly heterogeneous structures of different chemical classes, such as fucoidans (Phaeophyceae), sulfated heteropolysaccharides (Chlorophyceae) and sulfated galactans (mainly carrageenans and agar) (Rhodophyceae), varying among algal species (Pomin, 2012; Amorim et al., 2012; Rodrigues et al., 2013; Zhang et al., 2014). On a basis of abundance, Rhodophyceae produces high amounts of SPs (Prajapati et al., 2014) in comparison with Chlorophyceae (Rodrigues et al., 2013) and Phaeophyceae (Zhang et al., 2014) and other living organisms (Dantas-Santos et al., 2012; Chang et al., 2013).

The physical-chemical properties and bioactivities of SPs can vary according to the harvest time and collection site of the algae, negatively impacting some attributes, such as yield and purity, became thus the standardization of a commercial product still a challenge at industrial level (Pereira & Costa-Lotufo, 2012). The genera *Gracilaria* (agarophyte), *Hypnea* and *Kappaphycus* (carragenophytes) of edible red seaweeds are the main supplies to the world' hydrocolloid industry (Pereira & Costa-Lotufo, 2012; Prajapati et al., 2014). However, the continued exploitation of natural-beds of seaweeds has led to a gradual decline of these resources (Pereira & Costa-Lotufo, 2012) and studies on the mariculture of these organisms arise as a sustainable activity for hydrocolloids production in large scale and for the protection of natural areas (Hung et al., 2009; Rodrigues et al., 2011).

Although exhibiting very distinct structures and effects that cannot simulate the same ability of unfractionated heparin (UHEP) due to the evident absence of the specific pentasaccharide sequence with high antithrombin affinity, SPs from seaweeds may constitute alternatives of UHEP because it has consequences for clinical use, including bleeding and thrombocytopenia (Mourão, 2015). Traditionally, *in vitro* effects on coagulation have been measured using classical tests, such as the activated partial thromboplastin time (APTT) and the prothrombin time (TP), in which explore the intrinsic and extrinsic coagulation pathways, respectively (Rodrigues et al., 2011; Zhang et al., 2014). However, these methods do not indicate the participation of inhibitors, as well as do not reflect a clinical risk of circulatory disorder, e.g., bleeding and venous thrombosis; therefore, they do not have accuracy to identify the phases of clot formation and are only capable of detecting an amount of 5% generated thrombin in plasma (Castoldi & Rosing, 2011). Thrombin generation (TG)-based coagulation assays provide a more precise representation of the overall tendency of a plasma sample to form thrombin after *in vitro* activation in order to facilitate the interpretation of the clinical events related to hemostasis and analyzer anticoagulants (Castoldi & Rosing, 2011).

Some SPs from different aquatic organisms have been examined on TG assays to obtain additional data on their anticoagulant actions. For example, for a SP (fucosylated chondroitin sulfate) isolated from the sea cucumber *Ludwigothurea grisea*, Mourão et al. (2001) reported *in vitro* inactivation of TG. Nishino et al. (1999) revealed that a SP (fucoidan) from the brown seaweed *Ecklonia kurome* had inhibitory effect on TG. It was demonstrated by Glauser et al. (2009) that the SP isolated from the red seaweed *Botryocladia occidentalis* inhibited TG by the prothrominase complex. More recently, Zhang et al. (2014) described that the SP (fucoidan) from the brown seaweed *Fucus vesiculosus* displayed both anti- and procoagulant effects on *in vitro* TG assays.

The edible red macroalga *Gracilaria birdiae* Plastino & Oliveira (Gracilariales, Rhodophyta) is distributed from Ceará State to Espírito Santo State and has been cultured and commercialized for agar extraction by fishing communities in Northeastern of Brazil (Maciel et al., 2008; Bezerra & Marinho-Soriano, 2010). Infrared and nuclear magnetic resonance analyses of this SP revealed

repeating units of β-D-galactopyranose; 3,6-anhydro-α-L-galactopyranose and a-L-galactopyranose-6-sulfate and other backbone components (Maciel et al., 2008; Fidelis et al., 2014). Studies on its antioxidant (Souza et al., 2012), anti-inflammatory (Vanderlei et al., 2011) and anticoagulant (Fidelis et al., 2014) potentials without toxicity *in vivo* (Vanderlei et al., 2011) were also demonstrated by other scientific groups. No analysis of its SPs on *in vitro* TG assays has been reported so far.

Current investigation was to obtain and compare SPs from Brazilian samples of *G. birdiae* collected and cultured on the coastal line of Flecheiras beach regarding their yield, physical-chemical characteristics and their anticoagulant potentials on TG in 60-fold diluted human plasma using chromogenic method by continuous detection system.

Material and Methods

G. BIRDIAE OBTAINING AND ISOLATION OF SPS AND PHYSICAL-CHEMICAL ANALYSES

Specimens of the Rhodophyceae *G. birdiae* were collected in natural-bed and cultured in the sea open using *long line* structures located at 200 m from coastal line of Brazil (03°13'06"S, 39°16'47"W) (Flecheiras beach, Trairí, Ceará) (Maciel et al., 2008), and then they were taken to the Carbohydrates and Lectins laboratory (CarboLec), Federal University of Ceará, in plastic bags. A voucher specimen (no. 40781) was deposited in the Herbarium Prisco Bezerra of the Department of Biological Sciences, Federal University of Ceará, Brazil. After collection, the algal samples were washed with distillated water and cleaned to eliminate residues and epiphytes, followed by dehydration at room temperature (Rodrigues et al., 2011; Vanderlei et al., 2011). The analyses of the *G. birdiae* SPs were performed at Connective Tissue laboratory, Federal University of Rio de Janeiro (FURJ), Brazil.

Two grams of dehydrated algal tissue were cut into small pieces and subjected to papain digestion (60°C, 24 h) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM), as previously published elsewhere (Vanderlei et al., 2011). A sample of each 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) equilibrated with the same buffer. The fractionation was conducted using a stepwise gradient from 0 to 1.5 M NaCl at 0.25 M intervals in the same buffer. Fractions of 2.5 mL were collected and analyzed for SPs using the metachromatic assays containing dimethymethylene blue with an Amersham Bioscience Ultrospec 3100 spectrosphotometer at 525 nm (Farndale et al., 1986). The metachromatic fractions were further dialyzed and freeze dried. Quantitative determination for sulfate, hexoses and proteins (Vanderlei et al., 2011) and detection of SPs on agarose (Dietrich & Dietrich, 1976) and polyacrylamide (Fidelis et al., 2014) gels by sequential staining with toluidine blue/Stains-All (Volpi & Maccari, 2002) were also performed 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., 2013). A more detailed analysis on *in vitro* coagulation was conducted with the polysaccharide fractions (named Gbnb-I and Gbc-I) that proved higher SPs yields.

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, 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.

ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) AND PROTHROMBIN (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 of the *in vitro* TG assay. For APTT assay, a mixture of 100 µL of plasma and concentrations of SPs (0-1 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 per mg (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, containing: 10 µL of cephalin (contact-activator system) or thromboplastin (1083 µg $well\text{-}plate^{-1}$, factor tissue-activator system) + 30 µL of 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 µL of SPs (*G. birdiae* SPs fractions: 0, 4.1, 41.6 or 83.3 $\mu g.well\text{-}plate^{-1}$ or UHEP: 2 or 4 $\mu g.well\text{-}plate^{-1}$) + 60 µL of 20 mM $CaCl_2$/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 $well\text{-}plate^{-1}$) (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) (Rodrigues et al., 2016).

STATISTICAL ANALYSES

The data of extract yields were expressed as mean ± S.E.M., followed by analysis of Student's test, considering $p < 0.05$ as significant. In the classical APTT and PT assays, the analysis of variance (Anova) was performed, followed by Tukey's test for unpaired data. with $p < 0.05$ as statistically significant.

Results and Discussion

The dehydrated raw tissue of *G. birdiae* was digested with papain to obtain SPs, followed by both cethlypiridinium chloride and ethanol precipitations in order to compare their yields and physical and chemical characteristics for samples collected in natural-bed and cultured. The values of yield and composition of the crude SPs extracts differed between the Brazilian samples of *G. birdiae* obtained from the two field conditions.

The crude SP extraction yield (27.46 ± 1.1.66%) revealed 10.1% sulfate and 69.2% hexoses from the quantitative analyses for cultured samples of *G. birdiae*. This yield was about 1.75-fold higher compared with samples collected from natural-bed (15.70 ± 1.822% yield) ($p < 0.05$) that had levels of 7.2% and 65.7% for sulfate and hexoses, respectively. These yields were not in concordance with those previously found for the same species collected from the Flecheiras beach by Vanderlei et al. (2011), applying the same method of current study, obtained a crude SP of 4.66% exhibiting 8.3% sulfate and 68.2% hexoses, respectively, and by other scientific groups that found different levels of yield (0.52-8.26%), sulfate (6.4-8.4%) and hexoses (85.6%) using proteolysis, NaOH, ultrasound or water under different conditions to obtain *G. birdiae* SPs (Maciel et al., 2008; Souza et al., 2012; Fidelis et al., 2014). On the basis of sulfate content, values were in conformity with those revealed for agar-producer *Gracilaria* species (4-10%) (Amorim et al., 2012; Pereira & Costa-Lotufo, 2012). Bezerra & Marinho-Soriano (2010) found yields ranging from 15 to 22%, depending on the season, for samples of *G. birdiae* cultured on the coastal of Rio Grande do Norte State, Brazil.

On the base of these observations, studies on the *G. birdiae* SPs composition are required focusing on the seasonality (Pereira & Costa-Lotufo, 2012), which would also lead to identify new biologically active SPs (Fidelis et al., 2014). As expected, papain-assisted extraction removed proteins in analyzed raw polymer samples (Vanderlei et al., 2011) since carbohydrate-protein complexes occur in the seaweed extracellular matrix (Fidelis et al., 2014).

G. BIRDIAE SUGGESTS OVERPRODUTION SPS IN ITS CELL-WALL UNDER GROWING CONDITION IN THE SEA

A comparative analysis, by anion-exchange chromatography on DEAE-cellulose column between a sample of each crude SPs extract obtained from *G. birdiae*, was further carried out, and the representative chromatograms are illustrated in Figure 1A.

The profiles of DEAE-cellulose chromatography showed differences between the samples of *G. birdiae* obtained from the two environments under analysis. Increasing molarities of NaCl steadily reduced the metachromasy, yield and number of fractions eluted from the column, reflecting heterogeneity of the SPs obtained (Rodrigues et al., 2011; Vanderlei et al., 2011). On a basis of mass-to-mass, 0.50 and 0.75 M NaCl fractions (Gbnb-I→II and Gbc-I→II), which were eluted at the beginning of the salt gradient, accounted almost the total material recovered from the DEAE-cellulose column (60.5 and 88.5%, respectively) (Table 1).

Results corroborated with their respective metachromasy due to a large number of anionic groups present these molecules (Athukorala et al., 2006), as was also previously observed for samples of *G. birdiae* collected from the same beach by Vanderlei et al. (2011). However, it was important to mention that two more fractions (Gbc-III→IV) were, respectively, eluted with 1 and 1.25 M of NaCl, from cultured samples of *G. birdiae*, although presenting very scarce yields (Figure 1A and Table 1). In fact, *G. birdiae* synthesized other extracellular matrix SPs when cultured in sea open since that the amount of SPs recovered from the column for samples collected in natural-bed was also 1.60-fold lower (Table 1). Hung et al. (2009) and Pereira & Lotufo (2012) reported that the standardization of a commercially-important natural product from the industry is very difficult, especially with regard to the collection of these organisms in different sites or at different seasons.

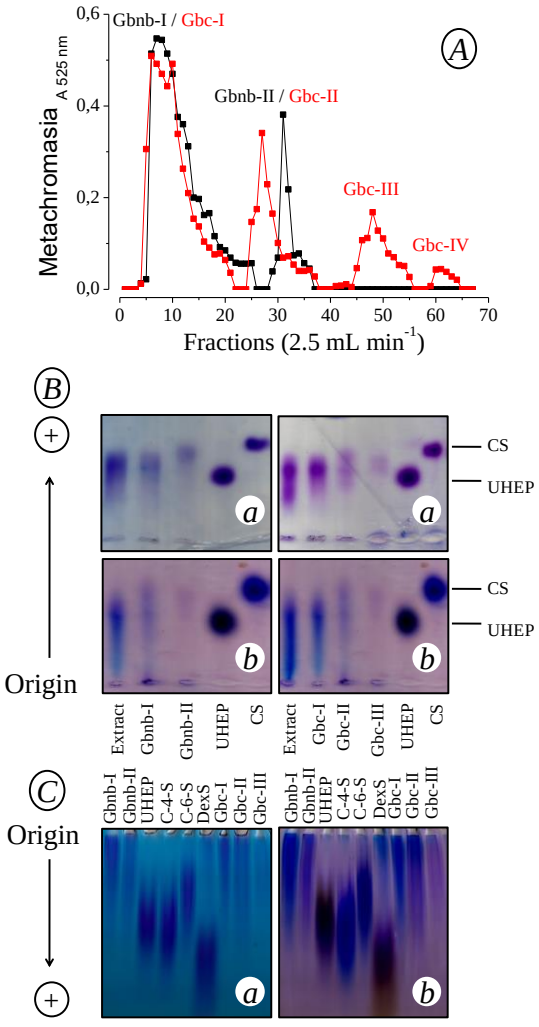

Figure 1 (*A*) Fractionation of SPs from *Gracilaria birdiae* collected in natural-bed (Gbnb) (■—■) and cultured in the sea (Gbc) (■—■) by DEAE-cellulose. Fractions were collected and checked by metachromasia using 1,9-dimethylmethylene blue. Agarose (*B*) or polyacrylamide (*C*) gel electrophoresis of extracts and fractions (Gbnb-I→II and Gbc-I→IV) 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 unfractionated heparin (UHEP, 14 kDa) present on gels were stained with 0.1% toluidine blue (*a*) or Stains-All (*b*).

Table 1 Yield of SPs fractions obtained by ion-exchange chromatography (DEAE-cellulose) from the red seaweed *Gracilaria birdiae* collected from the two environments.

Environment	Fractions	NaCl (M)[a]	Yield (%)[b]
Cultivation	Gbc-I	0.50	80.00
	Gbc-II	0.75	8.50
	Gbc-III	1.00	7.50
	Gbc-IV	1.25	*
Natural-bed	Gbnb-I	0.50	55.50
	Gbnb-II	0.75	5.00

* Scarce material for analysis.

Physical-chemical analyses of the SPs isolated from *G. birdiae* on agarose or polyacrylamide gel electrophoresis are shown in Figures 1B and C. The staining pattern of extracts and fractions on agarose gel was individualized according to the stepwise of NaCl as a result of differences in terms of sulfation of the glycans obtained (Rodrigues et al., 2011; Mourão, 2015). Comparatively, they revealed polydisperse bands with motilities close to UHEP and presenting strong metachromasy for the fractions eluted at 0.5 and 0.75 M (Gbc-I→II, respectively) (Figures 1A and Ba) (Vanderlei et al., 2011). As the SPs from *G. birdie* interacted with the 1,3-diaminopropane/acetate buffer, it was also speculated same structural conformation and charge/mass ratio because extracts and fractions showed similar migrations on agarose gel after 60 min (Dietrich & Dietrich, 1976; Fidelis et al., 2014).

Interestingly, sequential staining with toluidine blue and Stains-All improved the limit of detection for other polysaccharides that were not revealed by staining with toluidine blue alone as visualized by staining for cyan, especially for extract and fraction F I (eluted at 0.5 M) obtained from cultivated samples (Figure 1Bb). This observation allowed us to identify nonSPs on the same analyzed polymer samples (Volpi & Macocari, 2002) containing complex structures of agarophytes which are made of a neutral fraction (agarose) and an anionic fraction (agaropectin), with low sulfate content (Maciel et al., 2008; Amorim et al., 2012). Similarly, polyacrylamide analysis also showed more intense polysaccharides on gel, when large molecular size distribution SPs (> 100 kDa) were revealed (Figure 1C), as a common features for SPs from seaweeds (Athukorala et al., 2006; Pomin, 2012; Mourão, 2015).

These combined observations suggested glucuronic acid-rich SPs. Fidelis et al. (2014) evaluated the effect of sonication to extract the SPs from *G. birdiae* and detected a high amount of glucuronic acid according to the monosaccharide composition, and spectral signals by infrared evidenced carboxyl groups of uronic acid in their structures. Another hypothesis for our findings would be an overproduction of *G. birdiae* cell-wall polysaccharides as a defense mechanism in response to environment changes, when cultured under field conditions (Bezerra & Marinho-Soriano, 2010; Pereira & Costa-Lotufo, 2012), but further studies must show these questions. The approach allowed us to detect other polysaccharides within complex glycans preparations derived from Brazilian samples of *G. birdiae* as previously demonstrated for glycosaminoglycans from animals tissues (Volpi & Maccari, 2002); therefore, given additional data on the complexity of algae SPs (Prajapati et al., 2014; Mourão, 2015).

SPS FROM *G.*. *BIRDIAE* DO NOT ALTER APTT AND TP TESTS, BUT MODIFY THE TG RESPONSE

In order to evaluate the *in vitro* effect of the SPs from *G. birdiae* collected and cultured on the coastal of Flecheiras beach, routine coagulation tests were employed using 193 IU.mg^{-1} UHEP standard, as shown in Table 2.

Table 2 Evaluation of fractions obtained by anion-exchange chromatography (DEAE-cellulose) from the red seaweed *Gracilaria birdiae* on classical coagulation tests.

			APTT (s)*	PT (s)**		
Environment	Fractions	NaCl (M)	1 mg.mL^{-1}***		$T_1.T_0^{-1}$&	IU.mg^{-1}#
Cultivation	Gbc-I	0.50	44.80 ± 0.35*	12.45 ± 0.31	1.33	0.51
	Gbc-II	0.75	34.20 ± 0.06	12.30 ± 0.05	1.01	0.19
	Gbc-III	1.00	35.93 ± 0.20	12.85 ± 0.14	1.07	0.39
Natural-bed	Gbnb-I	0.50	33.40 ± 0.10	12.35 ± 0.14	1.00	0.38
	Gbnb-II	0.75	36.30 ± 0.11	12.55 ± 0.02	1.08	0.41

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}: 2.5 and 100 µg.mL^{-1} for APTT (42.15 ± 0.6 s[a]) and PT (20.30 ± 0.7 s), respectively); Controls: 33.53 ± 0.14 s and 12.57 ± 0.18 s for APTT and PT tests, respectively (n = 3, ANOVA, Tukey's test, $p < 0.05$* vs. control).

The tested fractions did not inhibit ($p > 0.05$), at a concentration of 1 mg.mL^{-1}, the normal human plasma coagulation time since APTT (33.40 ± 0.10-34.06 ± 0.10 s) and PT (12.30 ± 0.05-12.85 ± 0.14 s) values were not prolonged in comparison with those of controls without SPs, except 0.5 M NaCl fraction (Gbc-I) that modestly extended ($p < 0.05$) about 1.33-fold higher the APTT test (44.80 ± 0.35 s, 0.51 IU.mg mL^{-1}) from cultured samples of *G. birdiae*, virtually capable of acting on the intrinsic and/or common pathways of the coagulation (Rodrigues et al., 2011). UHEP still showed, at concentrations of 2.5 and 100 µg.mL^{-1}, respectively, *in vitro* anti-clotting effects on the APTT (42.15 ± 0.6 s) and PT (20.30 ± 0.7 s) tests vs. control times (APTT: 33 ± 0.14 s; PT: 12.57 ± 0.18 s).

The most intriguing result of our study was the lack of *in vitro* anticoagulant action of the *G. birdiae* SPs on both APTT and PT tests because the main peak eluted at 0.5 M (Gbnb-I and Gbc-I) had highest charge density as revealed by DEAE-cellulose and electrophoresis analyses (Figures 1A and B) and high molecular size (Figure 1C). These requisites have been well-documented for seaweeds SPs display anticoagulation, not only for fucans from brown seaweeds (Athukorala et al., 2006; Pomin, 2012; Zhang et al., 2014), but also in sulfated galactans from green and red seaweeds (Rodrigues et al., 2013; Mourão, 2015). Fidelis et al. (2014) demonstrated that a decrease in the molecular mass of the SPs from *G. birdiae*, when extracted by different conditions, was accompanied by a reduction in their anticoagulant effects as measured by *in vitro* APTT test. The anticoagulation was only displayed with a molecular mass of over 45 kDa.

In our case, the chemical structures of the SPs (*G. birdiae*) were not affected by papain digestion because it has a wide spectrum to eliminate proteins associated with SPs (Rodrigues et al., 2011; Vanderlei et al., 2011) and polyacrylamide analysis confirmed large molecular masses SPs (Figure 1C) (Pomin, 2012; Mourão, 2015). The protease treatment also helped in the solubility of the *G. birdiae* fractions in water for their evaluation by classical tests (Table 2) (Rodrigues et al., 2011) since the levels of sulfation and associated cations (mainly calcium) may influence the intrinsic abilities of gelling and stabilizing of the SPs (Prajapati et al., 2014). It was possible suggest in this study that the SPs were not able to form complexes with thrombin and plasma inhibitors (Pomin, 2012; Mourão, 2015) due to relatively low charge density on their structural composition (Figure 1Ba) (Vanderlei et al., 2011) when compared to other studied algae SPs (Mourão, 2015) or accuracy of the classical coagulation methods to detect any anticoagulant response (Castoldi & Rosing, 2011). Glucuronic acid composition could also influence the ability of SPs to displays anticoagulation (Athukorala et al., 2006). In these circumstances, are the *G. birdiae* SPs capable of inhibiting TG? In order to evaluate this hypothesis, the fractions Gbnb-I and

Gbc-I, both eluted at 0.5 M (Figure 1A and Table 1), were further analyzed on an *in vitro* TG system, as shown in Figure 2.

Comparison between Gbnb-I and Gbc-I (data not shown) on our TG method allowed more precise analysis the response of these molecules on the coagulation *in vitro*. Gbc-I acted on both contact-activated and thromboplastin systems leading to a concentration-dependent decrease in TG using 60-fold diluted human plasma, as observed by PTh and/or TPeak parameters and by delays in the lag phase, with basis the amydolytic activity of thrombin that decayed rapidly until a plateau was reached (29 or 19 min) (Figures 2A and B) (Mourão et al., 2001; Rodrigues et al., 2016). No activator response of TG in plasma in the absence of cephalin or thromboplastin (negative control) was observed *in vitro* during 60 min.

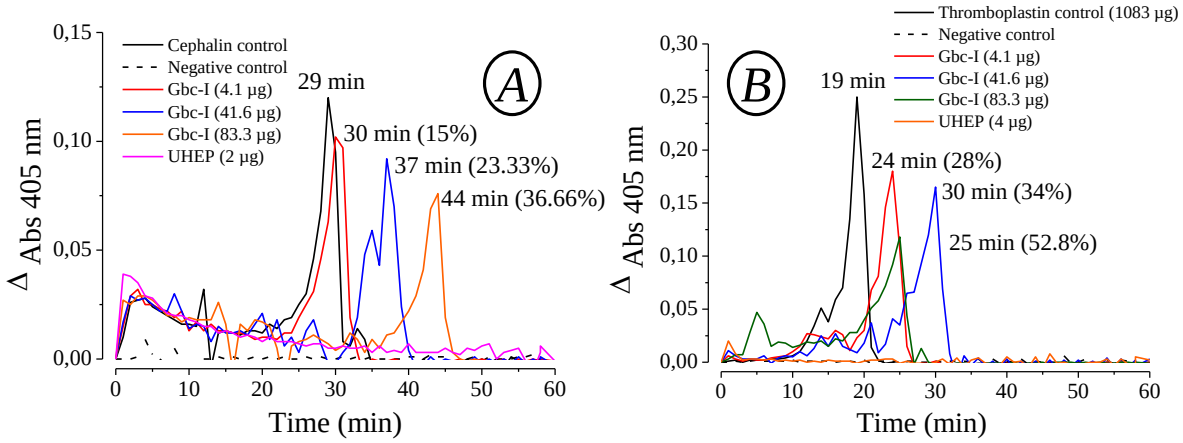

Figure 2 Effect of different concentrations of Gbc-I, obtained by DEAE-cellulose, from the red seaweed *Gracilaria birdiae* on cephalin (*A*)- or thromboplastin (*B*)-triggered TG in 60-fold diluted human plasma using chromogenic method by a continuous detection system.

Its most inhibitory effect was revealed to be on thromboplastin-activated TG where at different concentrations of SPs (4.1→83.3 µg.well-plate^{-1}) inactivated, respectively, by 28, 34 and 52.8% PTh. Similarly to the SP from the brown seaweed *E. kurome* that reduced TG in the intrinsic and extrinsic pathways dependently of concentration. However, its *in vitro* inhibitory effects of TG by intrinsic activation was observed to be at least 2-fold higher than from our study and results also suggested that the polysaccharide inhibited the system in normal diluted plasma to varying extent (Nishino et al., 1999).

High concentration of Gbc-I induced about 50% inhibition of TG, although with a concentration 20.82-fold higher than UHEP, taking the fact that it abolished TG on this *in vitro* system due to its mechanism of thrombin inhibition that requires antithrombin-binding pentasaccharidic sequence which is not found in other SPs-rich sources (Mourão, 2015). The disability of Gbc-I to fully inhibit TG could be related to its mechanism of action which could be sterospecific (Mourão, 2015) to interact with the active coagulation factors converted by thrombin in plasma (Rau et al., 2007). UHEP abolished extrinsic pathway-induced TG when a concentration 2-fold higher was required (Figure 2B) in coherency with the PT assay where a relatively higher amount of it (at least 100 µg) was necessary for more modestly inhibit the extrinsic pathway factors (Table 2), confirming its most inhibitory effect to be on intrinsic pathway-activated coagulation than extrinsic one (Mourão, 2015). Observation contrasted with the findings of Glauser et al. (2009), who reported no inhibition of TG by intrinsic pathway in plasma in the presence of UHEP up to 10 µg. Our TG system could be more sensitive to analysis of anticoagulant compounds (Figure 2) (Rodrigues et al., 2016). Similar *in vitro* inhibitory effects were found for fraction Gbnb-I compared to Gbc-I (data not shown), postulating the same level of contribution on both coagulation pathways. This is important because differences in the relative proportions of sulfate

and composition impact the bioactivity of the algae SPs (Athukorala et al., 2006; Pomin, 2012; Mourão, 2015).

The physical-chemical characteristics of the fractions (Figure 1) did not limit the anticoagulant action on our *in vitro* TG system to facilitate the interpretation of the effects on the clot inhibition (Figure 2) (Nishino et al., 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014) compared with conventional assays (APTT and PT) (Castoldi & Rosing, 2011). Decreased TG by *G. birdie* SPs could interplay on the anti-inflammatory actions of these glycans (Vanderlei et al., 2011) because the concentration of SPs was about 120,000-fold lower in the anticoagulant response. 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 et al., 2007).

Given the importance of *Gracilaria* species for hydrocolloid industry worldwide and in biotechnology (Maciel et al., 2008; Bezerra & Marinho-Soriano, 2010; Amorim et al., 2012; Pereira & Costa-Lotufo, 2012), TG assay constituted as a useful tool to guide combined analyses of SPs, extracted from Brazilian samples of *G. birdiae* collected and cultured on the coastal line of Flecheiras beach, regarding their anticoagulant dynamics to prevent thrombosis *in vitro* (Nishino et al., 1999; Mourão et al. 2001). Complementary studies on the mechanisms underlying involved in their anticoagulant actions on TG in both intrinsic and extrinsic pathways are still required to better understanding of the mode of action by which it would modulate the circulatory dysfunctions.

Conclusion

The rhodophyceae *Gracilaria birdiae* distributed on the coastal line of Flecheiras beach change its polysaccharide content, based on anion-exchange chromatography (DEAE-cellulose) and electrophoretic procedures, when obtained from cultured samples in the sea. In support to these findings, sequential staining with toluidine blue and Stains-All reveals as a sensitive approach to detect other polysaccharides produced by this species, as verified by both agarose and polyacrylamide analyses. Polysaccharides have high molecular sizes (> 100 kDa), but on classical coagulation tests (APTT and PT), they do not show anticoagulation. However, when one fraction of each sample is tested on an *in vitro* thrombin generation assay, it inhibits concentration-dependent both intrinsic and extrinsic pathways, with 50% inhibition to be on thromboplastin-activated thrombin generation, although less potent than heparin, when in 60-fold diluted human plasma. Results allow us to suggest that *G. birdiae* contains glycans capable of preventing thrombosis since them alter thrombin generation parameters, as a promising source of natural anticoagulants to modulate coagulation system disorders.

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