Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v40i1.34463

BIOTECHNOLOGY

Extraction, physical-chemical characterization and *in vitro* inhibitory potential of thrombin generation of crude sulfated polysaccharides from Brazilian tropical seaweeds

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ABSTRACT. The biotechnological value of macroalgae for screening assays of thrombin generation-TG using sulfated polysaccharides-SPs as substitutes to heparin has been poorly explored. Five Brazilian species of macroalgae (*Gracilaria birdiae*, *Acanthophora muscoides*, *Halymenia* sp., *Caulerpa cupressoides* and *C. racemosa*) were analyzed and compared for their abundance, physical-chemical characteristics and *in vitro* anticoagulant assays of activated partial thromboplastin time-APTT, prothrombin time-PT and TG. Papain extraction yielded ($p < 0.001$) from 0.66±0.03% (*C. racemosa*) to 41.60±1.10% (*Halymenia* sp.) of crude SPs varying sulfate (8.41-42.60%) and total sugars (47.80-70.53%). Crude SPs showed difference in mobility and resolution pattern by agarose electrophoresis, while polyacrylamide analysis revealed SPs of > 100 kDa. These procedures, combined with the use of Stains-All, also indicated nonSPs. APTTs ranged from 2.81 (*A. muscoides*) to 21.30 IU (*Halymenia* sp.) vs. heparin (193 IU), and were dependent on sulfation of the crude SPs. PT was not altered. With respect to TG assay, crude SPs modified concentration-dependent and independently from molecular mass TG by both intrinsic/extrinsic pathways in 60-fold diluted human plasma, with total intrinsic inactivation using crude SPs from *A. muscoides* in parallel to heparin ($p < 0.05$). Thrombosis *in vitro* is differentially modulated by distinct crude SPs from Brazilian seaweeds.

Keywords: marine algae; polyglycans; coagulation tests; thrombin.

Extração, caracterização físico-química e potencial inibitório *in vitro* da geração de trombina de polissacarídeos sulfatados brutos de algas marinhas tropicais brasileiras

RESUMO. O valor biotecnológico das macroalgas para ensaios de varredura de geração de trombina-GT pouco tem sido explorado usando polissacarídeos sulfatados-PSs como substitutos à heparina. Foram analisadas e comparadas cinco espécies brasileiras de macroalgas (Gracilaria birdiae, Acanthophora muscoides, Halymenia sp., Caulerpa cupressoides e C. racemosa) quanto à abundância, às características físico-químicas e os ensaios anticoagulantes in vitro de tempo de tromboplastina parcial ativada-TTPA, ao tempo de protrombina-TP e a GT. A extração com papaína rendeu ($p < 0,001$) de 0,66±0,03% (C. racemosa) a 41,60±1,10% (Halymenia sp.) de PSs brutos variando sulfato (8,41-42,60%) e açúcares totais (47,80-70,53%). Os PSs brutos mostraram diferenças na mobilidade e resolução por eletroforese em agarose, enquanto pela análise em poliacrilamida revelou PSs brutos de >100 kDa. Esses procedimentos, combinados ao uso de azul de toluidina/Stains-All, indicaram também polissacarídeos-não sulfatados. Os TTPAs foram dependentes da sulfatação dos PSs brutos e variaram de 2,81 (A. muscoides) a 21,30 UI (Halymenia sp.) vs. heparina (193 UI). O TP não foi alterado. Com respeito ao ensaio de GT, os PSs brutos modificaram, dependente de concentração e independentemente de massa molecular, GT pelas vias intrínseca/extrínseca no plasma humano diluído 60 vezes, com inativação intrínseca total usando PSs brutos de A. muscoides em paralelo à heparina ($p < 0,05$). A trombose in vitro é modulada diferencialmente por PSs brutos distintos de algas marinhas brasileiras.

Palavras-chave: algas marinhas; poliglicanos; testes de coagulação; trombina.

Introduction

In Brazil, a wide diversity of marine life (e.g., cucumbers, fishes and seaweeds) is distributed along the more than 8000 Km of coastline, revealing many biologically active metabolites (e.g., polysaccharides, proteins and carotenoids) which have already been isolated and studied as anticancer (Costa-Lotufo et al., 2006), anticoagulant, antithrombotic (Mourão et al., 2001; Fonseca et al., 2008; Rodrigues et al., 2012a) and anti-inflammatory (Pomin, 2012; Fernandes, Oliveira, & Valetin, 2014) agents, and as ingredients for food preparations (e.g., polysaccharides-based edible films) (Fenoradosoa et al., 2009; Paula et al., 2015). As a result, in the last decades, various classes of marine organisms-derived polymers with healthy-related benefits have been well-documented by different Brazilian groups (Costa-Lotufo et al., 2006; Fernandes et al., 2014; Mourão, 2015).

There is an increasing demand for seaweeds extracellular matrix complex sulfated polysaccharides (SPs) and the main biomaterials of economic value are agar and carrageenans, representing a billionaire market of over US$ 6 billion dollars year (Pereira & Costa-Lotufo, 2012). Regarding their chemical classes, Rhodophyceae and Phaephyceae are the most common sources of sulfated galactans and fucoidan or fucan, respectively (Pomin, 2012; Mourão, 2015), while sulfated heteropolysaccharides frequently occur in Chlorophyceae (Pomin & Mourão, 2008; Rodrigues et al., 2013; Arata et al., 2015). Although revealing a high structural diversity among algal species, these marine glycans of large molecular masses (> 100 kDa), with variable sulfation on their chains, different linkages and high degree of branching, which contribute to their heterogeneity and complexity, have high levels of effectiveness for screening assays of anticoagulation of extracts from seaweeds to the identification of novel SPs with potent inhibitory actions of the coagulation (Athukorala, Jung, Vasanthan, & Jeon, 2006; Pomin, 2012; Mourão, 2015). To obtain seaweeds SPs, enzyme-assisted extraction methods allow high yield and enhanced quality on the physical-chemical and biological properties of these molecules (Athukorala et al., 2006), considering the effort to obtain a commercial product on a large scale (Pereira & Costa-Lotufo, 2012).

With the advance of glycomics, a substantial number of structurally diverse SPs from seaweeds displaying anticoagulant action has been extensively described (Shanmugam, Ramavat, Mody, Oza, & Tewari, 2001; Athukorala et al., 2006; Fonseca et al., 2008; Pomin, 2012; Mourão, 2015; Mansour et al., 2017). Their effects are stereospecifics and complexes and do not display only as a consequence of negative charge density and sulfate content, but also of structural composition, with anticoagulant mechanisms distinct from those revealed for heparin (HEP), a commercial drug obtained from pig and bovine tissues primarily used in anticoagulant therapy (e.g., thromboembolic disorders), as well as in extracorporeal circulation and hemodialysis, but known due to its episodes of extensive bleeding and HEP-induced thrombocytopenia. This functional class of glycosaminoglycan has specific pentasaccharide sequence with high antithrombin affinity, which is not present in other SPs-expressing living organisms to achieve the same effect of HEP (Mourão, 2015). Both the activated partial thromboplastin time (APTT) and the prothrombin time (PT) tests are conventionally employed, respectively, for intrinsic and extrinsic coagulation pathway examination. However, these screening assays have limited value to predict risk of circulatory disorder (e.g., bleeding and thrombosis) and, in the last years, thrombin generation (TG)-based coagulation methods have constituted as more representatives of the total clot formation process (Castoldi & Rosing, 2011; Duarte, Ferreira, Rios, Reis, & Carvalho, 2017) to give additional data about plasma alternative anticoagulants (Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014; Rodrigues et al., 2016a; 2017; Mansour et al., 2017; Salles et al., 2017).

The species of Rhodophyceae *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vicent, *Gracilaria birdiae* Plastino & Oliveira, and *Halymenia* sp. J. Agardh; and of Chlorophyceae *Caulerpa cupressoides* var. *lycopodium* C. Agardh and *C. racemosa* (Forsskal) J. Agardh are commonly found along the Northeastern coast of Brazil and studies demonstrated SPs with pharmacological value as anticoagulant (Fidelis et al., 2014; Quinderé et al., 2014; Rodrigues et al., 2011; 2012b; Rodrigues, Quinderé, Queiroz, Coura, & Benevides, 2012c; Rodrigues et al., 2013; 2016b), analgesic/anti-inflammatory (Vanderlei et al., 2011; Rodrigues et al., 2012d; Ribeiro et al., 2014) and antiviral (Vanderlei et al., 2016; Rodrigues et al., 2017) for biotechnology. No examination on the *in vitro* anticoagulant potentials of crude SPs from these species using TG assays has been revealed so far.

The present study was designed to enzymatically extract and compare the physical-chemical properties of anticoagulant crude SPs derived from five Brazilian macroalgae species (*G. birdiae*, *A. muscoides*, *Halymenia* sp., *C. cupressoides* and *C. racemosa*) using APTT and PT-based coagulation

models. In addition, crude SPs were analyzed for their *in vitro* inhibitory effects of TG.

Material and methods

Marine macroalgae samples and analyses of the crude SPs

The Brazilian samples of Rhodophyceae *G. birdiae* Plastino & Oliveira, *A. muscoides* (Linnaeus) Bory de Saint-Vicent and *Halymenia* sp. J. Agardh; and of Chlorophyceae *C. cupressoides* var. *lycopodium* C. Agardh and *C. racemosa* (Forsskal) J. Agardh were collected in September 2011 on seashore from the Flecheiras beach, Trairí, Ceará State, and then they were taken to the Carbohydrates and Lectins Laboratory (CarboLec), *Universidade Federal do Ceará*, in plastic bags. Voucher of each algal species (# 40781, 46093, 56149, 4977 and 52418, respectively) was also deposited in the Herbarium Prisco Bezerra of the Department of Biological Sciences, *Universidade Federal do Ceará*, Brazil. After collection, the algal samples were washed with distilled water and cleaned to remove residues and macroscopic epiphytes, followed by dehydration at room temperature (25±0.05ºC) (Rodrigues et al., 2011; Quinderé et al., 2013; Ribeiro et al., 2014). The experimental analyses of the macroalgae crude SPs were performed at Connective Tissue Laboratory, *Universidade Federal do Rio de Janeiro* (UFRJ), Brazil.

Two grams of each dehydrated algal tissue were cut into small pieces and then incubated with papain (60°C, 24 hours) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM), as previously published elsewhere (Rodrigues et al., 2011; Vanderlei et al., 2011; Rodrigues et al., 2012c; Quinderé et al., 2013). Briefly, the incubation mixture was then filtered through a nylon membrane and the homogenate was saved. The residue was washed with 50 mL distilled water and the SPs in mixture were precipitated with 6.4 mL of 10% cetylpyridinium chloride (CPC) solution. After 24 hours at room temperature, the mixture was centrifuged at 2,560 × *g* at 5°C for 30 min. The SPs in the pellet were washed with 200 mL of 0.05% CPC solution, dissolved with 100 mL of a 2 M NaCl: Ethanol (100: 15, v v^{-1}) mixture and precipitated with 200 mL of absolute ethanol. After 24 hours at 4°C, the precipitate was collected by centrifugation (2,560 × g at 5°C for 30 min), washed twice with 200 mL of 80% ethanol and washed once with 150 mL absolute ethanol. The final precipitate was oven dried at 60°C for 24 hours. After that, the crude SPs extract obtained for each algal species was quantified as a percentage (%) of the dehydrated matter and analyzed for its contents of sulfate (Dogson & Price, 1962), total sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and proteins (Bradford, 1976).

The physical-chemical characterization of complex polysaccharides was performed by agarose (Dietrich & Dietrich, 1976) and polyacrylamide (Rodrigues et al., 2013; Fidelis et al., 2014; Quinderé et al., 2014) electrophoresis procedures using sequential staining with toluidine blue/Stains-All (Volpi & Maccari, 2002) by comparison with the electrophoretic mobility of the standard glycosaminoglycans dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa), chondroitin-6-sulfate (~ 60 kDa), dermatan sulfate and/or heparan sulfate. On a manufacturer basis, all the reagents of these respective protocols were purchased from Sigma-Aldrich or analytical grade.

In vitro anticoagulant assays

APTT and PT tests

Crude SPs were assessed by both the *in vitro* APTT and PT tests, using normal citrated human plasma (10 different donors, University Hospital Clementino Fraga Filho, UFRJ) according to the manufacturers' specifications, for evaluating a possible anti-clotting effect in a coagulometer Amelung KC4A before the *in vitro* TG assays. For the APTT test, a mixture of 100 µL plasma and different concentrations of SPs (0-1 mg mL^{-1}) was incubated with 100 µL APTT reagent (kaolin bovine phospholipid reagent) from Wiener Lab (Rosario, Argentina). After 2 min of incubation at 37°C, 100 µL of 25 mM $CaCl_2$ from Wiener Lab (Rosario, Argentina) was added to the mixtures, and the clotting time was recorded. Regarding the PT assay, a mixture of 100 µL plasma and various concentrations of SPs (0-1 mg mL^{-1}) was incubated at 37°C for 1 min. After that, 100 µL PT reagent from Wiener Lab (Rosario, Argentina) was added to the mixtures, and the clotting time was recorded using the same coagulation equipment. The polysaccharide concentrations and the curve of unfractionated heparin (UHEP) (with 193 international units per mg (IU mg^{-1}) from Europharma Lab, São Paulo, Brazil, on both tests were determined as described by Rodrigues et al. (2011). Tests were performed in triplicate.

TG inhibition assay

This assay was performed in a microplate format, containing: 10 μL APTT reagent (cephalin, contact-activator system) or PT reagent (thromboplastin, 830 μg well-plate^{-1}, factor tissue-activator system) from Wiener Lab (Rosario, Argentina) with 30 μL of 0.02 M Tris HCl/PEG-buffer (pH 7.4), 10 µL SPs (0, 4.1, 8.3, 41.6 or 83.3 μg well-plate^{-1}; UHEP: 2 or

4 μg well-plate^{-1}) and 60 µL a solution containing 20 mM $CaCl_2$ from Wiener Lab (Rosario, Argentina) and 0.33 mM chromogenic substrate S2238 from Chromogenix (Mölndal, Sweden) (10:50 ratio, v v^{-1}). 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 inhibitory response of TG by SPs was determined by peak thrombin (PTh) and time to peak (TPeak) (Rodrigues et al., 2016a). Graphics were processed using Origin Program version 8.0, USA, as the Statistical Analysis Software.

Statistical analysis

Analyses were normalized and performed using a statistical software program (GraphPad Prism version 5.0.1, USA). Values (mean±S.E.M., n = 3) of the classical coagulation tests and the TG parameters were subjected to Analysis of Variance (One or Two-way ANOVA), followed by Tukey's test for unpaired data, at level of $p < 0.05$.

Results and discussion

In this study, samples of five different Brazilian seaweed species [*G. birdiae*, *A. muscoides* and *Halymenia* sp. (Rhodophyta); *C. cupressoides* var. *lycopodium* and *C. racemosa* (Chlorophyta)] collected in Flecheiras beach were digested with papain to obtain crude SPs and compare their respective physical-chemical characteristics by electrophoresis. After incubation, followed by both CPC and ethanol precipitations and drying, the percentage of crude SPs recovered from the dehydrated matter varied among the benthic macroalgae species ($p < 0.05$), as shown in Figure 1A.

The crude SPs yields ranged from 0.66±0.03% in the green seaweed *C. racemosa* to 41.60±1.10% in the red seaweed *Halymenia* sp. (Table 1). Depending on the phylum, yields from 13.57±1.72 to 41.60±1.10% in Rhodophyta species were considered higher compared with those of Chlorophyta species ($p < 0.001$), as a typical biosynthetic profile of these marine organisms (Pomin & Mourão, 2008).

The highest yield in *Halymenia* sp. was consistent with that of Rodrigues et al. (2012b), who previously demonstrated a high level of crude SPs from this species (46% yield); it was also at least 2.77-fold higher than those obtained by aqueous (18%) and alkaline (15%) conditions containing 43-44% sulfate content from the red seaweed *Halymenia durvillei* (Fenoradosoa et al., 2009).

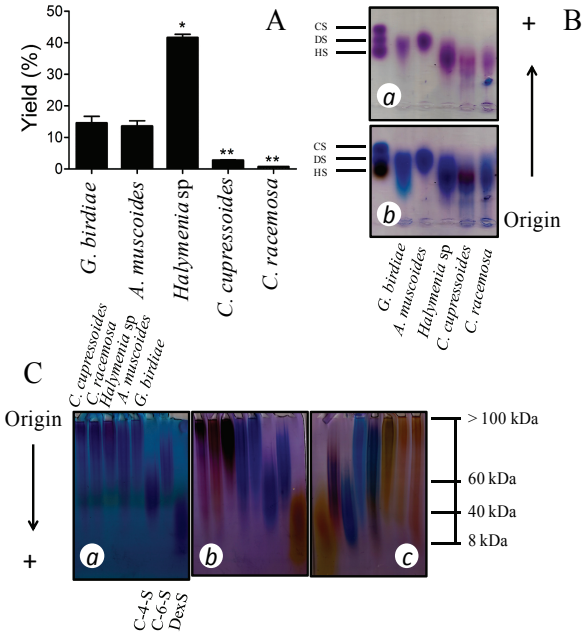

Figure 1. (A) Yield (%, n = 3) of the crude SPs extracts obtained from Brazilian tropical seaweeds collected at Flecheiras beach. *p < 0.05 reveals significant difference between species and **p < 0.001 indicates significant difference from *A. muscoides* (One-way ANOVA, Tukey's test). Agarose (B) or polyacrylamide (C) gel electrophoresis of crude SPs extracts and standards chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa), dextran sulfate (DexS, 8 kDa), dermatan sulfate (DS) and heparan sulfate (HS) present on gels were stained with 0.1% toluidine blue (a) and/or Stains-All (b and c).

Table 1. Chemical composition (%) of crude SPs extracts obtained from the Brazilian tropical seaweeds collected at Flecheiras beach compared with other studies.

Species	Sulfate[a]	Total sugars[b]	Total sugars/ sulfate[c]	Proteins[d]	Reference
Rhodophyta					
G. birdiae	8.41	70.53	8.38	-	This study
	8.38	68.20	8.13	-	Vanderlei et al. (2011)
	-	-	5.10-15.00	0.00-2.90	Fidelis et al. (2014)
A. muscoides	32.54	56.76	1.74	-	This study
	31.80	54.00	1.69	-	Quinderé et al. (2013)
Halymenia sp.	42.60	58.71	1.37	-	This study
H. durvillei	43-44	*	*	*	Fenoradosoa et al. (2009)
Chlorophyta					
Caulerpa cupressoides	20.24	49.71	2.45	-	This study
	22.30	47.23	2.11		Rodrigues et al. (2013)
Caulerpa racemosa	23.00	47.80	2.07	-	This study
	15.17	*	*	-	Ribeiro et al. (2014)

[a]Dosage by Dodgson and Price' method using $NaSO_3$ as standard; [b]Dosage by Dubois et al.' (1956) method using D-galactose as standard; [c]Ratio of hexose and sulfate of each crude SPs extract; [d]Dosage by Bradford' method using bovine serum albumin (- not detected). *Not determined.

However, similar yields ($p > 0.05$) for *G. birdiae* and *A. muscoides* (Rhodophyta) were observed; likewise, for samples of *A. muscoides* collected in the same region by Quinderé et al. (2013), who obtained

11.7% extraction yield (Table 1). *G. birdiae* presented a yield 3.12-fold higher than that of Vanderlei et al. (2011), while varying levels of yields (0.52-8.26%) and total sugars/sulfate ratio (5.10-15.00) were found by Fidelis et al. (2014), applying proteolysis, NaOH, ultrasound or water under different extraction conditions, to obtain crude SPs from *G. birdiae* collected in Rio Grande do Norte State, Brazil.

Crude SPs of *Caulerpa* species (order Briopsidales) yielded between 0.66±0.03 and 2.74±0.13% ($p > 0.05$), although 4.66-fold higher in *C. cupressoides*, but showing both contents of sulfate (20.24-23.00%) and total sugars (47.80-49.71%) similarities between both species (Rodrigues et al., 2011; Ribeiro et al., 2014; Rodrigues et al., 2017). It has been reported that green seaweeds belonging to the order Bryopsidales produce low yields (0.3-33.7%) of SPs (Shanmugam et al., 2001; Arata et al., 2015).

All crude SPs extracts had no proteins, confirming papain digestion of seaweeds to enhance the release of SPs within the cell-wall (Quinderé et al., 2013; Rodrigues et al., 2013; Ribeiro et al., 2014; Rodrigues et al., 2016b), where carbohydrate-protein complexes (proteoglycans) are naturally found (Rodrigues et al., 2011; Fidelis et al., 2014).

Electrophoretic analyses of the crude SPs extracts on agarose or polyacrylamide gel are illustrated in Figure 1B and C, and all algal extracts revealed SPs with toluidine blue staining.

As shown in Figure 1Ba, agarose analysis showed distinct mobility among the seaweeds crude SPs, as well as in polydispersion and metachromasy. Bands corresponding to crude SPs from *G. birdiae* and *A. muscoides* migrated as dermatan sulfate, where that of *A. muscoides* revealed a single band; therefore, a homogeneous crude SPs extract in charge density than that of Quinderé et al. (2013) and that from *G. birdiae* (Vanderlei et al., 2011) which showed polydispersion and relatively weaker metachromasy; while that of *Halymenia* sp. had mobility close to the heparan sulfate, and highly charged (Rodrigues et al., 2012b). *Caulerpa* species showed polydisperse crude SPs extracts with relatively low charge density, as expected (Rodrigues et al. 2012c).

These data confirmed the differences on the physical-chemical aspects among the macroalgae crude SPs (Figure 1 and Table 1), but some revealed contrasting characteristics compared to previously described results for both Rhodophyta *A. muscoides* (Quinderé et al., 2013) and *Halymenia* sp. (Rodrigues et al., 2012b), which showed essentially heterogeneous crude SPs by agarose analysis. As the diamine interacted with the algae crude SPs through their sulfated groups, distinct crude SPs were suggested with basis on their differences in charge/mass ratio (Table 1) and structural conformation, as showed by respective electrophoretic profiles (Figure 1Ba) (Dietrich & Dietrich, 1976).

Polyacrylamide analysis revealed a wide dispersion in their molecular masses (> 100 kDa), typical for SPs from seaweeds (Pomin, 2012; Fidelis et al., 2014; Mourão, 2015), except *C. cupressoides* that had low molecular weights SPs (from 8 to > 100 kDa), as observed in Figure 1Ca; likewise, for a fraction isolated from this same algal species by Rodrigues et al. (2013).

Our conflicting observations are justified by the influence of climatic conditions on biosynthesis of other SPs, life cycle of the algae, and/or use of various protocols for obtaining these compounds (Pereira & Costa-Lotufo, 2012; Rodrigues et al., 2016b; 2017). As presented in Figure 1Ab, sequential staining with toluidine blue and Stains-All led to a improved sensitivity of detection not only SPs, but also non SPs, speculating the presence of carboxylated groups in their chemical structures, as also demonstrated for glycosaminoglycans (Volpi & Maccari, 2002; Salles et al., 2017).

Using the polyacrylamide technique, low molecular masses SPs (*C. cupressoides*) were also detected (Figure 1Cb and Cc) (Rodrigues et al., 2013), although with low amounts of polysaccharide species in complex mixtures (Volpi & Maccari, 2002). Taking with literature data, previous studies revealed acid polysaccharides in *G. birdiae* (Fidelis et al., 2014) and in *C. cupressoides* (Rodrigues et al., 2013; 2017); and pyruvate in *H. durvillei* (Fenoradosoa et al., 2009) and in *A. muscoides* (Quinderé et al., 2014; Rodrigues et al., 2016b). Our combined findings led to a suggestion that Stains-All could be useful to detect other structural components in the algae extracellular matrix polysaccharides, as well as in analysis of purity of these complex glycans preparations (Volpi & Maccari, 2002; Rodrigues et al., 2017; Salles et al., 2017).

Evaluation of *in vitro* anticoagulant effect of the macroalgae crude SPs using APTT, PT and TG assays

Samples of each macroalgae crude SPs extract were further assessed for their *in vitro* anticoagulant effects by APTT and PT tests compared with that of 193 IU mg^{-1} standard UHEP before the *in vitro* TG assays. As shown in Figure 2, the APTT coagulation test revealed intrinsic pathway inhibition by algae

crude SPs extracts ($p < 0.05$), except for *G. birdiae* crude SPs extract (32.95±0.31 s, $p > 0.05$) that at a concentration of 1 mg mL^{-1} did not modify the normal APTT values (33.5±0.08 s).

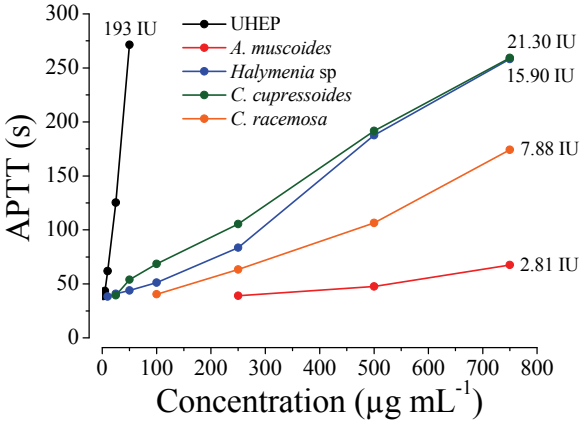

Figure 2. *In vitro* anticoagulant effect by APTT assay of the crude SPs extracts, obtained by papain digestion, from Brazilian tropical seaweeds.

The most extended APTT effects had positively related to the sulfation (Table 1) and were of the order of 21.30 (*C. cupressoides*) and 15.90 IU mg^{-1} (*Halymenia* sp.) ($p > 0.05$), but 9- to 12-fold lower than that of UHEP, which had a marked increment on intrinsic system already at low concentrations. Using these same macroalgae species, Rodrigues et al. (2011; 2012b) recorded effects of 24.62 and 72.66 IU mg^{-1} from major polysaccharidic fractions, respectively. The tested samples of *A. muscoides* and *C. racemosa* showed modest inhibitory effects corresponding to 2.81 and 7.88 IU mg^{-1}, respectively, as described by Quinderé et al. (2014) and Rodrigues et al. (2016b; 2012c), who previously reported reduced anticoagulation induced by their SPs extracted with papain. The serpin-dependent or independent anticoagulation of both *C. cupressoides* (Rodrigues et al., 2013) and *A. muscoides* (Quinderé et al., 2014) SPs was previously demonstrated in purified systems. According to Fonseca et al. (2008) and Mourão (2015), slight differences in the proportion and/or distribution of sulfated residues in polysaccharide chains could be critical for the interaction of proteases, inhibitors and activators of the coagulation system, resulting in a distinct pattern of anti- and pro-coagulant effects and in antithrombotic action. Regarding the PT test, treatment of the human plasma with crude SPs extracts (1 mg mL^{-1}) did not modify the normal values in the extrinsic coagulation pathway (data not shown - Rodrigues et al., 2011).

An intriguing observation of our study was the lack of *in vitro* anticoagulant effect by APTT assay of the crude SPs extract from *G. birdiae* based on a more recently published report by Fidelis et al. (2014), who detected with the same standard method anti-clotting effect of different crude SPs from this species, when obtained by various experimental conditions. The inhibitory response by crude SPs decreased in parallel with their molecular sizes and a molecular mass of over 45 kDa was required to display anticoagulation. In our case, the length of the sugar chain (> 100 kDa) was not affected by protease digestion (Figure 1C - Pomin, 2012; Mourão, 2015).

Additionally, a high solubility of the crude SPs preparations from the seaweeds in water was obtained (Athukorala et al., 2006) because the gel formation in solution is greatly dependent on the amount of 3,6-anhydrogalactose and sulfation, as well as associated cations with carbohydrates, as calcium (Fenoradosoa et al., 2009; Paula et al., 2015). As the addition of calcium chloride to display the APTT had no influence on the evaluation by *in vitro* testing (Figure 2), limited values of detection by *in vitro* APTT test in human plasma treated with *G. birdiae* crude SPs extract could be speculated because this classical test measures only 5% of thrombin formed in plasma, and the production of thrombin and fibrin could be still occurring *in vitro* (Castoldi & Rosing, 2011; Duarte et al., 2017).

Although no considering as an automated method (Duarte et al., 2017), our alternative technique have shown able to evaluate TG *in vitro* (Rodrigues et al., 2016a; Salles et al., 2017).

The accuracy of the algae crude SPs on a TG system in 60-fold diluted human plasma triggered at 37°C for 60 min, using in parallel the HEP as a function of its inhibitory reference, was further examined in this study. Differential inhibition patterns in correspondence to the TG parameters (PTh and TPeak) were observed under the conditions used (Figure 3 and Table 2).

The respective crude SPs extracts added to plasma modified the total amount of thrombin formed and, in the presence of the crude SPs extract from *A. muscoides*, it was totally abolished on the range of concentrations used (4.1-83.3 μg plate-well^{-1}, 100% inhibition, $p < 0.05$) vs. others classes of algal polyglycans and at almost the same level of concentration than UHEP in the contact-activated pathway (Figure 3A and Table 2), which has an antithrombin-specific pentasaccharide sequence not found in other SPs-rich sources (Mourão, 2015). The inhibition of the extrinsic pathway (63.68% PTh inhibition) by its crude SPs extract required a concentration of at least 4.1 μg plate-well^{-1} since the

Δ_{abs} of control was 2-fold higher than that from intrinsic system (Figure 3A and D) and UHEP added to plasma abolished TG at same concentration in this assay (Figure 3D).

The species *Halymenia* sp. (Figure 3B and E) and *C. racemosa* (Figure 3C and F) had crude SPs extracts that acted in different dose-response curves on both cephalin-induced and thromboplastin-induced systems, with ranges for TPeak and PTh inhibition from 32 to 45 min and from 51.88 to 81.58%, respectively (Table 2), based on maximum Δ_{abs} of active thrombin generated (Figure 3B and E, 3C and F) (Mourão et al., 2001). By contrast, lower inhibitory effects of TG in plasma in the presence of both crude SPs extracts from *G. birdiae* and *C. cupressoides* were recorded ($p > 0.05$) (Table 2).

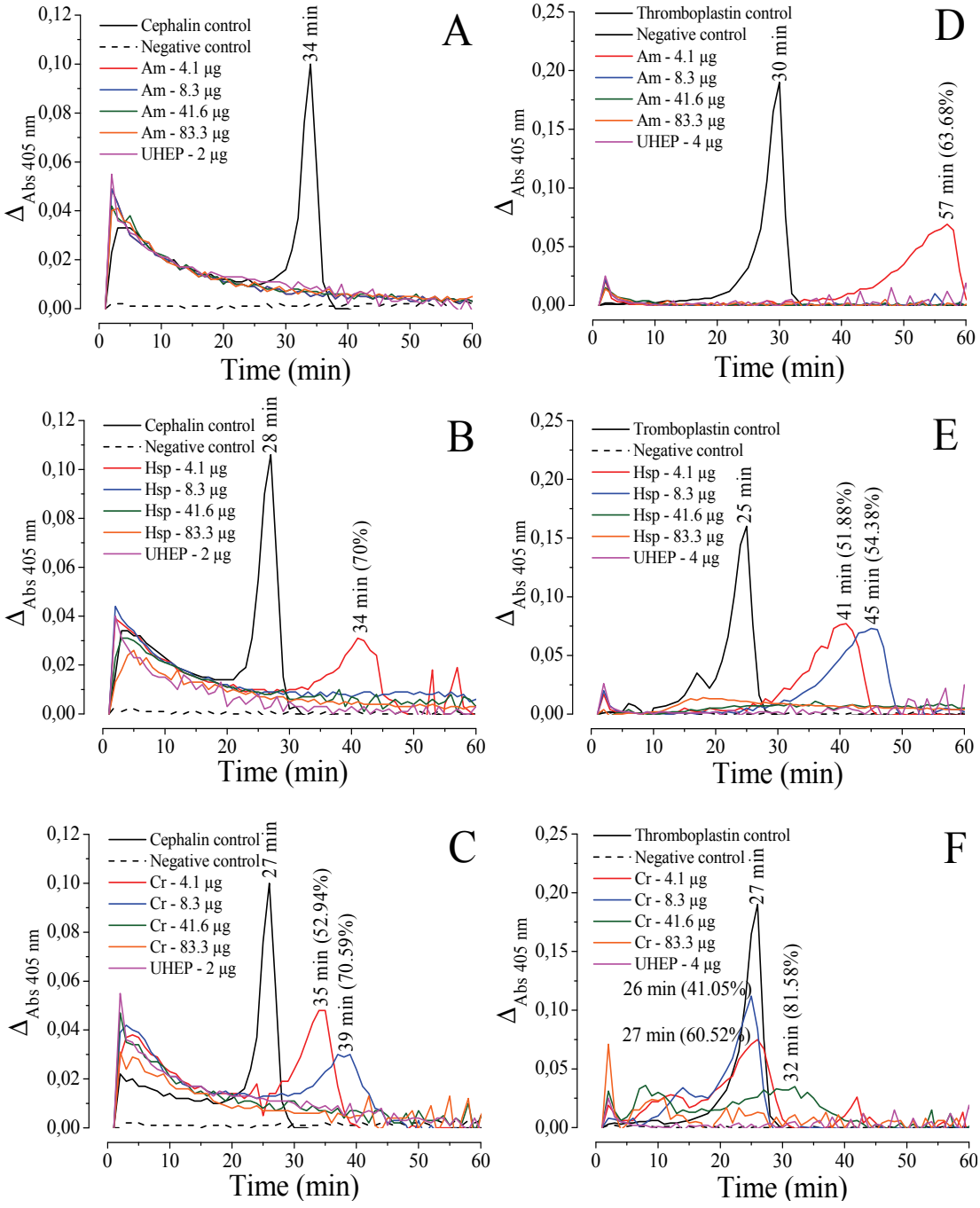

Figure 3. Effect of different concentrations of crude SPs extracts from the Rhodophyta *A. muscoides* (Am) (A and D) and *Halymenia* sp. (Hsp) (B and E); and from the Chlorophyta *C. racemosa* (Cr) (C and F) on cephalin-activated or thromboplastin-activated TG systems in 60-fold diluted human plasma using chromogenic method by a continuous detection system (405 nm, at 37°C for 60 min).

Table 2. TG parameters in diluted human plasma (peak thrombin [% PTh]) and time to peak (TPeak. min) in the presence of different concentrations of crude SPs from the Brazilian tropical seaweeds compared to UHEP.

Glycans (μg)	Am		Gb		Hsp		Cc		Cr	
	Intrinsic (Int.) and Extrinsic (Ext.) coagulation pathways									
	TPeak. min (% PTh)									
	(Int.)	(Ext.)	(Int.)	(Ext.)	(Int.)	(Ext.)	(Int.)	(Ext.)	(Int.)	(Ext.)
4.1	0±0.0 (100±0.0)^{a}	57±0.4 (63.68±0.7)*	31±0.5 (18±0.5)^{b}	28±0.5 (15±0.6)**	34±0.5 (70±0.8)^{c}	41±0.2 (51.88±0.4)*	32±0.6 (12±0.5)^{b}	28±0.5 (16±0.7)**	35±0.5 (52.94±0.3)^{d}	26±0.5 (41.05±0.6)***
8.3	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*	33±0.6 (20±0.2)^{b}	31±0.2 (19±0.7)**	0±0.0 (100±0.0)^{a}	45±0.8 (54.38±0.7)***	32±0.2 (13±0.1)^{b}	30±0.3 (18±0.9)**	39±0.3 (70.59±0.8)^{c}	27±0.2 (60.52±0.6)***
41.6	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*	36±0.3 (27±0.8)^{b}	33±0.4 (22±0.9)**	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*	33±0.5 (15±0.8)^{b}	31±0.6 (22±0.7)**	0±0.0 (100±0.0)^{a}	32±0.4 (81.58±0.8)***
83.3	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*	38±0.0 (32±0.6)^{b}	37±0.3 (25±0.7)**	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*	35±0.2 (21±0.6)^{c}	33±0.5 (22.1±0.2)**	0±0.0 (100±0.0)^{a}	0±0.0 (100±0.0)*

Am - *A. muscoides*; Gb – *G. birdiae*; Hsp - *Halymenia* sp.; Cc - *C. cupressoides*; Cr - *C. racemosa*. Letters or symbols at each polysaccharide concentration indicate difference between species ($p < 0.05$) (Two-way ANOVA). UHEP abolished intrinsic/extrinsic coagulation pathways-induced TG at 2 and 4 μg plate-well^{-1}, respectively.

The reduced TG in system reflected the strength of anticoagulation independently from molecular mass and sulfate content of the diverse classes of seaweeds SPs (Figure 1C and Table 1) (Salles et al., 2017), except *G. birdiae* and *C. cupressoides* that had the lowest sulfate contents among all macroalgal species (Table 1). Indeed, the complex effects of the crude SPs extracts on TG varied with macroalgal species and stimuli *in vitro* (Nishino et al., 1999; Glauser et al., 2009; Zhang et al., 2014), possibly due to their different anticoagulant mechanisms (Rodrigues et al., 2013; Quinderé et al., 2014). Recently, Mansour et al. (2017) examined a fucosylated chondroitin sulfate from the sea cucumber *Holothuria polii* body wall on TG using calibrated automated thrombography. This glycan manifested a dual effect, with a procoagulant tendency for low doses and an anticoagulant action when at higher doses.

Elevated TG is associated with the risk of venous thrombosis (Castoldi & Rosing, 2011; Duarte et al., 2017) and thrombin interplays between coagulation and inflammation (Pomin, 2012). The TG method led us to a more precise analysis of macroalgae anticoagulants than traditional methods (Castoldi & Rosing, 2011; Duarte et al., 2017). The diverse classes of SPs prevented the human plasma coagulability induced by both cephalin and thromboplastin and TG parameters would have potential to postulate their mechanisms underlying *in vitro*, due to the lack of structure-function relationship data of these compounds, as a biotechnological perspective (Nishino et al., 1999; Mourão et al., 2001; Glauser et al., 2009; Zhang et al., 2014). Furthermore, our data could be useful to analyze the molecular mechanisms behind the anti-inflammatory actions of macroalgae SPs, which involve multiple points during the leukocyte recruitment into inflamed tissues in order to regulate the inflammatory process (Vanderlei et al., 2011; Rodrigues et al., 2012d; Quinderé et al., 2013; Ribeiro et al., 2014) because an increase of TG is also associated to exposure of blood to tissue factor in the subendothelium (Rau, Beulieu, Huntington, & Church, 2007). These hypotheses combined in a more critical study deserve to be investigated for a mechanistic characterization of the polymers.

Conclusion

Availability of crude sulfated polysaccharides from five Brazilian tropical seaweed species shows Rhodophyta (*Gracilaria birdiae*, *Acanthophora muscoides* and *Halymenia* sp.) to contribute more than Chlorophyta (*Caulerpa cupressoides* and *C. racemosa*). The combined technique of agarose/polyacrylamide gel electrophoresis with sequential toluidine blue/Stains-all staining partially characterizes distinct sulfated polysaccharides and non-sulfated polyglycans presenting large molecular masses. The model of thrombin generation exhibits accurately anti-clotting effects by algae crude SPs than classical assays. Actions occur dependently of concentration regardless of molecular size on both intrinsic/extrinsic coagulation pathways, differentially preventing thrombosis in diluted human plasma, but with lower potential than heparin.

Acknowledgements

This study was funded by Brazilian funding agencies (Capes-PNPD, Funcap, Faperj, CNPq, MS and MCTI). Benevides, N. M. B and Mourão, P. A. S. are senior investigators of CNPq/Brazil.

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Received on December 05, 2016.
Accepted on June 09, 2017.

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