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Inhibition of coagulation proteases and thrombosis and sub-chronic toxicological study of a sulfated polysaccharidic fraction from the red alga *Gelidiella acerosa*

Ismael Nilo Lino de Queiroz[1], José Ariévilo Gurgel Rodrigues[1], Ana Luíza Gomes Quinderé[2], Márjory Lima Holanda[2], Maria Gonçalves Pereira[3] and Norma Maria Barros Benevides[2*]

*[1]Laboratório de Tecido Conjuntivo, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro, Rio de Janeiro, Brazil. [2]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. Mister Hull, s/n, 60455-970, Fortaleza, Ceará, Brazil. [3]Faculdade de Educação Ciências e Letras do Sertão Central, Universidade Estadual do Ceará, Quixadá, Ceará, Brazil. *Author for correspondence. E-mail: nmbb@ufc.br*

ABSTRACT. Metabolites isolated from *Gelidiella* species (Rhodophyta) have been few studied. We evaluated a sulfated polysaccharidic fraction from *G. acerosa* collected from two Brazilian beaches on the northwestern coast of Brazil (Flecheiras-F and Pedra Rachada-PR) on coagulation proteases and thrombosis. Their toxicity *in vivo* was also assessed. Enzymatic extractions yielded 1.40%, and similar chromatographic profiles (DEAE-cellulose) were obtained, with fractions (Ga-I→V) containing differences among the relative proportions of sulfate (5-42%), and revealing charge density patterns by electrophoresis. Ga-IV-PR had a discrete effect (3.01 IU mg^{-1}) on normal human coagulation compared with heparin (193 IU mg^{-1}) and was tested on coagulation proteases (thrombin and factor Xa) in the presence of antithrombin and in a model of venous thrombosis in rats using thromboplastin as the thrombogenic stimulus. The systems were inhibited; but at higher doses (>1.0 mg kg^{-1}), this fraction reverted the antithrombotic effect. Regarding the toxicological study, consecutive Ga-IV (9 mg kg^{-1}) for 14 days did not cause mortality in mice, but some biochemical and hematological parameters were discretely altered. Histopathological analysis revealed that increased liver and spleen sizes had no toxicological significance. Therefore, *G. acerosa* does not biochemically change its matrix polysaccharide composition and proved to be safe antithrombotic agent.

Keywords: Rhodophyta, polysulfated, *Gelidiella acerosa*, antithrombotic agent, bioactivities, *in vivo* toxicity.

Inibição de proteases da coagulação e trombose e estudo toxicológico subcrônico de uma fração polissacarídica sulfatada da alga vermelha *Gelidiella acerosa*

RESUMO. Poucos estudos mostram metabólitos isolados de rodofíceas de espécies *Gelidiella*. Avaliou-se uma fração polissacarídica sulfatada de *G. acerosa* coletada a partir de duas praias brasileiras do Nordeste do Brasil (Flecheiras-F e Pedra Rachada-PR) sobre proteases da coagulação e trombose, e em ensaio de toxicidade *in vivo*. Extrações enzimáticas renderam 1,40% e foram obtidos perfis cromatográficos semelhantes (DEAE-celulose), apresentando frações (Ga-I→V), contendo diferenças entre as proporções relativas de sulfato (5-42%), além de a eletroforese revelar diferenças na densidade de carga. A Ga-IV-PR apresentou discreto efeito (3,01 UI mg^{-1}) sobre a coagulação humana normal comparada à heparina (193 UI mg^{-1}) e foi testada sobre proteases da coagulação (trombina e fator Xa) na presença de antitrombina e em um modelo de trombose venosa em ratos usando tromboplastina com estímulo trombogênico, sendo inibidos esses sistemas. Entretanto, em elevadas doses (>1,0 mg kg^{-1}) o efeito antitrombótico foi revertido. No estudo toxicológico, Ga-IV (9 mg kg^{-1}) consecutiva durante 14 dias não causou mortalidade em camundongos, mas alterou discretamente alguns parâmetros bioquímicos e hematológicos. O aumento nos tamanhos do fígado e baço não apresentou significância toxicológica, segunda análise histopatológica. Portanto, *G. acerosa* não muda bioquimicamente a composição de polissacarídeo de sua matriz e detém agente antitrombótico seguro.

Palavras-chave: Rhodophyta, polissulfatados, *Gelidiella acerosa,* agente antitrombótico, bioatividades, toxicidade *in vivo*.

Introduction

Seaweeds (marine macroalgae) comprise a group of autotrophic organisms occurring in diverse marine ecosystems and are classified into three different phyla (Chlorophyta, Rhodophyta and Ochrophyta) (SIMPSON; ROGER, 2004). They have a wide variety of metabolites (e.g., carbohydrates, proteins, lipids and

micromolecules) for use in different fields, including chemical ecology (CARDOZO et al., 2007), bioproducts (CAMPO et al., 2009) and chemotaxonomy (RODRIGUES et al., 2012a), due to their broad spectrum of physical-chemical and physiological properties for environmental, human and animal uses (CARDOZO et al., 2007; PEREIRA et al., 2005; PEREIRA; LOTUFO, 2012; YANG et al., 2006).

In the last decades, the exploration of hydrocolloids (mainly agar and carrageenan) from seaweeds has established a worldwide multi-billion-dollar market (SMIT, 2004). Considerable interest by industries emerged to identify novel economically impacting agents for a wide variety of applications, mainly in foods, cosmeceuticals and pharmaceutics due to their gelling, stabilizing and emulsifying properties (CAMPO et al., 2009; CARDOZO et al., 2007; PRAJAPATI et al., 2014; THOMAS; KIM, 2013). Sulfated polysaccharides (SPs) are the main classes of structural components of seaweeds cell walls with physiological roles (KLOAREG; QUATRANO, 1988). Although polydispersity and heterogeneity contribute to their complexity, they have attracted the attention in glycomics (POMIN, 2012) by modulating various biological systems, such as thrombosis (FONSECA et al., 2008; RODRIGUES et al., 2011), coagulation (AMORIM et al., 2011; POMIN, 2012) and inflammation (QUINDERÉ et al., 2013; RODRIGUES et al., 2012b, 2014). However, their structural compositions depend on algae species, harvest time, location and extraction protocols (ARAÚJO et al., 2012; FARIAS et al., 2000). Sulfated galactans (Rhodophyta), fucoidan or fucan (Phaeophyta) and arabinogalactan (Chlorophyta) are the most common SPs classes in seaweeds (POMIN, 2012). Sea grasses (AQUINO et al., 2005), microalgae (CARDOZO et al., 2007) and animals (POMIN, 2012) have SPs. More recently, Dantas-Santos et al. (2012) and Chang et al. (2013) also detected SPs in freshwater plants and in the edible mushroom *Armillaria mellea* (Vahl:Fr.) Kummer, respectively.

SPs from seaweed have been widely described due to their effects on the haemostatic system. Their effects comprise 1) inhibition of the intrinsic and/or extrinsic pathways (RODRIGUES et al., 2009, 2011); 2) a catalytic effect rather than the formation of blood serpin-protease complexes (AMORIM et al., 2011; PEREIRA et al., 2005; RODRIGUES et al., 2013); 3) exhibition of antithrombotic effects (RODRIGUES et al., 2011; XIE et al., 2011) by stimulating the synthesis of antithrombotic heparan sulfate by endothelial cells (ROCHA et al., 2005); and 4) independent effect of serpin (QUINDERÉ et al., 2014). When tested in animal models for thrombosis, they also produce prothrombotic effects at high concentrations (FONSECA et al., 2008; QUINDERÉ et al., 2014; RODRIGUES et al., 2011). In addition, SPs from seaweeds have proved to be safe when their toxicity *in vivo* was evaluated and thus revealed their human and animal health-related importance (QUINDERÉ et al., 2013; RODRIGUES et al., 2012b; SIQUEIRA et al., 2011).

Heparin (HEP), one of the few underscored SPs drugs worldwide, consists of 1,4-linked residues of uronic acid and D-glucosamine (glycosaminoglycans family). It is widely employed in anticoagulant therapy (e.g., vascular surgery and hemodialysis) and is commercially obtained from pig and bovine intestine at low concentrations. On the other hand, its prolonged administration may produce several adverse consequences, including the monitoring of frequent activated partial thromboplastin time (APTT), the inability to inhibit thrombin bound to the clot, hemorrhage, anaphylaxis and the occurrence of thrombocytopenia in some patients. Possibility of prions and viruses may also occur. Systemically, its mechanism of action is mainly through the potentiation of antithrombin (AT), a serine protease inhibitor (serpin) which modulates the coagulation system, especially thrombin (factor IIa) and factor Xa (NADER et al., 2001; QUINSEY et al., 2004).

Species of the family Gelidiaceae (Rhodophyta, Florideophyceae) are usually found in tropical and subtropical waters of Brazil and others countries. Their spatial and temporal distribution has been correlated with seasonal changes (SANTELICES, 1978) for raw material supply (PRASAD et al., 2006) in the agar industry worldwide. This SP may also be found in Gracilariaceae, Phyllophoraceae and Ceramiaceae, and may be extracted by different protocols (ARMISEN; GALATAS, 1987; CARDOZO et al., 2007). In the case of the edible species *Gelidiella acerosa* (Forsskal) Feldman and Hammel, studies have been performed on its ecology (SANTELICES, 1978), physiological aspects, physical gel properties or molecular variations (ROLEDA et al., 1997), cultivation (GANESAN et al., 2011), antioxidant capacity and metal chelating potential (SUGANTHY et al., 2013). No study has described the biological properties of SPs from the *Gelidiella* species.

Current analysis isolated and compared the physical and chemical characteristics; it also assays the *in vitro* anticoagulant effect of SPs from *G. acerosa* collected from two localities (Flecheiras and Pedra Rachada beaches, Trairí-Paracuru, Ceará, Northeastern Region of Brazil). Further, a polysaccharidic fraction from samples of this same algal species from Pedra Rachada beach has been evaluated for its *in vitro* effects on coagulation proteases and *in vivo* using a rat venous thrombosis model. A toxicological assessment was also carried out on mice.

Material and methods

Marine alga, isolation of SPs and physico-chemical analyses

Samples of *G. acerosa* were collected on the seashore from two beaches (Flecheiras and Pedra Rachada, respectively) on the Northwestern coast of Brazil. The algae were cleaned from epiphytes and foreign organisms, washed with distilled water and stored at -20°C until use. A voucher specimen (n. 046094) was classified and archived by Ana Cecília Fortes Xavier at the Prisco Bezerra Herbarium (EAC), Federal University of Ceará, Fortaleza, Ceará State, Brazil.

For crude SPs extraction, five grams of dehydrated algal tissue were subjected to papain digestion (6 hours, 60°C) in 100 mM sodium acetate buffer (pH 5.0) containing cystein and EDTA (both 5 mM) (FARIAS et al., 2000), with some modifications based on Rodrigues et al. (2011). Lyophilized crude SPs extracts (56 mg) were then dissolved in 25 mL of 50 mM sodium acetate buffer (pH 5.0) and applied to DEAE-cellulose column (2 × 30 cm) equilibrated with the same solution. The column was developed using a stepwise gradient of 0 to 2 M NaCl at 0.25 M intervals in the same solution. The flow rate of the column was 5.4 mL $min.^{-1}$ and fractions were collected and analyzed for SPs using the metachromatic assay containing dimethylmethylene blue with an Amersham Biosciences Utrospec 1100 spectrophotometer at 525 nm (FARNDALE et al., 1986). The metachromatic fractions were then dialyzed and freeze-dried. The biological protocols were performed with a polysaccharide fraction from this algal species collected from Pedra Rachada that showed to have a better *in vitro* anticoagulant effect (Ga-IV).

Total sugars (TSs) content was estimated by phenol-sulfuric acid analysis using D-galactose as standard (DUBOIS et al., 1956). After acid hydrolysis of the soluble polysaccharides (1 mL of HCl for 5 hours at 100°C), the sulfate (S) content was measured by the $BaCl_2$ $gelatin^{-1}$ method (DOGSON; PRICE, 1962). The content of contaminant proteins (CPs) was measured by Bradford's method (BRADFORD, 1976) with bovine serum albumin as reference. The degree of polydispersion, the charge density of crude SPs and their fractions were checked by agarose gel electrophoresis as previously described (DIETRICH; DIETRICH, 1976).

In vitro anticoagulant assays

For tests, normal citrated human blood was obtained from different donors at the Hematology and Hemotherapy Center of Ceará (Hemoce) and the Activated Partial Thromboplastin Time (APTT) was measured according to manufacturer´s specifications. Clotting time was recorded in a coagulometer (Drake Quick Timer). Heparin with 193 international units per mg (IU mg^{-1}) of polysaccharide was used as standard. The assays for inhibition of thrombin or factor Xa by antithrombin (AT) with the Ga-IV fraction were performed as previously described by Fonseca et al. (2008) and Rodrigues et al. (2013) using incubations in 96-well plates.

Animals

For the *in vivo* experimental assays, a total of 40 Wister rats and 12 male Swiss mice were used. All procedures and animal treatments were performed according to guidelines for Institutional Animal Care and Use of the Federal University of Ceará, Ceara State, Brazil, previously approved by 80/10 protocol.

Effect on venous thrombosis

Antithrombotic effect was investigated in rats with rabbit brain thromboplastin as thrombogenic stimulus (FONSECA et al., 2008; RODRIGUES et al., 2011). Rats (200-250 g) (both sexes, 5 animals per dose) were anesthetized with an intramuscular injection of 100 mg kg^{-1} body weight of ketamine and 16 mg kg^{-1} body weight of xylazine. Each animal had the right vena cava exposed and dissected. Different doses of SPs fraction or heparin (fourth International Standard (85/502), National Institute for Biological Standards and Control, Potters Bar, UK) were intravenously infused and allowed to circulate for 5 min. Further, the inferior vena cava

was isolated and brain thromboplastin (5 mg kg^{-1} body weight) from Biolab-Merieux AS (Rio de Janeiro State, Brazil) was slowly injected intravenously; after 1 min., 0.7 cm of isolated vena cava was clamped off using distal and proximal sutures. The formed thrombus inside the occluded segment was pulled out after 20 min. stasis, and then washed with 0.15 M saline solution, dried (1 hour, 60°C) and weighted. Mean thrombus weight was obtained by the average weight from each group and expressed as weight percentage, with 100% representing complete inhibition of thrombosis formation (presence of Ga-IV). Data were expressed as mean ± standard error of mean (S.E.M.).

In vivo sub-chronic toxicological assessment

The assay was performed with male mice (20-26 g) for 14 consecutive days. Animals were previously weighed before daily Ga-IV (Pedra Rechada) administration. Ga-IV (9 mg kg^{-1}) was dissolved in 0.9% saline solution and then intraperitoneally administered (10 mL 10 g^{-1}, i.p.) in mice (6 animals $group^{-1}$). Control group received only saline solution (i.p.). Mice had free access to water and food during the experimental period. Clinical signs (body mass variation, survival rate, mucosa, eyes, hair erection, scratch or licking paws, freezing reactions, general behavior, among others) and biochemical (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate, creatinine and urea) and hematological (red blood cells count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), white blood cell count (WBC) and differential WBC (leukocytes, monocytes and neutrophils) parameters were measured, as previously described by Quinderé et al. (2013).

Statistical analyses

In the venous thrombosis assay, the one-way analysis of variance (ANOVA) was used to determine the differences among the groups, followed by 'post hoc' Tukey's test. Differences were considered statistically significant at $p < 0.01$ or 0.001. In the case of toxicological examination, data were given as mean ± standard error (SEM) of six animals per group. Analysis of variance (ANOVA) was performed, followed by Student's 't' test for unpaired rates, with $p < 0.05$ as statistically significant.

Results and discussion

The red seaweed *G. acerosa* SPs were isolated and compared to evaluate their abundance and their physical and chemical characteristics. The lyophilized crude SPs yields 6 hours after papain digestion (60°C) were, respectively, 1.43 and 1.41% for samples of the algal species collected from the two beaches Flecheiras and Pedra Rachada. In addition, quantitative analyses of sulfate (10.81 and 11.63%) and total sugars (87 and 80%), respectively, were almost equal among them from the two areas under analysis. Proteins were also removed during extraction process (ARAÚJO et al., 2012; QUINDERÉ et al., 2013; RODRIGUES et al., 2011, 2014).

There are very few studies concerning the biochemical aspects of phycocolloids from seaweeds, especially for SPs from *Gelidiella* species. The ecological aspects (COSTA et al., 2012; SANTELICES, 1978) and cultivation (GANESAN et al., 2011) of algae from this genus have been generally explored. Employing pre-treatment with acetic acid followed by pressure extraction, Roleda et al. (1997) showed crude SPs yields varying between 17.9 and 39.9%, with relatively low sulfate content (1.6-2.2%) from different life stages (vegetative and tetrasporic) of *G. acerosa* collected from two localities on the coast of the Philippines. Samples of *G. acerosa* collected from Gujarat coast, India, on different seasons, revealed rates of crude SPs yields ranging between 8.5 and 40.4% (PRASAD et al., 2006). According to Pereira et al. (2005), applying the same protocol of current study to obtain *Gelidium crinale* (Hare ex-Turner) Gaillon (Rhodophyta) crude SPs, resulted 2.60%. Herein, sulfate content values were in accordance with those found for *Gracilaria* species (4-10%), the raw material supplies for the world's agar industry (ARMISEN; GALATAS, 1987; CARDOZO et al., 2007). However, a more detailed analysis of *G. acerosa* SPs is needed which would identify new potentially active SPs (RODRIGUES et al., 2009; SIQUEIRA et al., 2011).

SPs from *G. acerosa* present similar physical and chemical characteristics

A sample of each crude SPs extract from the red seaweed *G. acerosa* collected from two areas (Flecheiras and Pedra Rachada) was submitted to ion-exchange chromatography on DEAE-cellulose column, as shown in Figure 1, and similar profiles were observed.

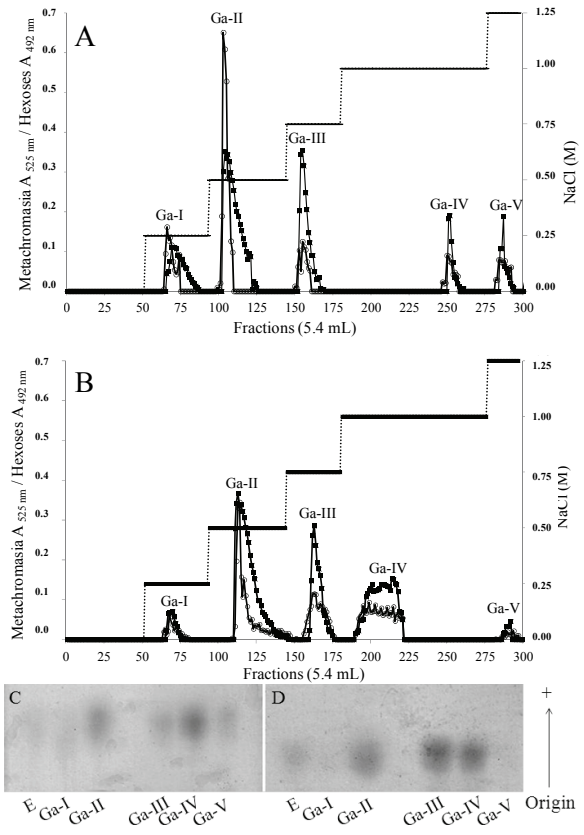

Figure 1. Separation of SPs (Flecheiras-A and Pedra Rachada-B beaches) from the red seaweed *Gelidiella acerosa* by DEAE-cellulose. Fractions were collected and checked by metachromasia using 1,9-dimethylmethylene blue (■—■) and phenol-H_2SO_4 (○—○). (-) NaCl concentration. Agarose gel electrophoresis (Flecheiras-C and Pedra Rachada-D beaches) of SPs from *G. acerosa*. Extracts (E) and fractions (Ga-I→V) present on gel were stained with 0.1% toluidine blue.

Five different SPs fractions (Ga-I, Ga-II, Ga-III, Ga-IV and Ga-V) were, respectively, eluted with 0.25, 0.50, 0.75, 1.00, and 1.25 M of NaCl. Ga-II (Flecheiras beach), eluted with 0.75 M of NaCl, also had the highest dosage of total sugars (hexoses) when compared with its metachromatic property (Figure 1A) and fraction Ga-II of *G. acerosa* collected on the Pedra Rachada beach (Figure 1B). According to Dubois et al.'s method, all other fractions, when obtained by DEAE-cellulose, showed results with a relatively low carbohydrate dosage. In fact, results suggest that neutral sugars could be capable of interacting with the DEAE-cellulose column, according to Araújo et al. (2012). The employment of DEAE-cellulose as a important matrix for seaweeds SPs separation has also been reported, revealing characteristics among different species, such as *Gelidium crinale* (PEREIRA et al., 2005) and *Halymenia pseudofloresia* F. S .Collins and M. A. Howe (RODRIGUES et al., 2009), *Lobophora variegata* (J. V. Lamouroux) Womersley ex E. C. Oliveira (SIQUEIRA et al., 2011) and *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vincent (QUINDERÉ et al., 2013).

The relative proportions of total sugars and sulfate varied among the fractions. Ga-I, Ga-II and Ga-IV had higher SPs yields and total sugars and sulfate contents for *G. acerosa* from both regions (Table 1). Values represented the heterogeneity of these compounds in this algal species (PEREIRA et al., 2005; QUINDERÉ et al., 2013; RODRIGUES et al., 2011).

Table 1. Yield of SPs fractions obtained by DEAE-cellulose from the red seaweed *Gelidiella acerosa* collected in the two localities (Flecheiras and Pedra Rachada beaches), Brazil.

Beaches	Fractions	Yield[a] (%)	Chemical composition		
			Total sugars[b] (%)	Sulfate[c] (%)	Proteins[d] (%)
Flecheiras	Ga-FI	9.80	9.16	7.74	-
	Ga-FII	27.45	23.39	42.18	-
	Ga-FIII	8.56	11.04	7.61	-
	Ga-FIV	8.91	3.34	7.81	-
	Ga-FV	3.39	1.80	5.44	-
Pedra Rachada	Ga-FI	15.69	5.69	11.23	-
	Ga-FII	41.53	17.09	40.56	-
	Ga-FIII	14.08	3.36	10.15	-
	Ga-FIV	13.55	7.64	27.71	-
	Ga-FV	8.38	2.13	11.38	-

[a]Yield calculated as the percentage from a sample of each extract applied on DEAE-cellulose column; [b]Dosage by Dubois et al.' method using D-galactose as standard; [c]Dosage by Dodgson and Price' method using $NaSO_3$ as standard; [d]Dosage by Bradford' method using bovine serum albumin (- not detected).

Agarose gel electrophoresis procedure was performed to verify the physical-chemical characteristics of SPs from *G. acerosa*. Interestingly, similar electrophoretic profiles of SPs from were obtained for samples of *G. acerosa* collected from both beaches. Single bands exhibiting high charge densities on agarose gel were revealed for Ga-II and Ga-IV when compared with extracts and others isolated fractions (Figures 1C and D). These findings were also corroborated by the sulfate content of the isolated SPs (Table 1). Roleda et al. (1997) reported that high gel strength could be related to the biochemical changes in the wall matrix SPs composition of *G. acerosa* in response to its physiological state. Prasad et al. (2006) found seasonal variations in the physico-chemical and rheological properties of agar from *G. acerosa* collected in India. On the other hand, physico-chemical correlations have been used as an auxiliary supplement for chemotaxonomic studies involving different species or the same algal species (RODRIGUES et al., 2009, 2012a). Our results are important because the quality and content of SPs depend on environmental parameters, growth and reproducible cycle of the algae (CARDOZO et al., 2007).

Effects of SPs from *G. acerosa* on APTT, coagulation proteases and thrombosis

APTT assay was performed, or rather, a standard plasma clotting test initiated by a mixture of contact activators and phospholipids (AMORIM et al., 2011; NADER et al., 2001; QUINDERÉ et al., 2014; PEREIRA et al., 2005), to initially evaluate the *in vitro* effect of SPs fractions from the red seaweed *G. acerosa* collected from two beaches (Flecheiras and Pedra Rachada) (Table 2).

Table 2. Anti-clotting effect of SPs fractions obtained by ion-exchange chromatography (DEAE-cellulose) from the red seaweed *Gelidiella acerosa* compared to HEP.

Beaches	Fractions	NaCl (M)	APTT test* / coagulation protease/serpin - IC_{50} (μg mL^{-1})****			
			1.00 mg mL^{-1}**	IU mg^{-1}***	IIa/AT	Xa/AT
Flecheiras	Ga-I	0.25	40.03 ± 0.87	1.69	-	-
	Ga-II	0.50	49.95 ± 1.06	2.11	-	-
	Ga-III	0.75	40.65 ± 1.06	1.72	-	-
	Ga-IV	1.00	51.55 ± 2.62	2.18	-	-
	Ga-V	1.25	44.26 ± 1.00	1.87	-	-
Pedra Rachada	Ga-I	0.25	42.00 ± 1.09	1.78	-	-
	Ga-II	0.50	48.15 ± 1.25	2.04	-	-
	Ga-III	0.75	52.70 ± 1.37	2.23	-	-
	Ga-IV	1.00	71.15 ± 1.85	3.01	~10	~8
	Ga-V	1.25	54.90 ± 1.42	2.32	-	-

NaCl – Sodium chloride; *Activated partial thromboplastin time (APTT); **SPs concentration to prolong the APTT in seconds; ***Anticoagulant effect expressed in international units (IU) per mg of SPs (IU mg^{-1}); HEP (193.00 IU mg^{-1}; 0.01 mg mL^{-1}; APTT: 45.5 ± 0.89 s); Control: 40.05 ± 1.19 s (n = 3); ****Thrombin (IIa) or Factor Xa/Antithrombin (AT) – Inhibitory concentration 50%: concentration required to inhibit IIa activity by 50%; - no measured.

Anti-clotting effects (APTT) have been detected from different SPs in the extracellular matrix of algae (RODRIGUES et al., 2009). SPs fractions from *G. acerosa* did not practically alter (1.29-3.01 IU mg mL^{-1}) the normal coagulation time since APTT values were not duplicated when compared to those of control (human plasma). Ga-IV, eluted with 1 M of NaCl (Pedra Rachada), had maximal anticoagulant effect, but at a high concentration (1 mg mL^{-1}, 3.01 IU mg^{-1}), and a lower potency than HEP (193 IU mg^{-1}). Results indicated that SPs from *G. acerosa* were virtually capable of acting on the intrinsic and/or common pathways of the coagulation (ARAÚJO et al., 2012).

SPs from seaweed form particularly stabilized complexes with positively charged groups in plasma proteins that determine their effects on the haemostatic system (AMORIM et al., 2011; PEREIRA et al., 2005; RODRIGUES et al., 2013). Based on this hypothesis, are the *G. acerosa* SPs capable of inhibiting coagulation proteases and thrombosis? Current analysis further evaluated the possible effect of Ga-IV (Pedra Rachada) using inhibition IIa or factor Xa by AT assays *in vitro*. Although revealing a discrete effect on APTT (Table 2), Ga-IV had ability on IIa inhibition through AT-dependent pathway (IC_{50} ~ 10 μg mL^{-1}), as well as on factor Xa inactivation by AT (IC_{50} ~ 8 μg mL^{-1}) (AMORIM et al., 2011; FONSECA et al., 2008; PEREIRA et al., 2005; RODRIGUES et al., 2013). The same properties were also confirmed for heparin (IC_{50} ~ 1 (IIa) and 1.5 (factor Xa) μg mL^{-1}, respectively).

As factor Xa inhibition (Table 2) has been associated with antithrombotic action (FONSECA et al., 2008), our studies were also directed to evaluate Ga-IV on a rat venous thrombosis model system (RODRIGUES et al., 2011). Our results showed that the administration of a single injection of Ga-IV significantly reduced the thrombus weight in this *in vivo* model, although two distinct effects were observed (Figure 2). Ga-IV (0.01, 0.1, 0.5, and 1 mg kg^{-1} body weight) prevented thrombus formation by 27.1 (3.84 ± 0.82 mg), 50.22 (2.30 ± 12 mg), 82.68 (0.84 ± 0.65 mg) and 94.37% (0.26 ± 0.11 mg), respectively, in comparison with saline solution (4.62 ± 0.66 mg) ($p < 0.01$ or 0.001). When high doses were tested (> 1 mg kg^{-1} body weight), the inhibitions were 55.84 (2.04 ± 0.46 mg) and 9.52% (4.02 ± 0.53 mg) (2 or 3 mg kg^{-1} body weight, $p > 0.05$, respectively). Similar effect profiles were also found for SPs from *Botryocladia occidentalis* (Børgesen) Kylin (Rhodophyta) and *Caulerpa cupressoides* (Vahl) C. Agardh (Chlorophyta) by Fonseca et al. (2008) and Rodrigues et al. (2011), respectively, whereas only an antithrombotic response was registered for the SP from the brown seaweed *Laminaria japonica* (XIE et al., 2011).

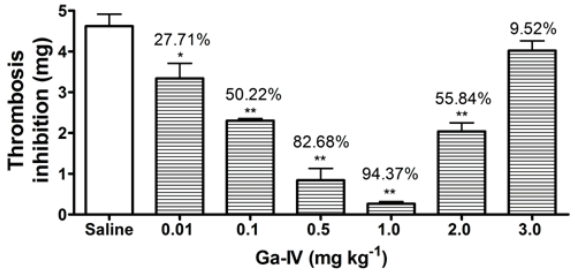

Figure 2. Antithrombotic effect after intravascular administration of Ga-IV in rats. Venous antithrombotic effect was investigated using a stasis thrombosis model that combined stasis and hypercoagulability. Different doses of Ga-IV were administered and allowed to circulate for 5 min. Thromboplastin (5 mg kg^{-1} body weight) was slowly injected intravenously and 0.7 cm of the isolated vena cava segment was tied off. The results of Ga-IV were expressed in mg of weight ($n = 5$, $p < 0.01$ or 0.001 *vs* control).

Results were actually intriguing because *G. acerosa* is an agarophyte (PRASAD et al., 2006; ROLEDA et al., 1997) and its colloid (agar) has been extensively used in food and cosmetic preparations as gelling agent due to the presence of 3,6-anhydro-

L-galactose (CARDOZO et al., 2007). It has also been reported that the water solubility of the SPs of algae depends on the levels of sulfate content and associated cations (e.g., calcium) (CAMPO et al., 2009). Rocha et al. (2005) also revealed that a sulfated galactofucan from the brown seaweed *Spatoglossum shroederi* (C. Agardh) Kützing had no anticoagulant activity on several *in vitro* assays (due to the presence of non-sulfate xylose units at the non-reducing terminal ends of the branches), but was capable of stimulating the synthesis of antithrombotic heparan sulfate by endothelial cells.

Our study revealed that the enzyme-assisted extraction (papain) resulted in high sulfate contents (Table 1) and possibly helped in the solubility of the SPs of *G. acerosa* fractions (CAMPO et al., 2009). In addition, when calcium chloride was added to display the APTT assay in detecting the anticoagulant potential, any influence in the evaluation of this test was noted (Table 2) (ARAÚJO et al., 2012). Therefore, Ga-IV interfered with the blood components (Table 2) and reduced venous thrombosis (Figure 2). In these circumstances, further studies are required to better clarify its structural requirements for these events (FONSECA et al., 2008; PEREIRA et al., 2005; QUINDERÉ et al., 2014).

Ga-IV does not cause toxicity in mice

In the case of toxicological assessment, variations in Ga-IV treatment for relative organ weight (liver-saline: 1.74 ± 0.09 g; Ga-IV: 5.78 ± 0.1 g; spleen-saline: 0.32 ± 0.00 g: Ga-IV: 0.66 ± 0.01 g) after 14 consecutive days were observed ($p < 0.05$). Repeated injections of Ga-IV (9 mg kg^{-1}, s.c.) were capable of exhibiting discrete signs of toxicity in mice at the biochemical (ALT-saline: 10.66 ± 0.6; Ga-IV: 13.10 ± 2.0) and hematological (HGB, MCV and MCH) (data not shown) parameters from their respective controls. However, histopathological analyses of the heart, kidney, spleen, thymus and lymph nodes removed from animals treated with a single injection of Ga-IV did not reveal any damage to the tissues. Although showing mild vacuolar pan-lobular degeneration of hepatocytes, there were no significant lesions in the liver where AT is produced (QUINSEY et al., 2004). The increased spleen size had no toxicological relevance and could be an immune function response. It has been suggested that the observed histopathological changes were reversible (data not shown) (QUINDERÉ et al., 2013; RODRIGUES et al., 2012b; SIQUEIRA et al., 2011).

Current findings revealed that combinatorial biochemistry techniques for studies of SPs from the red seaweed *G. acerosa* (Flecheiras and Pedra Rachada) would represent a good strategy for further safe antithrombotic drugs development. However, due to the great chemical diversity of these polymers among different algae species (CARDOZO et al., 2007; PRAJAPATI et al., 2014), the standardization of a commercial product from the industry is difficult, especially with regard to the collection of these organisms in different sites or at different seasons (PEREIRA; LOTUFO, 2012). Additional investigations are in progress by our research group to evaluate the physiological response of *G. acerosa* in different periods of the year.

Conclusion

The red seaweed *Gelidiella acerosa* collected from two Brazilian beaches (Flecheiras and Pedra Rachada) does not biochemically change its wall matrix sulfated polysaccharides composition, when analyzed by ion-exchange chromatography (DEAE-cellulose) and agarose gel electrophoresis procedures. The assessment by the anti-clotting test (APTT) supported the observations. When a polysaccharide fraction was tested on coagulation proteases, both thrombin and factor Xa activities by antithrombin were inhibited. The molecule also reduced venous thrombosis in rats, but reversed this status at high concentrations. No important systemic damage has been reported in mice.

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