Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v41i1.35655

Molecular characteristics of polysulfated fractions from the green seaweed *Caulerpa cupressoides* and actions on thrombin generation *in vitro*

José Ariévilo Gurgel Rodrigues[1*], Norma Maria Barros Benevides[2], Ana Maria Freire Tovar[1] and Paulo Antônio de Souza Mourão[1]

[1]Laboratório de Tecido Conjuntivo, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rua Rodolpho Paulo Rocco, 255, 21941-590, Rio de Janeiro, Rio de Janeiro, Brazil. [2]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. *Author for correspondence. E-mail: arieviloengpesca@yahoo.com.br

ABSTRACT. *Caulerpa cupressoides* produces sulfated polysaccharides (Cc-SPs) with serpin-dependent anticoagulant effect, but their actions on thrombin generation (TG) are unknown. This study aimed to partially characterize Cc-SPs and examine their potential as modulators of TG. Infrared analysis characterized extract containing three ulvan fractions (Cc-SP1, -SP2 and -SP3) separated by DEAE-cellulose chromatography, with differences in the relative proportions of sulfate (10.99-18.38%) and total sugars (46.59-51.12%), without presenting proteins. Charge density patterns and nonSPs varying from 8 to > 100 kDa on agarose and polyacrylamide gel electrophoresis by sequential staining with toluidine blue and stains-all were also confirmed by gel permeation chromatography. The molecular weight of Cc-SP2 was not altered after treatment with 0.4 M HCl up to 5 h. Only Cc-SP2 altered the activated partial thromboplastin time (15 ± 0.3 IU) vs. heparin (193 IU) and abolished at high concentrations (> 4.1 µg) TG by intrinsic pathway in 60-fold diluted human plasma, while at 4.1 µg attenuated TG by 33.87% delaying the lag phase (32 min.) vs. control (28 min.). Cc-SP2 induced concentration-dependent TG in system without cephalin. Heparin abolished TG at 4.15-fold lower amount, but did not stimulate TG. Therefore, Cc-SPs express dual effects on thrombosis *in vitro*.

Keywords: Caulerpaceae; marine alga; sulfated glycans; thrombosis.

Received on February 28, 2017.
Accepted on November 11, 2017.

Introduction

Cardiovascular diseases currently constitute the major global incidence of mortality and morbidity and the thromboembolic events arise in about one in 1,000 individuals per year, especially in the developed countries. Unfractionated heparin (UHEP) is routinely required to the treatment of hypercoagulable state (or thrombosis) and for the prevention of recurrent episodes of venous thromboembolism and death in numerous clinical trials. Structurally, UHEP comprises a functional class of glycosaminoglycans made of a long chain of repeating disaccharide unit of α-D-glucosamine alternating with α-L-iduronic acid to be highly sulfated. UHEP's specific pentasaccharide sequence depends on antithrombin (AT) to inactivate thrombin. Although effective against deep vein thrombosis, the prolonged use of UHEP as standard anticoagulant induces bleeding complications and thrombocytopenia because the response of individual patients is highly variable (Brieger, Mak, Kottke-Marchant, & Topol, 1998; Pineo & Hull, 1998), encouraging studies on new therapeutic tools (Athukorala, Jung, Vasanthan, & Jeon, 2006; Pomin & Mourão, 2008; Pomin, 2012; Mourão, 2015).

Seaweeds of the genus *Caulerpa* Lamouroux (1809) (Bryopsidales, Chlorophyta) comprise a green biomass overgrowing in eutrophicated waters generating environmental and economic impacts (Wang, Wang, Wu, & Liu, 2014). Opportunities to their use as food by coastal communities and human nutrition have been practiced in the Indo-Pacific region for centuries to reduce the risk of chronic diseases, such as neurodegenerative, inflammatory and cardiovascular complications, due to their substantial diversity of natural bioactive molecules with health benefits (Gaillande, Payri, Remoissenet, & Zubia, 2017) and as food ingredients (Kumar et al., 2018), besides *Caulerpa* cultivation on a large scale in Southeast Asia, such as *C. lentillifera*, where this species is also popularly consumed and explored in therapeutic medical applications (Maeda, Ida, Ihara, & Sakamoto, 2012).

Caulerpa species produce highly complex sulfated polysaccharides (SPs), known as ulvans (Patel, 2012; Wang et al., 2014), which may be used as emerging functional ingredients due to their anionic nature (S=O) acting as antiviral (Ghosh et al., 2004), anticoagulant (Hayakawa et al., 2000; Arenajo et al., 2017), antitumor (Ji, Shao, Zhang, Hong, & Xiong, 2008), immunostimulatory (Maeda et al., 2012), anti-promastigote (Pires et al., 2013), anti-antioxidant, and anti-inflammatory (Wang et al., 2014) agents. On a structural basis, these glycans have molecular masses of more than 100 kDa (Shanmugam, Ramavat, Mody, Oza, & Tewari, 2001; Maeda et al., 2012) and sulfation occurring at the C-6 of the hydroxyl group of galactose as the major sugar source, with xylose, uronic acid, glucose, arabinose and mannose appearing as common components (Ghosh et al., 2004; Ji et al., 2008; Patel, 2012; Wang et al., 2014; Liang, Liu, Chang, & Pan, 2015). Studies based on biology and structural evolution suggested that the SPs could be conserved among taxonomic groups and minor amounts occurring in Chlorophyta than Rhodophyta and Phaeophyta (Pomin & Mourão, 2008), which are the most common sources in sulfated galactans (mainly carrageenan and agaran) (Maciel et al., 2008; Pomin & Mourão, 2008) and fucan or fucoidan (Pomin & Mourão, 2008; Pomin, 2012), respectively.

Caulerpa cupressoides var. *lycopodium* (Vahl) C. Agardh is a particularly interesting benthic species of green seaweed endemic to the Ceará coast, Brazil, having nutritional value (Carneiro, Rodrigues, Teles, Cavalcante, & Benevides, 2014) and metabolites-derived physiological effects (Vanderlei et al., 2010; Rivanor et al., 2014; Wang et al., 2014). Studies performed by Rodrigues et al. (2011a), Rodrigues, Quinderé, Queiroz, Coura, and Benevides (2012a), Rodrigues et al. (2014a) isolated and partially characterized three SPs fractions (-SP1, -SP2 and Cc-SP3), of which only Cc-SP2 showed sulfation-dependent anticoagulant effect vs. UHEP. Cc-SP1 and Cc-SP3 had no *in vitro* anti-clotting effects (Rodrigues et al., 2011a), but Cc-SP1 revealed to be an *in vitro* anti-herpetic and anti-dengue agent (Rodrigues et al., 2017a). Subsequently, Cc-SP2 exhibited *in vivo* pro- and antithrombotic effects devoid of hemorrhagic risk (Rodrigues et al., 2011b) and protected experimentally-inflamed rats (Rodrigues et al., 2012b; Rodrigues et al., 2014b) without toxicity in mice (Rodrigues et al., 2012b). It was also demonstrated by Rodrigues et al. (2013) that Cc-SP2 containing low-molecular-size SPs inhibited coagulation proteases (thrombin and factor X) through both AT and/or heparin cofactor II-dependent pathways, respectively. However, the physical-chemical and structural features of its SPs have been still poorly investigated regarding their impacts on the haemostatic system.

Activated partial thromboplastin time (APTT) test is one of the most used to detect anticoagulant SPs (Shanmugam et al., 2001; Athukorala et al., 2006; Pomin & Mourão, 2008; Pomin, 2012; Fidelis et al., 2014; Mourão, 2015), but contradictions related to the restrict value on the total amount of thrombin formed have currently encouraged the use of thrombin generation (TG) assays as more sensitive tools to analyze the haemostatic status than the APTT method, on a significant prognostic view (Castoldi & Rosing, 2011; Luna-Záizar et al., 2014; Jun et al., 2014). TG-based coagulation tests would offer relevant information of plasma alternative anticoagulants to HEP in prevention of thrombosis *in vitro* (Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999; Glauser et al., 2009; Rodrigues et al., 2016; Salles et al., 2017) or predicting hypercoagulability (Zhang et al., 2014; Barcellos et al., 2018). Little evidence of green seaweeds SPs as inhibitors of TG has been demonstrated as a more refined step for the rational development of functional agents from these polymers (Barcellos et al., 2018; Rodrigues, Benevides, Tovar, & Mourão, 2017b).

Considering the above, the aim of this study was to examine the structural and physical-chemical features of complex SPs extracted from *C. cupressoides* using Fourier Transform Infrared spectroscopy experiment and combination of agarose/polyacrylamide gel electrophoresis and sequential staining with toluidine blue/stains-all, respectively. The *in vitro* inhibitory potential on contact-stimulated TG in 60-fold diluted human plasma by its fractions was also evaluated using a continuous detection system. An experimental approach using a fraction in plasma system without the intrinsic pathway activator was also conducted to analyze a possible TG induction.

Material and methods

Algal material and structural and physical-chemical analyses of SPs

Brazilian samples of fresh *C. cupressoides* var. *lycopodium* (Vahl) C. Agardh were carefully collected in August 2011 along the Flecheiras Beach (Trairí, Ceará State). In plastic bags, they were taken to the Carbohydrate and Lectins Laboratory, Department of Biochemistry and Molecular Biology, *Universidade Federal do Ceará* (UFC), Brazil. After transportation, macroscopic epiphytes were removed from the

specimens, followed by extensive washing with distilled water and storing at -20°C (Carneiro et al., 2014). Species was identified by PhD. José Ariévilo Gurgel Rodrigues from the Department of Fisheries Engineering (UFC) and a voucher specimen (# 4977) was deposited by Ana Cecília Fortes Xavier at the Prisco Bezerra Herbarium (UFC). The current study did not involve endangered or protected species and was conducted in accordance with the law MP 2.186-16/2001, resolution 29 of the Dispatch Component of Genetic Patrimony (Brazilian Regulatory Standard).

The dehydrated algal tissue (room temperature) was involved in plastic film and then taken to the Connective Tissue Laboratory, *Universidade Federal do Rio de Janeiro* (UFRJ), Brazil, for experimental analyses. Essentially, five grams were digested with papain at 60°C for 6 hours to obtain crude SP as described elsewhere (Rodrigues et al., 2011a). Nitrogen, carbon, hydrogen and sulfate contents and structural features of the crude SP were examined by elemental microanalysis (CHN equipment Perkin Elmer model 2400) (Maciel et al., 2008) and Fourier Transform Infrared (FT-IR) spectroscopy technique (SHIMADZU IR spectrophotometer model 8300) between 4000 and 500 or 900 and 400 cm^{-1} (Rodrigues et al., 2013; Rodrigues et al., 2014a), respectively.

Fractionation of the crude SP by anion-exchange chromatography on a DEAE-cellulose column using a stepwise of NaCl (0→1 M, with 0.25 M intervals) was performed and -SP1, -SP2- and Cc-SP3 (eluted with 0.5, 0.75, and 1 M NaCl, respectively) were obtained. Chemical determination of sulfate, total sugars and proteins of the fractions were carried out as previously described (Rodrigues et al., 2011a). Visualization of SPs on agarose (Dietrich & Dietrich, 1976) / polyacrylamide (Rodrigues et al., 2013) gel by sequential staining with toluidine blue and stains-all (Volpi & Maccari, 2002; Rodrigues et al., 2017b) was conducted by comparison with the electrophoretic mobility of standard compounds dextran sulfate (ca. 8 kDa), chondroitin-4-sulfate (ca. 40 kDa), chondroitin-6-sulfate (ca. 60 kDa) (Rodrigues et al., 2013), dermatan sulfate (DS) and/or heparan sulfate (HS) (Salles et al., 2017). Analysis of each fraction by gel permeation chromatography (GPC) (Shimadzu apparatus) was also performed using pullulan samples as standard (5.9×10^3, 1.18×10^4, 4.73×10^4, 2.12×10^5 and 7.88×10^5 g moL^{-1}) (Rodrigues et al., 2011a).

Analysis of mild-acid hydrolysis for generation of Cc-SP2-derived products

Native Cc-SP2 (10 mg) was dissolved in 1 mL 0.04 M HCl and maintained at 60°C for different periods. After this depolymerization procedure, the pH was neutralized by the addition of 1 mL ice-cold 0.04 M NaOH. The analysis of molecular masses of the products were examined by polyacrylamide gel electrophoresis at 100 V for ~50 min., by comparison with the electrophoretic mobility of the standard low-molecular-weight compounds dextran sulfate (~8 kDa), chondroitin-4-sulfate (~40 kDa), chondroitin-6-sulfate (~60 kDa) and/or UHEP (~14 kDa). After that, the SPs were stained with 0.1% toluidine blue in 1% acetic acid (Rodrigues et al., 2016).

In vitro coagulation examination

Blood samples

Coagulation analyses were conducted using venous blood samples collected in citrated Vacutainer tubes containing 3.2% sodium citrate from 10 different donors (University Hospital Clementino Fraga Filho, UFRJ), followed by centrifugation at 2000 × *g* for 15 min. prior to tests. Normal citrated human plasma aliquots of 1 mL were frozen and stored at - 70°C until use (Barcellos et al., 2018).

Activated partial thromboplastin time (APTT) test

The anticoagulant effect of the fractions was assessed by *in vitro* APTT test according to the manufacturers' specifications using a coagulometer Amelung KC4A before the *in vitro* TG assay. For the assay, a mixture of 100 µL plasma and concentrations of SPs (0-1 mg mL^{-1}) was incubated with 100 µL APTT reagent (kaolin bovine phospholipid reagent). After 2 min. incubation at 37°C, 100 µL 20 mM $CaCl_2$ was added to the mixtures, and the clotting time was recorded. Unfractionated heparin (UHEP) with 193 international units per mg (IU mg^{-1}) of polysaccharide was used as standard. All tests were performed in triplicate.

TG-based coagulation assay

Effect of fractions on TG in plasma after activation of the intrinsic pathway

This assay was carried out in a microplate format, containing: 10 µL APTT reagent (contact-activator system) + 30 µL 0.02 M Tris-HCl/PEG buffer (pH 7.4) + 10 µL SPs (*C. cupressoides* fractions: 0, 4.1, 41.6 or

83.3 µg well-plate^{-1}; UHEP: 2 µg.well-plate^{-1}) + 60 µL 20 mM $CaCl_2$/0.33 mM chromogenic substrate S2238 (10:50 ratio, v/v^{-1}). The *in vitro* reaction was triggered at 37°C by the addition of plasma (diluted 60-fold well-plate^{-1}, 10 µL), and the absorbance (405 nm) was recorded every 1 min. (60 min, 37°C) using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The inhibitory response of TG by SPs was analyzed by lag phase, peak thrombin (PTh) and time to peak (TPeak) (Rodrigues et al., 2016). Graphics were processed using Statistical Analysis Software version 8.0 Origin Program.

Effect of Cc-SP2 on TG in plasma system without cephalin

This assay was similar as mentioned above using a microplate format, but in the absence of cephalin, which was prepared as follow: 40 µL 0.02 M Tris-HCl / PEG buffer (pH 7.4) + 10 µL SPs (Cc-SP2: 0, 4.1, 41.6 or 83.3 µg well-plate^{-1}; UHEP: 2 µg.well-plate^{-1}) + 60 µL 20 mM $CaCl_2$ / 0.33 mM chromogenic substrate S2238 (10:50 ratio, v/v^{-1}). The *in vitro* reaction was triggered at 37°C by the addition of plasma (diluted 60-fold well-plate^{-1}, 10 µL), and the hydrolysis of the substract was detected at 405 nm every 1 min (120 min., 37°C) using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The stimulatory response of TG by Cc-SP2 was analyzed by absorbance of the assay. Graphic was processed using Statistical Analysis Software version 8.0 Origin Program.

Statistical analyses

The APTT values were expressed as mean ± S.E.M., and Two-way Analysis of Variance (Two-way ANOVA) test was run, followed by Tukey's test for unpaired data, with $p < 0.05$ as statistically significant. To verify statistically the active fraction and UHEP, it was applied the *Student*'s t-test, considering $p < 0.05$.

Results and discussion

Caulerpa cupressoides synthesizes ulvan-type SPs, but with galactose-2/6-sulfate

Sulfated glycans were extracted from macerated *C. cupressoides* coenocytic tissue by treating with protease (papain) at 60°C for 6 hours, resulting in 2.7% of crude SP extract from the dehydrated alga and after drying procedure (60°C, 24 hours). This here-reported crude SP yield was in concordance with the previously described (about 3%) for this same algal species (Rodrigues et al., 2011a; Rodrigues et al., 2012a), and other SPs-rich *Caulerpa* species (0.6-29%) using different protocols (Ghosh et al., 2004; Ji et al., 2008; Patel, 2012). Low yields of SPs (0.3-33.7%) belonging to the order Bryopsidales have been reported in literature (Shanmugam et al., 2001; Arata et al., 2015).

Figure 1 shows the structural nature of the crude SP derived from the green seaweed *C. cupressoides*, as well as in its extended spectral version further plotted. Spectral signals related to the presence of ester sulfate (at 1253 cm^{-1}, S = O) (Ghosh et al., 2004; Maciel et al., 2008; Zhang et al., 2008), uronic acid (at 1637 cm^{-1}, COO- or O-H) and/or proteins (amide band) (Wang et al., 2014), galactose-6-sulfate (at 811 cm^{-1}), arabinogalactan sulfate (at 1074 cm^{-1}) (Ghosh et al., 2004), carboxyl group of pyruvic acid (1383 cm^{-1}), O-H (at 3429 cm^{-1}) (Zhang et al., 2008) and O = S = O (at 619 and 468 cm^{-1}) (Figure 1A) were deduced as also found for the SPs fractions in another study (Rodrigues et al., 2014a).

Although ulvan has been characterized in green seaweeds (Wang et al., 2014), this investigation could allow contradictions among *Caulerpa* species that expressed sulfated xyloarabinogalactan and sulfated xylogalactan, such as in *C. racemosa* (Ghosh et al., 2004) and *C. lentillifera* (Maeda et al., 2012), respectively. However, it has been described that the structures of SPs produced by Chlorophyta belonging to the Bryopsidales galactans predominate (Wang et al., 2014; Arata et al., 2015). On an evolutionary basis, it has been proposed that galactans in marine organisms occur regarding preferential sulfation site based on a tendency toward 2-sulfation for animals, 4-sulfation for algae and marine angiosperms, and in the case of 6-sulfation, as a dispersive distribution among phyla during evolution (Pomin & Mourão, 2008).

The occurrence of galactose-6-sulfate (a biological precursor to transform in 3,6-anhydrogalactose) in the polysaccharide structure of *C. cupressoides* coenocytic tissue (Rodrigues et al., 2013, 2014a), as being more evidenced in extended spectrum (at 815 cm^{-1}) (Figure 1B). It could also indicate its importance for the food industry because alkaline treatment has a positive effect on the functional properties in the attempt to obtain specific gel formation under different conditions displaying as thickening, gelling and stabilizing agents for food application (Pomin & Mourão, 2008; Patel, 2012). According to Liang et al. (2015), the use of

alkali extraction resulted in β-1, 3-xylan as a component of cell-wall polysaccharide from the green seaweed *C. lentillifera*.

Curiously, the almost imperceptible vibration band at 835 cm^{-1} in the extended spectrum of the crude SP (Figure 1B) supported the suggestion above as 2-sulfate of galactose residues typically assigned to agarocolloids, besides the signal at 779 cm^{-1}, but these findings were uniquely described for SPs from red seaweeds (Maciel et al., 2008; Pomin & Mourão, 2008; Mourão, 2015) because the region attributed to 800-850 cm^{-1} is also commonly used to infer the position of sulfate group (Maciel et al., 2008). Signals from 538 to 885 cm^{-1} were also assigned to ulvan polysaccharide (Wang et al., 2014). The reexamined infrared spectral analysis for the current study hypothesized that SPs from *C. cupressoides* would resemble glycosaminoglycans in proteoglycans obtained from mammalian connective tissue (Pomin & Mourão, 2008; Wang et al., 2014).

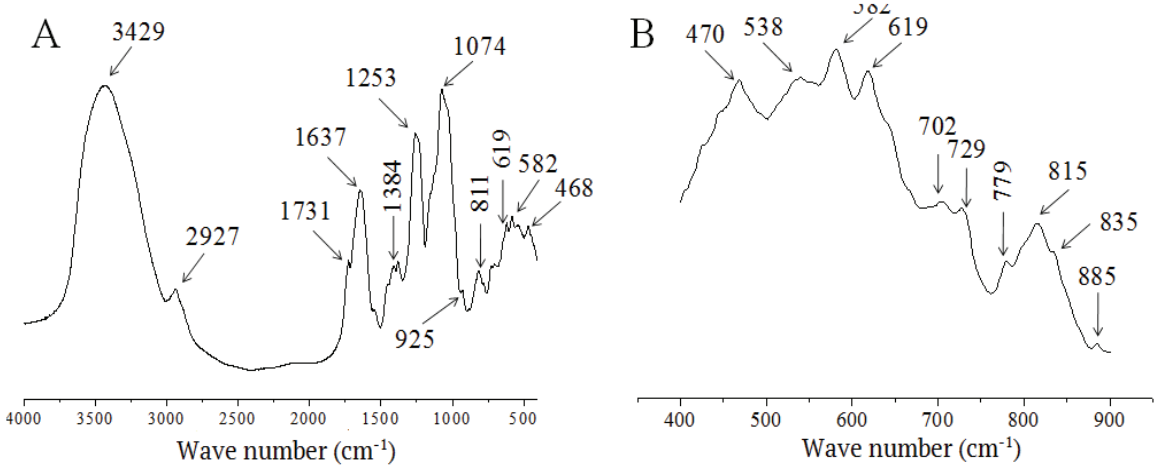

Figure 1. Infrared spectrum (A) and its extended version (B) of the crude SP from the green seaweed *Caulerpa cupressoides* at 4000 and 500 or 900 and 400 cm^{-1} in kBr pellets.

On the basis of elemental microanalysis, crude SP extract from *C. cupressides* showed sulfate content (4.36%) similar to its SPs fractions (2.16-4.55%) as analyzed in previous studies (Rodrigues et al., 2013, 2014a), but was about 2.18-fold higher compared with aqueous SPs extract from the red seaweed *Gracilaria birdiae* (Maciel et al., 2008). In fact, this combined result reinforced the use of proteases to obtain biologically-active *C. cupressoides* SPs (Rodrigues et al., 2012b; Rodrigues et al., 2013; Rodrigues et al., 2014a; Wang et al., 2014) as revealed by infrared that detected spectral signals at 815, 835 and 1253 cm^{-1} (Figure 1) (Rodrigues et al., 2013; Rodrigues et al., 2014a). Sulfate content of cold and hot water extracts of five species of Indian marine Chlorophyta (*C. racemosa*, *C. taxifolia*, *C. scalpelliformis*, *C. veravalensis* and *C. peltata*) ranged from 4.00 to 7.66% according to Shanmugam et al. (2001).

The presence of nitrogen (2.31%) was also comparatively distinct from that of cold extract containing SPs from *G. birdiae* (Maciel et al., 2008) and confirmed the hypothesis of a polysaccharide-protein complex (proteoglycan) making part of the structure of the compound based on literature (Ghosh et al., 2004; Rodrigues et al., 2013; Rodrigues et al., 2014a; Wang et al., 2014). This observation led us to speculate amino acids of the proteins (Ghosh et al., 2004; Ji et al., 2008), as an already estimated protein content to be 20.79 ± 0.58 g 100 g^{-1} dehydrated weight (Carneiro et al., 2014), presents in the residual sulfated glycans of *C. cupressoides*, of which they were generated by papain treatment (Rodrigues et al., 2011a), and perhaps supporting that amide band (at 1637 cm^{-1}) observed in the spectrum (Figure 1A) (Rodrigues et al., 2013).

Analyses of SPs fractions by physical-chemical approaches reveal similar masses

The crude SP extract from the green seaweed *C. cupressoides* was fractionated by DEAE-cellulose anion-exchange chromatography applying a stepwise of NaCl, yielding three SPs fractions (-SP1, -SP2 and Cc-SP3 eluted at 0.50, 0.75 and 1.00 M NaCl, respectively), based on their respective metachromatic properties (Rodrigues et al., 2011a).

The chemical composition of the DEAE-cellulose column-bound SPs eluted at intermediate salt concentration (Cc-SP2) showed highest levels of both sulfate (18.38%) and total sugars (51.12%) vs. Cc-SP1

and Cc-SP3 revealing sulfation (10.99 and 14.86%) and total sugars (46.59 and 48.67%), respectively; therefore, heterogeneous composition in agreement with the SPs from *C. cupressoides* (Rodrigues et al., 2011a; Rodrigues et al., 2014a) and other seaweeds species SPs (Ji et al., 2008; Wang et al., 2014; Mourão, 2015). Besides, no protein contamination by colorimetric method was detected (Rodrigues et al., 2011a; Rodrigues et al., 2011b; Rodrigues et al., 2013; Rodrigues et al., 2014b), although postulating the presence of amino acids from the analyzed crude SP extract sample as shown by infrared (Figure 1) and nitrogen content also mentioned above.

Since the separation by DEAE-cellulose of a complex mixture of sulfated glycans was possible (Rodrigues et al., 2011a), the further step was to physical-chemically characterize by using electrophoretic techniques the degree of purity and molecular mass of the *C. cupressoides* SPs fractions, as shown in Figure 2.

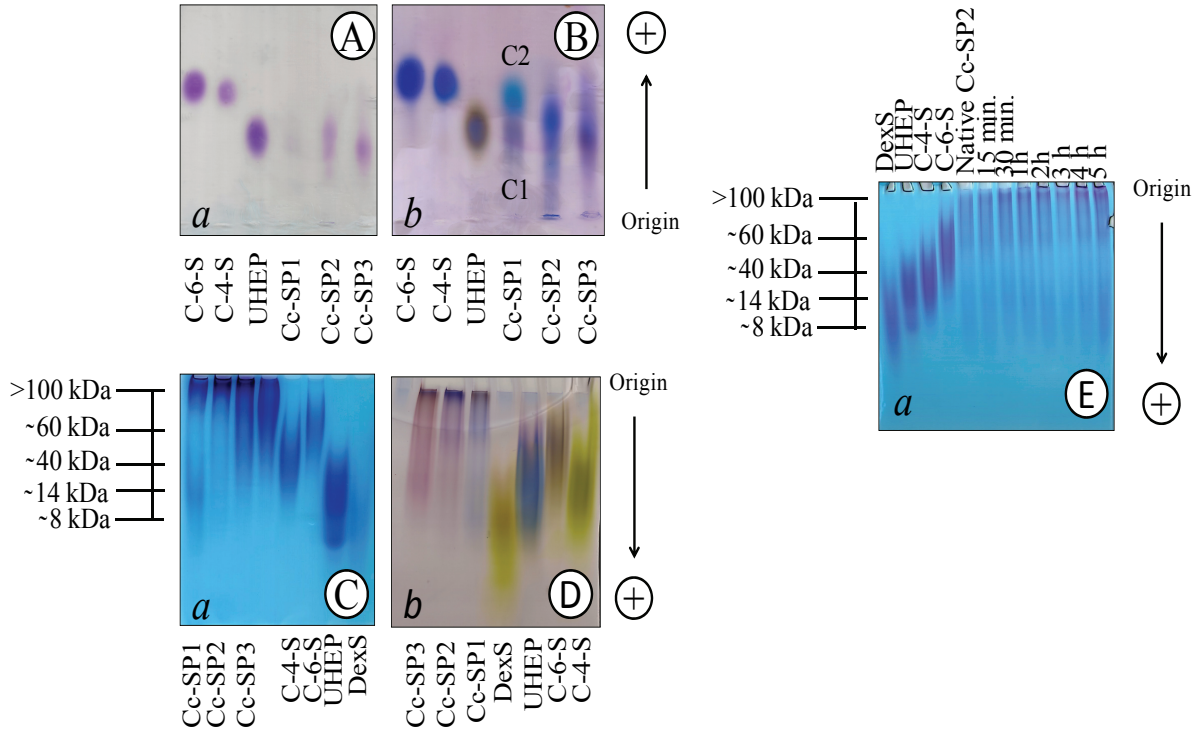

Figure 2. Agarose (A, B) and polycrylamide (C, D, E) gel electrophoreses of *Caulerpa cupressoides* SPs fractions, obtained by DEAE-cellulose, and standard glycosaminoglycans chondroitin-6-sulfate (C-6-S, 60 kDa), chondroitin-4-sulfate (C-4-S, 40 kDa), dextran sulfate (DexS, 8 kDa), unfractionated heparin (UHEP, 14 kDa) present on gels were stained with 0.1% toluidine blue (a) or stains-all (b). (E) Polyacrylamide analysis after the mild-acid hydrolysis procedure with 0.04 HCl reveals stability of Cc-SP2.

Treatment with toluidine blue dye showed SPs with polydispersion and variables charged among the fractions co-migrating as UHEP with basis on their respective metachromasy from the agarose gel analysis (Figure 2Aa).

The interaction between the *C. cupressoides* SPs through their sulfate radicals and diamine deduced same structural conformation and charge/mass ratio of these compounds showing electrophoretic mobility in diamino propane/acetate buffer similar to animal matrix glycosaminoglycans (Dietrich & Dietrich, 1976; Fidelis et al., 2014; Salles et al., 2017).

The degree of density charge and sulfation quantity of the glycans were associated with the FT-IR analyses of the fractions as expected (Rodrigues et al., 2014a); the band intensity at 1259 cm^{-1} (ester sulfate groups) related to the sulfate of Cc-SP1 becomes lowest in comparison with others fractions in this study (data not shown). Furthermore, the values determined by elemental microanalysis could be appropriate as also previously described by Rodrigues et al. (2014a). Therefore, the *C. cupressoides* SPs charge mainly originates from attached sulfate radicals (Maciel et al., 2008; Rodrigues et al., 2014a).

On the contrary, this gel staining pattern contrasted with the results for the fraction Cc-SP3 eluted with 1 M NaCl that did not appear on the agarose gel in the study of Rodrigues et al. (2011a), who formerly physical-chemically investigated its SPs. Findings supported herein the sulfate profile already mentioned as an important correlation to suggest that *C. cupressoides* biochemically change its coenocytic tissue

polysaccharide composition (Rodrigues et al., 2017a) according to the ecophysiological growth conditions (Pomin & Mourão, 2008; Wang et al., 2014).

Since fraction Cc-SP1 was weakly visualized on the gel by staining with toluidine blue (Figure 2Aa), it was intended to more accurately analyze this SPs preparation using stains-all as another cationic dye for detection of glycans (Volpi & Maccari, 2002; Rodrigues et al., 2017b; Salles et al., 2017). Figure 2Bb allowed an improved examination of standards and fractions, especially Cc-SP1 that clearly exhibited two polysaccharide components stained distinctly on gel, named C1 and C2, where the first one co-migrated as UHEP and the second close to CS because ulvan resemble glycosaminoglycans containing uronic acid and sulfate (Wang et al., 2014).

The occurrence of these two components in fraction Cc-SP1 could be 'polymeric blocks' of uronic acid in the native polysaccharide, as supported by the preliminary spectral values found by solution ^{1}H RMN experiment of Cc-SP1 in another investigation (Rodrigues et al., 2014a). Fractions Cc-SP2 and Cc-SP3 primarily showed here electrophoretic mobilities compared to UHEP. These observations suggested that *C. cupressoides* produced other polysaccharides that were not detected after staining with toluidine blue alone (Volpi & Maccari, 2002; Salles et al., 2017; Rodrigues et al., 2017a; Rodrigues et al., 2017b).

These combined observations would determine the speculation of a compositional variability among the fractions containing nonSPs based on infrared spectral study that also indicated pyruvylated sugar residues at 1383 cm^{-1} (Figure 1) (Ghosh et al., 2004; Zhang et al., 2008; Rodrigues et al., 2014a), naturally found in SPs from green seaweeds (Wang et al., 2014; Arata et al., 2015). Therefore, the monosaccharide composition of the *C. cupressoides* SPs deserve to be determined in future studies.

Polyacrylamide gel analysis presented a wide dispersion of molecular masses (Rodrigues et al., 2013) vs. respective standards, as revealed by toluidine blue (Figure 2Ca). This electrophoretic behavior was shown to be in Cc-SP1 and Cc-SP2, which were eluted (at 0.5 and 0.75 M NaCl, respectively) at the beginning of the NaCl stepwise, accounting for the total material recovered from the DEAE-cellulose column. By contrast, Cc-SP3 contained SPs with molecular masses of more than 100 kDa as usually observed for SPs from seaweeds (Pomin & Mourão, 2008; Pomin, 2012; Mourão, 2015). Ghosh et al. (2004) examined, by size exclusion chromatography on a Sephacryl S-100 column, a hot water crude SP extract obtained from *C. racemosa* and determined the apparent molecular weight of two major peaks (F 1 and F 2) to be 120 and 70 kDa, respectively. More recently, Maeda et al. (2012) extracted SPs from *C. lentillifera* by treating with water and the purification by size-exclusion chromatography (Superdex 200 HR 10/30) yielded a xylogalactan with a molecular size of > 100 kDa. Pires et al. (2013) examined the molecular size, using a TSKgel G3000SW column equilibrated with phosphate buffer, of a fraction obtained on a DEAE-cellulose ion-exchange chromatography for *C. racemosa* SPs solution in sodium acetate buffer. This analytical approach characterized the polymer to be 25 kDa.

As this investigation indicated a preponderance of SPs with distributions of molecular weight ranging from 8 to > 100 kDa in the coenocytic structure of *C. cupressoides* (Figure 2Ca) (Rodrigues et al., 2013), fractions were also examined by GPC and the results corroborated with those of the polyacrylamide analysis predicting low-molecular-size SPs (Rodrigues et al., 2017a). Considering the above, a heterogeneous dispersion for -SP1 (4.12×10^4 and 2.53×10^4 g moL^{-1}), -SP2 (2.5×10^4 and 2.1×10^4 g moL^{-1}) and Cc-SP3 (4.12×10^4 and 2.53×10^4 g moL^{-1}), respectively, was found from the analyzed polymer samples and reflected its molecular nature, as shown in Table 1. On the basis of these results, it was observed that all fractions had two molecular mass peaks as a consequence of molecular homogeneity along the algal tissue (Rodrigues et al., 2011a).

Table 1. Molar mass (Mw) of SPs fractions, analyzed by gel permeation chromatography, from the green seaweed *Caulerpa cupressoides*.

Fractions	NaCl (M)	Peak	Elution volume (mL)	Mw (g moL^{-1})
Cc-SP1	0.5	I	8.43	4.12×10^4
		II	10.02	2.53×10^4
Cc-SP2	0.75	I	10.05	2.5×10^4
		II	10.45	2.1×10^4
Cc-SP3	1	I	8.43	4.12×10^4
		II	10.02	2.53×10^4

After the acid treatment (0.4 M HCl), no impact on the polysaccharide chains up to 5 h was noted; therefore, Cc-SP2 was resistant to hydrolytic action based on electrophoretic mobilities of the known glycosaminoglycans (Figure 2Ea), demonstrating the stability of this heterogeneous system of polyglycans vs. that of SPs from the red seaweed *Acanthophora muscoides*, which resulted in sulfated oligosaccharides using this same experimental assay in another study (Rodrigues et al., 2016). Liang et al. (2015) extracted β-1,3-xylan from the green seaweed *C. lentillifera* by using alkali treatment, and the hydrolysis by *Escherichia coli* ClearColi BL21 (DE3)-β-1,3-xylanase XYLII produced < 3 kDa β-1,3-xylooligosaccharide. Our findings were advantageous to obtain large quantities of low-molecular-weight fractions of SPs because the application of controlled depolymerization processes are more laborious (Pomin & Mourão, 2008; Zhang et al., 2008; Wang et al., 2014).

Staining of the *C. cupressoides* SPs fractions with the use of stains-all also improved visualization of the samples vs. standards from the polyacrylamide gel analysis (Figure 2Db), including small amounts of low-molecular-mass nonSPs (Salles et al., 2017), as particularly useful for a rapid detection of polymeric complexes to their quality control of algae carbohydrates-based products; likewise, as low-molecular-size glycosaminoglycans generated by chemical depolymerization procedures commonly applied in biomaterial industry (Volpi & Maccari, 2002).

Low-molecular-size SPs-containing fractions from *C. cupressoides* more accurately interfere TG than the conventional APTT test

The *in vitro* anticoagulant efficacy of the *C. cupressoides* SPs fractions were initially verified on APTT clotting assays in human plasma of healthy donors and were compared with UHEP (193 IU mg^{-1}, ca. 14 kDa) as a reference.

The typical APTT test confirmed that SPs only eluted at intermediate concentration of NaCl (Cc-SP2) from the DEAE-cellulose column were capable of prolonging the normal coagulation time compared with the other polysaccharide fractions isolated from the green seaweed *C. cupressoides* as previously observed (Rodrigues et al., 2011a; Rodrigues et al., 2011b; Rodrigues et al., 2013). On a weight-to-weight basis, the dose-response curve of Cc-SP2 demonstrated intrinsic coagulation pathway inactivation of at least 100 μg mL^{-1} (39.60 ± 0.30 s), but doubling the APTT at a concentration of 500 μg mL^{-1} (87.35 ± 0.31 s) on the basis of the T_1 T_0^{-1} ratio from the citrate-treated plasma control without SPs (31.80 ± 0.10 s).

This anticoagulant action of Cc-SP2 was revealed to be 15 ± 0.3 IU mg^{-1} (Figure 3A), whereas UHEP (193 IU mg^{-1}) still altered the APTT test when a minimum concentration of 2.5 μg mL^{-1} (42.15 ± 0.60 s) was used (Salles et al., 2017; Rodrigues et al., 2017b) as inhibitor of the contact-pathway ($p < 0.01$) (Pomin, 2012; Mourão, 2015). As also expected, fractions Cc-SP1 and Cc-SP3 had no effect on APTT, when a high concentration of SPs (1 mg mL^{-1}) was tested *in vitro* ($p > 0.05$) (data not shown) (Rodrigues et al., 2011a).

Anticoagulation of seaweeds SPs has been correlated with sugar type, sulfation pattern, anomeric configuration, glycosidic linkage and molecular mass (Pomin, 2012; Mourão, 2015). Some SPs isolated from *Caulerpa* species have been described as blood coagulation inhibitors (Hayakawa et al., 2000; Wang et al., 2014), when evaluated by APTT method (Ghosh et al., 2004; Liang et al., 2015; Arenajo et al., 2017). They delayed the APTT dependent mostly on the sulfate content and charge density (Shanmugam et al., 2001; Rodrigues et al., 2012a; Wang et al., 2014).

Although revealing a sulfation level (18.38%) of about 1.30-fold lower than that previously quantified (23.79%) in another investigation (Rodrigues et al., 2011a), Cc-SP2 required herein a sulfation quantity of at least 1.24-fold higher than the other fractions to display anticoagulation due to its highest density in the sulfate group as also observed by agarose gel (Figure 2A). Such findings demonstrated an active level (60.92%) of about 1.64-fold lower in terms of IU based on a previous study (24.62 IU) that confirmed the influence of sulfate content for the anticoagulant action (Rodrigues et al., 2011a). The bioactivity of the algae SPs may be affected by environmental factors of the marine habitat (Pomin & Mourão, 2008; Patel, 2012; Wang et al., 2014).

Seaweeds have become promising sources of alternative ingredients to supplement food with functional compounds to enhance the safety and quality of diverse industrial preparations. Within this scenario of modern lifestyle, more specific biological assays are useful approaches to elaborate high-quality formulations of biotechnological interest (Patel, 2012; Gaillande et al., 2017; Kumar et al., 2018) as gelling and stabilizing agents (Pomin & Mourão, 2008).

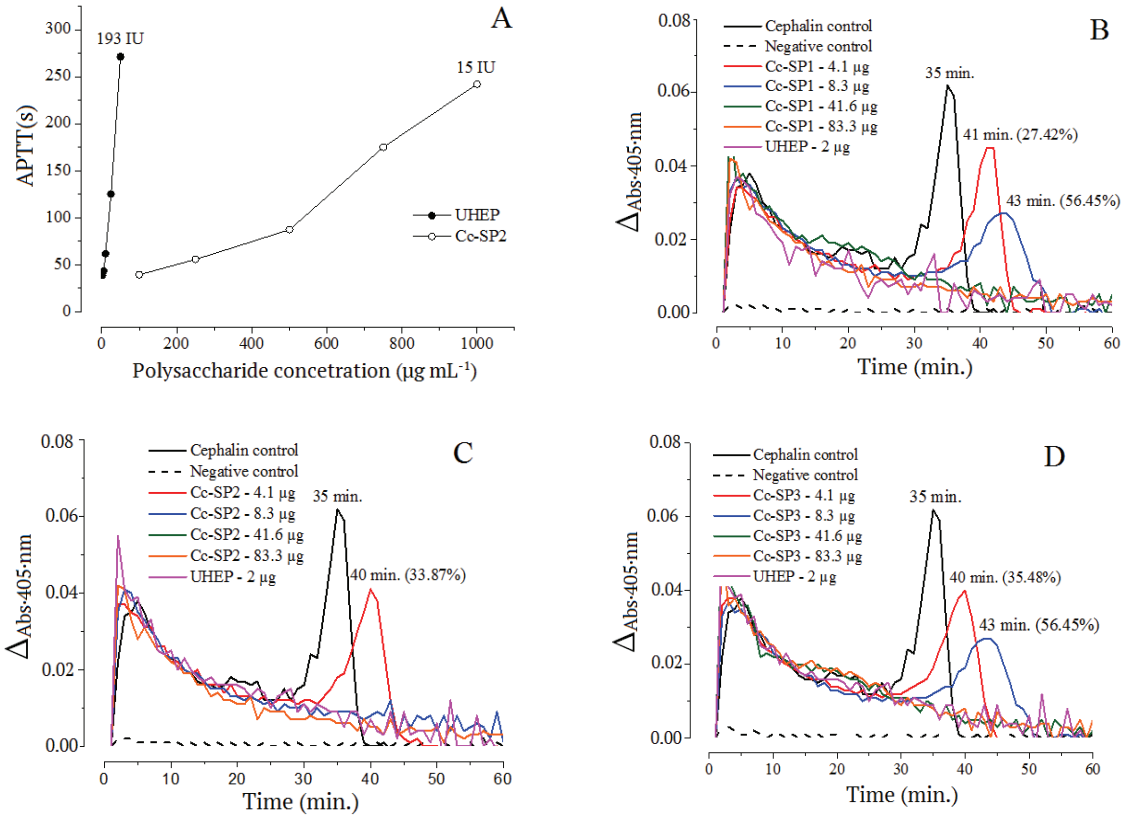

Figure 3. (A) Concentration dependence of the fraction Cc-SP2 added to plasma slightly modifies the *in vitro* classical APTT coagulation assay vs. UHEP. Effect of different concentrations of the fractions -SP1 (B), -SP2 (C) and Cc-SP3 (D) and standard UHEP on cephalin-triggered TG in 60-fold diluted normal human plasma using chromogenic method by a continuous detection system (37°C, 60 min.).

To more accurately explore the effect of the sulfate content on the functionality of the algal SPs, an *in vitro* TG model was employed in this study as another analytical tool. Using fractions at different concentrations (4.1→83.3 µg well-plate^{-1}) (Rodrigues et al., 2016; Salles et al., 2017; Rodrigues et al., 2017b), our results showed that sulfate differentially interfered with the TG response in 60-fold diluted human plasma activated by cephalin (intrinsic coagulation pathway) as recorded continually at 37°C for 60 min., in comparison with UHEP used as a reference. No activator response of TG in plasma in the absence of cephalin (negative control) was found *in vitro* during 60 min (Figures 3B, C, D) (Rodrigues et al., 2016).

All the three SPs fractions (-SP1, -SP2 and Cc-SP3) from *C. cupressoides* in the range of concentrations tested in the experiment prevented thrombosis *in vitro* in a concentration-dependent manner with basis on the amidolytic activity of thrombin that decreased rapidly until a plateau was reached (controls: 35 min.) using normal diluted plasma as a similar almost inhibitory profile found for other classes of polyglycans (Rodrigues et al., 2016; Salles et al., 2017), although also using other experimental protocols (Nishino et al., 1999; Glauser et al., 2009).

Clearly, Cc-SP2 had the strongest TG inhibition compared to the other fractions tested, totally abolishing it at highest concentrations (> 4.1 µg well-plate^{-1}) as analyzed by the PTh parameter compared with the control without SPs (TPeak: 35 mim.), whereas at 4.1 µg.well-plate^{-1} this fraction modulated PTh by 33.87% at 40 min. and showed a lag time for 32 min. vs. cephalin control (28 min.) (Figure 3C). Similar behavior for some SPs from seaweeds that manifested *in vivo* antithrombotic actions was also reported in literature (Mourão, 2015).

Interestingly, the effect of Cc-SP1 and Cc-SP3 on TG induced by the intrinsic pathway activator in diluted human plasma was shown to be similar (Figures 3B, D). At low concentrations (4.1 and 8.3 µg well-plate^{-1}), these fractions inhibited TG by 27.42→35.48 and 56.45%, respectively, considering PTh from 41 to 43 min., and prolonging the lag time (28→34 min.) vs. controls (26 min.), revealing a direct correlation with the TG parameters (Rodrigues et al., 2016; Salles et al., 2017). A complete suppression of TG by fractions

was observed at high amounts (41.6 and 83.3 μg.well-plate^{-1}) from this plasma system. This *in vitro* inhibition degree of the fractions on the intrinsic coagulation pathway of TG stimulated by cephalin did not occur on the same elution order, where fraction Cc-SP3 (eluted at 1 M NaCl) with high sulfation exhibited almost equal inhibitory effects compared with the fraction Cc-SP1, which revealed a comparatively lower sulfation.

It could be a consequence of seaweeds SPs showing similar structure (Rodrigues et al., 2014a) and equivalent size chain (Table 1), but with slight differences in sulfation levels (Mourão, 2015). As a result, it speculating specific bindings of these glycans with plasma proteins to their molecular interactions in the haemostatic system preventing thrombin formation *in vitro* at differential inactivation levels (Nishino et al., 1999; Pomin, 2012; Rodrigues et al., 2013; Mourão, 2015), but without totally excluding any influence of the molecular weight on these processes since their actions may require length of the polysaccharide chain necessary to decrease TG (Rodrigues et al., 2016).

Since thrombin exerts an essential role in thrombosis and hemostasis (Castoldi & Rosing, 2011; Duarte, Ferreira, Rios, Reis, & Carvalho, 2017), our observations did not support the APTT results that were of limited value to measure thrombin formation in plasma (Figure 3A) (Castoldi & Rosing, 2011; Jung et al., 2014). It was raised the hypothesis that galactose-rich SPs displayed as potent thrombin inhibitors (Pomin, 2012; Wang et al., 2014; Mourão, 2015) due to the presence of 6-sulfate (Figure 1) as a structural feature for anticoagulant action in *Caulerpa* species (Hayakawa et al., 2000; Ghosh et al., 2004; Rodrigues et al., 2013; Wang et al., 2014).

For this reason, the effects of the fractions may be attributed to complex interactions with thrombin-mediated both by HCII and AT (Rodrigues et al., 2013), whereas UHEP was capable of binding with high affinity for AT due to its pentasaccharide not found in other natural sources (Pomin, 2012; Mourão, 2015) to act on TG (Jung et al., 2014). In fact, UHEP (2 μg well-plate^{-1}) abolished TG with an amount of SPs of at least 4.15-fold lower than those of Cc-SP2 (8.3→83.3 μg well-plate^{-1}) (Figures 3C), as observed in terms of concentration of SPs from the red seaweed *A. muscoides* (Rodrigues et al., 2016) and from the skin of Nile tilapia (*Oreochromis niloticus*) (Salles et al., 2017) as modulators of TG.

The algal SPs concentration was 487.80-fold lower than the antithrombotic dose in another study. It was previously documented an AT-dependent SP (Cc-SP2) from *C. cupressoides* with *in vivo* antithrombotic effects using a model of venous thrombosis in rats, but without presenting *in vivo* hemorrhagic effect (Rodrigues et al., 2011b). As the extension of plasma coagulability displayed by *C. cupressoides* SPs was referred to as an anticoagulative response (Figure 3), the investigation of their anticoagulant dynamics employing *in vitro* TG assay seemed to be of practical use because *in vivo* models of thrombosis in experimental animals are always a laborious methodology (Pomin & Mourão, 2008; Pomin, 2012; Mourão, 2015).

It is possible that the specificity of these low-molecular-weight fractions of *C. cupressoides* SPs added to diluted normal human plasma delaying the TG may also be involved in their *in vivo* anti-inflammatory effects blocking the function of adhesion molecules, such as P- and L-selectins (Rodrigues et al., 2012b; Rodrigues, Chaves, Alves, Filgueiras, Bezerra, & Benevides, 2014), because thrombin interplays between coagulation and inflammation (Pomin, 2012). On the basis of pharmacological application, our *in vitro* TG alternative assay could perhaps be an additional tool for developing *C. cupressoides* SPs-based functional biomaterials with benefits in prevention of circulatory disorders (Patel, 2012; Mourão, 2015), since thrombin activity in a plasma sample is also monitored by automated methods revealing clinical potential (Durate etal., 2017).

Seaweeds usually express large-molecular-size SPs as activators of Factor XII predicting hypotension and this property has been found as a prothrombotic response of polyglycans at high concentrations, when assessed in experimental models of venous thrombosis *in vivo* (Pomin, 2012; Rodrigues et al., 2011b; Mourão, 2015). As low-molecular-weight fragments are more promising as new antithrombotic drugs (Glauser et al., 2009; Rodrigues et al., 2016), our study was also directed to examine the fraction Cc-SP2 on the *in vitro* TG system without cephalin as contact-activator and continually analyze regarding its possible stimulatory response, as illustrated in Figure 4.

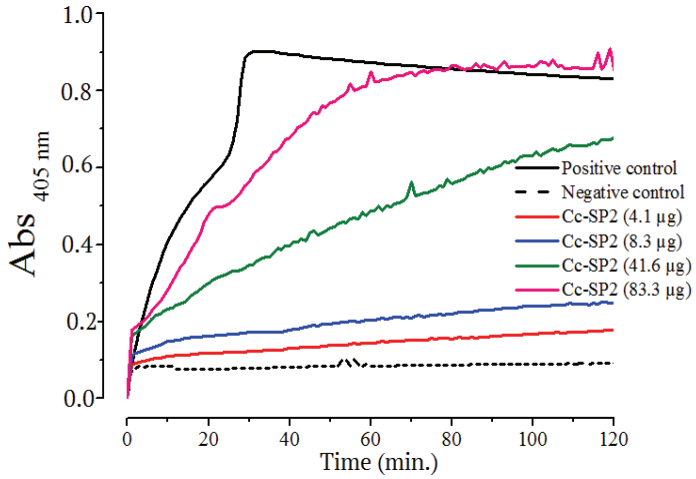

Figure 4. Profile of the TG-inducing fraction Cc-SP2 from the green seaweed *C. cupressoides*. Illustration shows the absorbance curves of the different concentrations of polysaccharide activating continually the coagulation *in vitro* in 60-fold diluted human plasma by cleavage of the substrate (120 min., 37°C).

Our results showed that Cc-SP2 was an important tool for predicting hypercoagulability with increasing concentrations of the sample in diluted plasma. As verified by absorbance of the thrombin formed (about 33 min, positive control), the total stimulation pattern of the coagulation by fraction Cc-SP2 was achieved at 83.3 µg well-plate^{-1} of polysaccharide (100% induction) (Figure 4). This activation of Cc-SP2 in plasma occurred in a concentration-dependent manner, possibly in connectivity with its high-molecular-mass SPs (Figure 2C, D and Table 1) (Pomin & Mourão, 2008; Mourão, 2015). UHEP did not induce this system because of its potent antithrombin-mediated anticoagulant effect (data not shown) as expected (Jung et al., 2014; Barcellos et al., 2018) vs. others polysaccharides that are known to activate coagulation via the contact pathway at high concentrations (Zhang et al., 2014; Mourão, 2015; Barcellos et al., 2018).

A substantial delay in TG initiation by fraction Cc-SP2 at concentrations < 83.3 µg well-plate^{-1} compared with the positive control absorbance (cephalin) was also noted, but with total activation of the coagulation by fraction Cc-SP2 at 83.3 µg well-plate^{-1} (ca. 80 min.) vs. the baseline values of the negative control without cephalin. Comparison of TG profiles in the presence and absence of cephalin confirmed those dual effects found for the SPs from *C. cupressoides* preventing or inducing venous thrombosis *in vivo* (Rodrigues et al., 2011b), but under other point of view, because TG study may be limited since the *in vitro* conditions do not reflect the physiological environment (Luna-Záizar et al., 2014).

Recently, Rodrigues et al. (2017a) revealed that the fraction Cc-SP1 from *C. cupressoides* simultaneously modulated viral infections and TG *in vitro*. Current findings pointed to an almost concomitance of the biological responses on TG by *C. cupressoides* SPs. Fraction Cc-SP2, with large-molecular-size distribution and highest sulfate content, had an effective concentration range as a function of density charge for TG inhibition and possibly contact pathway-stimulated TG properties associated in parallel with the molecular size distribution, and the balance of these two effects *in vitro* deserve be better clarified in future studies (Mourão, 2015). On the other hand, our TG system demonstrated to be a starting phase for detecting distinct biological effects using SPs from Chlorophyta species, allowing an essential step for the development of safe new antithrombotic drugs (Barcellos et al., 2018) and for other prognostics (Castoldi & Rosing, 2011; Mourão, 2015; Duarte et al., 2017).

Given the food importance and environmental of *Caulerpa* species in several regions of the world, *C. cupressoides* features SPs arise as a promising source of functional glycans in the modulation of blood coagulation disorders, since an increase of plasma prothrombin's referential values increase the TG after activation by the intrinsic pathway (Castoldi & Rosing, 2011; Rodrigues et al., 2016). Studies of this nature could contribute to a potential strategy to marine macroalgae-derived functional polymer design to enhance the security of food preparations, reducing the risks of clotting dysfunctions (Pomin & Mourão, 2008; Patel, 2012; Gaillande et al., 2017). Nevertheless, our observations also allow to assumption the fact to the hypercoagulant effect manifested by native polymer present in *C. cupressoides*.

Conclusion

The coenocytic green seaweed *Caulerpa cupressoides* contains a heterogeneous system of complex glycans varying in sulfation and molecular mass (from 8 to > 100 kDa). Acid treatment (0.4 M NaCl) reveals stability of this polysaccharide system. In 60-fold diluted human plasma, the sulfated polysaccharides act as inhibitors of thrombin generation *in vitro* by the intrinsic pathway dependent on charge less potent than unfractionated heparin. However, increasing concentrations of the polysaccharides stimulate thrombin generation in the absence of cephalin, predicting a risk of thrombosis. This combined information arises as another analytical approach *in vitro* to investigate natural antithrombotics.

Acknowledgements

We thank the funding from PNPD/CAPES, FAPERJ, CNPq, and MCTIC. Benevides, N. M. B. and Mourão, P. A. S. are senior investigators of CNPq/Brazil.

References

Arata, P. X., Quintana, I., Canelón, D. J., Vera, B. E., Compagnone, R. S., & Ciancia, M. (2015). Chemical structure and anticoagulant activity of highly pyruvylated sulfated galactans from tropical green seaweeds of the order Bryopsidales. *Carbohydrate Polymers, 122*(1), 376-386. doi: 10.1016/j.carbpol.2014.10.030

Arenajo, A. R., Ybañez, A. P., Ababan, M. M. P., Villajuan, C. E., Lasam, M. R. M., Young, C. P., & Reyes, J. L. A. (2017). The potential anticoagulant property of *Caulerpa lentillifera* crude extract. *International Journal of Health Sciences, 11*(3), 29-32. PMID: 28936148

Athukorala,Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydrate Polymers, 66*(2), 184-191. doi: 10.1016/j.carbpol.2006.03.002

Barcellos, P. G., Rodrigues, J. A. G., Queiroz, I. N. L., Araújo, I. W. F., Benevides, N. M. B., & Mourão, P. A. S. (2018). Structural and physical-chemical analyses of sulfated polysaccharides from the sea lettuce *Ulva lactuca* and their effects on thrombin generation. *Acta Scientiarum. Biological Sciences, 40*(1), e349161-12. doi: 10.4025/actascibiolsci.v40i1.34916

Brieger, D. B., Mak K-H., Kottke-Marchant, K., & Topol, E. J. (1998). Heparin-induced thrombocytopenia. *Journal of American College of Cardiology, 31*(7), 1449-1459. doi: 10.1016/S0735-1097(98)00134-X

Carneiro, J. G., Rodrigues, J. A. G., Teles, F. B., Cavalcante, A. B. D., & Benevides, N. M. B. (2014). Analysis of some chemical nutrients in four Brazilian tropical seaweeds. *Acta Scientiarum. Biological Sciences, 36*(2), 137-145. doi: 10.4025/actascibiolsci.v36i2.19328

Castoldi, E., & Rosing J. (2011). Thrombin generation tests. *Thrombosis Research, 127*(Suppl. 3), S21-S25. doi: 10.1016/S0049-3848(11)70007-X

Dietrich, C. P., & Dietrich, S. M. C. (1976). Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Analytical Biochemistry, 70*(2), 645-647. doi: 10.1016/0003-2697(76)90496-6

Duarte, R. C. F, Ferreira, C. N., Rios, D. R. A., Reis, H. J., & Carvalho, M. G. (2017). Thrombin generation assays for global evaluation of the hemostatic system: perspectives and limitations. *Brazilian Journal of Hematology and Hemotherapy*, *39*(3), 259-265. doi: 10.1016/j.bjhh.2017.03.009

Fidelis, G. P., Camara, R. B. G., Queiroz, M. F., Costa, M. S. S. P., Santos, P. C., Rocha, H. A. O., & Costa, L. S. (2014). Proteolysis, NaOH and ultrasound-enhanced extraction of anticoagulant and antioxidant sulfated polysaccharides from the edible seaweed. *Gracilaria birdiae*. *Molecules, 19*(11), 18511-18526. doi: 10.3390/molecules191118511

Gaillande, C., Payri, C., Remoissenet, G., & Zubia, M. (2017). *Caulerpa* consumption, nutritional value and farming in the Indo-Pacific region. *Journal of Applied Phycology, 29*(5), 2249-2266. doi: 10.1007/s10811-016-0912-6.

Ghosh, P., Adhikari, U., Ghossal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B., & Ray, B. (2004). *In vitro* anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*. *Phytochemistry, 65*(23), 3151-3157. doi: 10.1016/j.phytochem.2004.07.025

Glauser, B. F., Rezende, R. M., Melo, F. R., Pereira, M. S., Fransischetti, I. M. B., Monteiro, R. Q., ... Mourão, P. A. S. (2009). Anticoagulant activity of a sulfated galactan: Serpin-independent effect and specific interaction with factor Xa. *Thrombosis and Haemostasis, 102*(6), 1183-1193. doi: 10.1160/TH09-04-0273

Hayakawa, Y., Hayashi, T., Lee, J. B., Srisomporn, P., Maeda, M., Ozawa, T., & Sakuragawa, N. (2000). Inhibition of thrombin by sulfated polysaccharides isolated from green algae. *Biochimica et Biophysica Acta, 1543*(1), 86-94. doi: 10.1016/S0167-4838(00)00193-X

Ji, H., Shao, H., Zhang, C., Hong, P., & Xiong, H. (2008). Separation of the polysaccharides in *Caulerpa racemosa* and their chemical composition and antitumor activity. *Journal of Applied Polymer Science, 110*(3), 1435-1440. doi: 10.1002/app.28676

Jun, W., Ru, Z. H., Ying, Z. H., Ling, G. Y., Shuang, Q., Jie, Z., ... Song, S. (2014). Thrombin generation increasing with age and decreasing with use of heparin indicated by calibrated automated thrombogram conducted in Chinese. *Biomedical and Environmental Sciences, 27*(5), 378-384. doi: 10.3967/bes2014.063

Kumar, A., Krishnamoorthy, E., Devi, H. M., Uchoi, D., Tejpal, C. S., Ninan, G., & Zynudheen, A. A. (2018). Influence of sea grapes (*Caulerpa racemosa*) supplementation on physical, functional, and anti-oxidant properties of semi-sweet biscuits. *Journal of Applied Phycology*, 30(2), 1393-1403. doi: 10.1007/s10811-017-1310-4

Liang, W. S., Liu, T. C., Chang, C. J., & Pan, C. L. (2015). Bioactivity of β-1,3-xylan extracted from *Caulerpa lentillifera* by using *Escherichia coli* ClearColi BL21 (DE3)-β-1,3-xylanase XYLH. *Journal of Food and Nutrition Research, 3*(7), 437-444. doi: 10.12691/jfnr-3-7-5

Luna-Záizar, H., Beltrán-Miranda, C. P., Esparza-Flores, M. A., Soto-Padilla, J., Bergés-García, A., Rodríguez-Zepeda, M. D. C., ... Jaloma-Cruz, A. R. (2014). Thrombin generation as objective parameter of treatment response in patients with severe haemophilia A and high-titre inhibitors. *Haemophilia, 20*(1), e7-e14. doi: 10.1111/hae.12309

Maciel, J. S., Chaves, L. S., Souza, B. W. S., Teixeira, D. I. A., Freitas, A. L. P., Feitosa, J. P. A., & Paula, R. C. M. (2008). Structural characterization of cold extracted fraction of soluble sulfated polysaccharides from red seaweed *Gracilaria birdiae*. *Carbohydrate Polymers, 71*(4), 559-565. doi: 10.1016/j.carbpol. 2007.06.026

Maeda, R., Ida, T., Ihara, H., & Sakamoto, T. (2012). Immunostimulatory activity of polysaccharides isolfated from *Caulerpa lentillifera* on macrophage cells. *Bioscience Biotechnology and Biochemistry, 76*(3), 501-505. doi: 10.1271/bbb.110813

Mourão, P. A. S. (2015). Perspective on the use of sulfated polysaccharides from marine organisms as a source of new antithrombotic drugs. *Marine Drugs, 13*(5), 2770-2784. doi: 10.3390/md13052770

Nishino, T., Fukuda, A., Nagumo, T., Fujihara, M., & Kaji, E. (1999). Inhibition of the generation of thrombin and factor Xa by a fucoidan from the brown seaweed *Ecklonia kurome*. *Thrombosis Research, 96*(1), 37-49. doi: 10.1016/S0049-3848(99)00060-2

Patel, S. (2012). Therapeutic importance of sulfated polysaccharides from seaweeds: updating the recent findings. *3 Biotech, 2*(3), 171-185. doi: 10.1007/s13205-012-0061-9

Pineo, G. F., & Hull, R. D. (1998). Heparin and low-molecular-weight heparin in the treatment of venous thromboembolism. *Baillière's. Clinical Haematology, 11*(3), 621-637. PMID: 10331096

Pires, C. L., Rodrigues, S. D., Bristot, D., Gaeta, H. H., Toyama, D. O., Farias, W. R L., & Toyama, M. H. (2013). Evaluation of macroalgae sulfated polysaccharides on the *Leishmania (L.) amazonensis* promastigote. *Marine Drugs, 11*(3), 934-943. doi: 10.3390/md11030934

Pomin, V. H. (2012). Fucanomis and galactanomics: Current status in drug discovery, mechanisms of action and role of the well-defined structures. *Biochimica et Biophysica Acta, 1820*(12), 1971-1979. doi: 10.1016/j.bbagen.2012.08.022

Pomin, V. H., & Mourão, P. A. S. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology, 18*(12), 1016-1027. doi: 10.1093/glycob/cwn085

Rivanor, R. L. C., Chaves, H. V., Val, D. R., Freitas, A. R., Lemos, J. C., Rodrigues, J. A. G., ... Benevides, N. M. B. (2014). A lectin from the green seaweed *Caulerpa cupressoides* reduces mechanical hyper-nociception and inflammation in the rat temporomandibular joint during zymosan-induced arthritis. *International Immunopharmacology, 21*(1), 34-43. doi: 10.1016/j.intimp.2014.04.009

Rodrigues, J. A. G., Queiroz, I. N. L., Quinderé, A. L. G., Vairo, B. C., Mourão, P. A. S., & Benevides, N. M. B. (2011b). An antithrombin-dependent sulfated polysaccharide isolated from the green alga *Caulerpa*

cupressoides has *in vivo* anti- and prothrombotic effects. *Ciência Rural, 41*(4), 634-639. doi: 10.1590/S0103-84782011000400014

Rodrigues, J. A. G., Vanderlei, E. S. O., Bessa, E. F., Magalhães, F. A., Paula, R. C. M., Lima, V., & Benevides, N. M. B. (2011a). Anticoagulant activity of a sulfated polysaccharide isolated from the green seaweed *Caulerpa cupressoides*. *Brazilian Archives of Biology and Technology, 54* (4), 691-700. doi: 10.1590/S1516-89132011000400007

Rodrigues, J. A. G., Quinderé, A. L. G., Queiroz, I. N. L., Coura, C. O., & Benevides, N. M. B. (2012a). Comparative study of sulfated polysaccharides from *Caulerpa* spp. (Chorophyceae). Biotechnological tool for species identification? *Acta Scientiarum. Biological Sciences, 34*(4), 381-389. doi: 10.4025/actascibiolsci.v34i4.8976

Rodrigues, J. A. G., Vanderlei, E. S. O., Silva, L. M. C. M., Araújo, I. W. F., Queiroz, I. N. L., Paula, G. A., ... Benevides, N.M.B. (2012b). Antinociceptive and anti-inflammatory activities of a sulfated polysaccharide isolated from the green seaweed *Caulerpa cupressoides*. *Pharmacological Reports, 64*(2), 282-292. doi: 10.1016/S1734-1140(12)70766-1

Rodrigues, J. A. G., Neto, E. M., Teixeira, L. A. C., Paula, R. C. M., Mourão, P. A. S., & Benevides, N. M. B. (2013). Structural features and inactivation of coagulation proteases of a sulfated polysaccharidic fraction from *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae, Chlorophyta). *Acta Scientiarum. Technology, 35*(4), 611-619. doi: 10.4025/actascitechnol. v35i4.16709

Rodrigues, J. A. G., Chaves, H. V., Alves, K. A., Filgueiras, A. A., Bezerra, M. M., & Benevides, N. M. B. (2014b). Structural features and assessment of zymosan-induced arthritis in rat temporomandibular joint model using sulfated polysaccharide. *Acta Scientiarum. Biological Sciences, 36*(2), 127-135. doi: 10.4025/actascibiolsci.v36i2.19342

Rodrigues, J. A. G., Neto, E. M., Santos, G. R. C., Paula, R. C. M.; Mourão, P. A. S., & Benevides, N. M. B. (2014a). Structural analysis of a polysaccharidic fraction obtained from the coenocytic green seaweed *Caulerpa cupressoides* var. *lycopodium*. *Acta Scientiarum. Technology, 36*(2), 203-210. doi: 10.4025/actascitechnol.v36i2.17866

Rodrigues, J. A. G., Queiroz, I. N. L., Quinderé, A. L. G., Benevides, N. M. B., Tovar, A. M. F., & Mourão, P. A. S. (2016). Mild-acid hydrolysis of a native polysulfated fraction from *Acanthophora muscoides* generates sulfated oligosaccharides displaying *in vitro* thrombin generation inhibition. *Acta Scientiarum. Biological Sciences, 38*(1), 7-15. doi: 10.4025/actascibiolsci.v38i1.28257

Rodrigues, J. A. G., Eloy, Y. R. G., Vanderlei, E. S. O., Cavalcante, J. F., Romanos, M. T. V., & Benevides, N. M. B. (2017a). An anti-dengue and anti-herpetic polysulfated fraction isolated from the coenocytic green seaweed *Caulerpa cupressoides* inhibits thrombin generation *in vitro*. *Acta Scientiarum. Biological Sciences, 39*(2), 149-159. doi: 10.4025/actascibiolsci.v39i2.28081

Rodrigues, J. A. G., Benevides, N. M. B., Tovar, A. M. F., & Mourão, P. A. S. (2017b). *In vitro* inactivation of thrombin generation by polysulfated fractions isolated from the tropical coenocytic green seaweed *Caulerpa racemosa* (Caulerpaceae, Bryopsidales). *Acta Scientiarum. Biological Sciences, 39*(3), 283-292. doi: 10.4025/actascibiolsci.v39i3.32095

Salles, T. C., Rodrigues, J. A. G., Barcellos, P. G., Amaral, G. F., Araújo, I. W. F., & Mourão, P. A. S. (2017). Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*. *Agrária - Revista Brasileira de Ciências Agrárias, 12*(1), 98-104. doi: 10.5039/agraria.v12i1a5412

Shanmugam, M., Ramavat, B. K., Mody, K. H., Oza, R. M., & Tewari, A. (2001). Distribution of heparinoides-active sulfated polysaccharides in some Indian marine Green algae. *Indian Journal of Marine Sciences, 30*(4), 222-227.

Vanderlei, E. S. O., Patoilo, K. K. N. R., Lima, N. A., Lima, A. P. S., Rodrigues, J. A. G., Silva, L. M. C. M., ... Benevides, N. M. B. (2010). Antinociceptive and anti-inflammatory activities of lectin from the marine green alga *Caulerpa cupressoides*. *International Immunopharmacology, 10*(9), 1113-1118. doi: 10.1016/j.intimp.2010.06.014

Volpi, N., & Maccari, F. (2002). Detection of submicrogram quantities of glycosaminoglycans on agarose gels by sequential staining with toluidine blue and Stains-All. *Electrophoresis, 23*(24), 4060-4066. doi: 10.1002/elps.200290021

Wang, L., Wang, X., Wu, H., & Liu, R. (2014). Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years. *Marine Drugs, 12*(9), 4984-5020. doi: 10.3390/md12094984

Zhang, H. J., Mao, W. J., Fang, F., Li, H. Y., Sun, H. H., Chen, Y., & Qi, X. H. (2008). Chemical characteristics and anticoagulant activities of a sulfated polysaccharide and its fragments from *Monostroma latissimum*. *Carbohydrate Polymers, 71*(3), 428-434. doi: 10.1016/j.carbpol.2007.06.012

Zhang, Z., Till, S., Jiang, C., Knappe, S., Reutterer, S., Scheiflinger, F., ... Dockal, M. (2014). Structure-activity relationship of the pro- and anticoagulant effects of *Fucus vesiculosus* fucoidan. *Thrombosis and Haemostasis, 111*(3), 429-437. doi: l0.1160/TH13-08-0635