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An anti-dengue and anti-herpetic polysulfated fraction isolated from the coenocytic green seaweed *Caulerpa cupressoides* inhibits thrombin generation *in vitro*

José Ariévilo Gurgel Rodrigues[1*], Ygor Raphael Gomes Eloy[2], Edfranck de Sousa Oliveira Vanderlei[3], Jéssica Figueiredo Cavalcante[4], Maria Tereza Villela Romanos[4] and Norma Maria Barros Benevides[1*]

*[1]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Avenida Mister Hull, s/n., 60455-970, Fortaleza, Ceará, Brazil. [2]Centro de Ciências da Saúde, Universidade de Fortaleza, Fortaleza, Ceará, Brazil. [3]Faculdade Nordeste, Fortaleza, Ceará, Brazil. [4]Laboratório de Drogas Antivirais e Citotóxicas, Departamento de Virologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil. *Author for correspondence. E-mail: arieviloengpesca@yahoo.com.br; nmbb@ufc.br*

ABSTRACT. The antiviral potency of *Caulerpa cupressoides* (Chlorophyta) sulfated polysaccharidic fractions (Cc-SP1→3) associated with thrombin generation (TG) assay is unknown. This study analyzed the structure of Cc-SP1 and its effects against herpes viruses (HSV-1 and HSV-2), dengue-virus (DENV-1), human-metapneumovirus (HMPV) and adenovirus (AdV) in Vero and mosquito C6/36 cell-lines and as inhibitor of TG in 60-fold diluted human plasma using continuous system. Infrared analysis indicated ulvan containing 11% sulfate, 40.16% total sugars and 6% uronic acid. Procedures of agarose/polyacrylamide gels electrophoresis revealed two major components presenting sulfate and non SPs, with molecular dispersion ranging from 8 to > 100 kDa, respectively, as confirmed by gel permeation chromatography. Cc-SP1 did not cause cytotoxicity up to 1000 μg mL^{-1} and was more effective inhibitor of DENV-1 (96%, 0.35 μg mL^{-1}) compared with HSV-1 (90%, 27 μg mL^{-1}) and HSV-2 (99.9%, 0.9 μg mL^{-1}) in Vero cell-line, whose selectivity indexes were > 714, > 9.2 and > 131, respectively, but was inactive against HMPV, AdV and C6/36 cell-line using plate reduction assay. Cc-SP1 required concentration 20.8-fold higher of SPs than heparin for abolishes intrinsically TG, but at concentrations so far the anti-HSV-1. Therefore, TG assay provides data to guide studies of Cc-SP1 on anticoagulant/antiviral effects.

Keywords: Bryopsidales, marine glycans, cytotoxicity, viral infection, thrombin.

Uma fração polissulfatada anti-dengue e anti-herpética isolada da alga marinha verde cenocítica *Caulerpa cupressoides* inibe geração de trombina *in vitro*

RESUMO. A potência antiviral de frações polissacarídicas sulfatadas de *Caulerpa cupressoides* (Chlorophyta) (Cc-PS1→3) é desconhecida e associada com ensaio de geração de trombina (GT). Analisaram-se de Cc-PS1 estrutura e efeitos contra herpesviroses (HSV-1 e HSV-2), vírus da dengue (DENV-1), metapneumovírus-humano (MPVH) e adenovírus (AdV) em linhagens de células Vero e de mosquito C6/36 e como inibidora de GT em plasma humano diluído 60 vezes usando sistema contínuo. Análise de infravermelho indicou 'ulvana' contendo sulfato (11%), açúcares totais (40,16%) e ácido urônico (6%). Procedimentos de electroforese em géis de agarose/poliacrilamida revelaram dois componentes majoritários apresentando sulfato e polissacarídeos não sulfatados, com dispersão molecular variando de 8 a > 100kDa, respectivamente, confirmada por cromatografia de permeação em gel. Cc-PS1 não causou citotoxicidade até 1000 μg mL^{-1} e se mostrou, em linhagem de célula Vero usando ensaio de redução de placas, inibidora efetiva de DENV-1 (96%; 0,35 μg mL^{-1}) comparada com HSV-1 (90%; 27 μg mL^{-1}) e HSV-2 (99,9%; 0,9 μg mL^{-1}), cujos índices de seletividade foram > 714; > 9,2 e > 278, respectivamente, enquanto, demonstrou-se ineficaz sobre HMPV, AdV e linhagem de célula C6/36. Foi requerida concentração 20,8 vezes maior de Cc-PS1 que heparina para abolir GT intrinsicamente, mas em concentrações diferentes da anti-HSV-1. Portanto, ensaio de GT fornece dados para estudos direcionados sobre efeitos anticoagulante/antiviral de Cc-PS1.

Palavras-chave: Bryopsidales, glicanos marinhos, citotoxicidade, infecções virais, trombina.

Introduction

Dengue virus (DENV) is an enveloped virus (family Flaviviridae) transmitted by the mosquitoes *Aedes aegypti* and *A. albopictus* that cause several infectious diseases (e.g., dengue fever and dengue hemorrhagic fever), especially in tropical regions. Four different serotypes (DENV-1→4) have been

identified as a result of changes in its ecology/resurgence caused by the demographic impact of modern societies in the last decades, where more than two billion people are at risk of infection (Gubler, 2002). Herpes viruses types I (HSV-1) (also known as herpes labialis) and 2 (HSV-2) (also known as genital herpes) have also been widely recognized as important human pathogenic agents, being commonly transmitted through direct contact (e.g., lesions on the lips, eyes, or genitalia) (Kleymann, 2005). Acute respiratory infections caused by human metapneumovirus (HMPV) and adenovirus (AdV) are considered as a public health problem (Monto, 2002). However, no specific pharmacological agent for treatment and prevention of DENV infection has been established due to hemorrhage and toxicity manifested by therapeutics available. In addition, acyclovir is currently used in systemic or topical therapy for the HSVs treatment; but, prolonged therapies of patients have resulted in emergence of drug-resistant viruses (Kleymann, 2005). No antiviral therapy is available for HMPV and AdV infections. Thus, there is a demand to develop alternative agents (Ghosh et al., 2009, Vo, Ngo, Ta, & Kim, 2011).

Algae (macro and microalgae), sponges, tunicates, echinoderms, mollusks, shrimps, bacteria and fungi contain diverse classes of metabolites (e.g., sulfated polysaccharides-SPs, alkaloids, polyphenols, terpenes and glycolipids) (Ghosh et al., 2009, Vo et al., 2011, Patel, 2012) of great economical importance (Cardozo et al., 2007, Gupta & Abu-Ahannam, 2011, Toskas et al., 2011) to develop pharmaceutics for various diseases (Vo et al., 2011, Pomin, 2012, Mourão, 2015). Of all bioproducts, SPs from algae have revealed as potent tools against several viruses, including human immunodeficiency virus type 1 in MT-4 cell-line (Hasui, Matsuda, & Okutami, 1995), HSV-1 and HSV-2 in Vero cell-line (Mazumder et al., 2002, Ghosh et al., 2004, Cassolato et al., 2008, Vanderlei et al., 2016), white spot syndrome virus in black tiger shrimp *Penaeus monodon* (Chotiaget, Tongsupa, Supamataya, & Phongdara, 2004), DENV-2→4 in Vero, HepG2 or PH cell-line (Talarico et al., 2005, Talarico, Noseda, Ducatti, Duarte, & Damonte, 2011, Pujol, Ray, Ray, & Damonte, 2012), duck enteritis virus (Song et al., 2013) and Rift valley fever virus (Gomaa & Elshoubaky, 2016) in Vero cell-line, and in other types of viruses as described elsewhere, depending on the structural class of complex polysulfated (Ghosh et al., 2009, Vo et al., 2011, Patel, 2012, Pérez, Falqué, & Dominguez, 2016).

Although having various beneficial effects (e.g., antioxidant, antithrombotic and anti-inflammatory effects) (Gupta & Abu-Ahannam, 2011, Pomin, 2012, Mourão, 2015), SPs are highly complex and heterogeneous polymers varying according to algal species (Cardozo et al., 2007, Toskas et al., 2011, Pomin, 2012, Rodrigues et al., 2017), and are not well-explored (Ghosh et al., 2009, Pomin, 2012, Mourão, 2015). Sulfated galactans occur in Rhodophyceae (Mazumder et al., 2002, Cardozo et al., 2007, Amorim et al., 2012, Souza et al., 2015), whereas fucoidans or fucans are available in Phaeophyceae (Chotigeat et al., 2004, Athukorala, Jung, Vasanthan, & Jeon, 2006, Pomin, 2012). The genera of green algae *Ulva*, *Enteromorpha*, *Monostroma*, *Codium, Caulerpa* and *Gayralia* contain ulvan-type heteropolysaccharides (Patel, 2012, Wang, Wang, Wu, & Liu, 2014, Pérez et al., 2016). Other living organisms have also SPs (Mourão et al., 2001, Aquino, Landeira-Fernandez, Valente, Andrade, & Mourão, 2005, Dantas-Santos et al., 2012, Chang, Lur, Lu, & Cheng, 2013).

Testing on classical clotting assays, like activated partial thromboplastin time (APTT), SPs frequently display anticoagulation by inhibition of the initiation phase of coagulation (Ghosh et al., 2009, Dantas-Santos et al., 2012, Pomin, 2012, Fidelis et al., 2014), but these tests do not reflect the coagulation intrinsic factors and the accurately thrombin generated in plasma (Castoldi & Rosing, 2011); therefore the *in vitro* effects on these experimental systems are related to their sulfate content and molecular mass (Pomin, 2012). An approach to appropriately evaluate the coagulant status are thrombin generation (TG) assays for preclinical screening (e.g., bleeding or thrombotic risk) (Castoldi & Rosing, 2011) and development of diverse classes of new anticoagulants (Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999, Mourão et al., 2001, Rodrigues et al., 2016, Rodrigues et al., 2017, Salles et al., 2017) since the thromboembolic events require primarily anticoagulant therapy with limited use of heparin due to its high rates of bleeding episodes and other risk factors (e.g., thrombocytopenia) (Mourão, 2015). However, no literature datum has shown association between *in vitro* tests of viral infection and TG using SPs.

Caulerpa genus J. V. Lamouroux (1809) (order Bryopsidales) plays a relevant role in various coastal ecosystems worldwide. Massive *Caulerpa* blooms lead to ecological disturbances on coral reefs and lagoons as a result of anthropogenic land-based nutrient pollution (Lapointe & Bedford, 2010); however, they are promising sources of structurally

diverse antiviral metabolites (Ghosh et al., 2004, Pujol et al., 2012, Wang et al., 2014). From the species *C. cupressoides* C. Agardh, there are still few data on its chemodiversity and pharmacology (Carneiro, Rodrigues, Teles, Cavalcante, & Benevides, 2014a, Rodrigues et al., 2014b). Focusing on its SPs, three fractions (Cc-SP1, Cc-SP2 and Cc-SP3) have been isolated (Rodrigues et al., 2011b). Cc-SP2 had anticoagulant effect (Rodrigues et al., 2011b) dependent of serpins (Rodrigues et al., 2013a), anti-/prothrombotic (Rodrigues et al., 2011a), antinociceptive and anti-inflammatory (Rodrigues et al., 2012) effects (*in vitro* and/or *in vivo*). Cc-SP1 and Cc-SP3 showed no *in vitro* anticoagulant actions on APTT assays (Rodrigues et al., 2011b), but *in vivo* antinociceptive and anti-inflammatory efficacies of Cc-SP1 have been more recently reported as biological responses at doses that did not influence the normal APTT (Rodrigues et al., 2013b, 2014a) and the administered dose for the lack of toxicity in mice (Rodrigues et al., 2013b).

In this study, the efficacy of Cc-SP1 was tested *in vitro* against some viruses (DENV-1, HSV-1, HSV-2, HMPV and AdV) and the anticoagulation by an *in vitro* TG assay; it was also analyzed the type of SP present in its structure by Fourier Transform Infrared (IR) Spectroscopy.

Material and methods

Marine algae and Cc-SP1 isolation

Caulerpa cupressoides C. Agardh was manually collected at Flecheiras beach, municipality of Trairí, Ceará State, in the Northeastern Brazilian coast in July 2010, and then separated from macroscopic epiphytes, washed in distilled water to eliminate marine salt, and stored (-20°C) until use. Species was identified by PhD. José Ariévilo Gurgel Rodrigues from the Department of Fisheries Engineering (UFC) and a voucher specimen (#4977) was then deposited at the Prisco Bezerra Herbarium of the *Universidade Federal do Ceará*. The current study did not involve endangered or protected species and was conducted in accordance with the law MP 2186-16/2001, resolution 29 of the Dispatch Component of Genetic Patrimony.

The dehydrated *C. cupressoides* tissue at room temperature was subjected to papain digestion (30 mg mL^{-1}, 60°C, 6 hours) (Vetec Química), and then a sample of the crude SP extract was fractionated on a DEAE-cellulose column (Sigma Chemical) using stepwise elution with 50 mm acetate sodium (Vetec Química) buffer (pH 5.0) containing 0→1.0 M NaCl (Vetec Química). Fractions Cc-SP1, Cc-SP2 and Cc-SP3 were monitored by the metachromasy assay and Cc-SP1 was freeze-dried in a Labonco FreeZone 4.5 apparatus and analyzed for sulfate, total sugars, contaminant protein and uronic acid. Electrophoretic techniques on agarose gel and polyacrylamide gel (PAGE) were performed and sulfate and nonSPs were revealed by sequential staining with toluidine blue/stains-all (Sigma Chemical) (Volpi & Maccari, 2002) by comparison with the electrophoretic mobility of standard compounds dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa), chondroitin-6-sulfate (~ 60 kDa) (Rodrigues et al., 2013a) and/or unfractionated heparin (UHEP, ~ 14 kDa) (Sigma Chemical) (Volpi & Maccari, 2002). Its peak molar masses were estimated by gel permeation chromatography (GPC) using pullulan 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}) (Sigma Chemical). Protocols were conducted as previously published (Rodrigues et al., 2011b, 2013a, 2014a) and some characteristics of ulvan were identified by IR spectroscopy (Toskas et al., 2011).

Cells and viruses

Vero (African green monkey kidney, Rio de Janeiro Cell Bank) cell-line monolayers were grown in Eagle's minimum essential medium (Eagle-MEM) supplemented with 2 mm L-glutamine, 50 μg mL^{-1} garamicin, 2.5 μg mL^{-1} fungizon, 10 mm HEPES plus, 10% of heat-inactivated fetal bovine serum (FBS) (Sigma Chemical) (Schmidt, 1979) and maintained at 37°C in an atmosphere of 5% CO_2. DENV-1 was obtained from Laboratory of Respiratory Virus, and Enteric Eye, Department of Virology, *Universidade Federal do Rio de Janeiro*, Brazil. HSV-1 and HSV-2 were isolated from typical lip lesion and genital lesion, respectively, in the Virology Department of the Federal University of Rio de Janeiro, Brazil. HMPV and AdV were kindly provided by ViroNovative BV, Erasmus University Rotterdam, Netherlands. The C6/36 HT mosquito cell-line from *A. albopictus* (Rio de Janeiro Cell Bank) was cultured in L-15 medium (Leibovitz) + RPMI supplemented with 0.3% tryptose phosphate broth, 1% Eagle-MEM non-essential amino acids solution and 5% fetal calf serum. Virus stocks were obtained by propagation in Vero and C6/36 HT cell-lines, and they were titrated by plaque forming assay.

In vitro cytotoxicity and antiviral assays

The cytotoxicity assay was performed prior to antiviral tests by incubating triplicate Vero or C6/36 HT cell-line monolayers cultured in 96-well

microplates with two-fold serial dilutions of the Cc-SP1 (0-1000 μg mL^{-1}) and acyclovir at 37°C for 48 hours (5% CO_2) (Vanderlei et al., 2016). The morphological alterations of the treated cells were observed in an inverted optical microscope. Cell viability was evaluated by the neutral red dye-uptake method (Borenfreund & Puerner, 1985). The 50% cytotoxic concentration (CC_{50}) was calculated as the dilution that caused a reduction of 50% in the number of viable cells (Vanderlei et al., 2016).

The antiviral effect of Cc-SP1 and acyclovir was evaluated by the titer reduction (Garrett et al., 2012). The virus titers were calculated using the Reed and Muench statistical method and expressed as 50% tissue culture infective dose ($TCID_{50}$) per mL. Vero or C6/36 HT cell-line monolayers were treated with the Cc-SP1 or acyclovir (0-1000 μg mL^{-1}) at the maximum non-toxic concentration (MNTC) (Pérez et al., 2016) and 100 $TCID_{50}$ mL^{-1} of DENV-1, HSV-1, HSV-2, HMPV or AdV suspensions were added to treated and untreated cell cultures and incubated at 37°C for 48 hours (5% CO_2). After incubation, the supernatant was collected and virus titers in treated and untreated cells were determined. The antiviral effect was expressed as percentage inhibition (PI) using antilogarithmic $TCID_{50}$ values, as follows Equation 1:

$$PI = [1-(\text{antilogarithmic test value}/\text{antilogarithmic control value})] \times 100 \quad (1)$$

The dose-response curve was established starting from the MNTC, and the 50% effective concentration (EC_{50}) was defined as the concentration required for 50% protection against virus-induced cytopathic effects. The selectivity index (SI) was determined as the ratio between CC_{50} and EC_{50}. The experiment was performed in triplicate (Vanderlei et al., 2016).

Activated partial thromboplastin time (APTT) and TG assays

Cc-SP1 was tested *in vitro* on the APTT using normal citrated human plasma (different donors), according to the manufacturer specifications, for confirming primarily the lack of anti-clotting effect (Rodrigues et al., 2011b) in a coagulometer Amelung KC4A before of the *in vitro* TG assay. UHEP (193 IU mg^{-1}) (fourth International Standard (85/502)) from the National Institute for Biological Standards and Control (Potters Bar, UK) was used as a reference.

TG assay was performed in a microplate format, containing: 10 μL of cephalin (contact-activator system) + 30 μL 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 µL SPs (Cc-SP1: 0, 4.1, 8.3, 41.6 or 83.3 μg well-plate^{-1} or 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 addition of plasma (diluted 60-fold well-plate^{-1}) (10 µL), and the absorbance (405 nm) was recorded for 80 min (Molecular Devices, Menlo Park, CA, USA). The inhibitory response of TG by SPs was determined by *lag* time, peak thrombin and time to peak (Rodrigues et al., 2016).

Results and discussion

A *C. cupressoides* polysulfated fraction (Cc-SP1), eluted at 0.5 M NaCl from the DEAE-cellulose column, which contained 11 sulfate, 40.16 total sugars, 6% uronic acid and no contaminant proteins, revealed SPs with similar structural features compared to those described by Rodrigues et al. (2011b, 2013a, 2014b), who previously examined Cc-SP1 regarding its separation and partial characterization. This chemical analysis of Cc-SP1 was in accordance with those found for ulvan-yielded Chlorophyta (sulfate: 9-29.5%; total sugars: 39.8-79.9%; uronic acid: 4-19%) (Ghosh et al., 2004, Cassolato et al., 2008, Toskas et al., 2011, Wang et al., 2014). Treatment with protease eliminated the high crude protein level as previously estimated (20.79 ± 0.58 g 100 g^{-1} dehydrated weigh) by Carneiro et al. (2014a) and was also important to note that the use of cetylpyridinium chloride as cationic solvent (Rodrigues et al., 2011b) had a specific interaction with the ester sulfate groups of the SPs to discard other components of the algal biomass, including pigments and lipids, since these constituents require nonpolar organic solvents for their co-isolation with the SPs (Souza et al., 2015). The application of enzymes would result in purity and enhanced yield of bioactive compounds for biotechnology (Athukorala et al., 2006, Wang et al., 2014).

As expected, Cc-SP1 did not alter the normal APTT values (data not shown) (Rodrigues et al., 2011b). In contrast, our group also demonstrated the Cc-SP1 pharmacological value on chemically-induced nociception and inflammation devoid of systemic damage *in vivo* (Rodrigues et al., 2013b, 2014a). Because the use of anticoagulants could lead to a more extensive hemorrhage risk on the focus of clinical evaluation (Ghosh et al., 2009), the fraction Cc-SP1 (that also showed a good yield compared with Cc-SP2 and

Cc-SP3) (Rodrigues et al., 2013b) was chosen for the antiviral studies. In parallel, Cc-SP1 was more accurately investigated in relation to its possible actions on TG as another experimental strategy of biomedical approach for the rational development of biologically active products (Rodrigues et al., 2016).

On these combined hypotheses, the ulvan-type SP present in Cc-SP1 and its *in vitro* effects against viruses (DENV-1, HSV-1, HSV-2, HMPV and AdV) in Vero and C6/36 HT cell-lines were further investigated, as experimental models of mammalian and mosquito by a virus yield inhibition assay, respectively, and in a global coagulation test of TG in microplate format using the chromogenic method.

Cc-SP1 reveals physical and chemical characteristics of ulvan

As seen in the Figure 1, Cc-SP1, obtained by DEAE-cellulose, was examined by IR technique in order to partially characterize the structural nature of the polysaccharide present in *C. cupressoides*.

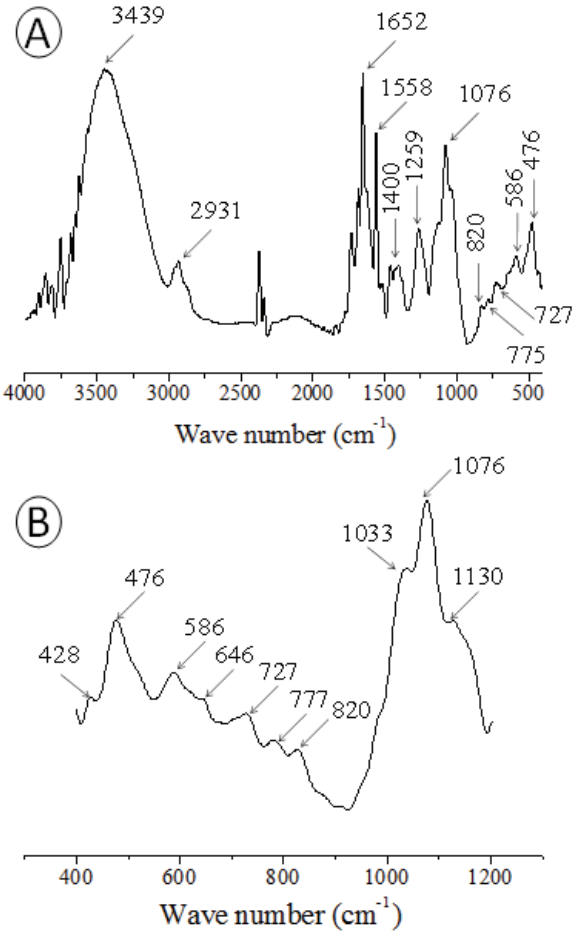

Figure 1. IR spectrum of Cc-SP1 (A) and its extended version (B) at 4000-500 cm^{-1} in KBr pellet.

The IR spectral analysis (Figure 1A) revealed complex acid sugar residues presenting main original absorption bands related to the occurrence of ester sulfate (S = O, at 1259 cm^{-1}), galactose-6-sulfate (at 820 cm^{-1}), uronic acid carboxylate groups (-COO or OH, at 1652 cm^{-1}), arabinogalactan sulfate backbone (at 1076 cm^{-1}) and CH_2 (at 2931 cm^{-1}); as well as carboxyl group of the pyruvic acid (at 1400 cm^{-1}) and O-H (at 3439 cm^{-1}) as previously inferred by Rodrigues et al. (2013a, 2014a, 2014b) and in conformity with those found for the SPs from the green macroalgae *Caulerpa racemosa* (Ghosh et al., 2004) and *C. mexicana* (Carneiro et al., 2014b).

To facilitate the IR assignment to ulvan, the spectrum of Cc-SP1 was extended (Figure 1B). As a result, it was observed IR spectral signals at 777 (C-O-S), 838 (C-C or S = O), 1558 (B-O group, speculating borate in the native polymer or $RO\text{-}SO^{-3}$) and 1076 cm^{-1} (-COO, carboxylate groups of UAs), suggesting the presence of ulvan-type acidic polysaccharide in Cc-SP1, as recorded during comparisons with published data for ulvan (Cassolato et al., 2008, Toskas et al., 2011). Other original absorption bands at 428, 476, 586, 617, 646, 727, 775, 887, 931 and 1033 cm^{-1} were also assigned to the spectrum of ulvan (Ghosh et al., 2004, Carneiro et al., 2014b, Rodrigues et al., 2014a) as a highly conserved structural sugar residue identity in the *Caulerpa* genus (Patel, 2012, Wang et al., 2014, Pérez et al., 2016).

The staining pattern conducted by toluidine blue revealed SPs from the Cc-SP1 sample after its separation by agarose gel electrophoresis (Rodrigues et al., 2011b, 2017, Salles et al., 2017) as illustrated in Figure 2A.

Although polydispersion naturally occurs in SPs from seaweeds (Pomin, 2012, Fidelis et al., 2014, Mourão, 2015, Rodrigues et al., 2017), the current study contrasted with that previously performed, where Cc-SP1 did not appear on agarose gel (Rodrigues et al., 2011b) allowing us to identify here at least two major sulfated components (SC-1 and SC-2) in this fraction with profiles of migration compared to condroitin-4-sulfate and UHEP, respectively, when they were used as standards (Volpi & Maccari, 2002, Salles et al., 2017).

In PAGE system (Figure 2C), Cc-SP1 revealed at least four SPs (SP-1→4) of distinct heterogeneous molecular weights ranging from ~ 8 to > 100 kDa. For SP-1 and SP-2, the molecular sizes were, consecutively, > 100 and ~ 100 kDa (Pomin, 2012, Fidelis et al., 2014), while the average molecular masses of the subfractions SP-3 and SP-4 were estimated by ~ 60 and ~ 8 kDa, respectively (Wang

et al., 2014, Rodrigues et al., 2017), similarly to the fraction Cc-SP2 in another study (Rodrigues et al., 2013a). In addition, data of Cc-SP1 were corroborated by GPC analysis that showed heterogeneous behave ($2.53 \rightarrow 4.12 \times 10^4$ g moL^{-1}) similar to the SPs isolated from the green seaweed *Ulva rigida* (Toskas et al., 2011). In a study performed with a SP fraction isolated from the red seaweed *Acanthophora muscoides*, Vanderlei et al. (2016) revealed a system of sulfated glycans having low molecular mass, when GPC procedure was employed.

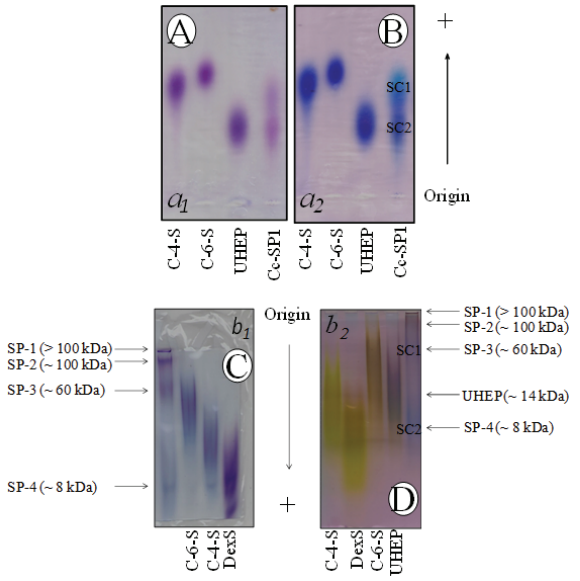

Figure 2. Agarose gel (A and B) and polyacrylamide gel (C and D) electrophoreses of Cc-SP1 and standards chondroitin-6-sulfate (C-6-S, ~ 60 kDa), chondroitin-4-sulfate (C-4-S, ~ 40 kDa), dextran sulfate (DexS, ~ 8 kDa) and/or UHEP (~ 14 kDa). SPs present on gels stained with toluidine blue (a_1 and b_1) or stains-all (a_2 and b_2).

When both electrophoretic procedures were associated with the use of stains-all, as another cationic dye (Volpi & Maccari, 2002, Salles et al., 2017), nonSPs were detected in a preparation of Cc-SP1, postulating the presence of carboxylate groups from the analyzed polymer sample that showed distinct components (SC-1 and SC-2) also in molecular masses of less than 100 kDa, as glycosaminoglycans (Volpi & Maccari, 2002) from the polyacrylamide analysis (Figure 2D) (Salles et al., 2017). These results were in concordance with those signals found by IR spectra that revealed acid polysaccharides in the chemical structure of Cc-SP1 (Figure 1), as supported by staining for cyan in comparison with standards (Figure 2B) (Volpi & Maccari, 2002). The PAGE analysis led us to the argument that the different classes of polysulfated would also contribute to a compositional variability of acidic components (Figure 2D) (Patel, 2012, Fidelis et al., 2014, Mourão, 2015) because ulvan resemble glycosaminoglycans from animal tissues (Wang et al., 2014).

The combined interpretations suggested 'polymeric blocks' of uronic acid with basis on preliminary spectral values found by solution ^1H RMN experiment of Cc-SP1 (Rodrigues et al., 2014a). Another hypothesis here would be a possible biochemical change in *C. cupressoides* polysaccharide composition at different times of algal collection (Cardozo et al., 2007, Wang et al., 2014, Rodrigues et al., 2017) comparing with published data on this species since the physiological role of these glycans in Caulerpaceae is still unclear (Rodrigues et al., 2011b, Patel, 2012, Rodrigues et al., 2014a, Wang et al., 2014).

The selective antiviral efficacy of Cc-SP1 has no cytotoxicity *in situ*

Table 1 summarizes the effect on the cell viability (Vero or C6/36 HT cell-line) of Cc-SP1 using plate assay. No *in situ* cytotoxic effects on cell viability were detected with Cc-SP1 at CC_{50} up to 1000 μg mL^{-1} for cultured cells, suggesting biocompatibility *in vitro* (Vanderlei et al., 2016). In addition, no microscopically visible change of normal cellular morphology was observed after the use of neutral red for distinguishing living, dead or damaged cells (Garrett et al., 2012, Gomaa & Elshoubaky, 2016, Vanderlei et al., 2016). Acyclovir did not exhibit cytotoxicity up to 200 μg mL^{-1}.

Table 1. Cytotoxicity assay with Vero and C6/36 HT cell-lines in the presence of Cc-SP1.

Compounds	Cell-lines	Cytotoxicity (CC_{50}, μg mL^{-1})
Cc-SP1	Vero	> 1000
	C6/36 HT	> 1000
Acyclovir	Vero	> 200

CC_{50} – 50% cytotoxic concentration; Acyclovir – standard compound.

SPs from seaweeds have low toxicity to cultured cells (Hasui et al., 1995, Mazumder et al., 2002, Ghosh et al., 2004, Cassolato et al., 2008, Ghosh et al., 2009, Vanderlei et al., 2016). This observation on Vero cell-line (African green monkey kidney) was in accordance with *C. cupressoides* SPs-intraperitoneally treated Swiss mice for 14 consecutive days or 72 h. No toxicological significance at the level of some biochemical parameters of blood (AST, ALT and urea) and histological structure of organs (liver, kidney, heart, spleen, thymus and lymph nodes) was observed (Rodrigues et al., 2012, 2013a). Studies on toxicity of *Caulerpa* SPs have revealed that they are tolerated by

experimental animals (Carneiro et al., 2014b, Ribeiro et al., 2014, Wang et al., 2014).

Given the lack of cytotoxicity (Table 1), the *in vitro* effects of Cc-SP1 against DENV-1, HSV-1, HSV-2, HMPV and AdV in cultured Vero and mosquito C6/36 HT cell-lines were further evaluated. The potent efficacy of the test sample (Cc-SP1), when analyzed by virus plate reduction assay, was only able to affect DENV-1 multiplication and both serotypes of HSV in cultured Vero cell-line (Table 2), suggesting that Cc-SP1 interfered with virion envelope structures or masking viral structures, once they are necessary for adsorption or entry into cells (Mazumder et al., 2002, Talarico et al., 2011, Pujol et al., 2012, Vanderlei et al., 2016). High values of SI (CC_{50}/EC_{50}) against these viruses were found representing the degree of safety of Cc-SP1 (Ghosh et al., 2004, Cassolato et al., 2008, Vanderlei et al., 2016), especially for DENV-1 (SI > 714).

Table 2. Antiviral potency and selectivity index of Cc-SP1.

Agents	HSV-1		HSV-2		DENV-1	
	EC_{50} ($\mu g\ mL^{-1}$)	SI (CC_{50}/EC_{50})	EC_{50} ($\mu g\ mL^{-1}$)	SI (CC_{50}/EC_{50})	EC_{50} ($\mu g\ mL^{-1}$)	SI (CC_{50}/EC_{50})
Cc-SP1	27	> 9.2	0.9	> 278	0.35	> 714
Acyclovir	0.8	> 250	1.38	> 145	-	-

HSV-1 – herpes simplex virus type 1; HSV-2 – herpes simplex virus type 2; EC_{50} – effective concentration to reduce virus titers by 50%; SI – selectivity index; Acyclovir – standard compound.

Additionally, the results showed an important efficacy (percentage inhibition (PI)) of Cc-SP1 (PI = 90 and 99.9% for HSV-1 and HSV-2, respectively) in comparison with acyclovir (PI = 99%), especially in terms of SI (> 278) for HSV-2, when an EC_{50} of about 14-fold higher of Cc-SP1 for HSV-1 compared with HSV-2 inhibition was detected. The antiviral effect of Cc-SP1 against DENV-1 infection in Vero cell-line was 96% (PI), but it was not an inhibitor of virus multiplication in mosquito cell-line (C6/36 HT). No polysaccharide concentration tested of Cc-SP1 (up to 1000 $\mu g\ mL^{-1}$) was able to inactive both HMPV and AdV replications in cultures. Collectively, these findings could be related to the molecular peculiarities of Cc-SP1 as visualized in Figures 1 and 2 (Vanderlei et al., 20106).

Antimicrobial agents, like seaweed SPs (Pérez et al., 2016), have been widely used to control shrimp disease (Chotiaget et al., 2004) and in the hydrocolloid industry (Cardozo et al., 2007, Gupta & Abu-Ahannam, 2011, Amorim et al., 2012). The anionic features of the algal SPs, as well as their molecular sizes, have been positively correlated with the antiviral potency against viruses (Ghosh et al., 2009, Vo et al., 2011). Our findings for *in vitro* anti-herpetic actions (EC_{50}) were comparable with those of SPs from Rhodophyta *G. corticata* (0.19-27.5 $\mu g\ mL^{-1}$) (Mazumder et al., 2002), but higher than that obtained for the Chlorophyta *C. racemosa* (3 $\mu g\ mL^{-1}$) (Ghosh et al., 2004). Cassolato et al. (2008) obtained EC_{50} values ranging from 0.27 to 0.3 $\mu g\ mL^{-1}$ for HSV-1 and values 10-fold lower for HSV-2 of a sulfated heterorhamnan from the green seaweed *Gayralia oxysperma*. A study conducted by Hasui et al. (1995) reported that the SPs from the marine microalga *Cochlodinium polykrikoides* had an anti-HSV-1 action (EC_{50} = 4.5 $\mu g\ mL^{-1}$). More recently, Vanderlei et al. (2016) evaluated the anti-HSV ability of three SPs from Brazilian tropical seaweeds (*A. muscoides*, *Gracilaria birdiae* and *Solieria filiformis*) and the most inhibitory effect (1.63 $\mu g\ mL^{-1}$) was exerted by *A. muscoides* SPs that had the highest sulfate content and the lowest molecular size vs. other two species studied. Gomaa & Elshoubaky (2016) found an *in vitro* anti-infectivity of aqueous SP from the red seaweed *A. spicifera* on intracellular HSV-1 (80.5 $\mu g\ mL^{-1}$) replication.

Interestingly, Cc-SP1 was a more effective inhibitor against DENV-1 multiplication compared with herpes virus infection in cultures (Table 2) and even totally inactive against HMPV and AdV infections. This variation in its inhibitory action depending on the viral serotype and host cell (Vero and mosquito C6/36 HT cells) may be ascribed to a difference in the virus-cell interaction leading to virus entry (Ghosh et al., 2009).

Talarico et al. (2005, 2011) and Pujol et al. (2012) evaluated the antiviral potency of commercial carrageenans (iota, kappa and lambda), SPs that differ on the sulfate position per disaccharide repeating unit and constituted of a combination of ionic and hydrophobic regions in the same polymer (Cardozo et al., 2007, Gupta & Abu-Ahannam, 2011, Patel, 2012) and found highest selectivity for anti-DENV-2. Studies on the anti-DENV effects revealed that SPs isolated from *Gymnogongrus griffithsiae*, *Cryptonemia crenulata* (Rhodophyta), *Stoechospermum marginatum, Cystoseira indica* (Phaeophyta) and *C. racemosa* (Chlorophyta) acted as inhibitors of four virus serotypes of DENV. Depending on the structural class of SP, a low presence of heparan sulfate (a polysulfated present in some types of cells, especially mammalian) on the cell surface could be indicative of a differential involvement for DENV entry in these cells. DENV-2 was the most susceptible to all polymers (EC_{50} = 0.12-20 $\mu g\ mL^{-1}$). Ghosh et al. (2009) reported that the efficacy of the SPs on the infection

process would involve their abilities to interfere with the attachment of the virion to the host cell surface thought their structural nature.

As observed from a structural, physical and chemical point of view (Figures 1 and 2), the tested compound (Cc-SP1) displayed strong antiviral effects (Table 2), supporting the hypothesis that the sulfation of galactose residues on C-6 might also be important and the inhibition of infections would require a specific sulfation pattern (Ghosh et al., 2009). The amount of sulfation was of about 1.8-fold greater than the uronic acid content and the effect of the chain length of molecular sizes of less than 100 kDa could contribute in parallel to the *in vitro* antiviral actions of Cc-SP1 (Ghosh et al., 2009, Pérez et al., 2016, Vanderlei et al., 2016).

Although recognized as important antiviral agents, SPs from seaweeds frequently exhibit anticoagulation activity due to their highly polyanionic character, which allow interactions with proteins and plasmatic regulators, displaying alternative mechanisms of action different from that of UHEP action (Pomin, 2012, Mourão, 2015). These mechanistic characteristics of the SPs would constitute a disadvantage in drug development for the prevention of infectious diseases, such as DENV (Ghosh et al., 2009). Conventionally, the APTT is a routine coagulation assay that could not reflect the overall coagulation function and the continuous interest for TG inhibition assays to more precisely analyze the profile of an anticoagulant in plasma has increased worldwide (Castoldi & Rosing, 2011).

Anticoagulation of Cc-SP1 by measuring TG in human plasma *in vitro*

In our global assay (Figure 3), which is characterized by a continuous measurement of TG potential in 60-fold diluted plasma per well-plate using the chromogenic method, Cc-SP1 was less effective than UHEP on TG in human plasma after stimulation by cephalin-activated system (Mourão et al., 2001, Rodrigues et al., 2016, Rodrigues et al., 2017), but was overall anticoagulant at concentrations so far the anti-HSV-1 effect (Table 2) based on Mazumder et al. (2002) and Ghosh et al. (2004).

This ability of Cc-SP1 was manifested in a concentration-dependent manner (Nishino et al., 1999, Rodrigues et al., 2016, Rodrigues et al., 2017, Salles et al., 2017), and required a concentration of 20.8-fold higher of SPs than UHEP for totally abolish the contact-activated pathway since the TG curve (positive control) indicated high values of absorbance until reaching a plateau (~ 24 min) (Figure 3).

The *in vitro* inhibitory response of Cc-SP1 was consistent with the TG parameters in diluted plasma (Nishino et al., 1999, Mourão et al., 2001, Rodrigues et al., 2016, Rodrigues et al., 2017, Salles et al., 2017), showing to be more than 50% in terms of thrombin peak at 4.1 (26 min) and 8.3 (32 min) μg well-plate^{-1}, whereas at high concentrations of SPs (41.6 and 83.3 μg well-plate^{-1}) TG was abolished compared with the positive control.

Current data would allow us to a more refined comparison between the APTT and TG assays (Castoldi & Rosing, 2011, Rodrigues et al., 2016, Rodrigues et al., 2017, Salles et al., 2017). This marked difference of Cc-SP1 on both experimental coagulation protocols could be possibly explained by its lack or less specificity of interaction with the plasmatic proteases or regulators (antithrombin and/or heparin cofactor II), displaying inhibition of the tenase and prothrombinase systems and/or the formation of a direct complex with thrombin, as revealed for other algal SPs that showed very distinct structures and effects than that of UHEP (a SP that contains a specific pentasaccharide sequence with high antithrombin affinity) on the coagulation (Mourão, 2015).

Although it is described that the galactose-6-sulfate content (Figure 1) influences the serpin-dependent *C. cupressoides* SPs anticoagulation (Rodrigues et al., 2013a, 2014b, Wang et al., 2014), the APTT test would reveal limited values of thrombin formed in plasma (Castoldi & Rosing, 2011). On the basis of this experimental limitation, when a TG continuous detection assay was used, the *dynamic* role of anticoagulation of Cc-SP1 was revealed as a more precise action of the algal SPs on the haemostatic system than the traditional APTT method (Nishino et al., 1999, Mourão et al., 2001, Rodrigues et al., 2016, 2017, Salles et al., 2017).

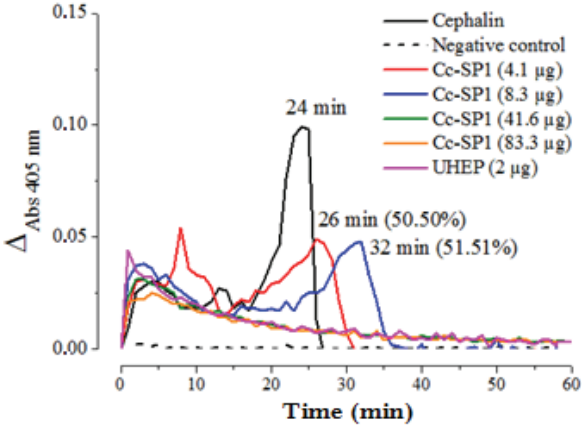

Figure 3. Effect of different concentrations of Cc-SP1, obtained by DEAE-cellulose, from the green seaweed *C. cupressoides* on contact-activated system in 60-fold diluted human plasma using the chromogenic method in a continuous system (37°C, 60 min).

Overall, the introduction of this novel experimental approach could be useful to guide complementary studies on Cc-SP1 as antiviral tool in parallel with the prevention of thrombosis *in vitro* because the concentrations of SPs were so far the anti-HSV-1 action, except for the inhibition level of HSV-2 and DENV-1, which required low levels (Table 1). Therefore, our investigation contributed to increase the knowledge of a novel antiviral agent concerning its biological capacities for simultaneously modulating infections and coagulation *in vitro*.

Conclusion

An ulvan-revealing polysulfated fraction from *Caulerpa cupressoides* (Chlorophyta) has different molecular peculiarities in comparison with others algal sulfated polysaccharides. It has no *in situ* cytotoxicity effect against Vero and mosquito C6/36 cell-lines and reveals as a more effective inhibitor (*in vitro*) of dengue virus type 1 compared with herpes simplex virus types 1 and 2, but even totally ineffective against in C6/36 cell-line and human metapneumovirus and adenovirus infections. Analysis by means of a thrombin generation method might provide data to guide further studies on this polysaccharide as antiviral tool in concomitance to modulation of thrombosis.

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