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Comparative study of sulfated polysaccharides from *Caulerpa* spp. (Chlorophyceae). Biotechnological tool for species identification?

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ABSTRACT. Studies on macromolecules isolated from marine algae suggested sulfated polysaccharides (SPs) as possible molecular markers for species. We evaluated isolated and fractionated SPs from the green marine algae *Caulerpa cupressoides*, *C. prolifera* and *C. racemosa* collected at Pacheco Beach, as possible taxonomic molecular indicators. Total SPs were extracted with papain in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM), followed by ion-exchange chromatography on DEAE-cellulose using a NaCl gradient. The obtained fractions were analyzed by 0.5% agarose gel electrophoresis. Anticoagulant assays employing normal human plasma and standard heparin (193 IU mg^{-1}) by the activated partial thromboplastin time (APTT) test were also performed as comparison parameters. Low yields, and similar chromatographic profiles were found among species' SPs, but electrophoresis revealed distinct SPs resolution patterns. The changes in APTT of SP fractions were dependent on charge density as showed by electrophoresis profiles. Activities were 17.37 (*C. cupressoides*), 22.17 (*C. racemosa*) and 25.64 (*C. prolifera*) IU mg^{-1}, respectively, similar to a previous study using the first and second species. The results suggest that comparative studies of SPs isolated from seaweeds may be an important tool for the identification of Caulerpaceae.

Keywords: chlorophyta, sulfated polysaccharides, molecular markers, APTT test.

Estudo comparativo dos polissacarídeos sulfatados de clorofíceas *Caulerpa* spp. (Chlorophyceae). Ferramenta biotecnológica na identificação de espécies?

RESUMO. A utilização de macromoléculas isoladas de organismos marinhos sugere correlacionar características em estudos taxonômicos e a investigação comparativa de polissacarídeos sulfatados (PSs) de algas despertam seu interesse como marcadores moleculares. Objetivou-se avaliar PSs isolados e fracionados das algas marinhas verdes *Caulerpa cupressoides, C. prolifera e C. racemosa*, coletadas na Praia do Pacheco, Estado do Ceará, como possíveis indicadores moleculares taxonômicos. Os PSs totais foram extraídos com papaína em tampão acetato de sódio 100 mM (pH 5,0) contendo cisteína e EDTA (ambos 5 mM), seguido por cromatografia de troca iônica em coluna de DEAE-celulose utilizando um gradiente de NaCl. As frações obtidas foram analisadas por eletroforese em gel de agarose a 0,5%. Ensaios anticoagulantes, utilizando o teste do tempo de tromboplastina parcial ativada (TTPA) com plasma humano normal e heparina padrão (193 UI mg^{-1}), também foram realizados como parâmetros de comparação. Verificaram-se baixos rendimentos e semelhantes perfis cromatográficos entre os PSs das espécies, porém revelando, por eletroforese, diferenças moleculares marcantes. As alterações no TTPA das frações de PS foram dependentes da densidade de cargas negativas mostradas nos perfis eletroforéticos, cujas atividades foram 17,37 (*C. cupressoides*), 22,17 (*C. racemosa*) e 25,64 (*C. prolifera*) UI mg^{-1}, respectivamente, e tal propriedade justificou um estudo já realizado utilizando a primeira e segunda espécies. Os resultados sugerem que estudos comparativos de PSs isolados de algas marinhas possam vir a ser uma ferramenta importante na identificação de Caulerpaceae.

Palavras-chave: chlorophyta, macromoléculas sulfatadas, marcadores moleculares, teste do TTPA.

Introduction

Seaweeds are used for millennia as food source and in medicine by several eastern peoples. They also provide byproducts for several applications in Biotechnology (ARAÚJO et al., 2008; AMORIM et al., 2012; CAMPO et al., 2009; CARVALHO et al., 2009; GHOSH et al., 2004; RODRIGUES et al., 2010a; SOUZA et al., 2008; VANDERLEI et al., 2010; ZHANG et al., 2003).

The numerous classes of compounds found suggest that algae have undergone adaptive changes to the environment throughout its evolution on Earth. We believe that the macromolecules from primary metabolism are related with ancient

processes that have been preserved for billions of years, whereas those involved in secondary functions become more complex and diversified, allowing marine organisms to colonize new environments. The isolation and structural elucidation of different chemical compounds present in marine organisms also lead to new knowledge about their biosynthetic mechanisms, such as those related with reproduction (pheromones), anti-herbivory agents and against other organisms, thus leading to the interest about the ecological and evolutionary role of these metabolites, as well as its usefulness as important in studies of taxonomy, phylogeny and biogeography, thus serving as a valuable tool in understanding the limits of differentiation between taxa (AMORIM et al., 2012; MAO et al., 2011; TEIXEIRA, 2002).

Found at high concentrations in the marine algae, the sulfated polysaccharides (SPs) are complex and heterogeneous macromolecules made up by sugars repetitive units and negatively charged, comprising the extracellular matrix. Probably these compounds in algae are related to mechanical, ionic and osmotic regulation, favoring the survival of these organisms in the marine environment (KLOAREG; QUATRANO, 1988). The environmental conditions and the study on different species of seaweeds provide a great structural variety of their polysaccharides, leading to particular pharmacological implications, when these compounds are used in several models of biological activities, in prospect of discovering new drugs (ASSREUY et al., 2008; FONSECA et al., 2008; GHOSH et al., 2004; JI et al., 2008; PEREIRA et al., 2005; RODRIGUES et al., 2009; RODRIGUES et al., 2010b; SILVA et al., 2010; ZHANG et al., 2003).

Green marine algae of the genus *Caulerpa* Lamouroux (1809) are commonly found on the Brazilian coast (RODRIGUES et al., 2010c; RODRIGUES et al., 2011a) and are characterized by filamentous coenocytic organization, with a creeping stem of macroscopic shape, attached to substrate by a rhizomatous portion. According to TRI (2009), it has been reported that *Caulerpa* is an important contributor to the algal biomass of coral reefs and laggons. Depending on the species, over its entire rhizomatous portion, we may found erect shoots presenting, for example, shape of leaves, feathers, pine trees, or bunch of grapes (JOLY, 1965; TRI, 2009). Evidence has shown that the morphology of their branches may present great plasticity, varying within the same species depending on environmental conditions (OHBA; ENOMOTO, 1987). Hence, different species may be described ineptly (OLIVEIRA et al., 2005).

Some studies involving taxonomy and distribution of algae of the genus *Caulerpa* collected on Brazilian coast describe the high polymorphism of this group, suggesting the use of molecular analyses (BRAYNER et al., 2008). A number of researches have been contributing on the use of SPs for such purpose (AMORIM et al., 2012; RODRIGUES; FARIAS, 2009).

In this context, the objective of this study was to use the comparative biochemistry in the study of SPs isolated from green marine algae of the genus *Caulerpa*, as auxiliary taxonomic tool for the differentiation of these organisms, contributing to new proposals for research involving chemotaxonomy.

Material and methods

Collection and identification of seaweeds

Specimens of green marine algae *Caulerpa cupressoides* (Vahl) C. Agardh, *Caulerpa racemosa* (Forsskal) J. Agardh and *Caulerpa prolifera* (Forsskal) Lamouroux, belonging to the genus *Caulerpa*, were collected in the intertidal zone at Pacheco Beach, municipality of Caucaia, State of Ceará, and the research developed in the laboratory of Carbohydrates and Lectins (CarboLec) from the Department of Biochemistry and Molecular Biology, Federal University of Ceará State. In the laboratory, the algae were cleaned to remove epiphytes and/or other fouling organisms, washed with distilled water, dehydrated at room temperature (25°C) and milled to extract the total SPs (TSPs).

Caulerpa cupressoides (Figure 1A) is characterized by a creeping axis fixed to the substrate by rhizoids pinnules arranged in the opposite direction, endowed with erect shoots (assimilative) pine tree-shaped distributed around the main axis (TRI, 2009). Their varieties are distinguished by the morphology of their branches (ramulosa) and/or by the presence of tristica branching in the pointed branches (BRAYNER et al., 2008), macroscopically differing them from the species *C. racemosa* (Figure 1B), which presents the structure of their branches club-shaped or cylindrical-shaped, and ending in an almost spherical portion (JOLY, 1965), and *C. prolifera* (Figure 1C), the latter being characterized by branches that form thin laminar expansions (leaves), lanceolate-shaped, smooth margins, apex rounded to slightly emarginated throughout its rhizomatous portion.

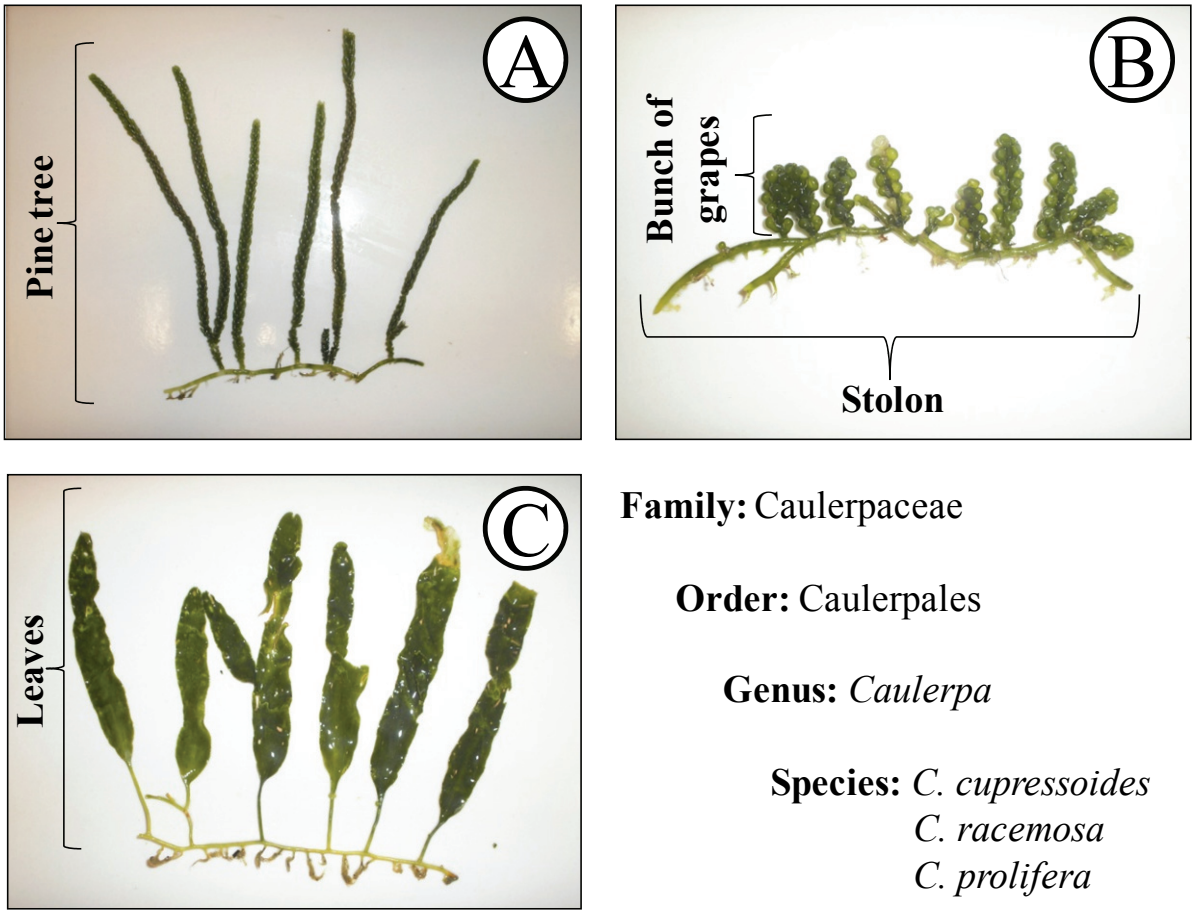

Figure 1. Green marine algae of the genus *Caulerpa*. Highlights the creeping stem (stolon) compounded of a rhizomatous portion with tufts of rhizoids, coenocytic at all portions. *C. cupressoides* (A) showing its branches pine tree-shaped, *C. racemosa* (B), evidencing the branches in the shape of bunches of grapes, and branches in leaves, characteristic of *C. prolifera* (C).

SPs extraction

Total SPs (TSPs) were obtained through the methodology previously described by Rodrigues et al. (2010c). Initially, dried and milled seaweed (25°C, 5 g) were rehydrated with 250 mL of 100 mM sodium acetate buffer (AcNa, pH 5.0) containing cysteine and EDTA (5 mM). Next we added 17 mL of a solution of crude papain (30 mg mL^{-1}) to extract TSPs in a water-bath (MARCONI, model MA 159) at 60°C for 6 hours. After incubation the materials were filtered, centrifuged (5,000 × *g*, 4°C, 30 min.) and to the supernatant we added 16 mL of a solution of cetylpyridinium chloride (CCP) (Sigma Chemical) at 10% for precipitation of the TSPs (25°C, 24h). The precipitates obtained were washed with 200 mL of 0.05% CCP, dissolved in 100 mL of 2 M NaCl: absolute ethanol (100:15, v:v) and submitted to precipitation with the addition of 80 mL of absolute ethanol (4°C, 24h). After precipitation, TSPs were centrifuged, subjected to two washes with 100 mL of commercial ethanol at 80%, and a third wash with ethanol of commercial degree (100 mL). Finally, the TSPs were dried in an air circulation oven (60°C, 4h) (RODRIGUES et al., 2010a).

Ion-exchange chromatography on a DEAE-cellulose column

TSPs (30 mg) were dissolved in buffer AcNa 50 mM (1 mg mL^{-1}) and subjected to ion-exchange chromatography on DEAE-cellulose column balanced and percolated with the same 50 mM AcNa buffer until complete removal of retained non-polysaccharides, followed by the fractioning of TSPs by elution with buffer AcNa containing NaCl at different concentrations (0.50, 0.75 and 1.00 M). The obtained fractions (5 mL) were monitored by metachromasia with 1.9-dimethylmethylene blue (FARNDALE et al., 1986) on an Elisa reader (AMERSHAM BIOSCIENCES, model BIOTRAK II) set at 525 nm. Then metachromatic fractions were lyophilized for further testing.

Agarose gel electrophoresis

The TSPs and the SP fractions (25 μg) obtained from the species were characterized amongst themselves regarding the density and patterns of negative charges by agarose gel electrophoresis. For this, the samples were applied on the 0.5% agarose gel (Bioagency) prepared in 50 mM 1.3-diaminopropane acetate buffer (Sigma-Aldrich) (pH 5.0). The procedure was accomplished using constant voltage (110 V, 60 min.). After the procedure, the SPs present in the gel were fixed with a solution of 0.1% N-cetyl-N, N, N-trimethyl ammonium bromide (Vetec Química) for 24 hours. Then, the gel was stained with 0.1% toluidine blue (Vetec Química) and bleached with a solution containing absolute ethanol, distilled water and concentrated acetic acid (4.95: 4.95: 0.1, v:v:v) according to Dietrich and Dietrich (1976).

In vitro anticoagulant assay

Still, as a way of comparison between species, we assessed the anticoagulant activity of SPs by the activated partial thromboplastin time (APTT) test, according to manufacturer's specifications. Initially, citrated human blood was obtained from twelve healthy donors of the Hematology and Hemotherapy Center of Ceará State (HEMOCE), and then centrifuged (73.75 × g; 15 min.) to obtain platelet-poor plasma. To accomplish the test, 50 μL of plasma were incubated at 37°C for 3 min. with 10 μL of SPs solution and 50 μL of the APTT reagent (CLOT, Bios diagnosis). After incubation we added to the mixture, 50 μL of 25 mM calcium chloride (CLOT, Bios diagnosis) in order to activate the coagulation cascade. The assays were performed in triplicate, and the clotting time automatically recorded in a coagulometer (DRAKE, model QUICK-TIMER) and the anticoagulant activity expressed as international units (IU) per mg of polysaccharide using the non-fractionated HEP (193 IU mg-1) as standard-curve.

Results and discussion

The employment of the methodology using the enzymatic digestion of proteins by proteolytic enzymes (papain) resulted in distinct yields of TSPs among species. The greater amount of TSPs was obtained for *C. racemosa* (4.00%), while *C. cupressoides* resulted in a maximum of 2.54% of TSPs, from algae dried at room temperature (25°C). A quite low yield of TSPs was obtained for *C. prolifera* (0.60%). Nevertheless, the morphophysiology of the used algae (Figure 1) suggests the difference in the TSPs yields obtained, and the values found were generally similar when compared to those obtained by Rodrigues et al. (2010c), using the first two species.

Several methods can be applied to extract TSPs from marine algae, such as aqueous, basic and enzymatic (AMORIM et al., 2012; FONSECA et al., 2008; GHOSH et al., 2004). The enzyme papain has been widely used to obtain these compounds (ASSREUY et al., 2008; BEZERRA-NETO et al., 2008; PEREIRA et al., 2005; RODRIGUES et al., 2009; RODRIGUES et al., 2010a). The TSPs yields also could be improved through sequential extractions. For example, the use of papain in consecutive extraction of TSPs from the algae Chlorophyta *C. sertularioides* (BEZERRA-NETO et al., 2008) and *C. cupressoides* (RODRIGUES et al., 2011b); and Rhodophyta *Halymenia pseudofloresia* and *Halymenia* sp. (RODRIGUES et al., 2009, 2010b) enable to perform three digestions of tissue of these species.

In this way, the use of sequential extractions suggests the identification of new natural biopolymers for biotechnology. Some seaweed species have SPs (known as phycocolloids) with properties thickener, gelling agent and emulsifier of great importance for industries of food (CAMPO et al., 2009), pharmaceutical (RODRIGUES et al., 2009; SILVA et al., 2010) and/or other economic sectors (ARAÚJO et al., 2008), suggesting the use of this technique using subsequently the species *C. racemosa* and *C. prolifera*.

Ion-exchange chromatography (DEAE-cellulose)

Chromatographic profiles obtained in column of ion-exchange (DEAE-cellulose) were similar between species, which indicated the separation of three different fractions of SP (F I, F II and F III) eluted at NaCl concentrations of 0.50, 0.75 and 1.00 M, respectively (Figure 2). However, the metachromatic intensity of the SP fractions varied between the species. The greatest yield of SP was obtained in F II, eluted with 0.75 M of NaCl, compared with other observed in the algae (Table 1).

Different and/or similar profiles of SPs were obtained, in DEAE-cellulose, from the use of distinct species, such as between Rhodophyta *Gelidium crinale* (PEREIRA et al., 2005), *Champia feldmannii* (ASSREUY et al., 2008), *H. pseudofloresia*, *Halymenia* sp. (RODRIGUES et al., 2009; 2010b) and *S. filiformis* (RODRIGUES et al., 2010a); and Chlorophyta *C. sertularioides* (BEZERRA-NETO et al., 2008) and *C. cupressoides* (RODRIGUES et al., 2011b).

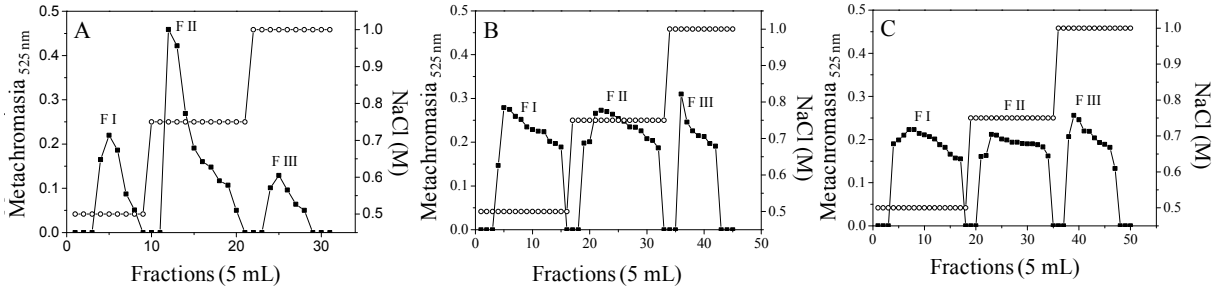

Figure 2. Ion-exchange chromatography (DEAE-cellulose) of TSPs from green marine algae *Caulerpa racemosa* (A), *Caulerpa prolifera* (B) and *Caulerpa cupressoides* (C). The column was balanced and eluted with 50 mM sodium acetate buffer (pH 5.0). The SPs adsorbed on the gel were eluted with the addition of NaCl (0.50, 0.75 and 1.00 M). (■—■) metachromasia; (○—○) NaCl concentration (M).

Table 1. Yield of SP fractions obtained by ion-exchange chromatography (DEAE-cellulose) of the green marine algae of the genus *Caulerpa* spp.

Species	NaCl (M)	Fractions	PS (%)
C. cupressoides	0.50	F I	12.66
	0.75	F II	24.66
	1.00	F III	4.17
C. prolifera	0.50	F I	18.67
	0.75	F II	20.33
	1.00	F III	3.33
C. racemosa	0.50	F I	7.49
	0.75	F II	27.33
	1.00	F III	5.23

In the present study, green marine algae *C. cupressoides* and *C. racemosa* presented similar chromatographic profiles of SPs between them and when compared to those obtained for these same species examined by Rodrigues et al. (2010c), emphasizing the use of this tool in comparative studies of these compounds in species of the genus *Caulerpa*. According to Marinho-Soriano and Bourret (2003), the collection of these organisms during different seasons may cause variations in yield and quality of these molecules.

Agarose gel electrophoresis

The agarose gel electrophoresis revealed remarkable molecular differences between species (Figure 3). *C. cupressoides* presented SP fractions (F I, F II and F III) distinct from each other in charge density on gel, where the F II fraction, eluted with 0.75 M of salt, presented a quite homogeneous pattern, denoting the efficiency of DEAE-cellulose column in the polysaccharide resolution. On the other hand, the fractions F I and F III, eluted with 0.50 and 1.00 M of salt, were practically not observed in the gel, suggesting a lower presence of sulfate groups in their chemical structures (Figure 2A), while the electrophoretic profile of SPs from *C. racemosa* resulted in a metachromatic band quite polydisperse in negative charges (F I), when compared to F II and F III, both eluted with 0.75 and 1.00 M of salt, being this latter discretly observed in the gel (Figure 2B). Such characteristics also justified a study with these species performed by Rodrigues et al. (2010c). In relation to *C. prolifera*, we observed practically the occurrence of the same mobility pattern of SP fractions, although F II revealing heterogeneous and highly charged, in comparison to the other polysaccharide fractions (F I and F III) obtained (Figure 2C).

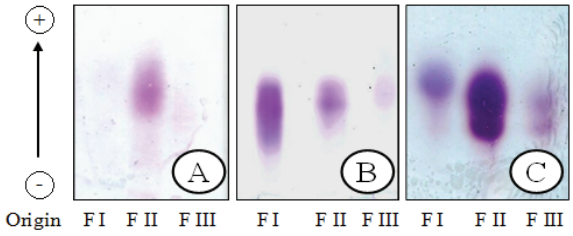

Figure 3. Agarose gel electrophoresis of SP fractions obtained through ion exchange chromatography (DEAE-cellulose) from green marine algae *Caulerpa cupressoides* (A), *Caulerpa racemosa* (B) and *Caulerpa prolifera* (C). The fractions (F I, F II and F III) present in the gel were stained with 0.1% toluidine blue.

The high pattern of resolution observed through the electrophoretic profiles for some SP fractions of used species was different from the first extraction of TSPs from the algae of the genus *Halymenia* (Rhodophyta), when the TSPs and the SP fractions showed a quite polydisperse pattern in negative charges. The accomplishment of two new digestions with papain, during the process of sequential extractions, resulted in more homogeneous molecules (RODRIGUES et al., 2009, 2010b). Therefore, the characteristics of these isolated compounds vary from species to species. Such descriptions are likely to be useful for Caulerpaceae, given the great polymorphism of this group, leading in some cases to wrong description of species of this genus (OLIVEIRA et al., 2005; TRI, 2009).

Anticoagulant activity

The anticoagulant potential of SPs isolated from marine macroalgae native of Ceará State coast has been researched (ASSREUY et al., 2008; FONSECA et al., 2008; PEREIRA et al., 2005; RODRIGUES et al., 2009, 2010b) and some studies report important biological activities of SPs isolated from green marine algae of the genus *Caulerpa*, such as anticoagulant, antithrombotic, prothrombotic and antiviral (GHOSH et al., 2004; RODRIGUES et al., 2011a). The interest in studying these compounds as anticoagulant and antithrombotic agents is justified by the therapeutic use of HEP and its complications in medical practice, with APTT test being one of the most used in the measurement of SPs with anticoagulant activity, which accurately indicates the anticoagulant potential of the compound isolated (PEREIRA et al., 2005).

In this context, this test can also be used as an additional parameter of evaluation for the algae characterization (RODRIGUES et al., 2010c). The species presented SP fractions able to change the APTT of normal human plasma (Figure 4).

We observed that the minimum concentration of SP to prolong the APTT was 0.10 mg mL^{-1} for the fractions F II (0.75 M of salt), whose activities were 17.37 and 22.17 IU mg^{-1}, respectively, for the species *C. cupressoides* and *C. racemosa* (Figures 4A and B). The fractions F I and F III, eluted with 0.50 and 1.00 M of salt, respectively, at a high concentration of SP (1 mg mL^{-1}), obtained from *C. cupressoides*, not prolonged the APTT of human plasma, whereas those obtained from *C. racemosa*, under these same concentrations of NaCl (F I and F III) presented, respectively, anticoagulant activity in the magnitude of about 7.10 and 3.59 IU mg^{-1}. The occurrence of activity for these fractions also had justified a previous study (RODRIGUES et al., 2010c). *C. prolifera* also presented PS fractions with anticoagulant activity (2.90 (F I); 25.30 (F II) and 5.64 (F III) IU mg^{-1} (Figure 4C). In this way, the anticoagulant potential of SPs isolated from used species were inferior to non-fractionated HEP (193 IU mg^{-1}).

The results suggest that the anticoagulant activity of SP fractions obtained from these species was, in general, dependent on the charge density present in the chemical structures of these macromolecules (AZEVEDO et al., 2009; SILVA et al., 2010), when observed by electrophoresis (Figure 3) (RODRIGUES et al., 2010c). Meantime, the action of these compounds on the coagulation does not occur merely as a function of charge densities (MOURÃO, 2004), and further studies are necessary to elucidate their mechanisms of anticoagulant action (AZEVEDO et al., 2009; FONSECA et al., 2008; PEREIRA et al., 2005). The use of animal models would also be indicated to evaluate the particular role of these polysaccharides in biological systems and its correlation with other pharmacological activities of interest in Biomedicine (ASSREUY et al., 2008; FONSECA et al., 2008; GHOSH et al., 2004; JI et al., 2008; SILVA et al., 2010; ZHANG et al., 2003). The methodology employing consecutive extractions of TSPs, to identify new macromolecules with anticoagulant activity (RODRIGUES et al., 2009, 2010b), of species in question, also has aroused interest in our research group.

In summary, the use of these molecules may provide new approaches regarding their application in taxonomy of marine algae. This fact may also help in better understanding of Caulerpaceae (RODRIGUES et al., 2010c, 2011b), since are algae microscopically without differentiation (JOLY, 1965), and as are restricted the studies involving chemotaxonomy of algae (MAO et al., 2011). For Teixeira and Kelecom (1991), phytochemical studies may provide important insights to understand natural systems among marine organisms, such as the action of sex pheronomes, the establishment of specific food chains and the analysis of evolutionary strategies responsible for the success of a given species.

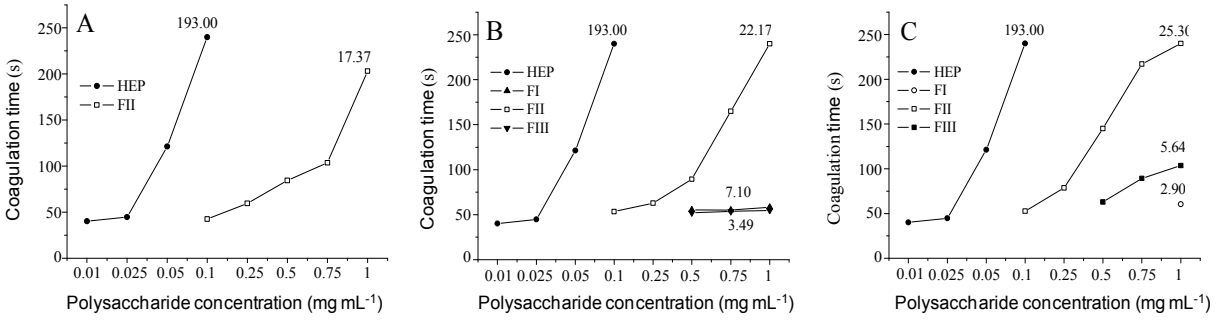

Figure 4. Anticoagulant activity (APTT) of SP fractions obtained by ion-exchange chromatography (DEAE-cellulose) from green marine algae *Caulerpa cupressoides* (A), *Caulerpa racemosa* (B) and *Caulerpa prolifera* (C) in relation to non-fractionated HEP (IU mg^{-1}). The points onto the curves represent the mean values of APTT from three determinations.

The isolation of algae metabolites suggests biogenetic routes and/or original functions, therefore being important correlations to understand the great structural diversity of natural products as possible taxonomic markers of low hierarchical levels. Besides that, such studies may also contribute to bioprospect new therapeutic agents (GHOSH et al., 2004; JI et al., 2008; RODRIGUES et al., 2011a; VANDERLEI et al., 2010), considering the biological invasion or accidental anthropogenic introduction (ballast water of ships, aquaculture, etc) of the genus *Caulerpa* on native benthic species and interfering on human activity worldwide (PIAZZI; CECCHERELLI, 2006; RUITTON et al., 2005).

Conclusion

Sulfated polysaccharides isolated by proteolytic digestion (papain) followed by ion-exchange chromatography (DEAE-cellulose) revealed, by electrophoresis, remarkable molecular characteristics between examined Caulerpaceae. The assessment by the anticoagulant test (APTT) emphasized the observations. We believe that more specific biochemical analyses may support such suggestions. Studies related to seasonal variation of these compounds would also be indicated.

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