Rev. Bras. Eng. Pesca (2025) 16 (2): 26-40
DOI: 10.18817/repesca.v16i2.3882

ARTIGO *REVISTA BRASILEIRA DE ENGENHARIA DE PESCA*

COMPARISON OF SEAWEEDS SULFATED POLYSACCHARIDES AND ANTIOXIDANT ACTION TO THOSE OF *Halymenia pseudofloresia*

COMPARAÇÃO DE POLISSACARÍDEOS SULFATADOS DE ALGAS MARINHAS E AÇÃO ANTIOXIDANTE DAQUELES DE *Halymenia pseudofloresia*

José Ariévilo Gurgel Rodrigues[1*], Arthur Borges Torres[1], Átila Dantas Maciel[1], Jefferson Silva Ferreira Mesquita[1], José Gabriel de Sousa Cândido[1], Thaís de Oliveira Costa[2], Johnny Peter Macedo Feitosa[3], Sandra de Aguiar Soares[3], Kelma Maria dos Santos Pires-Cavalcante[1] & Ianna Wivianne Fernandes de Araújo[1*]

[1]Departamento de Engenharia de Pesca, Universidade Federal do Ceará - UFC
[2]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará - UFC
[3]Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará - UFC

*e-mail: arieviloengpesca@yahoo.com.br , iwfaraujo@gmail.com

Recebido: (28/09/2024) / Publicado: (22/03/2025)

Abstract Taxonomic value of seaweeds sulfated polysaccharides (SPs) and antioxidant potential for genus *Halymenia* lack of studies. SPs from *Caulerpa cupressoides*, *C. racemosa*, *C. prolifera*, *Botryocladia occidentalis*, *Gracilaria birdiae*, *H. pseudofloresia* and *Solieria filiformis* were analyzed for their yields and structural features; and *H. pseudofloresia* SPs as antioxidants by DPPH, total antioxidant capacity and ferrous ion chelating (FIC) assays against BHT, ascorbic acid and EDTA, respectively. Papain extraction yielded ($p < 0.001$) from 0.42 ± 0.08% (*C. prolifera*) to 52.82 ± 6.16% (*H. pseudofloresia*) of crude SPs. Infrared specroscopy revealed ulvan (*Caulerpa* spp.), unrelated to agaran/carrageenan (*B. occidentalis*), agaran (*G. birdiae*), *lambda*-carrageenan (*H. pseudofloresia*) and *iota*-/*kappa*-carrageenan (*S. filiformis*) featuring basic structures. *H. pseudofloresia* had antioxidant SPs by all the *in vitro* assays, with most effectively by FIC property (propagation) than by other assays (initiation), although potent less than commercials. Therefore, SPs from Brazilian seaweeds suggested classes separated by phylum and/or species in chemotaxonomy and *H. pseudofloresia* a source in antioxidant SPs.

Key Words: macroalgae, morphology, glycans, chemical structure, reducing power.

Resumo O valor taxonômico de polissacarídeos sulfatados (PSs) carece de estudos de algas marinhas e o potencial antioxidante para o gênero *Halymenia*. PSs foram analisados de *Caulerpa cupressoides*, *C. racemosa*, *C. prolifera*, *Botryocladia occidentalis*, *Gracilaria birdiae*, *H. pseudofloresia* e *Solieria filiformis* quanto ao rendimento e características estruturais, e como antioxidantes de PSs de *H. pseudofloresia* pelos ensaios DPPH, capacidade antioxidante total e quelação de íon ferroso (QIF) contra HTB, ácido ascóbico e EDTA, respectivamente. A extração com papaína rendeu de PSs brutos ($p < 0,001$) de 0,42 ± 0,08% (*C. prolifera*) a 52,82 ± 6,16% (*H. pseudofloresia*). A espectroscopy de infravermelho revelou ulvana (*Caulerpa* spp.), distinto a agaran/carrageenan (*B. occidentalis*), agarana (*G. birdiae*), *lambda*-carragenana (*H. pseudofloresia*) and *iota*-/*kappa*-carragenana (*S. filiformis*) caracterizando estruturas básicas. *H. pseudofloresia*, por todos os ensaios *in vitro*, teve PSs com efetividade maior pela propriedade de QIF (propagação) que por outros ensaios (iniciação), embora menos potentes que os comerciais. Portanto, PSs de algas marinhas brasileiras sugeriram classes separadas por filo e/ou espécie em quimiotaxonomia e *H. pseudofloresia* uma fonte em PSs antioxidantes.

Palavras-Chave: macroalgas, morfologia, glicanas, estrutura química, poder redutor.

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Introduction

The reef systems are globally diverses as being also composed of macroalgae floras that commonly occur in the intertidal zone, especially in tropical and subtropical coastal waters (Joly, 1965; Reviers, 2006; Marinho-Soriano et al., 2009). The morphological diversity of macroscopic algae (seaweeds) found these regions is usually influenced by ecophysiological conditions that lead to spatial and temporal variability of coexistence groups of individuals occuping, until, a common area. Seaweeds are valuable ecological components to the coastal dynamic and marine life, since the global balance to the biochemical nature of the cell walls of coenocytic or multicellular benthic forms as relevant for their morphological plasticity to enviromental pressure. Wall biochemical of seaweeds has been a diverse field for biomechanical relationship studies regarding matrix sulfated polysaccharides (SPs) composition as molecular entities and how these components would generate taxonomic value for species identification and commercial applications (Stengel et al., 2011).

Seaweeds are classified into three higher taxa, known as Chlorophyta – green algae, Rhodophyta – red algae, and Ochrophyta – brown algae, respectively (Joly, 1965; Reviers, 2006). They have SPs conserved among phyla showing high degree of structural complexity/heterogeneity of fucan or fuicodan (Ochrophyta) and galactans (Rhodophyta), which are the most well-studied than those found in Chlorophyta (usually ulvan) by their abundance and biological, evolution and medical implications (Pomin & Mourão, 2008). The SPs composition varies *e.g.*, according to the species, extraction method (Campo et al., 2009) and enviromental factors (Cardozo et al., 2007); it also algal morphology by cell wall anatomy embbeded of SPs (Stengel et al., 2011) that could supplement such taxonomic issues (Usov et al., 1998). In Chlorophyta and Rhodophyta, the distribution, chemical class and comparison of SPs yield among different species have been still discussed on a chemotaxonomic criteria and evolutive position (Pomin & Mourão, 2008; Stengel et al., 2011). Structural biology of SPs has been few documented on a chemical perspective to complement the systematic classification of algae (Usov et al., 1998).

Rhodophyta produces β-galactoses with D-enantiomers, but α-galactose residues being D- (agaran), L- (carrageenan) (Pomin & Mourão, 2008) or DL-hybrid (Zibetti et al., 2005) configuration differing in the sulfation pattern and 3,6-anhydro bridge on the backbone structure. Such properties have been explored and applied for commercial use (Cardozo et al., 2007; Pomin & Mourão, 2008; Campo et al., 2009; Silva et al., 2010); and optimized from their matrixes (Zibetti et al., 2005; Rodrigues et al., 2009a). Chlorophyta has SPs in minor concentrations and high structural heterogeneity, became them a great challenge for the knowledge of their well-defined structures (Wang et al., 2014). Diverse structurally-bioactive SPs have been isolated as a major challenge in the glycomic field (Pomin & Mourão, 2008; Campo et al., 2009; Silva et al., 2010).

Seaweeds in hydrocolloid industry are natural sources of food and medicine products making them potential therapeutic ingredients largely screened for the obtaining of antioxidant extracts (Femi-Adepoju et al., 2023) to improve the biological defense against endogenous/exogenous factors associated to free-radicals (Barbosa et al., 2010) and alterantive to synthetic preservatives, such as butylatedhydroxytoluene (BHT) (Femi-Adepoju et al., 2023), which is a commercial antioxidant agent known as toxic (Panicker et al., 2014). Seaweeds SPs extracts have shown as natural inhibitors of free-radicals by various *in vitro* tests showing potential uses (Femi-Adepoju et al., 2023). Antioxidant-based SPs from ecologically-important seaweeds (Chlorophyta *Caulerpa cupressoides* var. *flabellata* by Costa et al. (2012) and *Caulerpa lentillifera* by Tesvichiana et al. (2024); Rhodophyta *Hypnea musciformis* by Alves et al. (2012), *Gracilaria birdiae* by Fidelis et al. (2014) and *G. caudata* by Alencar et al. (2019); and Ochorophyta *Turbinaria ornata* by Ananthi et al. (2010), *Lobophora variegata* by Paiva et al. (2011) and *Sargassum swartzii* by Vijayabaskar et al. (2012)) have already been recognized.

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Chlorophyta *C. cupressoides* var. *lycopodium* C. Agardh (Rodrigues et al., 2012, 2019), *C. racemosa* (Forsskcal) J. Agardh (Ghosh et al., 2004) and *C. prolifera* (Forsskal) Lamouroux, as coenocytic seaweeds (DeWreede, 2006); and Rhodophyta *Botryocladia occidentalis* (Børgesen) Kylin (Farias et al., 2000; Rodrigues et al., 2024), *G. birdiae* Plastino & Oliveira (Maciel el al., 2008; Fidelis et al., 2014), *Halymenia pseudofloresia* Collins & M. Howe (Rodrigues et al., 2009a, 2009b; Rodrigues & Farias, 2009) and *Solieria filiformis* (Kützing) P. W. Gabrielson (Araújo et al., 2011), as multicellular seaweeds, are most common along the Northeastern coast of Brazil, but there is no a structural comparison in chemotaxonomy among these species. This study was also focused to test the antioxidant potential of the SPs isolated from *H. pseudofloresia*, which is communly found as a delivery Rhodophyta on the beach zone, since that the genus have been still fewer studied (Fenoradosoa et al., 2009; Rodrigues et al., 2018).

Material and Methods

Seaweeds: collection sites and specimen identification

Different tropical seaweeds species (coenocytic Chlorophyta thalli *C. cupressoides* var. *lycopodium* C. Agardh, *C. racemosa* (Forsskcal) J. Agardh and *C. prolifera* (Forsskal) Lamouroux; multicellular Rhodophyta thalli *B. occidentalis* (Børgesen) Kylin, *G. birdiae* Plastino & Oliveira, *H. pseudofloresia* Collins & M. Howe) and *S. filiformis* (Kützing) P. W. Gabrielson were manually collected from two beaches popularly known to the State of Ceará in the Northeastern coast of Brazil. Specimens from *Caulerpa* and *Solieria* populations were obtained from Pacheco beach (municipality of Caucaia) and to other Rhodophyta populations were from Flecheiras beach (municipality of Traíri) during field expeditions carried out by our group.

All specimens were pre-selected in the environment from other algae species and then were transported in plastic bags to the Marine Biochemistry laboratory of the Aquaculture Biotechnology Center, Department of Fishing Engineering, Federal University of Ceará. Materials were washed with distillated water to remove impurities (*e.g.*, salt, sand and shells), necrotic parts and then stored -20°C according to Farias et al. (2000)' procedure.

A sample of each specimen was archived in the Herbarium Prisco Bezerra (Department of Biological Sciences, Federal University of Ceará, Brazil), except *H. pseudofloresia* that was archived in the Marine Sciences laboratory, Federal University of Ceará, Brazil. The algae materials were authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

In nature seaweeds from Northeastern Brazilian coast were firstly separated for macroscopic identification from a thallus sample of each species (Joly, 1965), using also the Marinho-Soriano et al. (2009)' catalogue. The three forms of *Caulerpa* taxa (Chlorophyta *C. cupressoides* var. *lycopodium*, *C. racemosa* and *C. prolifera* - Caulerpaceae, Caulerpales) are illustrated in figure 1.

They were collected from Pacheco beach and are composed by a creeping axis attached to the substratum by rhizoids structures, of which are endowed with erect shoots (known as assimilators) and that their morphologies separate the three species (DeWreede, 2006).

For *C. cupressoides* var. *lycopodium* (Figure 1A), it is characterized by the presence of tristica branching, whereas for *C. racemosa* and *C. prolifiera* (Figures 1B, C) by the presence of club-shaped or cylindrical-shaped (spherial portion) and by the presence of laminar expansions (leaves), lanceolate-shaped and smooth margins, respectively (Rodrigues et al., 2012).

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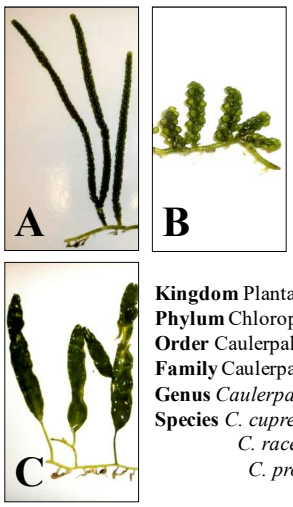

Figure 1. Coenocytic Chlorophyta showing stolon compounded of a rhizomatous portion in which tufts of rhizoids are found as structures that attach to the substratum. *C. cupressides* (A) with branches in pine three-shaped, *C. racemosa* (B) with branches in the shape of bunches of grapes; and *C. prolifera* (C) with branches in leaves (Rodrigues et al., 2012).

Regarding *in nature* Rhodophyta taxa, four species were collected on the two beaches (Pacheco and Flecheiras) and further identified are shown in figure 2.

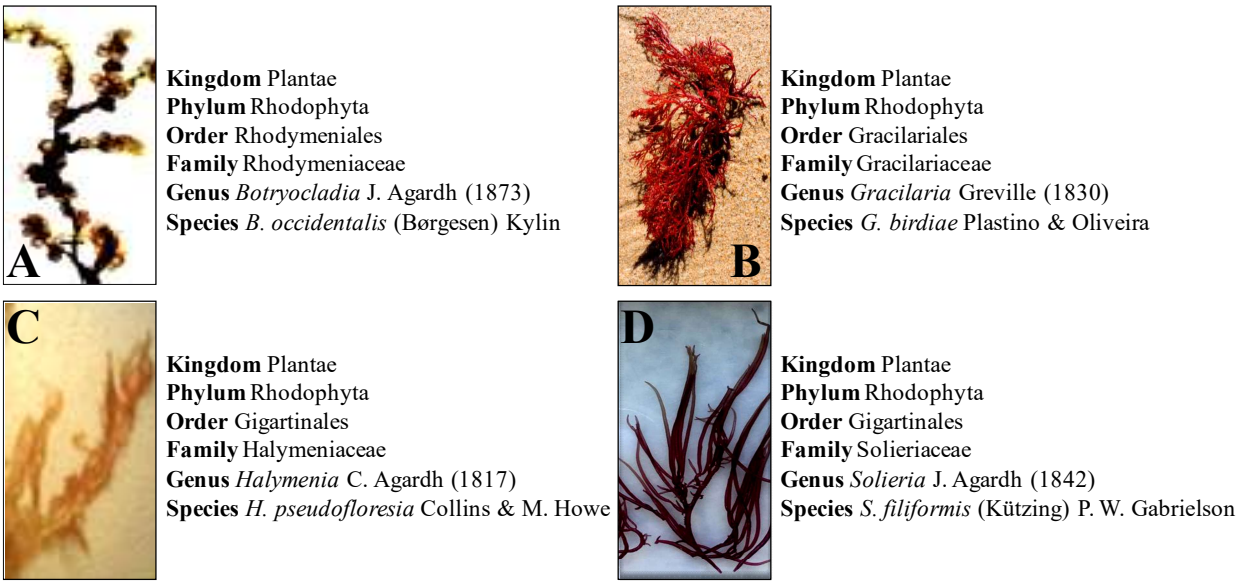

Figure 2. Images representing red specimens of *B. occidentalis* (Farias et al., 2000; Rodrigues et al., 2024) (A), *G. birdiae* (by Laura Primo, 2024) (B), *H. pseudofloresia* (Rodrigues & Farias, 2009) (C) and *S. filiformis* (by authors) (D). Species differ by morphological aspect and thallus texture collected from Pacheco or Flecheiras beach.

B. occidentalis (Figure 2A) is an arborescent species that has dark red/rose coloring with an anatomy of cell-wall that make up clusters of round pneumatic vesicles (Rodrigues et al., 2024). *G. birdiae* (Figure 2B) is a red species distributed on the Brazilian coast from Ceará State to Espirito Santo State and has a dark red thallus structure of pseudoparenchymatous shaped which is commercially exploited for the production of agar by coastal natives (Maciel et al., 2008). Even *H. pseudofloresia* (Figure 2C), also known as red sea lettuce, is characterized by a fleshy (gelatinose)

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texture and rosy-red colour with leaves branche form of variable length (some regions of dichotomies) showing semi-translucent aspect (Rodrigues & Farias, 2009). For *S. filiformis* (Figure 2D), it is a member of the genus *Solieria* found on the beaches of Atlantic, including Brazil, as some the most abundant in carrageenans for food, cosmetic or pharmaceutical potential (Campo et al., 2009; Araújo et al., 2011). It has a multicellular and filamentous structure of a red thallus and dichotomic branches presenting soft texture anchored to substratum (Joly, 1965).

Enzymatic extraction of the seaweeds SPs

Samples of different seaweeds thalli were initially dehydrated for 24 h in the sunlight and the each pretreated material was cut into small pieces prior to extraction of their matrix SPs according to the Farias et al. (2000)' procedure, with some modifications. For this (Figure 3), tritured tissue (5 g) of each species was used and then suspended in 100 mL of 100 mM sodium acetate buffer (pH 5.0), containing 5 mM EDTA and 5 mM cysteine, where each material in glass reactor flasks was enzimatically digested by adding of a crude papain solution (~31 mg mL^{-1}) at 60°C for 24 h, using a thermostatic bath.

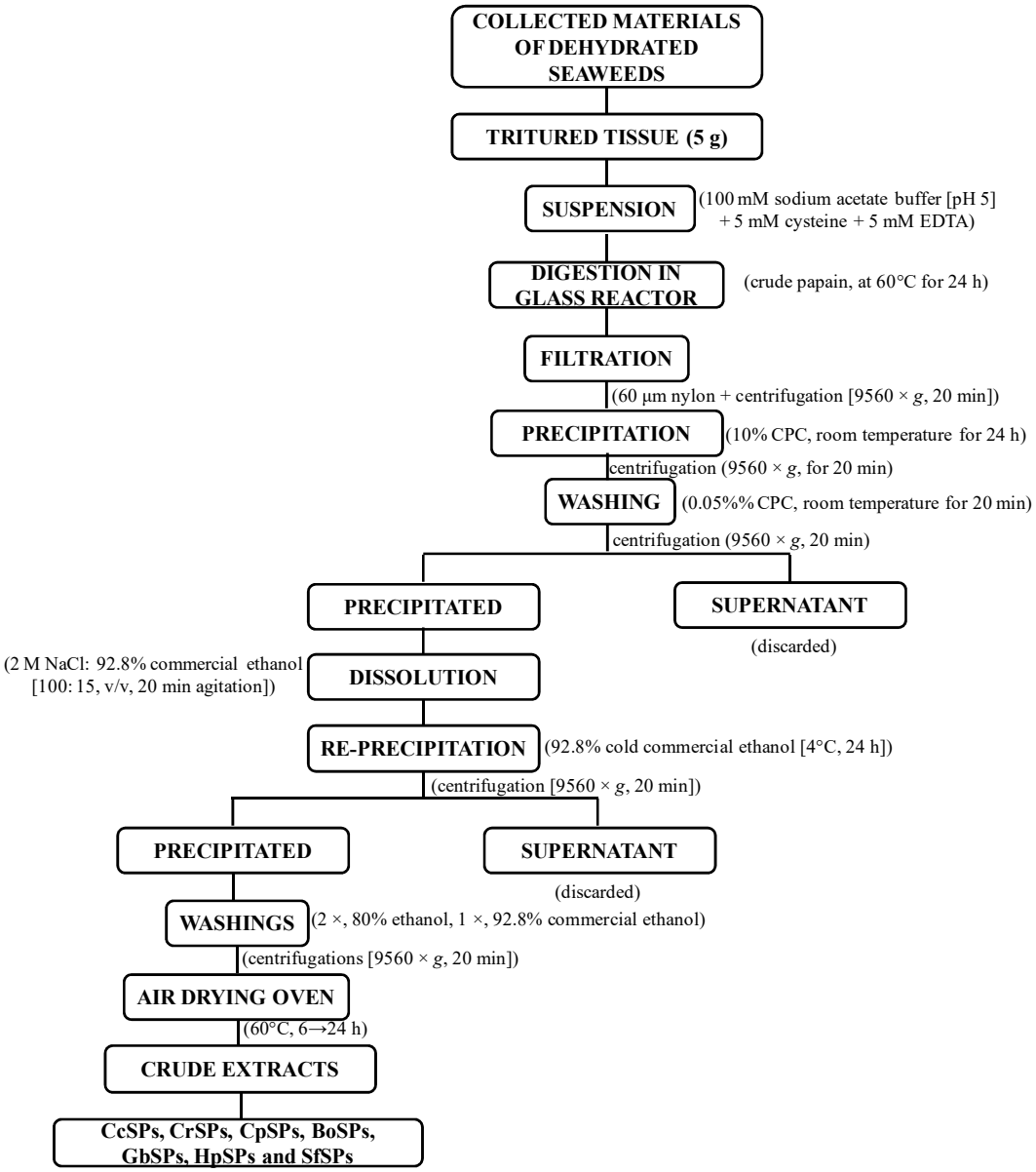

Figure 3. Protocol of obtaninig of SPs from seaweeds collected from Pacheco or Flecheiras beach.

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After seaweeds tissues-incubation period, each material was then separatelly filtered using a nylon net and the supernatant was saved. Seaweeds SPs that were present in solution were precipitated with 16 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature for 24 h and the material then collected by centrifugation (9.560 × *g*, for 20 min). The *pellet* containing seaweeds SPs was washed with 100 mL of 0.05% CPC solution, dissolved under agitation (for 20 min) in 100 mL of a 2 M NaCl:ethanol (100:15 ratio, v:v) solution, and then precipitated for 24 h at 4°C with addition of 100 mL of cold commercial ethanol (92.8%). The precipitate thus obtained was centrifugated (9.560 × *g* for 20 min), washed twice with 100 mL of 80% ethanol, and once with the same volume of 92.8% commercial ethanol.

Separatelly the materials were dried using an air drying oven (60°C, 6→24 h) until remove ethanol residue that varied with the total amount of seaweeds SPs. The yields of the seaweeds SPs (named CcSPs, CrSPs, CpSPs, BoSPs, GbSPs, HfSPs and SfSPs) were expressed as the percentage (%, n = 3) of each dehydrated matter.

Characterization by infrared (IR) spectroscopy

Extracted materials samples (CrSPs, CpSPs, BoSPs, GbSPs, HfSPs and SfSPs) were initially prepared before the analysis by IR recorded using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, 10 mg of each seaweed SPs sample was pressed in potassium bromide (KBr) *pellets*. The measurements were performed at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at 500-4000 cm^{-1}.

The spectral data and the graphicals were assigned and represented, respectively, using the Origin software version 8.0 as the Statistical Analysis Software (USA). All the graphicals of the samples were separatelly saved in Windows file to construct the integrated figure.

Analysis of the *in vitro* antioxidant potential by HpSPs

The test sample of HpSPs was chosen because these is no evidence on the antioxidant effects for the genus *Halymenia*. All the *in vitro* assays were assessed for antioxidant effects at the Seaweed II laboratory located at the Department of Biochemistry and Molecular Biology, FUC, and the *in vitro* methods are described below.

1,1-diphenyl-2-picryl-hydrazil (DPPH) scavenging effect

The effect of HpSPs to reduce DPPH was performed according to Blois (1958), with some modifications. For this test, different concentrations of HpSPs (0.125 to 4.0 mg mL^{-1}) were added to the methanol solution of DPPH (75 M). After 30 min, absorbance was measured at 517 nm. All reactions were performed in triplicates and BHT was used as a reference.

The DPPH scavenging effect was calculated using the following equation: scavenging activity (%) = $[A_0 - (A - A_b)/A_0] \times 100$, where A_0 = DPPH without sample; A = sample + DPPH; and A_b= sample without DPPH.

Total antioxidant capacity (TAC)

This assay was performed by the formation of the phosphomolybdate complex, based on Prieto et al. (1999). For this test, HpSPs (0.125 to 4.0 mg mL^{-1}) were added to a solution containing ammonium molybdate (4 mM), sulfuric acid (0.6 M), and sodium phosphate (28 mM), and were incubated at 95°C for 90 min. Absorbance was measured at 695 nm. All reactions were performed in triplicate and a 200 g mL^{-1} sample of ascorbic acid (AA) was used as a positive control and considered as 100% TAC.

The data were expressed as a percentage of TAC using the following formula: TAC (%) = $[(A_{sample}-A_{blank}) / (A_{ascorbic\ ac}-A_{blank})] \times 100$.

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Ferrous ion chelating (FIC) effect

This assay was based on methodology of Chew et al. (2008), with modifications. For this, different concentrations of HpSPs (0.125 to 4.0 mg mL^{-1}) were added to 0.1 mM ferrous sulfate ($FeSO_4$) and 0.25 mM ferrozine acid (3- (2-pyridyl) -5,6-diphenyl-1,2,4-triazine -p, p-disulfonic). The tubes were shaken 1 min, incubated 10 min and the absorbance measured at 562 nm. All reactions were performed in triplicates and EDTA was used as a positive control.

Data were expressed as a percentage of chelating effect according to the following formula: FIC effect (%) = $[A_0 - (A - A_b)/A_0] \times 100$, where A_0= $FeSO_4$ + Ferrozine without sample; A = sample + $FeSO_4$ + Ferrozine; and A_b = sample without $FeSO_4$ + Ferrozine.

Statistical analyses

All data were expressed as mean ± standard deviation (n = 3). For extraction yield comparison, statistical analysis was done by one-way ANOVA, followed by Tukey' test, applying $p < 0.05$ as significant. For *in vitro* antioxidant tests of HpSPs, data were also analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. The graphical representations of yield and IR spectroscopy were constructed using the Origin software version 8.0 as the Statistical Analysis Software (USA).

Results and Discussion

Comparison of extraction yield

The enzimatically-extracted products from dehydrated thalli samples of seven different Brazilian tropical seaweeds species, collected in Pacheco (Chlorophyta *C. cupressoides*, *C. racemosa* and *C. prolifera*; and Rhodophyta *S. filiformis*) or Flecheiras (Rhodophyta *B. occidentalis*, *G. birdiae* and *H. pseudofloeria*) beach, were compared on their yields of dried crude SPs. Thus, the extraction yield (%, w w^{-1}) was analyzed on the impact of the tritured seaweeds (algal morphology) to evaluate the stabilization condition used on crude SPs recovery (Rodrigues et al., 2018), avoiding variation on the values by other extraction techniques (Ghosh et al., 2004; Zibetti et al., 2005; Campo et al., 2009; Fidelis et al., 2014; Wang et al., 2014).

There were significant differences regarding crude SPs yield among the seaweeds (coenocytic and multicellular) species used and, mainly, considerating between phyla (Figure 4). *Caulerpa* taxa of benthic communities from Pacheco beach had the lower yields in SPs (between 0.42 ± 0.08 and 3.43 ± 0.61%, w w^{-1}), of which members of *C. prolifera* were the lowerst sources in CpSPs ($p < 0.05$), with a difference at least 6.52/8.16-fold lower than those found for CcSPs and CrSPs, respectively, similar profile to other studies by Rodrigues et al. (2012, 2018), who observed a low yield of crude SPs from the *Caulerpa* species using papain method. It was hypothetized that the greater amount of CrSPs suggested to a morphological difference of its coenocytic thallus with branches in the shape of bunches of grapes (Figure 1) devoid of transverse walls (multinucleate organism) (DeWreede, 2006), therefore, that could accumulate SPs in major concentration than other two Chlorophyta species of this genus (Rodrigues et al., 2012).

Rhodophyta of benthic communities from Pacheco or Flecheiras beach showed a different scenario regarding a high level of crude SPs extracted by papain digestion (Figure 4). Their crude SPs yields ($p < 0.001$) ranged from 9.07 ± 0.03% (*B. occidentalis*) to 52.82 ± 6.16% (*H. pseudofloresia*), of which the highest yield for the last species could be related to its highly gelatinose surface texture (Rodrigues & Farias, 2009) within all the taxa (Figure 2C). GbSPs and SfSPs revealed to be 24.97 ± 0.03% and 17.33 ± 3.32% ($p > 0.05$, w w^{-1}) yields from the respective dehydrated tissues. All red seaweeds yielded a significant level of crude SPs compared with those of *Caulerpa* species (Chlorophyta); therefore, as being at least 21.58-fold higher ($p < 0.001$). These extraction yields could be compared to studies performed by other authors using the same

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Rhodophyta species, with or not experimental variations (4%, w w^{-1} - Farias et al., 2000; 15 or 18%, w w^{-1} - Fenoradosoa et al., 2009; 40.5%, w w^{-1} - Rodrigues et al., 2009; 14.14%, w w^{-1} - Araújo et al., 2011; 0.52-8.26%, w w^{-1} - Fidelis et al., 2014; ~15%, w w^{-1} - Rodrigues et al. (2018); 5.81±0.71-10.75±0.42%, w w^{-1} - Rodrigues et al., 2024), reflecting different yields.

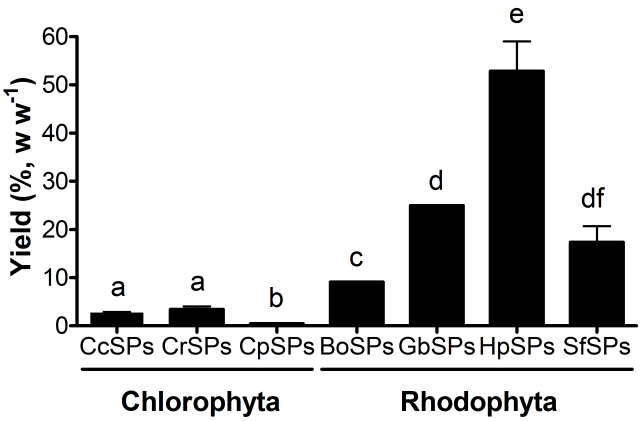

Figure 4. Yield of crude SPs extracted from Brazilian tropical seaweeds collected from two Northeastern beaches (Pacheco or Flecheiras) of Ceará State. Different letters on the bars indicate differences among the yields (ANOVA, Tukey' test, $p < 0.05$ or $p < 0.001$).

As can be noted, Rhodophyta taxa were more abundant in crude SPs than Chlorophyta ones, as communly found in the seaweeds (Pomin & Mourão, 2008; Rodtrigues et al., 2018). Rhodophyta wall biochemical rich in SPs could be explained by more complex structure of their anatomies and texture/composition of thalli (Joly, 1965; Reviers, 2006; Marinho-Soriano et al., 2009; Stengel et al., 2011) compared with coenocytic species, which are "acellulars" (DeWreede, 2006). This morphological plasticity of the cell walls between coenocytic and multicellular benthic forms would represent a chemical defense to enviromental pressure and how these SPs could be used as taxonomic value (Rodrigues & Farias, 2009, 2012; Stengel et al., 2011). Rhodophyta are valuable sources for commercially-important SPs production (Pomin & Mourão, 2008; Campo et al., 2009).

Chemical identification by IR spectroscopy

Further step was to identify the functional groups regarding class of SPs present in each crude extract sample in an attempt to obtain and compare structural bases by IR spectroscopy following taxonomic biology for species identification (Usov et al., 1998; Pomin & Mourão, 2008; Stengel et al., 2011; Campo et al., 2009; Marinho-Soriano et al., 2009; Rodrigues et al., 2012).

IR analysis would provide a comparison of CcSPs, CrSPs, CpSPs, BoSPs, GbSPs, HfSPs and SfSPs whether there is chemical relationship among the species (Ghosh et al., 2004; Maciel el al., 2008; Araújo et al., 2011; Fidelis et al., 2014; Rodrigues et al., 2019, 2024), as well as with other taxa (Alves et al., 2012; Costa et al., 2012; Ananthi et al., 2010; Silva et al., 2010; Paiva et al., 2011; Wang et al., 2014; Alencar et al., 2019; Tesvichiana et al., 2024).

Chlorophyta species

Crude extracts by papain digestion for *Caulerpa* taxa indicated by IR total sulfation (at 1246-1257 cm^{-1}, S=O) and uronic acid (at 1641-1647 and 1404-1458 cm^{-1}, COO^- or O-H) (Ananthi et al., 2010; Paiva et al., 2011) throughout the range from 500 to 4000 cm^{-1} (Figure 5), as expected for SPs of this genus (Ghosh et al., 2004; Costa et al., 2012; Wang et al., 2014; Rodrigues et al., 2019; Tesvichiana et al., 2024). O-H and C-H stretching vibrations were also noted as intense peaks within the range of 3431 to 3448 cm^{-1} and 2935 to 2937 cm^{-1}, as seaweeds SPs-related typical

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absorption features (Ghosh et al., 2004; Maciel et al., 2008; Araújo et al., 2011; Fidelis et al., 2014; Rodrigues et al., 2019; Tesvichiana et al., 2024).

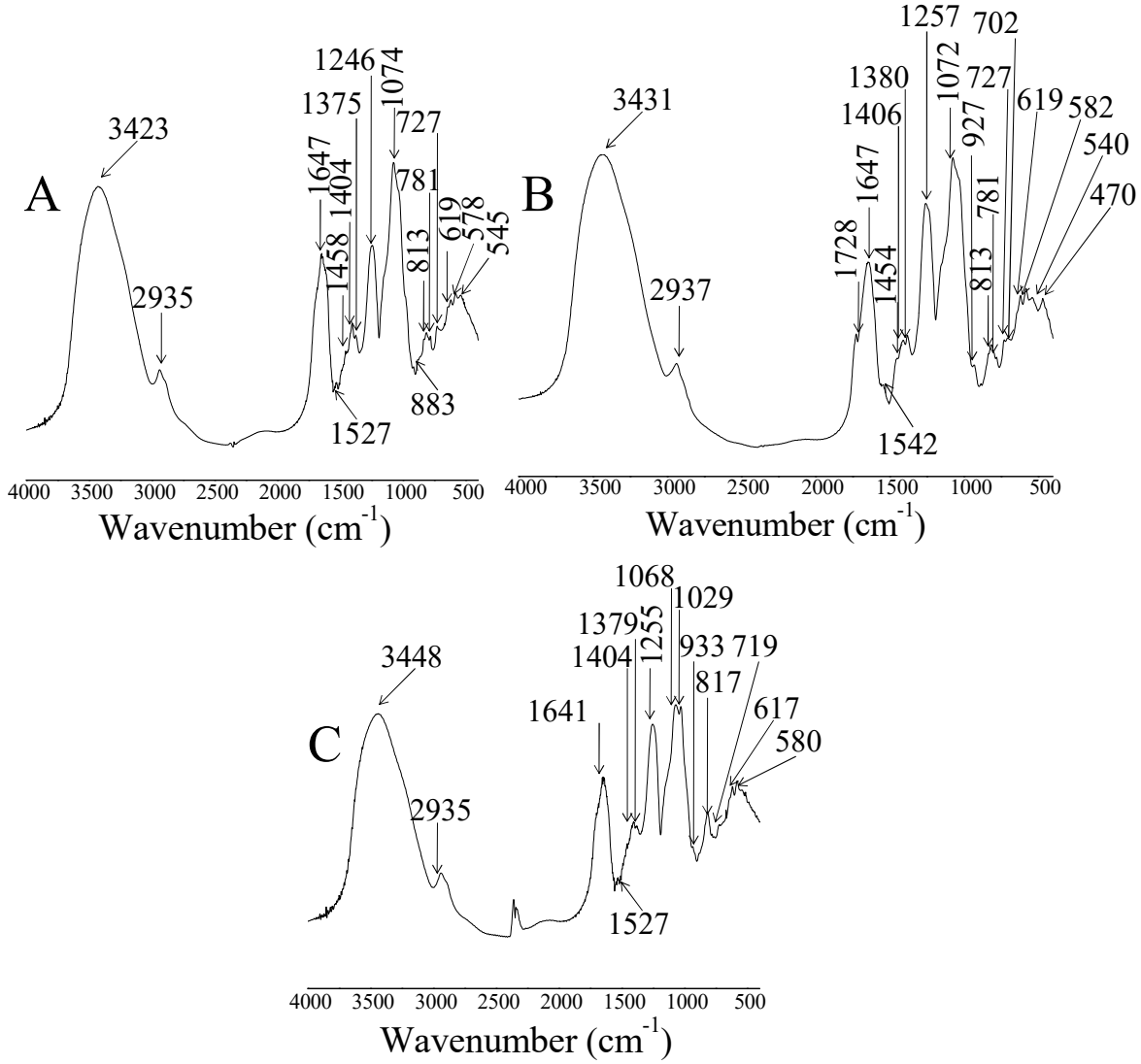

Figure 5. IR spectra of the Chlorophyta SPs (CcSPs [A], CrSPs [B] and CpSPs [C]).

The IR spectra for CcSPs, CrSPs and CpSPs suggested protein residues (undigested by papain) in the polysaccharidic structures (Rodrigues et al., 2019), galactose-6-sulfate (at 813-817 cm^{-1}) commun in Caulerpales (Wang et al., 2014) to generate 3,6-anhydrogalactose as in red seaweeds SPs (Araújo et al., 2011; Alves et al., 2012; Usov et al., 1998; Pomin & Mourão, 2008; Campo et al., 2009); and O=S=O (at 617-619 cm^{-1}) as sulfate (Rodrigues et al., 2019; Tesvichiana et al., 2024). Other signals (at 1527-1542, 1379-1380, 1068-1074, 719-727 and 578-582 cm^{-1}) were found in all samples, except at 1029 (CrSPs), 927-933 (CrSPs and CpSPs), 702 (CpSPs), 540-545 (CpSPs and CcSPs) and 470 (CpSPs) cm^{-1} that were present or not. Reports revealed variations of sulfation signals for *Caulerpa* SPs depending on the extraction method (Wang et al., 2014), such as in *C. cupressoides* var. *flabellata* by Costa et al. (2012) that observed 4-sulfate, 2-sulfate, and 6-sulfate of D-galactose units in SPs extracted with maxatase protease. *Caulerpa* SPs rich in sulfate-6-galactose residues have been implied for anticoagulant action (Wang et al., 2014). SPs from three *Caulerpa* species (Figure 1) had similar structural features, since there is still few evidence to establish a systematic criteria in terms of structure and seaweed order (Costa et al., 2012).

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Rhodophyta species

SPs biosysnthetized by multicellular red seaweeds (Figure 2) showed IR spectra at 3415-3456 (O-H), 2912-2937 (C-H), 1627-1637 (O-C-O), 1406-1419 (O-C=O) and 1251-1257 (S=O, total sulfate) cm^{-1}, as commun absorption features (Usov et al., 1998; Cardozo et al., 2007; Pomin & Mourão, 2008; Campo et al., 2009). Comparison among BoSPs, GbSPs, HpSPs and SfSPs indicated a distribution of different galactan structures and variable position of sulfation (Figure 6), since that structure of the HpSPs is still unknown.

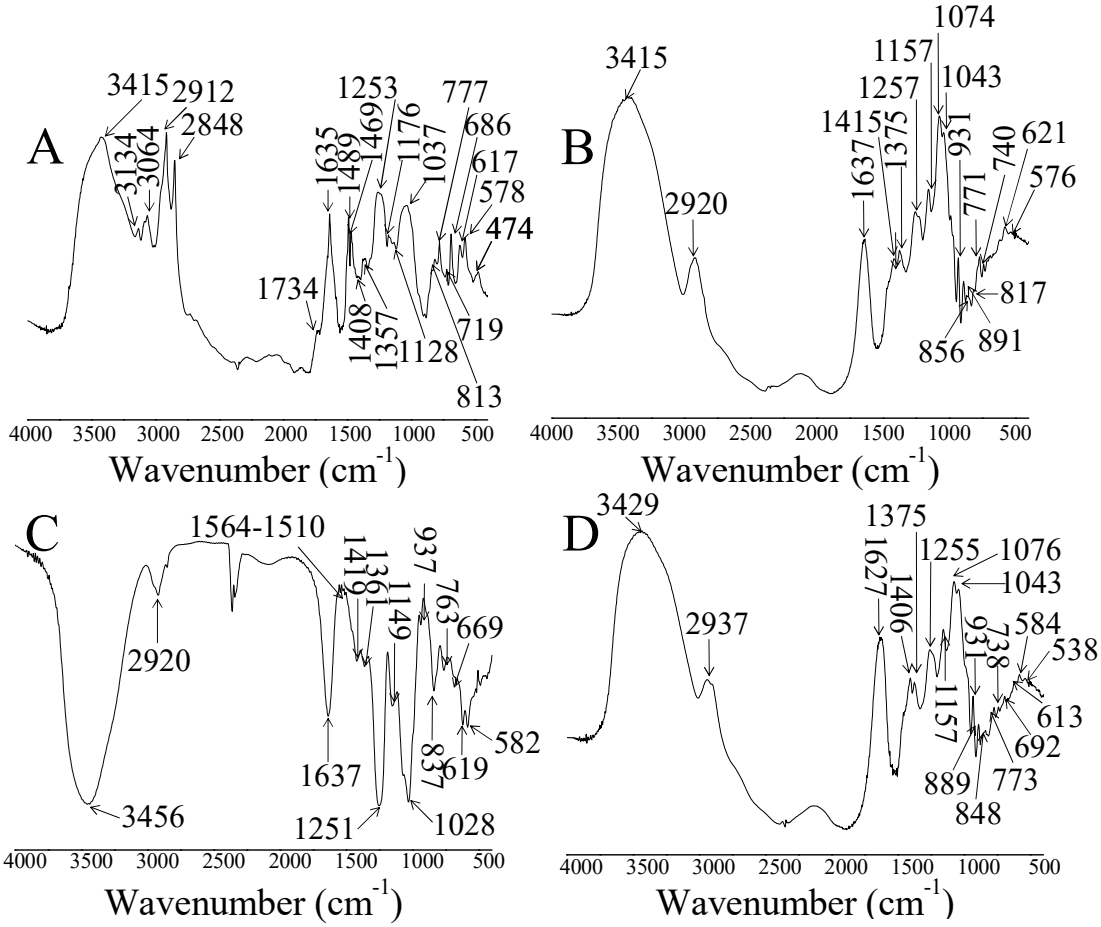

Figure 6. IR spectra of the Rhodophyta SPs (BoSPs [A], GbSPs [B], HpSPs [C] and SfSPs [D]).

BoSPs confirmed an agaran/carrageenan-unrelated galactan structure by absence of galactose-4-sulfate at 845 cm^{-1}, galactose-6-sulfate at 820 cm^{-1} and 3,6-anhydrogalactose-2-sulfate at 805 cm^{-1} (Rodrigues et al., 2024), although presenting alcholic residues (3064 to 3415 cm^{-1}) (Figure 6A).

GbSPs (Figure 6B) showed an agarocolloid-structure (at 1375, 1257, 1074, 931, 891 and 771 cm^{-1}) with basis on those values from aqueous extract of *G. birdiae* collected on the same region by Maciel et al. (2008) revealing sulfation at the C-4 of galactose (at 856 cm^{-1}), small degree on C-6 (at 817 cm^{-1}) and absence of 2-sulfate galactose (at 805 cm^{-1}). By contrast, Fidelis et al. (2014) chacterizated by IR five *G. birdiae*-derived extracts (proteolysis, NaOH and ultrasound) and found uronic acid for all samples as herein (at 1637 and 1415 cm^{-1}), revealing an acidic portion like in Chlorophyta (Wang et al., 2014) and in Ochrophyta (Ananthi et al., 2010; Paiva et al., 2011).

HpSPs (Figure 6C) suggested very complex galactan structural composition, but differences were based on commercially-used carrageenan (Silva et al., 2010). The analysis revealed at 1361 cm^{-1} (sulfate substitution), another with low intensity for 3,6-anhydrogalactose (at 937 cm^{-1}) and two absorptions bands at 837 and 825 (a discrete shoulder) cm^{-1}, whose both signals would

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represent the equatorial sulfate ester at O-2 and O-6 of galactose residues similar to *H. durvillei* SPs by Fenoradosoa et al. (2009), who characterized its SPs as *lambda*-carrageenan-structure, but with absence of signals around 850 and 805 cm^{-1} (axial sulfate ester). It was another description for SPs from the genus *Halymenia* belonging to *lambda* family, since fewer reports have been ascribed to family Halymeniaceae showing carrageenan *vs*. other genera (Zibetti et al., 2005; Cardozo et al., 2007; Pomin & Mourão, 2008; Campo et al., 2009; Silva et al., 2010).

IR analysis of the SfSPs confirmed both *iota*/*kappa*-carrageenan structures because total sulfate (at 1255 cm^{-1}) had the lowest intentity than that of lambda-carrageenan from HpSPs (Figures 6C, D) and signals at 848 (D-galactose 4-sulfate) and 805 (3,6-anhydrogalactose 2-sulfate, very discrete) cm^{-1} (Silva et al., 2010), suggesting *iota* preponderance (Araújo et al., 2011). Both *H. pseudofloresia* and *S. filiformis* produced different types of SPs (carrageenans) in their extracellular matrixes, revealing complex biochemical composition (Campo et al., 2009; Stengel et al., 2011).

Collectivelly, papain-assisted extraction partially characterized taxonomic entites in structure as supplements for chemical separation of seaweeds (coenocytic and multicellular). Galactose was the main sugar present in samples, but structural details presumed variations in sulfation, at least, within the Rhodophyta species (Figure 6), while well-conserved for *Caulerpa* SPs in position for the genus, independent of algal morphology (Figure 5). The feature of 6-sulfation was dispersive distribution among species and 4-sulfate restricted for SfSPs, and other small variations (Pomin & Mourão, 2008), presence or not of 3,6-anhydrogalactose that determine industrial uses, *e.g*., gelling and stabilizing properties (Usov et al., 1998; Campo et al., 2009).

In vitro effects of the HpSPs as antioxidants

HpSPs were capable of inhibiting the three *in vitro* assays (DPPH, FIC and TAC) on the oxidant event (Table 1), by two biological stages: initiation and propagation (Femi-Adepoju et al., 2023). DPPH-scavenging assay revealed that the HpSPs were potent less (from 0.55 ± 0.67 to 22.57 ± 0.09%, 0.12- 4.00 mg mL^{-1}, respectively) than the BHT systhetic antioxidant (92.70 ± 1.16% scavenging, at 4 mg mL^{-1}, $p < 0.05$). The standard action was about 4.10-fold higher in terms of *in vitro* inhibitory effect at highest concentration *vs*. test sample (HpSPs).

Studies on the antioxidant potential by seaweeds SPs by the DPPH method have showed some natural sources, such as in SPs from Chlorophyta *C. lentillifera* (18.41 ± 0.92 to 32.75 ± 0.05% for fractions at 0.02 mg mL^{-1} - Tesvichiana et al., 2024); from Rhodophyta *H. musciformis* (9.88 for fraction at 5 mg mL^{-1} - Alves et al., 2012); and from Ochrophyta *S. swartzii* (25.33 ± 2.52% for extract at 1 mg mL^{-1} *vs*. gallic acid: ~ 40% at 0.02 mg mL^{-1} - Vijayabaskar et al., 2012) and *T. ornata* (80.21 ± 2.50% for extract at 0.5 mg mL^{-1} *vs*. quercetin: 96.81 ± 1.23% at 0.125 mg mL^{-1} - Ananthi et al., 2010). Our results indicated that the HpSPs had a comparatively lower donator effect to display scavenging actions in the concentration range tested by the DPPH assay (Femi-Adepoju et al., 2023).

By the TAC, HpSPs acted on Mo to form a green phosphate/Mo complex total of the system (Prieto et al., 1999). The antioxidant effect of the sample increased with the concentration range, exhibiting an inibitory profile similar to DPPH, had a maximum reduction rate of up to ~33%, when at a HpSPs concentration of 4 mg mL^{-1} against the standard ascorbic acid (99.77% inhibition, at 0.4 mg mL^{-1}, $p < 0.05$). Thus, the antioxidant role by HpSPs required a concentration 10-fold higher for removing reative oxigen species by the TAC assay.

Several SPs from seaweeds have been reported as having antioxidant action by the TAC assay. Among them have those from Chlorophyta *C. cupressoides* var. *flabellata* (~ 20 equivalents - Costa et al., 2012), from Rhodophyta *G. caudata* (~ 90% at 4 mg mL^{-1} - Alencar et al., 2019); and from Ochrophyta *L. variegata* (75% at 5 mg mL^{-1} - Paiva et al., 2011) and *S. swartzii* (32.34 ± 1.42% at 0.02 mg mL^{-1} - Vijayabaskar et al., 2012). These studies involved SPs extracted by different protocols that varied the antioxidant response in relation to amnonium molyddate. Overall, they

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interacted with the reaction system more convincent sending electrons to reduce the free radical attack compared with the same ability of the HpSPs, when by the TAC assay (Fidelis et al., 2014). Therefore, both DPPH and TAC assays demonstrated that the HpSPs blocked the initiation phase of the *in vitro* reaction processes (Femi-Adepoju et al., 2023).

Table 1. Effects of HpSPs on DPPH, FIC and TAC assays.

HpSPs mg mL^{-1}	HpSPs		
	DPPH (%)	FIC (%)	TAC (%)
0.12	0.55 ± 0.67^{a}	19.85 ± 0.00^{a}	1.24 ± 1.88^{a}
0.25	0.93 ± 0.04^{a}	30.91 ± 0.22^{b}	2.03 ± 0.23^{a}
0,50	2.23 ± 0.13^{a}	39.20 ± 0.30^{c}	4.01 ± 0.18^{b}
1.00	5.24 ± 0.32^{b}	45.74 ± 0.08^{d}	9.53 ± 0.25^{c}
2.00	11.63 ± 0.09^{c}	50.24 ± 0.08^{e}	15.94 ± 0.25^{d}
4.00	22.57 ± 0.09^{c}	54.85 ± 0.30^{e}	33.42 ± 0.31^{e}
BHT 4 mg mL^{-1}	92.70 ± 1.16^{d}	-	-
EDTA 4 mg mL^{-1}	-	100.00 ± 0.00^{f}	-
ascorbic acid 0.4 mg mL^{-1}	-	-	99.77 ± 0.00^{f}

Different letters indicate significant differences at level of 5% (ANOVA, Tukey' test, $p < 0.05$).

Regarding FIC assay, HpSPs exhibited a preponderant effect within all the assays (Table 1), with a concentration-dependent response, where the sample significantly acted up to 54.85 ± 0.30% at 4 mg mL^{-1}. This profile, however, was around 50% inhibition against the EDTA antioxidant (100.00 ± 0.00% at 4 mg mL^{-1}, $p < 0.05$) used as a reference, which had an inhibitory property almost 2-fold higher than HpSPs. It meant that the HpSPs inhibited the propagation chain from this assay (Femi-Adepoju et al., 2023).

Metal ion-chelating property of SPs to prevents oxyradical formation has been reported, including from Chlorophyta *C. cupressoides* var. *flabellata* (44% at 2 mg mL^{-1}- Costa et al., 2012); and from Rhodophyta *H. musciformis* (8% at 5 mg mL^{-1} - Alves et al., 2012) and *G. caudata* (69.80% at 4 mg mL^{-1} - Alencar et al., 2019). Our results sugested that, by highest intensity of sulfation recorded by IR spectrum (Figure 6C), HpSPs could be dependent of their charge density (specific sulfation sites) for display antioxidation, as already well-known feature of *lambda*-carrageenan regarding their anticoagulant and inflammatory actions (Silva et al., 2010). Anticoagulant role (Rodrigues et al., 2009a) and non-toxicity in shrimps (Rodrigues et al., 2009b) were already described for HpSPs. In-depth studies on the sterospecific features, monossacaridic composition, glycosylation sites, aromericity, and spartial conformation of the HpSPs must be done in order to better evaluate the function-structure relationship of these marine molecules (Pomin & Mourão, 2008), because there is few researches to understand the antioxidant properties of natural molecules to determine the most effective agents and their pathways (Femi-Adepoju et al., 2023).

As there were marked differences in yield and structural features of the SPs between phyla or, until, among species (Figures 4, 5 and 6), current investigation bring a new challenge to chemotaxonomy of seaweeds as strategy for studies of the wall biochemical and how this perspective would generate as tool for identification of groups. Algal anatomy and distribuition of biactive SPs are still relevant topics related to physiological activity caused by enviromental pressure and, in parallel, how bringing biotechnological perspectives.

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Conclusion

Papain extraction showed that the availability of crude sulfated polysaccharides from seven Brazilian tropical seaweeds species collected from Pacheco (three *Caulerpa* species and *Solieria filiformis*) and Flecheiras (*Botryocladia occidentalis*, *Gracilaria birdiae* and *Halymenia pseudofloresia*) beaches reveal Rhodophyta quite abundant sources than Chlorophyta. Infrared analysis indicated different basic structures: ulvan-type polysaccharide (Chlorophyta taxa), agaran/carrageenan-unrelated polysaccharide (*B. occidentalis*), agaran (*G. birdiae*), *lambda*-carrageenan (*H. pseudofloresia*) and *iota*-/*kappa*-carrageenan (*S. filiformis*) as classes separated by phylum and, until, different species in morphological terms. Antioxidant testing of the *H. pseudofloresia* polysaccharides demonstrated inhibitory effects on all the *in vitro* assays, being most effective by propagation (ion-chelating) stage than initiation (reducing power) one.

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