Rev. Bras. Eng. Pesca (2025) 16(3): 81-96
DOI: 10.18817/repesca.v16i3.3696

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AQUEOUS EXTRACTION OF ANTIOXIDANT *KAPPA*-CARRAGEENAN FROM *Hypnea musciformis* (RHODOPHYTA) MARICULTURE

EXTRAÇÃO AQUOSA DE *KAPPA*-CARRAGENANA ANTIOXIDANTE DE MARICULTURA DE *Hypnea musciformis* (RHODOPHYTA)

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Recebido: 11/05/2024 / Publicado: 03/07/2025

Abstract Mariculture of *Hypnea musciformis* (Rhodophyta) on the northeast coast of Brazil has been an alternative in bioactives sulfated polysaccharides (HmSPs). This study aimed to extract in hot-water HmSPs to quantification and determination of their structural and antioxidant properties. After cold-water pretreatment (1.5%, w v^{-1}), algal residue was reextracted in water (80°C, 4 h) and HmSPs characterized by agarose/polyacrylamide gels electrophoreses *vs.* standard glycosaminoglycans stained with toluidine blue/Stains-All; and then by infrared analyzed. HmSPs were *in vitro* tested for antioxidant effects by DPPH, total antioxidant capacity (TAC) and ferrous ion chelating methods against BHT, ascorbic acid and EDTA, respectively. Refined aqueous extraction (20.00 ± 3.25%, w w^{-1}) revealed *kappa*-carrageenan made of a polydisperse charge system (>100 kDa) with structural peculiarities, but without presenting chemical degradation. HmSPs had antioxidant effects by all the tests, but revealing prepondetant role by TAC initiation phase than in propagation one with less efficacy than respective synthetics. Therefore, mariculture of *H. musciformis* may be a valuable alternative to antioxidant SPs obtaining.

Key words: open sea, red alga, molecular properties, reductor potential.

Resumo Maricultura de *Hypnea musciformis* (Rhodophyta) tem sido uma alternativa em polissacarídeos sulfatados (HmPSs) bioativos na costa nordeste do Brasil. Este estudo objetivou a extrair HmPSs em água quente para quantificação e determinação de suas propriedades estruturais e antioxidantes. Após pretratamento aquoso a frio (1.5%, w v^{-1}), foi re-extraído o resíduo algáceo em água (80°C; 4 h) e caracterizados HmPSs por electroforeses em géis de agarose/poliacrilamida *vs*. glicosaminoglicanos padrões corados com azul de toludina/"Stains-All" e, posteriormente, analisados por infravermelho. Foram testados *in vitro* os HmSPs para efeitos antioxidantes pelos métodos DPPH, capacidade antioxidante total (CAT) e quelação de íon ferroso contra BHT, ácido ascórbico e EDTA, respectivamente. A extração aquosa refinada (20,00 ± 3,25%; w w^{-1}) revelou *kappa*-carragenana constituída de um sistema de carga polidisperso (>100kDa) com peculiaridades estruturais, porém sem apresentar degradação química. HmPSs tiveram efeitos antioxidantes por todos os testes, porém revelando papel preponderante pela fase CAT inicial que na fase de propagação com eficácia menor que os sintéticos respectivos. Portanto, maricultura de *H. musciformis* pode ser uma alternativa valiosa para obtenção de PSs antioxidantes.

Palavras-Chave: mar aberto, alga vermelha, propriedades moleculares, potencial redutor.

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Introduction

Mariculture of seaweeds (macroalgae) has been an economically-important activity and for aquatic science studies developed by numerous tropical countries (*e.g.*, México - Munõz et al., 2004; Indian - Ganesan et al., 2006; and Brazil - Rodrigues et al., 2011), involving native (*e.g.*, *Hypnea musciformis* - Ganesan et al., 2006; Rodrigues et al., 2011a, 2011b; *Solieria filiformis* - Araújo et al., 2012; and *Gracilaria birdiae* - Maciel et al., 2008; Rodrigues et al., 2017) or introduced (*Kappaphycus alvarezii* - Munõz et al., 2004) species, that continues grow in order to offers technological, biological and social bases for the hydrocolloid industry (Cardozo et al., 2007; Maciel et al., 2008; Campo et al., 2009). Seaweeds explotation in the world has increased in the last decades bringing negative impacts for the population structures regarding the sustainability of natural stocks, since that they are also valuable sources for biologically actives sulfated polysaccharides (SPs, known as phycocolloids) obtaining as non-toxic agents in cosmetic and pharmaceutical formulations (Cardozo et al., 2007; Campo et al., 2009).

Cell-walls of red seaweeds (Rhodophyta) is composed by a fibrillar matrix embebed of SPs complexed to proteins (like proteoglycans) playing structural and physiological functions (Pomin & Mourão, 2008). These ultrastructures of amorphous matrixes are rich in sulfated galactans (carrageenan and agaran), which are hydrophilic colloids of high molecular masses (>100 kDa) and highly charged (S=O) (Pomin, 2012) composed of alternating 3-linked-β-D-galactopyranose unit (substituted with methly or sulfate ester radicals) and the 4-linked-3,6-anhydro-α-L-galactopyranose unit. β-galactoses are D-enantiomers, whereas the α-galactose residues may be D- for carrageenan, L- for agaran or DL-hybrid on the backbone of the polymer (Pomin & Mourão, 2008). These configurations also vary in sulfation pattern and occurence of 3,6-anhydrosugars, whose structural features would influence their extensive functionalities (*e.g.*, anticoagulant, antithrombotic, antiviral, anticancer, immuno-inflammatory, antioniceptive, anti-inflammatory and antioxidant effects) (Cardozo et al., 2007; Femi-Adepoju et al., 2023). Regarding carrageenan-structures, six basic forms are well-known (*iota*-, *kappa*-, *lambda*-, *mu*-, *nu*- and *theta*-carrageenans) for the commercial use and to identification of different Rhodophyta species, *e.g.*, genera *Hypnea*, *Chondrus*, *Eucheuma* and *Gigartina* (Campo et al., 2009).

Free radicals are generated by endogenous or exogenous sources that affect the cellular metabolism, changing biochemical nature and destroying cell membrane, whose impacts can lead to biomolecular oxidation with the development of diverse diseases (*e.g.*, cancer, atherosclerosis and respiratory) and degenerative processes (Barbosa et al., 210). The interest of natural antioxidants is also justified due to common use of synthetics substances (like butylatedhydroxytoluene-BHT) in food technology to a variety of processed products (Femi-Adepoju et al., 2023), although recognized as toxics (Panicker et al., 2014). On these questions, intense studies on the SPs-based antioxidants have shown with potentials in various *in vitro/in vivo* experiments. From different origins, antioxidant SPs have already been isolated and characterized not only in Chlorophyta (*Caulerpa cupressoides* var. *flabellata* - Costa et al. (2012), *Caulerpa lentillifera* - Tesvichian et al. (2024) and other genera (Wang et al., 2014)); Rhodophyta (*Gracilaria birdiae* - Fidelis et al. (2014) and *G. caudata* - Alencar et al. (2019)); and Ochrophyta (*Laminaria japonica* - Wang et al. (2008), *Turbinaria ornata* - Ananthi et al. (2010)), *Lobophora variegata* - Paiva et al. (2011) and *Sargassum swartzii* - Vijayabaskar et al. (2012)), but also in seagrasses (Silva et al. (2012)), by-products of fishes (Jridi et al., 2019; Nascimento et al., 2021; Santiago et al., 2024) and cyanobacteria (Ai et al., 2023). However, eco-friendly obtained bioactives from mariculture activity of red seaweeds species arise as a sustainable platform to new phytopolymers for hydrocolloid industry like novel functional foods and medicinal use (Rodrigues et al., 2011a, 2011b; Araújo et al., 2012; Femi-Adepoju et al., 2023).

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Hypnea musciformis (Wulfen) J. V. Lamouroux (Florideophyceae, Gigartinales) is a carrageenophyte epiphyte mainly cultured in tropical countries (Ganesan et al., 2006; Campo et al., 2009) and that has been experimentally produced with *Solieria filiformis* (Kützing) P. W. Gabrielson (Gigartinales, Solieraceae) along the northeastern Brazilian coast (Flecheiras Beach, Trairí, Ceará State) (Rodrigues et al., 2011a). This algal species is a valuable source in non-toxic *kappa*-carrageenan (Campo et al., 2009; Rodrigues et al., 2011b) already explored for Biotechnological studies, including anticoagulant (Rodrigues et al., 2011a), antioxidant (Alves et al., 2012; Souza et al., 2018), antimicrobial, neuroprotective (Souza et al., 2018) and rheological (Cosenza et al., 2014) properties. As the native seaweeds cultivation in open sea is an activity that promotes social inclusion of people considered in poverty situation in litoral areas (Ganesan et al., 2006; Maciel et al., 2008), in this study, the *H. musciformis* SPs (HmSPs) obtained by refined aqueous extraction were chemically/physically/structurally examined by electrophoreses and by Fourier Transform Infrared (FT-IR) spectroscopy; and then *in vitro* tested for their *in vitro* antioxidant effects, contributing with the efforts to the sustainable production and biochemical knowledge of this algal species developed on the northeast coast of Brazil.

Material and Methods

Harvest and processing of *H. musciformis*

Samples of the epiphyte *H. musciformis* (Figure 1) (Rhodophyta) were mannually removed from experimental mariculture of *S. filiformis* developed on the northeast coastal of Brazil (Flecheiras beach, Trairí, Ceará State) using *long-line* structures anchored and submersed at 200 m from littoral line (03°13'06"S, 39°16'47"W). After acquisition from the Carbohydrates and Lectins Laboratory, Department of Biochemistry and Molecular Biology, Federal University of Ceará (FUC), they were transported in plastic bags and then processed at the Marine Biochemistry laboratory (MaBio) of the Aquaculture Biotechnology Center, Department of Fishing Engineering, FUC, in which were separated from other macroscopic algae and then extensivelly washed with distilled water to removing impurities (*e.g.*, salt, sand and shells) and necrotic parts of the raw material before frozen at -20°C until analyses (Rodrigues et al., 2011a, 2011b; Araújo et al., 2012).

Kingdom Plantae
Phylum Rhodophyta
Class Florideophyceae
Order Gigartinales
Family Cystcloniaceae
Genus *Hypnea*
Species *H. musciformis* (Wulfen) J. V. Lamouroux, 1813

Figure 1. Red seaweed *H. musciformis* harvested as an opportunistic species on *S. filiformis* cultured on the Brazilian coastal of Flecheiras Beach. It is easily identified by the flattened at the end of branches.

In the MaBio laboratory, fresh samples of *H. musciformis* dark-red coloring with intact shape of the thallus were then macroscopically identified using the Marinho-Soriano et al. (2009)' catalogue. It is a marine species well-known and characterized by a cartilaginous thalli with branches commonly occuring a main axis of bifurcated shape (Joly, 1965) and its biomass production is influenced by climatic variations (Ganesan et al., 2006). Specimen was reposted in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil. The use of the algal material was authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

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Refined aqueous extraction of HmSPs

Total fresh biomass samples stored of *H. musciformis* mariculture carefully cleaned was then prepared before the HmSPs extraction. For this, raw material was dehydrated on filter paper at laboratory temperature (at 25-28°C) and kept overnight for obtaining of the pretreated tissue of the seaweed. Initially (Figure 2*a*), dehydrated first-matter was tritured and sample (5 g) was kept in water (1.5%, w v^{-1}) under mechanical stirring for 24 h at laboratory temperature (25-28°C) for removing precursors, pigments and other non-gel promoting structural elements as previously described (Rodrigues et al., 2011a, 2011b; Araújo et al., 2012). After that (Figure 2*b*), the algal residue was separated and resubmitted to a thermostatic bath (4h at 80ºC) in the same solvent at 1.5% (w v^{-1}). Then, the residue was filtered in a nylon net (60 µm), and the supernatant obtained precipitated (24 h, 20°C) with 92.8% ice-cold commercial alcohol (1:3, v:v) for aqueous SPs obtaining. The alcoholic colloid was exaustivelly dialyzed against destilated water and lyophilized yielding the crude SPs extract. Finally, HmSPs were weighed and quantified as the percentage (%, n = 3) of the dehydrated matter (w w^{-1}).

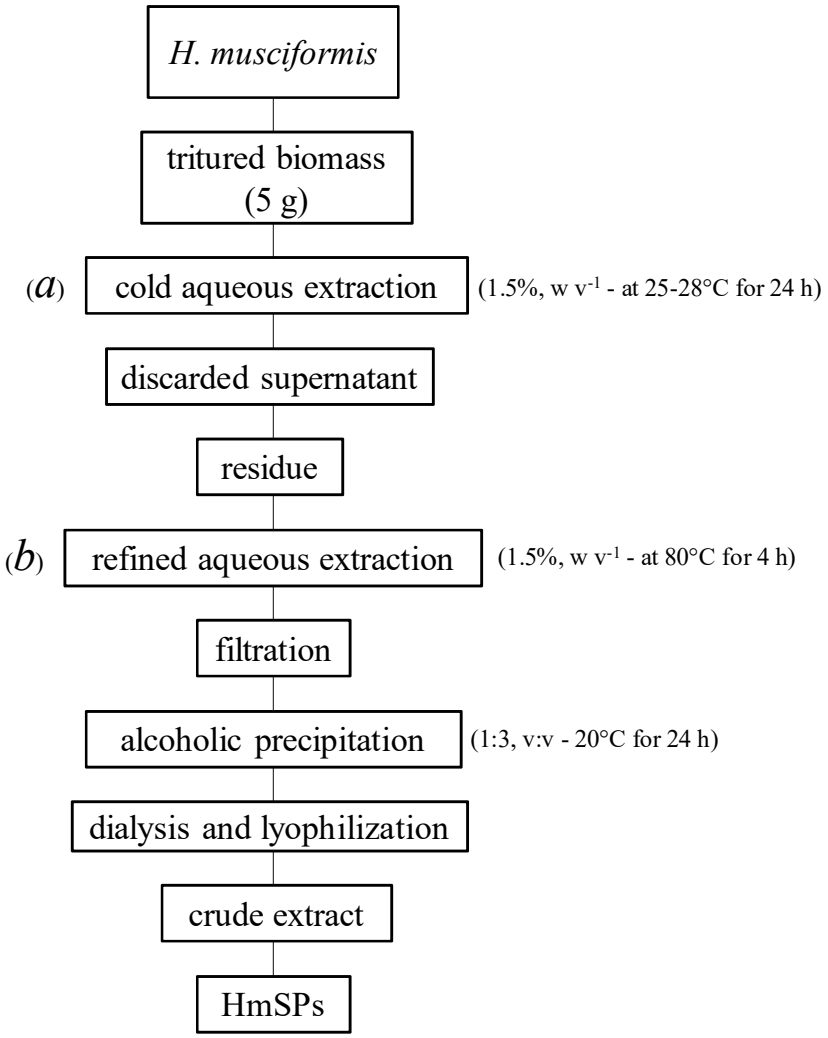

Figure 2. Procedures by two sequential steps (*a* and *b*) for obtaining of the aqueous SPs from *H. musciformis* mariculture.

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Physical and chemical characterization by electrophoreses

A crude sample of HmSPs was previously weighed and then dissolved in destillated water under stirring before its application from aliquot (~30 µg) in two different buffer systems of electrophoretic procedures. These assays analyzed, at initial level, the impact of the hot aqueous extraction on the molecular features (charge and mass) of the colloid-polymer.

Agarose gel electrophoresis

For the agarose analysis, HmSPs were characterized on the polydispersion pattern and charge density. The sample was carefully applied to a 0.5% agarose gel prepared in 0.05 M 1,3-acetate diaminopropane buffer (pH 9.0) and the run was carried out at constant voltage (100 V, 1 h). After the run, the HmSPs were then fixed on gel with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for 24 h and then dehydrated at 50-60°C for ~6h (Dietrich & Dietrich, 1976).

Polyacrylamide gel electrophoresis (PAGE)

In relation to apparent molecular mass distribution of HmSPs, PAGE was used in which the sample was carefully applied to a 6% polyacrylamide gel using 0.02 M Tris/HCl buffer (pH 8.6) and the run was scheduled at 500 mA for 1 h (Santiago et al., 2024).

To the visualization of the sample, the HmSPs present in both gels were detected with 0.1% toluidine blue cationic reagent and, subsequently, the gels were destained with a solution containing absolute ethanol, distilled water and acetic acid or using distilled water only. On a comparative basis, known markers of molecular mass (glycosaminoglycans: chondroitin-6-sulfate (C-6-S,~ 60 kDa), chondroitin-4-sulfate (C-4-S,~ 40 kDa), sulfated dextran (DexS,~ 8 kDa), dermatan sulfate (DS,~ 40 kDa) and/or UHEP (~ 15 kDa)) were used as animal standards (Dietrich & Dietrich, 1976; Santiago et al., 2024).

FT-IR spectroscopy

The qualitative analysis by FT-IR technique of the HmSPs was obtained using a spectrometer (IRPrestige-21 Shimadzu, Japan). About 10 mg of 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}.

***In vitro* antioxidant assays**

HmSPs were *in vitro* evaluated for their antioxidant effects at the Seaweed II laboratory located at the Department of Biochemistry and Molecular Biology, FUC, using different concentrations (0.125→4.0 mg mL^{-1}) by classical methods of oxidant reaction as described below.

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

The effect of HmSPs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of HmSPs 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 effect (%) = $[A_0 - (A - A_b)/A_0] \times 100$, where A_0 = DPPH without sample; A = sample + DPPH; and A_b= sample without DPPH.

Total antioxidant capacity (TAC)

This method was performed by the formation of the phosphomolybdate complex, based on Prieto et al. (1999). HmSPs 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

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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 (%) = $[(A_{sample}-A_{blank}) / (A_{ascorbic\ ac}-A_{blank})] \times 100$.

Ferrous ion chelating (FIC) effect

This method was conducted based on methodology of Chew et al. (2008), with modifications. Different concentrations of HmSPs 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 the 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 of HmSPs were expressed as mean ± standard deviation (n = 3). Values of the *In vitro* antioxidant assays analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. The graphical representations of FT-IR were also constructed using the Origin software version 8.0 as the Statistical Analysis Software (USA).

Results and Discussion

Refined aqueous extraction yield of HmSPs

Tritured tissue, of dehydrated *H. musciformis* (Rhodophyta) epiphyte biomass harvested and prepared from experimental mariculture (Figure 1), was submitted to cold water (1.5%, w v^{-1}) under constant agitation for 24 h at 25-28°C initially yielding a material that was discarded (Cosenza et al., 2014) because it was rich in precursors, photosynthetic pigments and other non-gel promoting structural elements (Rodrigues et al., 2011a, 11b; Araújo et al., 2012). Then, the algal residue was reextracted with the same solvent at 80°C for 4 h yielding on average 20.00 ± 3.25% (w w^{-1}) of aqueous SPs extract. This yield differed from those of HmSPs from algal samples obtained on the northeast coast of Brazil, when obtained by different conditions (Table 1).

On a comparative basis (Table 1), *H. musciformis* varied in SPs yield (20.00 ± 3.25%→49.05 ± 0.38%, w w^{1}) as a result of the employment of different extraction protocols (temperature, time / medium of extraction, and treatment of the first matter) (Araújo et al., 2012) and/or in response to ecophysiological conditions of the marine system located the cultivation (Munõz et al., 2004; Cardozo et al., 2007; Rodrigues et al., 2017). Thus, HmSPs extraction yielded, at least, ~2.45-fold lower than those of liquid nitrogen-macerated samples (*H. musciformis*) digested with protease (Rodrigues et al., 2011b; Souza et al., 2018) and was from ~1.21 to 1.97-fold higher for hot water extracted-tritured/milled samples of the same algal species cultured (Rodrigues et al. (2011b) or collected in Brazil (Cosenza et al., 2014), respectively. Liophylized HmSPs extract yield in this study was lowest and conflicted to other authors (Table 1) and the alcoholic precipitation could also corecovery floridean starch affecting the carrageenan quality (Munõz et al., 2004).

High extraction contact (at 80°C) employed for HmSPs obtaining in this study impacted on the total yield (20.00 ± 3.25%, w w^{-1}) based on Cosenza et al. (2014), who obtained on average 39.40% (w w^{-1}) of HmSPs after liophylization procedure, since that the long water extraction / high extraction temperature must also be considered (Ai et al., 2023). In fact, increasing temperature would lead to a high amount of SPs due to a trend of this factor negatively impact on the wall-

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matrix structure releasing the native SPs into the water by an abrupt difference of heating *H. musciformis* tissue (Rodrigues et al., 2011b; Araújo et al., 2012).

Table 1. Yields of SPs obtained from *H. musciformis* harvested on the northeast coast of Brazil.

Seaweeds/Locality	SPs	Extraction	Tissue	Yield/Drying (w w^{-1})	Reference
H. musciformis (mariculture)	HmSPs	refined water (80°C, 4 h)	tritured	20.00 ± 3.25% (liophylization)	This study
H. musciformis (mariculture)	HmSPs	water (80°C, 4 h) protease (60°C, 6 h)	tritured	44.77 ± 0.91% (oven) 49.05 ± 0.38% (oven)	Rodrigues et al. (2011a)
H. musciformis (mariculture)	HmSPs	protease (60°C, 6 h) water (80°C, 4 h) refined water (80°C, 4 h)	macerated	20.80 ± 0.36% (liophylization) 36.80 ± 1.27% (liophylization) 24.20 ± 0.87% (liophylization)	Rodrigues et al. (2011b)
H. musciformis (coast of Natal)	HmSPs	protease (0.15M, pH 8.0)	crushed	n.i	Alves et al. (2012)
H. musciformis (coast of Natal)	HmSPs	refined water (90°C, 8 h)	milled	39.40% (liophylization)	Cosenza et al. (2014)
H. musciformis (coast of Ceará)	HmSPs	protease (60°C, 6 h)	macerated	28.00% (liophylization)	Souza et al. (2018)

Yield was calculated as percentage (%) with basis of the dehydrated algal tissue; n.i: not informed.

In addition, the precipitated aqueous SPs by adding / washing of 92.8% cold alcohol was as a eco-friendly approach (green-based method) that has widely been used by the hydrocolloid industry (Cardozo et al., 2007; Campo et al., 2009) or by other researches (Munõz et al., 2004; Maciel et al., 2008) to recovery raw materials at a lowest cost, although usually existing some deficiencies by water extraction regarding an algal biomass rate of 1.5% (w v^{-1}) and contact time of 4 h for liophylized SPs obtaining (Figure 2). However, as there is a high risk for degradation of water-extracted SPs when at high temperatures (Ai et al., 2023; Tesvichian et al., 2024), the further step was to examine, by electrophoretic and FT-IR techniques, the impact of the applied extraction conditions on the molecular features of the liophylized product (HmSPs).

Partial characterization of the HmSPs by electrophoreses

After aqueous extraction, HmSPs were partially characterized by both agarose / PAGEs compared to animal glycosaminoglycans (Figure 3), as well-known standards (Santiago et al., 2024). Liophylized sample of HmSPs, present on agarose gel stained with toluidine blue, essentially indicated as a polydisperse component that confirmed for *H. musciformis*-derived crude SPs (Rodrigues et al., 2011a, 2011b; Souza et al., 2018) and other Rhodophyta species (Araújo et al., 2012; Rodrigues et al., 2017), typical profile of unfractionated anionic polymers (Fidelis et al., 2014). It complexed in gel made of diamine buffer system due to its charge density (Dietrich & Dietrich, 1976) visualized from origin to standards DS/HEP (Figure 3A), which had different eletrophoretic mobilities revealing a strong staining pattern in the presence of the dye based on Santiago et al. (2024). Different extraction methods could cause important differences on the molecular features of the algal extracted constituints for biotechnological use by varying their bioactivities (Rodrigues et al., 2011b; Araújo et al., 2012; Wang et al., 2014).

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From Stains-All dye application (Figure 3B), agarose gel showed us to a higher sensitivity to detect the material in the same preparation. Staining of HmSPs after the electrophoretic procedure with Stains-All treatment revealed to an intense and distinct coloration of bands (sample *vs.* standards). These results suggested to a compositional difference by each polysaccharide examined from different origins in present system, suggesting that the *H. musciformis* could synthetize amounts of other sugar residues that were not observed after treatment with toluidine blue alone (Figure 3A) (Fidelis et al., 2014). Therefore, Stains-All cationic dye produced a more stable and visible complex of HmSPs to evaluate, at initial level, the physical-chemical quality of the aqueous extraction of a non-animal product (Santiago et al., 2024).

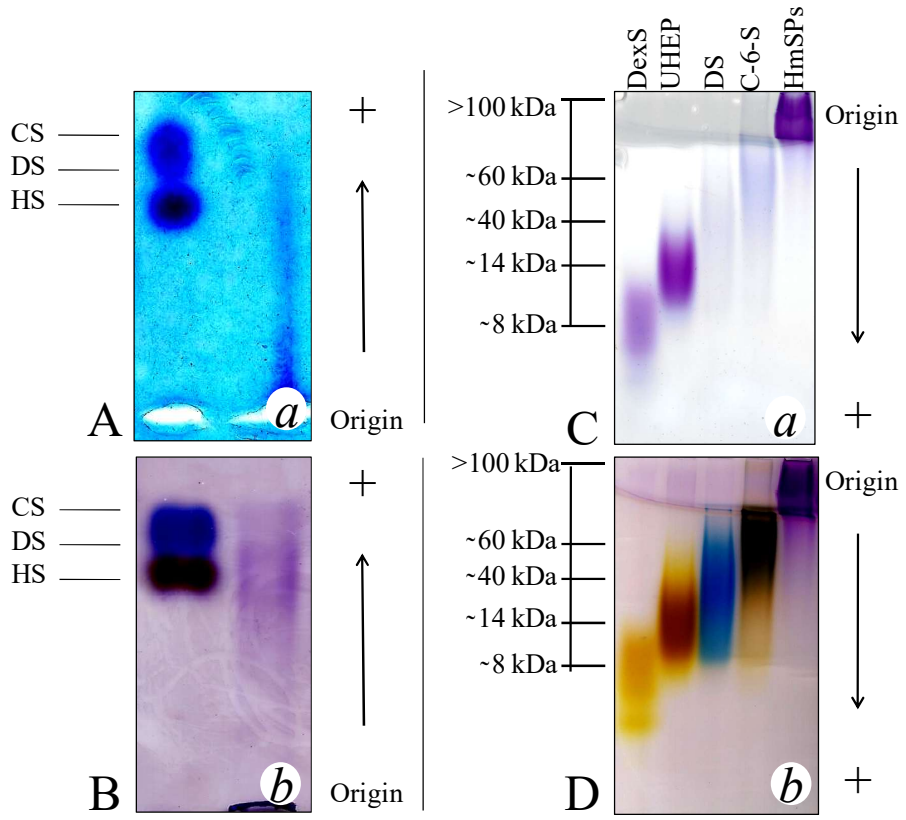

Figure 3. Agarose (A and B) / polyacrylamide (C and D) gels electrophoreses of *H. musciformis* SPs (HmSPs) extract and standards (glycosaminoglycans: chondroitin-6-sulfate (C-6-S,~ 60 kDa), dermatan sulfate (DS,~40 kDa), dextran sulfate (DexS,~ 8 kDa) and/or heparin (HEP,~ 15 kDa)) present on gels were stained with 0.1% toluidine blue (*a*) or (*b*) Stains-All cationic dye.

Regarding its molecular mass distribution, HmSPs aqueous extract revealed to be a metachromatic band of >100 kDa as observed by PAGE after toluidine blue treatment, since that it did not migrate on electrophoresis gel (Figure 3C), similarly to those SPs isolated from *G. birdiae* experimentally cultured on the same region of *H. musciformis* harvested (Rodrigues et al., 2017). In the case of staining with Stains-All alone, HmSPs aqueous extract, obtained by hot water extraction, the method stained the heterogeneous system of the material in a distinct coloring with basis on specific colors of each particular standard and their respective mobilities on gel (Santiago et al., 2024). On the basis of these observations, both electrophoretic procedures (agarose and polyacrylamide) did not apparently indicate any signal of chemical degradation of the HmSPs because no other polysaccharidic fragment was noted; and, therefore, being then analyzed their structural identities by FT-IR technique.

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Structural analysis by mean of FT-IR spectroscopy

The FT-IR spectrum of HmSPs informed that the main signals of functional groups were related to the presence of sulfated galactan-type polysaccharides during comparisons with other studies (Alves et al., 2012; Fidelis et al., 2014; Alencar et al., 2019). FT-IR analysis in the present study confirmed that the HmSPs were as a *kappa*-carrageenan structure based on Souza et al. (2018) who previously investigated by FT-IR the SPs-containing *H. musciformis* crude extract, when in papain digestion. On the spectral basis, bands at 3443, 2931, 1652, 1418, 1368, 1240, 1157, 1070, 930, 900 and 850 cm^{-1} were recorded from the analyzed polymer sample (Table 2).

Table 2. Main spectral signals recorded by FT-IR of the SPs from *H. musciformis* mariculture.

signal (cm^{-1})	functional group	reference
3443	O-H	Alves et al. (2012), Costa et al. (2012), Fidelis et al. (2014)
2931	C-H	Alves et al. (2012), Costa et al. (2012), Souza et al., 2018
1637-1639	O-C-O (uronic acid)	Fidelis et al. (2014), Ananthi et al. (2010)
1409-1419	O-C=O bending	Fidelis et al. (2014), Ananthi et al. (2010)
1240	S=O	Alves et al. (2012), Costa et al. (2012), Fidelis et al. (2014)
1153-1157	C-O-C (glycosidic linkages)	Ananthi et al. (2010)
1070	3,6-anhydrogalactose	Alves et al. (2012), Souza et al. (2018), Alencar et al. (2019)
931	C-O-C (3,6-anhydrogalactose)	Alves et al. (2012), Souza et al. (2018), Alencar et al. (2019)
900	non-sulfated β-D-galactose	Alves et al. (2012)
850	C-O-SO_4 bond in galactose C_4	Alves et al. (2012), Fidelis et al. (2014), Souza et al. (2018)

Band of high intensity at 1240 cm^{-1} was found as total sulfate (S=O, sulfate esters) in test sample (Alves et al., 2012; Costa et al., 2012, Fidelis et al., 2014) and previously confirmed the charge density of the HmSPs by electrophoretic profiles shown in figure 3 (Souza et al., 2018). Absorptions of 3,6-anhydrogalactose C-O bond were observed at 930 and 1070 cm^{-1} based on SPs extracted from *H. musciformis* which were enzymatically digested with maxatase (Alves et al., 2012) or papain (Souza et al., 2018); and SPs obtained from Rhodophyta *G. caudata* using papain method (Alencar et al., 2019). Signals at 900 (Alves et al., 2012) and 850 (Alves et al., 2012; Fidelis et al., 2014) cm^{-1} were assigned to non-sulfated β-D-galactose and a C-O-SO_4 bond of high intensity in galactose C_4, respectively, whereas *H. musciformis*-derived SPs in papain extraction yielded an almost absence peak at 900 cm^{-1} according to Souza et al. (2018). These spectral variations in terms of sulfation could be the result on the different time of harvest of *H. musciformis* on the coast of Brazil and its biochemical response to climatic fluctuations in which this species was also sujected based on studies of Ganesan et al. (2006) utilizing *H. musciformis* in open sea, Indian.

Values of FT-IR at 3443 and 2931 cm^{-1} were attributed to the stretching vibration of O–H (Alves et al., 2012, Costa et al., 2012, Fidelis et al., 2014; Souza et al., 2018) and C–H (Alves et al., 2012, Costa et al., 2012; Souza et al., 2018), respectively. Interestingly, 1652, 1418 and 1157cm^{-1} also suggested the presence of carboxyl group of uronic acid, O-C=O bending and C-O-C, respectively, based on Ananthi et al. (2010) and Fidelis et al. (2014). Taking with literature data, there is no

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information on the occurence of acid polysaccharides in *H. musciformis* (Campo et al., 2009; Alves et al., 2012; Souza et al., 2018), suggesting a more refined structural investigation by Nuclear Rossanance Magnetic analytical method compared to commercial carrageenans (Campo et al., 2009).

As the FT-IR analysis confirmed their structural integrity of *kappa*-carrageenan during the water extraction process, HmSPs were further investigated for their *in vitro* antioxidant effects.

Effects of HmSPs on *in vitro* oxidant assays

As the carrageenans are galactan-based polysaccharides that have water solubility (Campo et al., 2009), different aqueous solutions of HmSPs (0.125→4 mg mL^{-1}) were done prior to *in vitro* assays, which further explored HmSPs as natural neutralizers of oxidant reactions in comparison with the commercial synthetic antioxidants.

DPPH-scavenging effect

The effects of HmSPs on the DPPH radicals are shown in table 3 and the *in vitro* results indicated a low antioxidant potential. HmSPs inhibited dependently of concentration ($p > 0.05$), but only with a maximum inhibitory effect of 5.32 ± 0.41% (at 4 mg mL^{-1}) against the standard BHT that potentially reduced by 95.03 ± 0.63% the *in vitro* oxidant reaction, therefore, ~17.86-fold higher than the natural sample ($p < 0.05$). Thus, the reducing ability by HmSPs achieved up to ~ 5% as scavengers of the DPPH radicals.

Table 3. Effects of the *H. musciformis* SPs on DPPH, FIC and TAC assays.

HmSPs (mg mL^{-1})	*in vitro* assays		
	DPPH (%)	FIC (%)	TAC (%)
0.12	4.49 ± 0.39^{a}	15.87 ± 0.00^{a}	1.33 ± 0.18^{a}
0.25	4.07 ± 0.15^{a}	17.32 ± 0.19^{a}	0.78 ± 0.09^{a}
0.50	4.44 ± 0.09^{a}	23.43 ± 0.33^{b}	3.51 ± 0.038^{b}
1.00	4.44 ± 0.18^{a}	24.45 ± 0.25^{b}	18.51 ± 0.02^{c}
2.00	4.59 ± 0.09^{a}	27.31 ± 0.44^{b}	45.49 ± 1.91^{d}
4.00	5.32 ± 0.41^{a}	24.96 ± 0.25^{b}	70.51 ± 1.85^{e}
BHT (4 mg mL^{-1})	95.03 ± 0.63^{b}	-	-
EDTA (4 mg mL^{-1})	-	99.87±0.00^{c}	-
ascorbic acid (0.4 mg mL^{-1})	-	-	99.77±0.00^{f}

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

Some natural sources rich in SPs have shown free radical scavenging properties. Among them have isolated antioxidant SPs in maxatase digestion of seagrass *H. wrightii* (41.4% at 0.5 mg mL^{-1}; Silva et al., 2012); in hot water (90°C, 45 min) of Chlorophyta *C. lentillifera* (fractions SGP_{11}, SGP_{21} and SGP_{31}: 32.75 ± 0.05, 22.58 ± 0.27, and 18.41 ± 0.92%, respectively, at 31.25 mg mL^{-1}; Tesvichian et al., 2024); in hot water (90-95°C, 16 h) of Rhodophyta *S. swartzii* (25.33 ± 2.52% at 1 mg mL^{-1} *vs*. gallic acid: ~ 40% at 0.02 mg mL^{-1}; Vijayabaskar et al., 2012); in hot water (90-95°C, 3-4 h) of Ochrophyta *T. ornata* (80.21 ± 2.50% at 0.5 mg mL^{-1} *vs*. quercetin: 96.81 ± 1.23% at 0.125 mg mL^{-1}; Ananthi et al., 2010); and in alkaline/papain digestion (50 or 60°C, 12 or 24 h) of by-products of fishes (over > 40% at 3 and 5 mg mL^{-1}; Jridi et al., 2019 / 30.26 ± 2.80% at 4 mg mL^{-1}; Nascimento et al., 2021). Studies on the bioactivity of seaweeds SPs by the DPPH method

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showed that they were a natural eletrophilic substrate to donate electrons and produce *in vitro* scavenging actions (Jridi et al., 2019).

Results of the table 3 conflicted to those found by Souza et al. (2018) that isolated HmSPs in papain digestion (60°C, 6 h) of samples collected in natural-bed and showed no antioxidant effects by the DPPH method. These contrasting observations between studies by HmSPs on the DPPH method with regard to the origin of the species harvested in different field conditions could be ascribed by a critical lacking of active sites (Ai et al., 2023; Femi-Adepoju et al., 2023). In fact, in this study, HmSPs had some molecular variations detected by electrophoreses and FT-IR analyses that contrasted those of Souza et al. (2018) that there were not antioxidant actions by HmSPs. Low chemical standardization of natural products became they very difficult for hydrocolloid industry (Cardozo et al., 2007; Campo et al., 2009), since that changes algal matrix SPs composition naturally occurs in marine habit, impacting or not their bioactivies (Pomin & Mourão, 2008; Rodrigues et al., 2017; Wang et al., 2014).

TAC effect

HmSPs from samples experimentally cultured were also *in vitro* tested by the TAC method (Table 3). Herein, HmSPs significantly reduced Mo to form a green phosphate/Mo complex total (Prieto et al., 1999) and exhibited a dose-dependent antioxidant profile, with reduction rates from 1.33 ± 0.18 to 70.51 ± 1.85% for 0.125→4 mg mL^{-1}, respectively. However, this action had less potency than the standard ascorbic acid (99.77% inhibition, at 0.4 mg mL^{-1}, $p < 0.05$) used as synthetic agent.

As can be seen, the antioxidant property by HmSPs was preponderant by TAC assay than in the DPPH one reveling that the native sample extracted in hot water was, at least, about 13.25-fold most effective to reduce Mo based on Prieto et al. (1999). Collectively, these data allowed us to ascribe that the HmSPs inhibited the initiation stage of the oxidant process (Silva et al., 2012), suggesting the presence of specific sulfation sites involved in the reducing potential (Femi-Adepoju et al., 2023). Other SPs isolated from different living organisms were reported to be antioxidants, such as those isolated from seagrass *H. wrightii* (15.21 equivalents; Silva et al., 2012); 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); 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); and from *O. niloticus* skin (25% inhibition, at 4 mg mL^{-1}; Nascimento et al., 2021). The heterogeneous structure and the complexity of the SPs varies with the species, origin and extraction conditions, generating impact on their antioxidant actions (Cardozo et al., 2007; Ai et al., 2023), whose agents could be used in numerous contexts (Femi-Adepoju et al., 2023).

FIC effect

As highlighted in table 3, HmSPs were also capable of chelanting the ferrous ions, indicating that they inhibited the propagation stage of the oxidant reaction (Silva et al., 2012). On this role, HmSPs exhibited *in vitro* antioxidant actions by the FIC method, but with low effective responses varying dependently of concentration from 0.78 ± 0.09 (at 0.25 mg mL^{-1}) to 24.96 ± 0.25% (at 4 mg mL^{-1}, $p < 0.05$), therefore, up to ~24% of neutralizing of the event. Even the standard EDTA produced a FIC effect by 99.87±0.00% at 4 mg mL^{-1} and was more effective ($p < 0.05$) than the HmSPs, at least, 4-fold higher in the assay.

On the basis of this mechanism, HmSPs had to ability on the chelating power to $Fe2^{+}$ by substitution of hydroxyl group with ester group present on the algal polysaccharides as already demonstrated by Alves et al. (2012) with the SPs of this same species, but extracted by maxatase enzyme, however, achieving only 8% at 5 mg mL^{-1}. From other sources, Chlorophyta *C. cupressoides* var. *flabellata* (44% at 2 mg mL^{-1}; Costa et al., 2012); Rhodophyta *G. caudata*

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(69.80% at 4 mg mL^{-1}; Alencar et al., 2019); cuttlefish skin and muscle (over > 90% at 1 mg mL^{-1}; Jridi et al., 2019); and *O. niloticus* skin (32.22 ± 0.10% at 2 mg mL^{-1}; Nascimento et al., 2021) also had SPs with antioxidant actions by the FIC assay.

Considerating all the explored assays (Table 3), it was demonstrated that the Rhodophyta *H. musciformis* cultured in open sea was a valuable source of natural antioxidant SPs when extracted in hot water, showing that a high temperature of extraction did not alter the water solubility (Araújo et al., 2012; Campo et al., 2009; Tesvichian et al., 2024) having a good reducing power by TAC mechanism from the tested system (Table 3). This finding was interesting since HmSPs were also effectives as anticoagulant (Rodrigues et al., 2011a), antimicrobial and neuroprotective (Souza et al., 2018) based on other studies, therefore, acting on other important biological systems (Femi-Adepoju et al., 2023). Furthermore, HmSPs showed rheological properties (Cosenza et al., 2014) with potential applicability considerating processed and/or fuctional food and pharmaceutical and/or chemical formulations as industrial option (Cardozo et al., 2007; Campo et al., 2009).

Chemical nature of the HmSPs is a *kappa*-carrageenan structure (Table 2) as also revealed by Souza et al. (2018). As there are important chemical differences among the main types of commercial carrageenans (*kappa*, *iota* and *lambda*), it has been well-known recongnized their roles on the coagulation and inflammation due to the number of sulfate radicals and the presence or absence of 3,6-anhydrogalactose-α-L-galactose residues on the chemical structure. Such differences produce impact on the degree of biological responses, including *in vivo* and *in vitro* models (Campo et al., 2009).

In this study, mariculture-derived *H. musciformis* synsthetized a *kappa*-carrageenan with relevant structural differences that possibly its composition affected the *in vitro* antioxidant response (Table 3), but without presenting chemical degradation by hot water extraction as observed in figure 3 and confirmed by FT-IR analysis (Table 2), by which could induce or not biological effects, bringing wrong interpretations (Araújo et al., 2012). As a rule, the bioactivities of seaweeds SPs have been greatly depend on the composition and chemical structure (charge density and molecular size) (Wang et al., 2008), but not always dependent in terms of sulfation degree (Pomin, 2012). Studies on the antioxidant actions of SPs have indicated that chemically-produced low mass molecules showed stronger effects than undegraded those to react with free radical species, but is still a controversial theme in the literature because would involve the spartial structure of the polymer (Wang et al., 2014; Tesvichian et al., 2024). In fact, in-depth studies on the structure-function relationship regarding the antioxidant action of SPs lack of investigation (Ai et al., 2023; Femi-Adepoju et al., 2023).

Overrall, experimentally produced *H. musciformis* in open sea on the northeast coast of Brazil could be extensivelly explored as an interesting source of antioxidant SPs for biological testing. Use of this cultived macroalgae species for production of phycocolloids could bring advantage to the food sector and to discovery of novel bioactive agents for human health-related application. Further investigations are still required to determine the most effective mechanisms of the HmSPs on the reducing power.

Conclusion

Utilization of *Hypnea musciformis* (Rhodophyta) epiphyte biomass experimentally obtained from *long line* structures of *Solieria filiformis* mariculture in the coastal zone of Ceará, Brazil, showed as an promising alternative to produce natural antioxidant sulfated polysaccharides. The large amount of liophylized sulfated polysaccharides extracted in hot water and then precipited with cold alcohol showed to be an eco-friendly alternative of lowest cost since that did not observe any chemical degradation in sulfation and molecular size based on experimental analyses of the *kappa*-carrageeenan identified by FT-IR spectroscopy. All the three *in vitro* assays detected the antioxidant

property by algal *kappa*-carrageeenan, but the structural variations observed suggested more effectivity than in other studies using the same species collected in natural bed. Although showing important actions by total antioxidant capacity mechanism, the molecule revealed to be significantly less potent than all the synthetic antioxidants in the assays.

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