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OPTIMIZED EXTRACTION OF ANTIOXIDANT AGAR FROM THE *Gracilaria domingensis* RHODOPHYTA CELL-WALL ULTRASTRUCTURE

EXTRAÇÃO OTIMIZADA DE ÁGAR ANTIOXIDANTE DA ULTRA-ESTRUTURA DE PAREDE CELULAR DE RODOFÍCIA *Gracilaria domingensis*

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Abstract Antioxidant potential of *Gracilaria domingensis* sulfated polysaccharides (GdSPs) has have few explored. This study sequentially extracted and compared structural and antioxidant properties of GdSPs. After tissue digested with papain, in 100mM sodium acetate buffer+(pH 5)+5mM cysteine/EDTA, GdSPs were quantified and analyzed the molecular features (GdSPs-1→3) by agarose/polyacrylamide gels electrophoreses *vs.* standard glycosaminoglycans stained with toluidine blue or Stains-All; and structurally by infrared. Evaluation of antioxidant effects were *in vitro* tested by DPPH, total antioxidant capacity (TAC) and ferrous ion chelating methods against BHT, ascorbic acid (AA) and EDTA, respectively. Total yield was 44.82% considering three extractions, but GdSPs-3 was lowest (0.04%±0.01%, $p<0.05$) within all. GdSPs revealed agaran-structures presenting uronic acid and regularity in charge/size and functional groups along the algal matrix. GdSPs-1/-2 had concentration-dependent antioxidant responses, but there was a preponderance in TAC method, whose inhibitions were 81.57±0.54/72.55±1.37% at 4 mg mL^{-1}, respectively, *vs.* AA (99.77% inhibition, 0.4 mg mL^{-1}). GdSPs revealed a system of antioxidant agar along the wall-ultrastructure.

Key Words: matrix anatomy, seaweed, polyanionic profile, oxidation.

Resumo Potencial antioxidante de polissacarídeos sulfatados pouco tem sido explorado de *Gracilaria domingensis* (PSsGd). Este estudo extraiu sequencialmente e comparou de PSsGd propriedades estruturais e antioxidantes. Após tecido digerido com papaína, em tampão acetato de sódio 100mM+(pH5)+cisteína/EDTA 5mM, PSsGd foram quantificados e analisadas as características moleculares (PSsGd-1→3) por eletroforeses em géis de agarose/poliacrilamida *vs*. padrões glicosaminoglicanos corados com azul de toludina ou "Stains-All"; e por infravermelho estruturalmente. Avaliação de efeitos antioxidantes foram testados *in vitro* pelos métodos DPPH, capacidade antioxidante total (CAT) e quelação de íon ferroso (QIF) contra BHT, ácido ascóbico (AA) e EDTA, respectivamente. Rendimento total foi 44,82% considerando três extrações, mas foi PSsGd-3 menor (0,04%±0,01%; $p<0,05$) dentre todas. PSsGd revelaram estruturas agaranas apresentando ácido urônico e regularidade em carga/massa e grupos funcionais pela matriz algal. PSsGd-1/-2 tiveram respostas antioxidantes dependentes de concentração, porém houve uma preponderância no método CAT, cujas inibições foram 81,57±0,54/72,55±1,37% em 4 mg mL^{-1}, respectivamente, *vs.* AA (inibição 99,77%; 0,4 mg mL^{-1}). PSsGd revelaram um sistema de ágar antioxidante pela ultra-estrutura de parede.

Palavras-Chave: anatomia de matriz, alga marinha, perfil polianiônico, oxidação.

Introduction

Seaweeds (macroalgae) are non-vascular photosynthetic organisms of diverse anatomies mainly occurring in the litoral zone as ecological part of various coastal regions (Joly, 1965; Marinho-Soriano et al., 2009). They are classified into three higher taxa (Chlorophyta – green alga, Ochrophyta – brown alga, and Rhodophyta – red alga) (Joly, 1965) and are under review continually (Reviers, 2006; Marinho-Soriano et al., 2009). Their population structures vary with the environmental changes, especially when are impacted by human activity (Martins et al., 2012). On the other hand, seaweeds have gained ground in several economic sectors (*e.g.*, pharmaceutical, food, cosmetic, agricultural technology and aquiculture) due to their potential chemical constituints (*e.g.*, sulfated polysaccharides-SPs, proteins, lipids and pigments) for industrial use and scientific development (Reviers, 2006; Cardozo et al., 2007; Marinho-Soriano et al., 2009).

In the reef system, seaweeds are natural stocks in extracellular matrix SPs presenting complex structurally composition varying not only by species, but also by extraction protocol used and the season and climatic conditions in which they are found (Cardozo et al., 2007; Pomin & Mourão, 2008; Farias et al., 2000). Seaweed biomass-derived SPs can account for >40% of the dry weight of the algal cell-walls; it would represent to be more abundant than those of animals (called glycosaminoglycans); and some theories on the biology, evolution and clinical aspects have been reported during decades (Pomin & Mourão, 2008). SPs are known as galactans in red species; Ochrophyta fucans or fuicoidans are among the most rich source in SPs; and in Chlorophyta species, heteropolysaccharides occuring in minor concentrations, since that the green ones are still little explored (Pomin & Mourão, 2008). Galactans expressed by Rhodophyta have β-galactoses with D-enantiomers, but the α-galactose residues may be D- (agaran), L- (carrageenan) (Pomin & Mourão, 2008) or DL-hybrid (Zibetti et al., 2005) configuration varying the sulfation pattern and the occurence of 3,6-anhydro bridge on the backbone structure, whose properties have been extensively reviewed (Cardozo et al., 2007; Pomin & Mourão, 2008) and evaluated along the extracellular matrix (Zibetti et al., 2005; Rodrigues et al., 2009, 2016). These SPs have usually >100 kDa of molecular masses and are predominantly constituted of galactopyranosyl units with high degree of sulfation making them polyanionics capable of binding to proteins displaying bioactivities, *e.g.*, antibacterial (Vijayabaskar et al., 2012), anti-inflammatory (Ananthi et al., 2010; Paiva et al., 2011), anticoagulant (Farias et al., 2000) and antiangiogenic effects (Pomin, 2012).

Certain aquatic organisms have been described to have antioxidant SPs, such as cyanobacteria (Ai et al., 2023), by-products of fishes (Jridi et al., 2019; Nascimento et al., 2021) and seagrasses (Silva et al., 2012). The use of seaweeds as a large source in non-animal SPs, to act as reducing agents of free radicals and neutralize the oxidative reactions, has been screened. Some species collected from natural beds or cultured with or not economic value (Chlorophyta *Caulerpa cupressoides* var. *flabellata* by Costa et al. (2012); Rhodophyta *Hypnea musciformis* by Alves et al. (2012), *Gracilaria birdiae* by Fidelis et al. (2014) and *G. caudata* by Alencar et al. (2019); and Ochrophyta *Laminaria japonica* by Wang et al. (2008), *Turbinaria ornata* by Ananthi et al. (2010), *Lobophora variegata* by Paiva et al. (2011) and *Sargassum swartzii* by Vijayabaskar et al. (2012)) have been recognized as producers of antioxidant SPs. Studies on the potential role of seaweed SPs have revealed like novel food additives (Cardozo et al., 2007) to synthetic antioxidants (*e.g.*, butylatedhydroxytoluene-BHT) which are toxics and develop cancer-related factors and other systhemic complications (Panicker et al., 2014). Such consequences are caused by free-radicals formation derived from the normal cell metabolism or by exogenous sources, attacking biological molecules (Barbosa et al., 210).

Rhodophyta Wettstein (1901) of the genus *Gracilaria* (Florideophyceae, Gracilariaceae) have red coloring and are found in several tropical regions around of the world (Reviers, 2006). They are well-known as producers of agar, both in wild and cultivated species, generating economic and

social impact in coastal communities, including in Brazil (Melo et al., 2002; Maciel et al., 2008; Amorim et al., 2012; Fidelis et al., 2014). Some studies in relation to effect of sazonality in the biomass and agar yield from *Gracilaria* species were already reported (Cardozo et al., 2007). *G. domingensis* (Kützing) Sonder ex Dickie (1874) also occurs on the Atlantic coast of Brazil and previous results suggested it for experimental cultivation in net cages (Salles et al., 2010) and SPs (GdSPs) as a safe supplement to the seminal cooling medium of fish semen (Sales et al., 2023). There is no investigation on the system of matrix SPs synthetized by *G. domingensis*, when extracted and analyzed sequentially in terms of optimization of yield and their structural features with antioxidant properties.

The aim of this study was to sequentially optimize agar-type SPs from *G. domingensis* cell-wall ultrastructure and analyze their molecular features by Fourier Transform Infrared (FT-IR) spectroscopy. Prospection of SPs on their antioxidant potential was also conducted using *in vitro* classical assays.

Material and Methods

Algal material and macro-microscopic identification

Rhodophyta samples of *G. domingensis* (Figure 1A) were manually collected at low tide from a calcarium reef of the natural region located at Pacheco beach (Caucaia, Ceará-Brazil) during a field expedition carried out by our group. The specimens, before pre-selected in the environment from other algae species, were transported (in plastic bags) to the Marine Biochemistry laboratory of the Aquaculture Biotechnology Center, Department of Fishing Engineering, Federal University of Ceará. Then, they were washed with distillated water to remove impurities (*e.g.*, salt, sand and shells) and necrotic parts and then stored -20°C based on Farias et al. (2000). As herbarium archive, a sample of this specimen was reposted in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil. The material was also authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

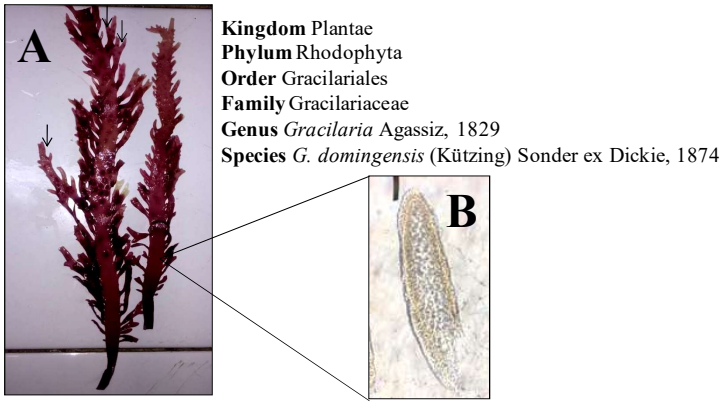

Figure 1. Morphological aspect showing cystocarps and some regions of dichotomies (arrows, A) and anatomy of the cell-wall (cross section, 400 ×) in which agar occurs embedded in the amorphous matrix (B) of the red seaweed *G. domingensis* (Kützing) Sonder ex Dickie

G. domingensis also occurs along the northeastern Brazilian coast and it was macroscopically identified from a part of its thallus following the Marinho-Soriano et al. (2009)' catalogue and Joly (1965)' identification key. Specimen shows a dark-red coloring in flat thallus with branches in an unique plan presenting some regions of dichotomies and cystocarps attached (Figure 1A). Using

light microscopy (Mateu, 1980), a sample of tissue was prepared and the material was characterized by numerous peripheral cells (corticals) of round shape and pigmented, while oval medullary cells of heterogeneous form and incolors making part of the central portion of the amorphous matrix as visualized from the cross section (Figure 1B).

Extraction of GdSPs and metachromasia assay

For SPs extraction, samples of *G. domingensis* were, initially, sujected to dehydration for 24 h to sunlight and then the material was cut into small pieces according to the Farias et al. (2000)' method, with some modifications. Briefly (Figure 2*a*), the pretreated tissue (5 g) was suspended in 100 mL of 100 mM sodium acetate buffer (pH 5.0), containing 5 mM EDTA and 5 mM cysteine, and digested by adding of a crude papain solution (~31 mg mL^{-1}) at 60°C for 3 h yielding GdSPs-1.

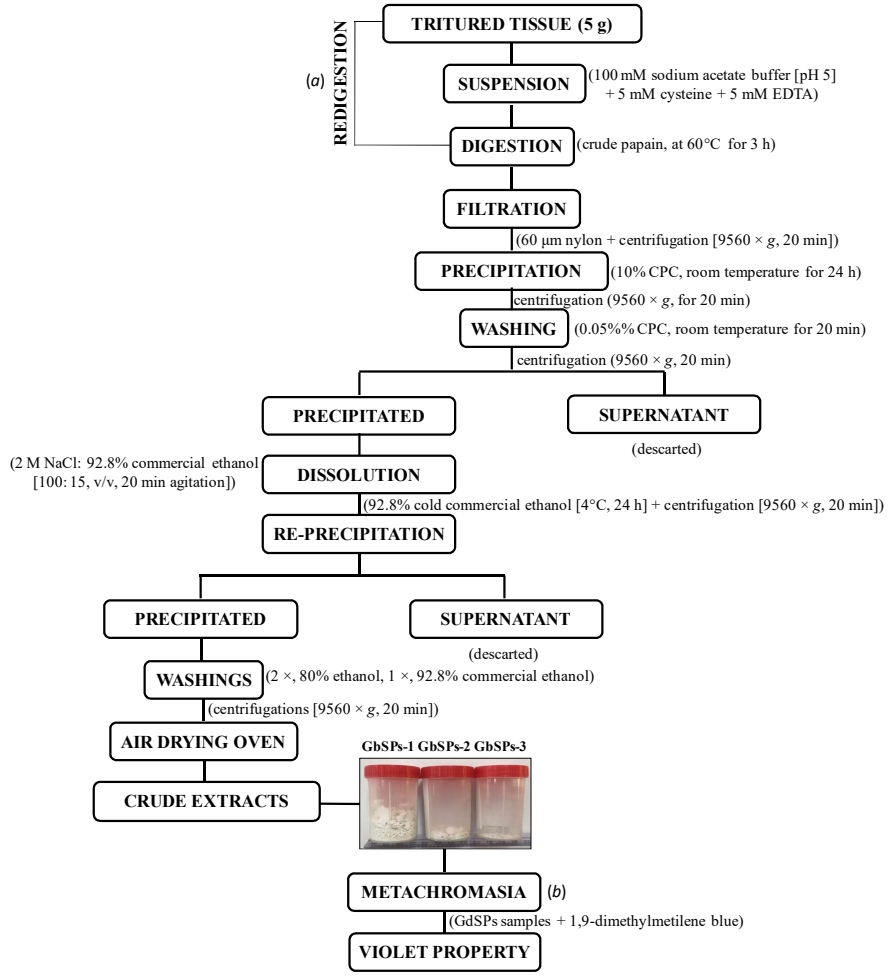

Figure 2. Protocols of obtaninig (*a*) and metachromasia (*b*) of SPs from the red seaweed *G. domingensis*.

The incubation mixture was then filtered using a nylon screen and the supernatant was saved. GdSPs-1 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 GdSPs-1 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 ice-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. Sequentially the *G. domingensis* residue was redigested twice with papain following the same procedure above yielding GdSPs-2 and GdSPs-3, respectively (Rodrigues et al., 2009, 2016). All the materials were finally dried using an air drying oven (60°C, 6 h) and the yields were expressed as the percentage (%, n = 3) of the dehydrated matter.

The metachromatic property of GdSPs was detected using the 1,9-dimethylmetilene (DMB) blue dye as an indicator of reaction by complex formed in violet thus revealing sulfation (Farndale et al., 1976). The preparation of GdSPs (9 µg) was tested in triplicate using glass tubes and the color observed to be specific for sulfated polyanions against the reagent used as a parameter (Figure 2*b*).

Physical and chemical analysis by electrophoreses

Initially, aliquots containing ~21 µg of each crude extract (GdSPs-1→3) were prepared in destillated water before the analysis by two systems of electrophoretic techniques that consisted of gels based on:

Agarose gel electrophoresis

For this system, GdSPs were analyzed by polydispersion pattern and charge density. All the samples were applied to a 0.5% agarose gel prepared with 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 GdSPs present in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for 24 h and then dehydrated (Dietrich & Dietrich, 1976).

Polyacrylamide gel electrophoresis (PAGE)

Regarding PAGE, GdSPs were examined by apparent molecular mass distribution. All the samples were applied to a 6% polyacrylamide gel using 0.02 M Tris/HCl buffer (pH 8.6) and the run was performed at 500 mA for 1 h as described by Rodrigues et al. (2016).

The GdSPs present in both gels were revealed with 0.1% toluidine blue or Stains-All cationic reagent and, subsequently, the gels were destained with a solution containing absolute ethanol, distilled water and acetic acid or using distilled water only. As known markers of molecular mass, 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 standard compounds (Andrade et al., 2017; Volpi & Maccari, 2002).

FT-IR spectroscopy

The values of FT-IR were obtained using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, 10 mg of GdSPs samples were 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**

The test samples (GdSPs-1→3) 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 GdSPs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of SPsAP (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). GdSPs (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 (%) = $[(A_{sample}-A_{blank}) / (A_{ascorbic\ ac}-A_{blank})] \times 100$.

Ferrous ion chelating (FIC) effect

This assay was based on methodology of Chew et al. (2008), with modifications. For this, different concentrations of GdSPs (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 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, data were also 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

Optimization of total extraction of GdSPs

G. domingensis collected, sequentially digested three times with the nonspecific protease (papain), for 3 h, at 60°C, combined with both CPC and ice-cold ethanol precipitations, yielded crude SPs extracts (GdSPs-1→3) from the dehydrated tissue. On a mass-to-mass basis (Figure 3A), GdSPs-1 had highest extraction yield (36.41% ± 3.21, w/w, $p < 0.05$) from 5 g of the seaweed. Regarding GdSPs-2 and GdSPs-3, they showed extraction yields ranging from 8.37% ± 4.60% to 0.04% ± 0.01% (w/w, $p < 0.05$), respectively, between them; therefore, the abundance of SPs drastically reduced along the process and accounted for 44.82% total yield of the dehydrated weight of the seaweed cell-wall.

This behaviour was the same in comparison with other red species rich in SPs, which are embedded in their wall-matrixes, applying with Rhodophyta *Halymenia pseudofloresia* (47.14%, w/w; Rodrigues et al., 2009) and *Acanthophora muscoides* (23.47%, w/w; Rodrigues et al., 2016) by papain-extraction sequence. It has been employed different methods to obtain SPs from seaweeds cell-walls (Zibetti et al., 2005; Cardozo et al., 2007; Paiva et al., 2011; Alves et al., 2012; Costa et al., 2012).

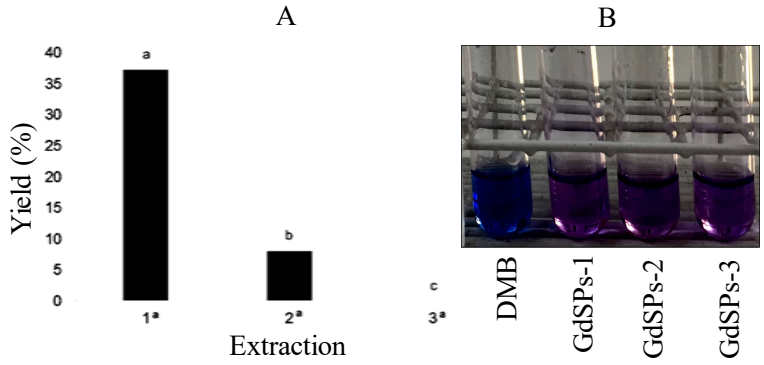

Figure 3. Yield (A) and metachromasia (B) of SPs extracted from *G. domingensis* matrix. Different letters on the bars indicate differences among the extractions (ANOVA, Tukey' test, $p < 0.05$); Violet-revealing sulfation.

SPs yield also varies with the algal species, such as for those of Rhodophyta *Botryocladia occidentalis* (4.00%, w/w) by papain digestion (Farias et al., 2000), *Gracilaria cornea* (21.40%, w/w) by papain digestion (Melo et al., 2002), *G. birdiae* (6.50%, w/w) by cold water (Maciel et al., 2008) or it (0.52-8.26%, w/w) by proteolysis, NaOH or ultrasound (Fidelis et al., 2014), *G. ornata* (9.20%, w/w) by cold/hot water-extraction sequence (Amorim et al., 2012); *G. caudata* (24.96%, w/w) by papain digestion (Alencar et al., 2019); and for those of Ochrophyta *L. japonica* (2.30%, w/w) by hot water (Wang et al., 2008), *T. ornata* (10.00%, w/w) by hot water (Ananthi et al., 2010) and *S. swartzii* (10.43%, w/w) by autoclaved water (Vijayabaskar et al., 2012). Other sources, such as from seagrasses (Silva et al., 2012), fish processing-discarded wastes (Jridi et al., 2019; Nascimento et al., 2021) and cyanobacteria (Ai et al., 2023), also SPs yielded, but in relatively lower amounts than seaweeds (Pomin & Mourão, 2008).

The histological preparation of *G. domingensis* tissue based on Mateu (1980) showed that its (multicellular) cell-wall anatomy is complex (Figure 1B) and suggested a matrix containing a large mucilage deposition of SPs on the surface texture, in which the polyanionics could be mainly located in matrix (Zibetti et al., 2005; Pomin & Mourão, 2008; Rodrigues et al., 2016); and it would reflex the highest extraction yield achieved by first (GdSPs-1) protease digestion (Figure 3A).

Papain-enzyme action sequence led to a progressive disorganization of the matrix structure embedded by SPs linked to proteins to obtain novel bioactives (Rodrigues et al., 2009, 2016). This proteolysis role for shorter-time (3 h) playing a sucessive attach on the *G. domingensis* cell-wall could reveals the composition of the crude extracts (GdSPs-1→3), since that the metachromasia of the analyzed polymer samples, detected by Farndale et al. (1976)' method, was similar at concentration tested (Figure 3B), although clearly showing different availably in terms of total yield as showed in figure 2A (Rodrigues et al., 2009, 2016).

Red seaweeds of the genus *Gracilaria* have a consistent thallus of edible biomass to be candidates to produce economic value products (Reviers, 2006; Marinho-Soriano et al., 2009) and are well-known sources in international market, being used as human foods, fertilizers and colloids (agar) (Cardozo et al., 2007). As a considerable biomass, they have also been ingredients by addition to food (including fresh seaweeds or their extracts) to take advantage of these functional properties or nutritional qualities (Reviers, 2006; Marinho-Soriano et al., 2009). However, the quality of the extracted product would determine its use as an industrial option (Cardozo et al., 2007). On this biotechnological perpective, GdSPs were further analyzed for their physical-chemical features using conventional biochemical techniques.

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Charge/mass analysis by electrophoreses

Analysis by mean of agarose / polyacrylamide gels electrophoreses is shown in figure 4 and revealed similar profiles among the SPs-rich crude extracts. Considerating agarose gel, in diaminopropane buffer system, it showed coincidences in migrations among the unfractionated extracts (GdSPs-1→3) and standard HEP after toluidine blue staining (Figure 4A). Each sample revealed metachromatic band of relatively visible blot on gel, with clear polydispersion as commonly found for seaweeds SPs (Fidelis et al., 2014; Rodrigues et al., 2009), but GdSPs-3 showing polysaccharides with a weak resolution pattern, suggesting a reduced density of ester sulfate groups in its backbone structure from the analyzed polymer sample (Costa et al., 2012; Rodrigues et al., 2016).

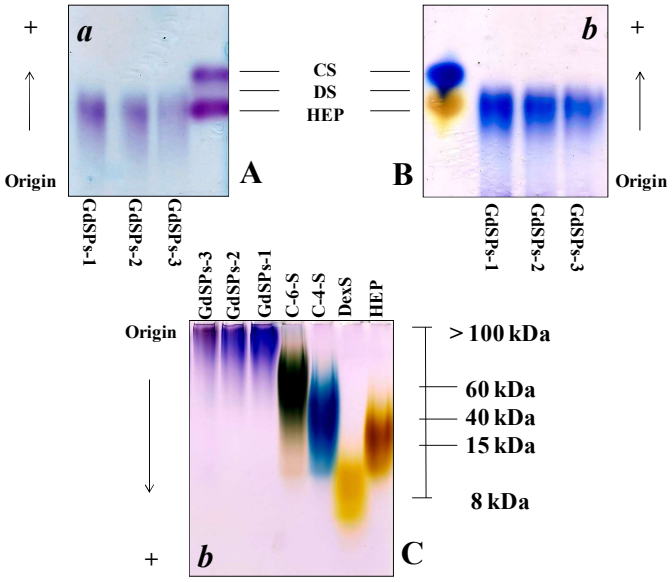

Figure 4. Agarose (A, B) / polyacrylamide (C) gels electrophoreses of *G. domingensis* SPs (GdSPs-1, -2 or -3) and standards chondroitin-6-sulfate (C-6-S,~60 kDa), chondroitin-4-sulfate (C-4-S,~40 kDa), dextran sulfate (DexS,~8 kDa), dermatan sulfate (DS,~ 40 kDa) and heparin (HEP,~15 kDa) present on gels were stained with 0.1% toluidine blue (*a*) or Stains-All (*b*).

This behaviour may corrobores with the extraction yield that significantly decreased along the technique used (Figure 3A) and, possibly, indicating any chemical difference at level of sulfation of these molecules from the *G. domingensis* matrix. It was noted that the GdSPs were complexed with their sulfated groups by diamine of the electrophoretic buffer by mean of their spacing of vicinal charges, although suggesting the same charge/mass ratio (Dietrich & Dietrich, 1976; Costa et al., 2012).

In the Stains-All dye-treated agarose gel (Figure 4B) evidenced strong intensities of the samples and standards in which suggested the presence of other carboxylated sugars or non charged residues in GdSPs as commonly demonstrated for animals SPs (Volpi & Maccari, 2002). As all the polymers interacted with the diamine contained in run buffer (Dietrich & Dietrich, 1976; Costa et al., 2012), the here sequentially extracted compounds would be the spatial configuration similar along the *G. domingensis* cell-wall ultrastructure as also reported for those of Rhodophyta *G. birdiae* (Fidelis et al., 2014) and *A. muscoides* (Rodrigues et al., 2016).

Interestingly, the visible complex of blue bands as standard CS deduced that the *G. domingensis* had important amounts of other structural components not detected by staining with toluidine blue alone (Fidelis et al., 2014) and more refined chemical studies are required by which these molecules

would react with the dye because each SP is unique in nature (Pomin & Mourão, 2008; Pomin, 2012). For instance, for the *Geoffroea spinosa* higher plant polysaccharide, Souza et al. (2015) visualized blue polydisperse band due to its composition rich of hexuronic acid, when revealed with Stains-All alone.

On the basis of our results, at initial level, GdSPs were also observed by PAGE to characterizing of their molecular masses along the algal extracellular matrix (Rodrigues et al., 2016). By staining with Stains-All dye alone (Figure 4C) (Andrade et al., 2017), all the samples (GdSPs-1→3) were estimated to be high molecular sizes (>100kDa) because they remained in the origin of the gel as expected for red seaweeds crude SPs (Fidelis et al., 2014; Rodrigues et al., 2016) *vs*. standards that showed a clear difference in their electrophoretic mobilities based on particular size (Andrade et al., 2017).

Combined observations led us to speculate to a "homogeneous" system of SPs present in *G. domingensis*. Demand for hydrocolloids of high quality by industry has significantly increased around the world for decades (Reviers, 2006; Cardozo et al., 2007; Marinho-Soriano et al., 2009) and experimental data revealed the cultivation of *G. domingensis* in Brazil (Salles et al., 2010).

Structural analysis by FT-IR

The spectral profile of functional groups presents in each extracted polymer sample (GdSPs-1→3) was obtained and compared by FT-IR spectroscopy as illustrated in Figure 5. This qualitative procedure offers structural data between SPs from diferent origins (Cardozo et al., 2007; Alves et al., 2012; Costa et al., 2012; Souza et al., 2012; Jridi et al., 2019; Ai et al., 2023).

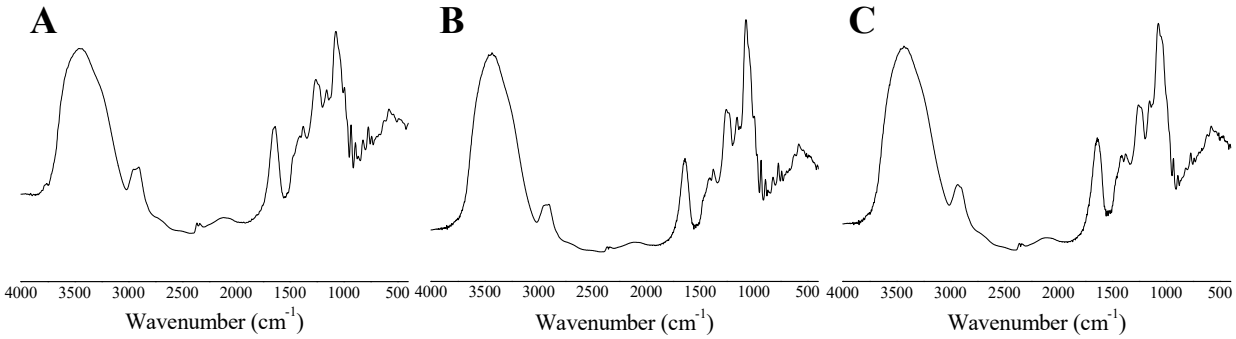

Figure 5. FT-IR spectra of the *G. domingensis* SPs (GdSPs-1 [A], -2 [B] and -3 [C]) at 500-4000 cm^{-1}.

G. domingensis from northeastern coast of Brazil contained extracellular matrix SPs that showed, at 500-4000 cm^{-1}, spectral signals (1375-1377, 1253-1257, 1072-1074, 931, 891 and 738-771 cm^{-1}) related to the presence of agarocolloids from the analyzed polymer samples (Melo et al., 2002; Maciel et al., 2008; Amorim et al., 2012; Fidelis et al., 2014; Alencar et al., 2019) as was also listed in table 1. Characteristic bands at 3431-3446 cm^{-1} and at 2902-2926 cm^{-1} were assigned for O-H stretching (Alves et al., 2012; Costa et al., 2012; Fidelis et al. 2014) and C-H (Maciel et al., 2008; Alves et al., 2012; Costa et al., 2012), respectively. It has been described that SPs from Rhodophyta would occur as agaran (L-series), carrageenan (D-series) or both (hybrids D-/L-series) depending on the species, season or locality (Pomin & Mourão, 2008).

In addition, it was identified in the FT-IR spectra of GdSPs the occurence of intense bands around 1637-1639 cm^{-1} and 1409-1419 cm^{-1} (O-C=O bending) corresponding to the carboxyl group of uronic acid based on Ananthi et al. (2010) and Fidelis et al. (2014), who formaly observed for *T. ornata* (Ochrophyta) and *G. birdiae* (Rhodophyta) SPs, respectively, using other specific extraction conditions. These spectral signals of acid form associated with the profile of blue bands from the

agarose/PAGE analyses were good to confirm the use of Stains-All as a dye to clearly detect other molecular complex regarding noncharged sugars residues (uronic acids) in seaweeds SPs structures compared with the mammalian CS used as a standard in this study (Figures 4B, C). Taking with literature studies, these values could indicate that the GdSPs may be an acidic portion in nature (Cardozo et al., 2007; Ananthi et al., 2010).

Table 1. Main spectral signals recorded by FT-IR of the *G. domingensis* matrix SPs (GdSPs-1→3).

signal (cm^{-1})	functional group	reference
3431-3446	O-H	Alves et al. (2012), Costa et al. (2012), Fidelis et al. (2014)
2902-2926	C-H	Maciel et al. (2008), Alves et al. (2012), Costa et al. (2012)
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)
1375-1377	C-O-C	Ananthi et al. (2010), Alencar et al. (2019)
1253-1257	S=O	Melo et al. (2002), Amorim et al. (2012), Costa et al. (2012)
1153-1157	C-O-C (glycosidic linkages)	Ananthi et al. (2010)
1072-1074	3,6-anhydrogalactose	Alves et al. (2012), Alencar et al. (2019)
931	C-O-C (3,6-anhydrogalactose-α-L-galactose)	Maciel et al. (2008), Amorim et al. (2012), Alencar et al. (2019)
891	glycosidic linkages	Ananthi et al. (2010)
817-823	C-6 of galactose	Melo et al. (2002), Maciel et al. (2008), Silva et al. (2012)

Absorptions around 1253-1257 cm^{-1} were for an asymmetric S=O stretching vibration (total sulfate) (Melo et al., 2002; Amorim et al., 2012; Costa et al., 2012), of which that of GdSPs-3 indicated lowest intensity among the samples as also verified by agarose gel analysis (Figure 4A); therefore, this difference found in the sulfation suggested the detection, at least, of two "populations" of extracellular matrix SPs as also found in *G. ornata* cell-wall (Rhodophyta) using cold/hot (25 and 80°C) water extractions (Amorim et al., 2012) and that the third redigestion during 3 h (GdSPs-3) was an important strategy to access minor amounts of *G. domingensis* SPs revealed by metachromasia (Figure 3).

The peaks at 1072-1074 cm^{-1} (Alves et al., 2012; Alencar et al., 2019) and at 931 cm^{-1} (Maciel et al., 2008; Amorim et al., 2012; Alencar et al., 2019) were caused by the bending vibration of 3,6-anhydrogalactose residue with C-O-C of α-L-galactose, respectively. On the basis of this result, its composition of sulfate group in the C-6 (equatorial sulfate) of galactose (at 817-823 cm^{-1}; Melo et al. 2002; Maciel et al., 2008) would be as a biological form to chemically generates anhydro-sugars for industrial applications (Cardozo et al., 2007). The presence of C-6 of galactose was also found in *H. wrightii* SPs (a seagrass) according to Silva et al. (2012).

The spectral bands at 891 (glycosidic linkage) and at 1153-1157 / 1375-1377 (C-O-C) cm^{-1} (Ananthi et al., 2010; Alencar et al., 2019) indicated as ring vibrations in the osidic cycles with or not sulfate. Other agar-related signals were virtually analyzed, but were not assigned (data not shown) (Alencar et al., 2019).

Collectivelly, our findings in this study partially characterized GdSPs, sequentially extracted by papain protease (3 h, 60°C) combined with both CPC and ice-cold alcoholic precipitations, as agar-structures occuring along the seaweed cell-wall based on Rodrigues et al. (2016), who studied the system of agar from the red seaweed *Acanthophora muscoides* by Nuclear Ressonance Magnetic spectroscopy. Herein, results evidenced certain structural regularity of the polymer for *in vitro* antioxidant studies.

Effects of GdSPs on *in vitro* oxidation processes

Based on figure 3A, GdSPs-1 and -2 samples were chosen for their *in vitro* antioxidant properties using standard assays and the results are listed in table 2. Both samples were explored on the two stages: initiation and propagation (Silva et al., 2012).

DPPH-scavenging assay

The DPPH scavenging effects of the samples (GdSPs-1 or -2) at concentration range (0.125 to 4 mg mL^{-1}) virtually showed a similar pattern of dose-dependent response, but GdSPs significantly inhibited no the *in vitro* process even at highest concentration tested (7.56 ± 0.22 and 5.34 ± 0.17% inhibitons for GdSPs-1 and -2, respectively); therefore, < 10% at 4 mg mL^{-1} and less actives than BHT synthetic antioxidant (94.15 ± 0.25% scavenging, at 4 mg mL^{-1}, $p < 0.05$) used as standard shown in table 2.

Table 2. Effects of *G. domingensis* SPs on DPPH, FIC and TAC assays.

GdSPs mg mL^{-1}	GdSPs-1			GdSPs-2		
	DPPH (%)	FIC (%)	TAC (%)	DPPH (%)	FIC (%)	TAC (%)
0.12	5.93±0.17^{a}	6.22±0.35^{a}	0.95±0.06^{a}	3.81±0.08^{a}	6.50±0.16^{a}	0.35±0.02^{a}
0.25	5.93±0.08^{a}	6.83±0.00^{a}	4.92±0.08^{b}	3.96±0.08^{a}	8.35±0.21^{b}	4.63±0.23^{b}
0.50	6.23±0.08^{a}	9.16±0.21^{b}	12.14±0.08^{c}	3.96±0.08^{a}	10.11±0.14^{c}	9.89±0.45^{c}
1.00	6.18±0.08^{a}	10.73±0.35^{b}	19.07±0.36^{d}	4.11±0.17^{a}	13.10±0.37^{d}	17.63±0.97^{d}
2.00	6.62±0.22^{a}	13.39±0.24^{c}	36.27±0.17^{e}	4.50±0.14^{a}	16.71±0.45^{e}	38.75±0.85^{e}
4.00	7.56±0.22^{a}	16.23±0.49^{d}	81.57±0.54^{f}	5.34±0.17^{b}	19.01±0.10^{f}	72.55±1.37^{f}
BHT 4 mg mL^{-1}	94.15±0.25^{b}	-	-	94.15±0.25^{c}	-	-
EDTA 4 mg mL^{-1}	-	99.56±0.00^{e}	-	-	99.56±0.00^{g}	-
ascorbic acid 0.4 mg mL^{-1}	-	-	99.77±0.00^{g}	-	-	99.77±0.00^{g}

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

The ability of the GdSPs in the DPPH assay was, at least, ~17.63-fold weaker than BHT at 4 mg mL^{-1}. Other sources rich in SPs had more important effects on DPPH method, such as those from seagrass *H. wrightii* (41.4% at 0.5 mg mL^{-1}; Silva et al., 2012); 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); 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 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). In fact, GdSPs were a limited substract to donate electrons and produce scavenging effects (Jridi et al., 2019). The antioxidant effect by SPs may not always be linked to their electronegativity and molecular size (Alves et al., 2012).

TAC assay

Considering the TAC method (Table 2), GdSPs reduced Mo to form a green phosphate/Mo complex total (Prieto et al., 1999) as an *in vitro* response. Both extracts (GdSPs -1 and -2) manifested stronger TAC potential with almost identical profiles of concentration-dependent mechanism, revealing both reduction rates of up to ~ 80% for GdSPs-1 and ~ 72% for GdSPs-2, respectively, at concentration of 4 mg mL^{-1} against the standard ascorbic acid (99.77% inhibition, at 0.4 mg mL^{-1}, $p < 0.05$); therefore, no difference between both tested polymer samples obtained from the *G. domingensis* matrix was noted.

Findings may be compared with other aquatic organisms SPs, including for those inhibitory effects of SPs 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). Studies demonstrated that the antioxidant action of SPs by TAC method depended on the species, origin and extraction conditions due to the heterogeneity of these compounds (Cardozo et al., 2007; Ai et al., 2023).

Both DPPH and TAC assays revealed that the GdSPs acted on the initiation phase of the *in vitro* oxidant process (Silva et al., 2012). Although suggesting a similar charge/mass ratio (GdSPs-1 and -2) from the electrophoresis and FT-IR analyses (Figures 4 and 5), it was speculated that the preponderant antioxidant effect of GdSPs on TAC could involve specific regions of the sulfation sites in the reducing potential (Alencar et al., 2019). In fact, this property was increased only for GdSPs-1 (1.12-fold higher than GdSPs-2) and this discrete variation in the bioactivity would be unlike to be a mere consequence of charge density effect, but thus probably on the spatial pattern of the molecules (Costa et al., 2012). It has been accepted that the sterospecific features, monossacaridic composition, glycosylation sites, aromericity, and spartial conformation are also important requeriments for the biological actions of SPs (Pomin, 2012).

FIC assay

Chelantin effect of GdSPs on ferrous ions was also dependent of concentration as listed in table 2. However, both extracts (GsSPs-1 and -2) showed no significant action between them, since at high concentrations (at 4 mg mL^{-1}) the maximum effects were only of 16.23 ± 0.49 and 19.01 ± 0.10%, respectively ($p > 0.05$), against the standard EDTA (99.56 ± 0.00% at 4 mg mL^{-1}, $p < 0.05$), which exhibited, at least, an *in vitro* antioxidant action of 6.13-fold higher than the examined polymer samples.

The low chelating power to $Fe2^{+}$ by GdSPs was due to weak ability of substitution of hydroxyl group with ester group present on the *G. domingensis* polysaccharides and, possibly, their ratio of charge/mass had no impact on such property based on discussion of Wang et al. (2008). Some SPs isolated from different origins have been reported as FIC agents, including from Chlorophyta *C. cupressoides* var. *flabellata* (44% at 2 mg mL^{-1}; Costa et al., 2012); 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); from cuttlefish skin and muscle (over > 90% at 1 mg mL^{-1}; Jridi et al., 2019); and from *O. niloticus* skin (32.22 ± 0.10% at 2 mg mL^{-1}; Nascimento et al., 2021).

It was suggested that the GdSPs are mainly localized in the peripheral region of its wall anatomy (Figure 1), in which they would playing a structural role and contributing to the irradiance protection-related physiological activity (Pomin & Mourão, 2008). Antioxidant effect of GdSPs, optimized extraction employing to release bioactives in *in vitro* tests, led us to the fact that marine plants are found in intertidal areas sujected to climatic disturbances, for instance, irradiation and water turbidity, as well as exposure to daily dehydration requering natural protection to cell injury and other physiological/structural damage-associated factors (Silva et al., 2012). Production of these

molecules by seaweeds would bring benefits to human and animal health and to coastal populations (Cardozo et al., 2007; Sales et al., 2023).

Conclusion

The system of matrix sulfated polysaccharides produced by *Gracilaria domingensis* (Rhodophyta) was found to be an agaran-structure of high molecular mass, whose total yield was extensively optimized by papain extraction sequence, and expressing *in vitro* antioxidant actions, although less potent than the commercial synthetic agents.

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