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UTILIZATION OF *Ulva fasciata* (CHLOROPHYTA) BIOMASS AS A SOURCE OF ANTIOXIDANT SULFATED POLYSACCHARIDES

APROVEITAMENTO DE BIOMASSA DE *Ulva fasciata* (CHLOROPHYTA) COMO UMA FONTE DE POLISSACARÍDEOS SULFATADOS ANTIOXIDANTES

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Abstract *Ulva fasciata* (Chlorophyta) from eutrophicated areas offers bioactives sulfated polysaccharides (UfSPs). In this study papain-extracted biomass was used to quantify and analyze structural and antioxidant properties of UfSPs. Sequentially the algal residues were redigested and UfSPs-1→3 characterized by agarose/polyacrylamide gels electrophoreses *vs.* standard glycosaminoglycans stained with toluidine blue; and then by infrared technique. UfSPs-1/-3 were *in vitro* evaluated for antioxidant effects by DPPH, total antioxidant capacity and ferrous ion chelating methods against BHT, ascorbic acid and EDTA, respectively. Total yield was 34.76% considering UfSPs-1→3 extracts, but reaching 27.6% within extracts ($p<0.05$). Molecular analyses of UfSPs characterized as ulvan of size >100 kDa, with low structural unregularity along the algal matrix. All the tests showed effects by UfSPs-1/-3, but revealing important variations in antioxidant response with less efficacy than respective synthetics. Therefore, *U. fasciata* biomass expresses antioxidant SPs along of its cell-wall.

Key words: cell-wall, green alga, molecular analyses, reductor power.

Resumo *Ulva fasciata* (Chlorophyta) oferece, de áreas entrofizadas, polissacarídeos sulfatados (UfPSs) bioativos. Neste estudo, biomassa extraída com papaína, foi usada para quantificar e analisar de UfPSs propriedades estruturais e antioxidantes. Foram redigeridos sequencialmente resíduos algáceos e caracterizados UfPSs-1→3 por eletroforeses em géis de agarose/poliacrilamida *vs*. glicosaminoglicanos padrões corados com azul de toludina e, posteriormente, por técnica de infravermelho. Foram avaliados *in vitro* os UfPSs-1/-3 para efeitos antioxidantes pelos métodos DPPH, capacidade antioxidante total e quelação de íon ferroso contra BHT, ácido ascórbico e EDTA, respectivamente. O rendimento total foi 34,76% considerando os extratos UfPSs-1→3, mas dentre extratos alcançando 27,6% ($p<0,05$). As análises moleculares caracterizaram de UfPSs como ulvana de tamanho >100kDa, com irregularidade estrutural baixa pela matriz algal. Efeitos em todos os testes mostraram por UfPSs-1/-3, porém revelando variações na resposta antioxidante com eficácia menor que os sintéticos respectivos. Portanto, biomassa de *U. fasciata* expressa PSs antioxidantes pela parede celular.

Palavras-Chave: parede celular, alga verde, análises moleculares, poder redutor.

Introduction

Macroscopic algae (seaweeds) are known as multicellular photosynthetic organisms that have benthic habitats where in their classification (Chlorophyta – green alga, Ochrophyta – brown alga, and Rhodophyta – red alga) (Joly, 1965) are also considered biochemistry, morphological and reproduction aspects (Reviers, 2006). These taxonomic groups have great commercial value as, *e.g.*, in pharmaceutics, cosmeceutics, food ingredients and aquaculture, due to their diverse chemical metabolites (sulfated polysaccharides-SPs, proteins, lipids and pigments) of potential interest for various industrial and scientific applications, like expressing health-related functionalities and medical products, without presenting important toxicity (Cardozo et al., 2007; Rodrigues et al., 2012).

Sulfated polysaccharides (SPs) are well-known matrix components by glycobiologists as an intrinsic class of biological macromolecules found in nature, not only in animals (called as glycosaminoglycans), but also in the cell-walls of the seaweeds (Pomin & Mourão, 2008). On a "nomenclature" basis, SPs are typically found as galactans in Rhodophyta, while fucans or fuicodan in Ochrophyta; and in Chlorophyta, they occur as sulfated heteropolysaccharides, especially ulvan-structures (Pomin & Mourão, 2008). As occur at the ultrastructural level, SPs from seaweeds matrixes have strongly anionic composition playing physiological functions and, when isolated SPs along the algal fibrilar wall, they would also represent as biological tools for diverse industrial applications (Cardozo et al., 2007; Rodrigues et al., 2009; Pomin, 2012). Seaweeds-derived SPs are of high molecular masses (> 100 kDa) and high content of sulfate groups on their backbone structures allowing electrostatic interations with basic proteins dependent or not from their sulfation pattern (Pomin, 2012) regarding biochemical and nutritional aspects, although with data still scarces (Cardozo et al., 2007). These molecules have a great structural diversity which are extensively studied as novel bioactives, including anticoagulant (Farias et al., 2000; Rodrigues et al., 2009, 2011; 2016), antiangiogenic (Pomin, 2012), antinociceptive and anti-inflammatory (Rodrigues et al., 2012) effects, but their actions in oxidation processes have been little explored.

The generation of reactive oxygen species causes cellular damage that has potential to develops systemic reactions in living organisms (*e.g.*, cancer) (Barbosa et al., 210). Hydrocolloid industry has interests on natural sources in antioxidant SPs (*e.g.*, thichening and gelling agents) for uses in foods (Cardozo et al., 2007) because the synthetic agents (like butylatedhydroxytoluene-BHT) are also known for their toxicities *in vivo* (Panicker et al., 2014). Some seaweeds species (Chew et al. 2008), as a large source in non-animal SPs (Pomin and Mourão, 2008), showed to play as free-radical scavengers or inhibitors from experimental assays acting as antioxidant tools. Isolated SPs by different protocols from Chlorophyta *Caulerpa cupressoides* var. *flabellata* (Costa et al., 2012); from Rhodophyta *Hypnea musciformis* (Alves et al., 2012), *Gracilaria birdiae* (Fidelis et al., 2014) and *G. caudata* (Alencar et al., 2019); and from 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) reduced free-radicals and neutralized the oxidative reactions when in *in vitro* and/or *in vivo* tests. Other SPs-rich sources also acted as antioxidants, such as in cyanobacteria (Ai et al., 2023), in by-products of fishes (Jridi et al., 2019; Nascimento et al., 2021) and in seagrasses (Silva et al., 2012).

Chlorophyta are benthic organisms (Reviers, 2006) belonging to nine genera (*Enteromorpha*, *Caulerpa*, *Codium*, *Monostroma*, *Ulva, Capsosiphon*, *Chaetomorpha*, *Bryopsis* and *Halimeda*) of green seaweeds that are commonly distributed in marine coastal regions. Some of them are well-known seaweeds as large biomasses usually found in eutrophicated areas generating impact in population structure (Wang et al., 2014). On the genera *Ulva* Linneus 1753, it is an important group of green seaweeds that has a low added-value as a natural source in nutraceutical and pharmaceutical fields and it could be more explored for massive production of novel structurally

diverse bioactive SPs (*e.g.*, anticoagulant, antiviral, antioxidative, antitumor, antinociceptive, anti-inflammatory immunomodulating, antihyperlipidemic and antihepatotoxic effects) (Wang et al., 2014; Araújo et al., 2016; Barcellos et al., 2018), since a largest number of the recorded species (38%) belong to *Ulva* (Wang et al., 2014) that is producer of SPs (Robic et al., 2008; Yaich et al., 2013; Barcellos et al., 2018) showing health benefits for human consumption or as ingredients in food formulations (Wang et al., 2014). As little is known about *U. fasciata* collected on the northeast coast of Brazil (Marinho-Soriano et al., 2009), the system of matrix SPs, known as ulvan, synthetized by this species has not been extensivelly examined by sequential extraction and analyzed on the yield, structural features and antioxidant actions, since that this bioactive property for some SPs from green seaweeds has been controversial on this topic (Wang et al., 2014).

The present study focused on sequentially optimize ulvan-type SPs produced from *U. fasciata* cell-wall ultrastructure and analyze their chemical features by electrophoreses and by Fourier Transform Infrared (FT-IR) spectroscopy. Antioxidant potential of the isolated SPs (UfSPs) along the process was also investigated using *in vitro* assays.

Material and Methods

U. fasciata collection and its macroscopic identification

Brazilian samples of *U. fasciata* (Figure 1), or populary known as limu palahalaha and sea lettuce, were manually collected from the litoral zone of an eutrophicated area located at Pacheco beach (Caucaia, Ceará-Brazil) during a field expedition carried out by our research group. Fresh biomass of algal thalli was found at low tide from specimens that colonized the substract in intertidal region with a population structure occuping a bloom of *Ulva* species and then was pre-selected in the environment from other algae species. After that, they were transported in plastic bags to the Marine Biochemistry laboratory of the Aquaculture Biotechnology Center, Department of Fishing Engineering, Federal University of Ceará, for washing with distillated water to removing impurities (*e.g.*, salt, sand and shells) and necrotic parts before frozen at -20°C (Farias et al., 2000) as an initial stabilization method of the raw material (Robic et al., 2008).

Kingdom Plantae
Phylum Chlorophyta
Class Ulvophyceae
Order Ulvales
Family Ulvaceae
Genus *Ulva* Linnaeus, 1753
Species *U. fasciata* Delile, 1813

Figure 1. Morphological aspect of grass green or dark green coloring of seaweed *U. fasciata* Delile with its thallus shaped similar to lanceolate flat attached to an appressoria.

On the arrival of *U. fasciata* at the laboratory, an algal sample was also macroscopically identified following the Marinho-Soriano et al. (2009)' identification list. It is a marine species of grass green or dark green coloring of thallus shaped similar to flat (long or lanceolate) which is attached on the substract by mean of an appressoria structure according to Joly (1965). The

specimen of figure 1 was reposted in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil. The algal material was authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

Sequential extraction of UfSPs and metachromasia detection

Total fresh biomass of *U. fasciata* was submitted to dehydration under continuous exposure to sunlight for 24 h prior to UfSPs extraction sequence. Then, the recovered material was cut into small pieces and weighed to obtain the algal sample pretreated required for extraction based on Farias et al. (2000)' method combined with Rodrigues et al. (2009). UfSPs extraction is illustrated in figure 2*a* and was performed from tritured material (5 g) using 100 mL of 100 mM sodium acetate buffer (pH 5.0) added with 5 mM EDTA and 5 mM cysteine, and the material initially digested in a thermostatic bath by adding of a crude papain solution (~31 mg mL^{-1}) at 60°C for 3 h resulting UfSPs-1.

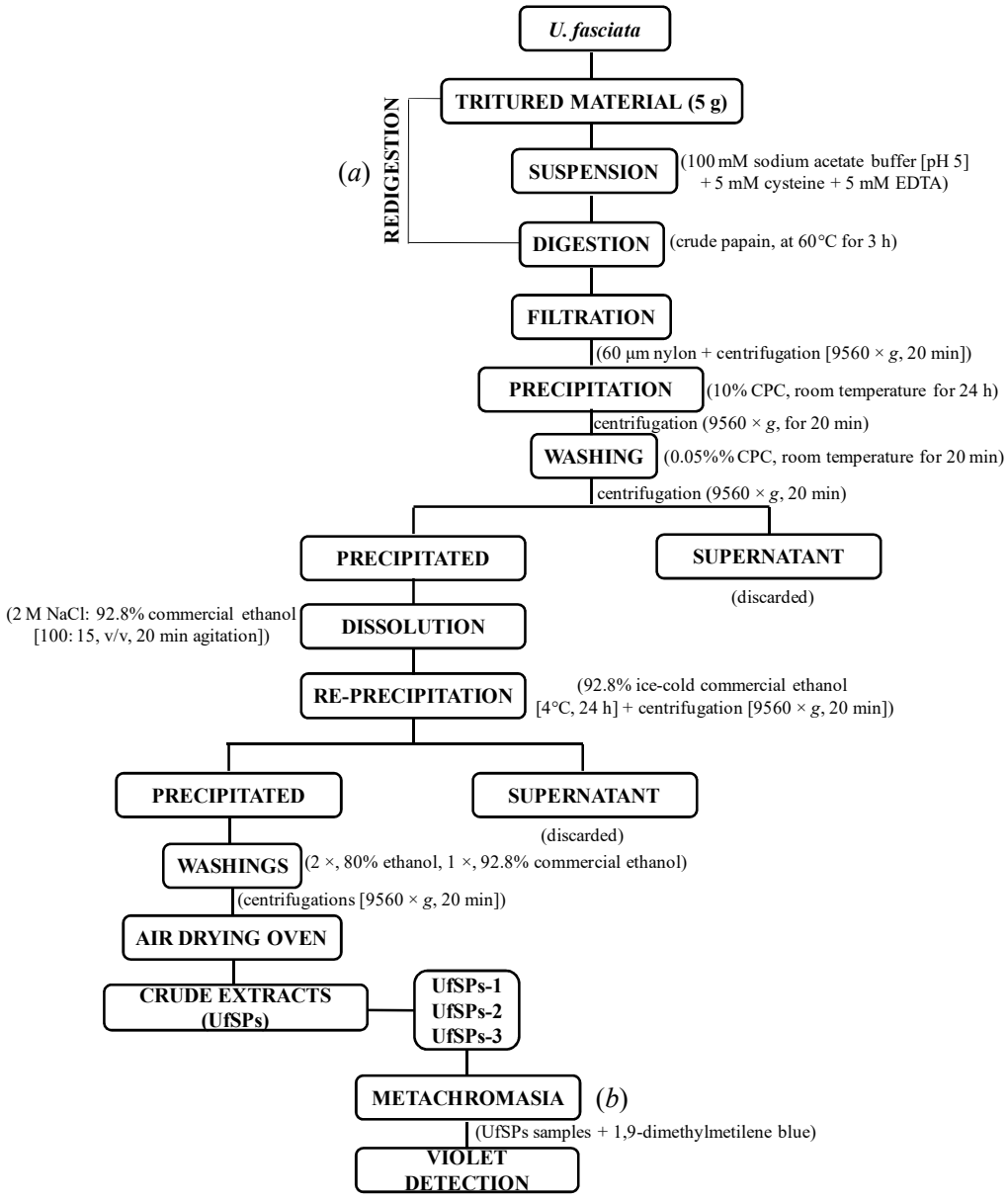

Figure 2. Procedures of obtaninig (*a*) and metachromasia (*b*) of SPs from the green seaweed *U. fasciata*.

Then, the digestion mixture was filtered with a nylon net and the supernatant was recovered. UfSPs-1 that were present in respective solution were precipitated with 16 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature for 24 h and the precipitated material then collected by centrifugation (9.560 × *g*, for 20 min). The *pellet* presenting UfSPs-1 was washed with 100 mL of 0.05% CPC solution, followed by dissolved under constant stirring for 20 min in 100 mL of a 2 M NaCl:ethanol (100:15 ratio, v:v) solution, and then again precipitated (for 24 h at 4°C) with adding of 100 mL of 92.8% ice-cold commercial ethanol. 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. Yield was sequentially optimized using *U. fasciata* residue redigested twice with papain incubation following the same procedure above resulting UfSPs-2 and UfSPs-3, respectively (Rodrigues et al., 2009, 2011, 2016). All the UfSPs extracts 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 presence of the metachromatic property in the UfSPs samples was *in vitro* tested using the 1,9-dimethylmetilene (DMB) blue dye as an indicator of reaction by complex formed by mean of violet coloring revealing sulfation (Farndale et al., 1976). The samples of UfSPs-1→3 (9 µg) were assayed in triplicate using glass tubes and the color visualized to be specific for sulfated polyanions compared with the cationic reagent used as a reference (Figure 2*b*).

Physical and chemical analysis by electrophoreses

Initially, aliquots containing ~21 µg of each crude extract (UfSPs-1→3) were prepared in destillated water before the physical-chemical analyses by two systems of electrophoretic procedures described below.

Agarose gel electrophoresis

For this system, UfSPs 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, UfSPs 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 (Rodrigues et al., 2016).

The UfSPs present in both gels were revealed with 0.1% toluidine blue cationic reagent and, subsequently, the gels were destained with a solution containing absolute ethanol, distilled water and acetic acid. 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 comparisons (Dietrich & Dietrich, 1976; Rodrigues et al., 2016).

FT-IR spectroscopy

The signals of FT-IR were obtained using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, 10 mg of UfSPs 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

All the test samples (UfSPs-1→3) were evaluated for antioxidant effects at the Seaweed II laboratory located at the Department of Biochemistry and Molecular Biology, FUC, and the *in vitro* protocols are described below.

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

The effect of UfSPs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of UfSPs (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). UfSPs (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 UfSPs (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 of UfSPs were expressed as mean ± standard deviation (n = 3). For extraction yield, statistical analysis was applied by one-way ANOVA, followed by Tukey' test, using $p < 0.05$ as significant. Regarding *in vitro* antioxidant assays, values 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

Optimized extraction yield of UfSPs monitored by metachromasia

Use of dehydrated *U. fasciata* biomass applying papain-assisted digestion sequence three times for shorter-time by heating tritured algal tissue (3h, 60°C) using a thermostatic bath, followed by both CPC and ice-cold ethanol precipitations revealed as a good approach for total yield of crude

SPs-rich extracts (UfSPs-1→3) based on other seaweeds studies (Rodrigues et al., 2009, 2011, 2016).

Under our conditions used (Figure 2*a*), the result of effect of pretreated raw matter and extraction parameters on the yield of UfSPs was highest in UfSPs-1 on average 29.20 ± 0.97% (w w^{-1}) of the drying (Figure 3A) which was different from those of *U. lactuca* yielding 2.21 and 13.13% from lyophilized samples extracted of the dehydrated raw biomass by Araújo et al. (2016) and by Barcellos et al. (2018), respectively, when treated with papain to obtain its crude SPs. These combined values would reflect the variation in yield of these molecules between *Ulva* samples globally used as bioresources to prepare SPs (Wang et al., 2014). Depending on the extraction conditions, yield of *Ulva* SPs could also be impacted by pH, temperature, extraction time and stabilization treatments of the algal biomass (Robic et al., 2008; Yaich et al., 2013).

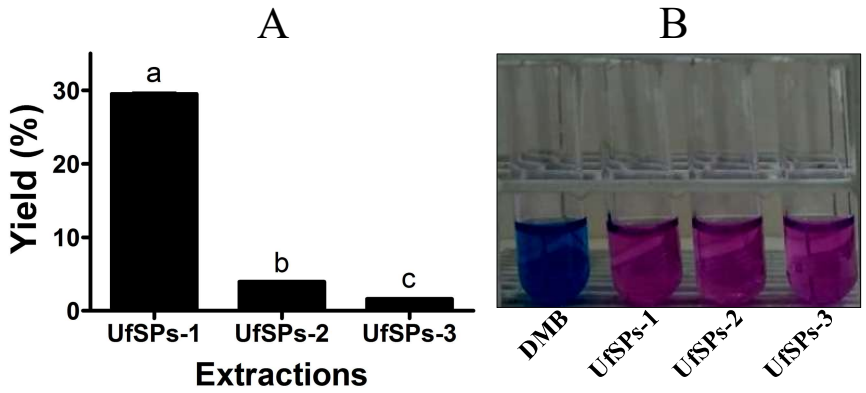

Figure 3. Yield (A) and metachromasia (B) of SPs sequentially extracted from the *U. fasciata* matrix. Different letters on the bars indicate differences among the extractions (ANOVA, Tukey' test, $p < 0.05$); Violet reaction showing sulfation in the presence of DMB dye.

U. fasciata residues redigested revealed extraction yields on average from 3.96 ± 0.20% to 1.60 ± 0.57% (w w^{-1}) of the drying for UfSPs-2 / -3, respectively, whose amounts of UfSPs drastically decreased ($p < 0.05$) among all the crude extracts recorded, accounting for 34.76% total yield (w w^{-1}), reaching 27.6% (w w^{-1}, UfSPs-1→3), from the tritured tissue samples of the seaweed. Similary, Rodrigues et al. (2009, 2011, 2016) observed the same behaviour in the total yield of SPs obtained from the Chlorophyta *Caulerpa cupressoides* var. *lycopodium* (4.61%, w w^{-1}); and from the Rhodophyta *Halymenia pseudofloresia* (47.14%, w w^{-1}) and *Acanthophora muscoides* (23.47%, w w^{-1}), respectively, applying papain-assisted extraction sequence. Diverse strategies (*e.g.*, enzymatic, water, alkaline, ultrasound and autoclaved methods) may be used for SPs extraction, yielding novel bioactives depending on the living organism (4.00% w w^{-1} - Farias et al., 2000; 2.30% w w^{-1} - Wang et al., 2008; 10.00% w w^{-1} - Ananthi et al., 2010; 0.52-8.26% w w^{-1} - Fidelis et al., 2014; 24.96% w w^{-1}- Alencar et al., 2019; 1.60% w w^{-1} - Jridi et al., 2019; 2.20% w w^{-1} - Nascimento et al., 2021; 15-20% w w^{-1} - Ai et al., 2023).

As the highest yield was achieved in first proteolytic digestion (UfSPs-1) of the seaweed extracellular matrix suggested that a high deposition of these molecules was concentrated on the surface texture where the polyanionics would be mainly located (Rodrigues et al., 2009, 2016). The unspecific influence of the protease for shorter-time (3 h) by sucessive actions on the *U. fasciata* cell-wall anatomy (UfSPs-1→3) generated a disorganization of the matrix structure in which SPs are linked to proteins isolating novel bioactives with different availably in yield (Figure 3A).

The composition of the extraction residues monitored by metachromatic property of the test samples (Figure 3B) indicated by Farndale et al. (1976)' method also evaluated the impact of the stabilization process on SPs recovery (Robic et al., 2008). On this basis, from dehydrated organic matter sujected to papain treatment could be an eco-friendly technique for rational utilization of residues from *U. fasciata* as industrial option (Cardozo et al., 2007) to take advantage of the functional properties or nutritional qualities of its SPs (Wang et al., 2014). As all the samples revealed the presence of sulfated compounds as checked by metachromasia (Figure 3B), the chemical quality of the extracted products (UfSPs) were further analyzed using traditional biochemical techniques.

Electrophoretic characterization of the UfSPs

UfSPs obtained from the extraction process were sujected to electrophoreses in agarose / polyacrylamide gels made of two different buffer systems to their charge/mass examination (Figure 4). By agarose gel analysis, all the samples (UfSPs-1→3) were clearly visualized and partially characterized as electrophoretic bands comigrating among themselves (Araújo et al., 2016; Rodrigues et al., 2011, 2016) in comparison to standards (DS/HEP) used as animal SPs references after toluidine blue staining (Figure 4A). This profile would indicate that the UfSPs have similar structural conformation based on their interaction with diamine buffer (Dietrich & Dietrich, 1976) and by other authors (Fidelis et al., 2014; Rodrigues et al., 2011, 2016). As each sample of UfSPs showed polydisperse blot of strong metachromasia pattern on gel (Rodrigues et al., 2009, 2011; Araújo et al., 2016) suggested that their densities of ester sulfate may be of high contents in the chemical structures of the analyzed molecules (Costa et al., 2012) and highly heterogeneous along the algal wall-matrix as an observation (Rodrigues et al., 2011), at initial level, of same charge/mass ratio based on *A. muscoides* (Rhodophyta) SPs (Rodrigues et al., 2016).

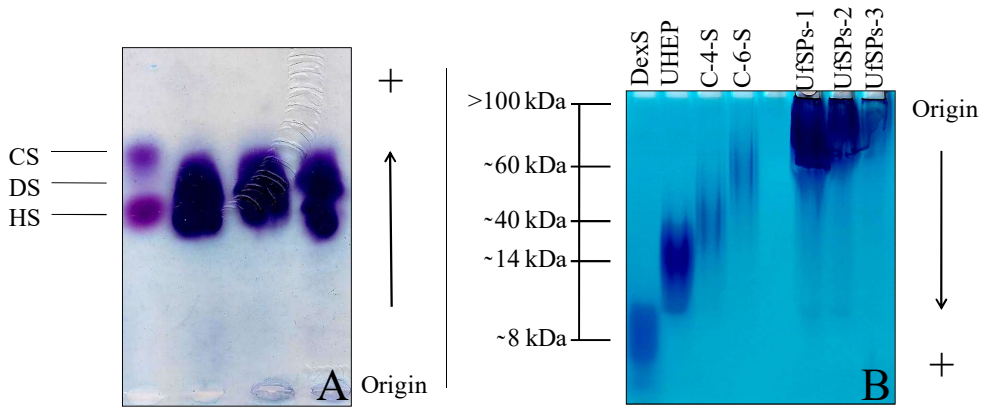

Figure 4. Agarose (A) / polyacrylamide (B) gels electrophoreses of *U. fasciata* SPs (UfSPs-1→3) and standards chondroitin-6-sulfate (C-6-S,~60 kDa), chondroitin-4-sulfate (C-4-S,~40 kDa), dextran sulfate (DexS,~8 kDa), dermatan sulfate (DS,~ 40 kDa) and heparin (HEP,~14 kDa) present on gels were stained with 0.1% toluidine blue.

However, molecular differences on the physical-chemical aspects have been reported for some seaweeds crude SPs, contrasting characteristics along the cell-wall ultrastructure (Rodrigues et al., 2009, 2011). SPs from *U. lactuca* (Chlorophyta) collected from different seasons and time of harvest showed conflicting results on their electrophoretic composition pattern when compared by agarose gel (Araújo et al., 2016; Barcellos et al., 2018). Such combined observations could perhaps are linked to the ecophysiological responses considerating mechanical, osmotic and ionic functions

where the seaweeds are found (Wang et al., 2014) and supporting more taxonomic, phylogenetic and biogeographic studies of these marine organisms (Rodrigues et al., 2011).

PAGE analysis was further employed to estimate the average molecular size of three UfSPs extracts and the result is shown in figure 4B. All the samples (UfSPs-1→3) exhibited high molecular masses of > 100 kDa after toluidine blue treatment, as expected for unfrationated polysaccharides, since they did not mobility present on gel *vs*. standard glycosaminglycans that showed as heterogeneous systems with distinct electrophoretic mobilities from their respective sizes (Fidelis et al., 2014; Rodrigues et al., 2016; Barcellos et al., 2018).

Combined techniques led us to speculate to a similar profile of SPs occuring in *U. fasciata* cell-wall matrix because there is a difficult to industrially produce a standardized product derived seaweeds with structural regularity and applicability for commercial use (Cardozo et al., 2007; Pomin & Mourão, 2008; Wang et al., 2014).

Structural investigation by FT-IR

The chemical identity of the UfSPs regarding the functional groups making part of each sample (UfSPs-1→3) was investigated and compared by FT-IR spectroscopy from other natural sources in SPs (Alves et al., 2012; Costa et al., 2012; Souza et al., 2012; Jridi et al., 2019; Ai et al., 2023). On a qualitative basis, spectral data were recorded at 500-4000 cm^{-1} from kBr *pellets* and the results are represented in figure 5.

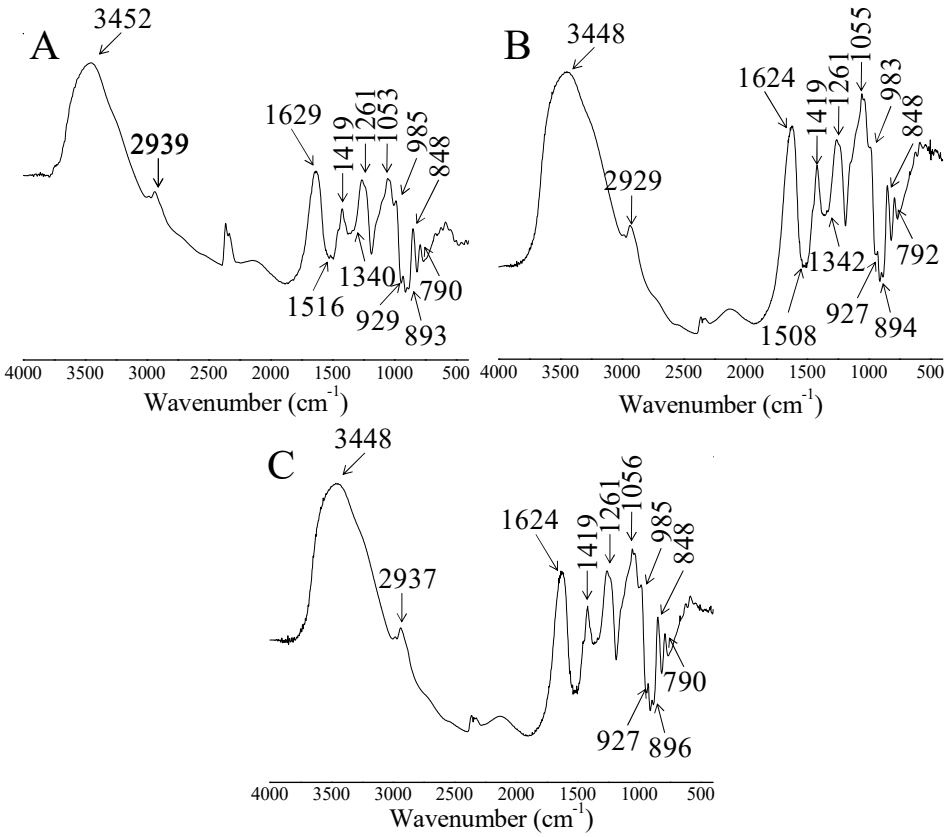

Figure 5. FT-IR spectra of the *U. fasciata* SPs (UfSPs-1 [A], -2 [B] and -3 [C]) at 500-4000 cm^{-1}.

Extracts UfSPs-1→3, which showed to be sultated materials from the biochemical analyses (Figures 3, 4), revealed structural features by FT-IR experiments related to ulvan-structures (Figure 5). Overall, the current investigation offered the observation that the spectra of UfSPs showed common peaks, but pointed out certain inconsistences among the analyzed polymer samples. On

this basis, SPs derived from *U. fasciata* (northeast coast of Brazil) showed main spectral values as those found in *Ulva* species (Wang et al., 2014; Araújo et al., 2016).

Absorption bands at 3448-3452 (Fidelis et al., 2014; Araújo et al., 2016; Alencar et al., 2019) and at 2929-2939 (Alves et al., 2012; Costa et al., 2012; Araújo et al., 2016) cm^{-1} were identified for axial deformations of hydroxyl groups (O-H stretching and C-H, respectively). Signals at 1637-1639 cm^{-1} and at 1409-1419 cm^{-1} were attributed to O-C=O bending, denoting the presence of carboxyl group of uronic acid as found in SPs isolated from *T. ornata* (Ochophyta) by Ananthi et al. (2010), *G. birdiae* (Rhodophyta) by Fidelis et al. (2014) and *U. lactuca* (Chlorophyta) by Araújo et al. (2016), respectively. Addictionally, 1340-1342 and 1053-1056 cm^{-1} were attributed to the ring vibrations in the osidic cycles with or not sulfate (Ananthi et al., 2010; Alencar et al., 2019).

Similar spectral regions around 1261 cm^{-1} were for an asymmetric S=O stretching vibration corresponding to total sulfate of the polymer (Paiva et al., 2011; Alves et al., 2012; Costa et al., 2012), indicating similar intensities of sulfation among the analyzed samples of UfSPs confirming agarose analysis (Figures 5, 4A), although with a significant difference among the yields of the metachromatic compounds (Figure 3). Common peaks of sulfation were also detected at 848 cm^{-1} (C-O-SO_4, in C-4 of sugar) from other authors (Alves et al., 2012; Costa et al., 2012; Araújo et al., 2016).

Interestingly, UfSPs analyzed by FT-IR spectroscopy also probably indicated to the relatively lower presence of 3,6-anhydrosugars residues C-O bond (927-929 cm^{-1}) discretaly varying among the samples based on SPs from red seaweeds (Alves et al., 2012; Alencar et al., 2019) and from cuttlefish skin and muscle (Jridi et al., 2019) known to have this structural component, but absence in *U. lactuca* (Chlorophyta) polysaccharides (Araújo et al., 2016). Addictional shoulders around 983-985, 893-896 and peaks at 790-792 cm^{-1} could be assigned to glycosidic linkages with or not sulfated regions (Ananthi et al., 2010; Alencar et al., 2019).

Collectivelly, our results pointed out to a peculiar ulvan-structure *vs*. other SPs-rich *Ulva* species (Wang et al., 2014). Of all the FT-IR spectra of UfSPs, it was also observed to the absence of signals around 1500 and 100 cm^{-1} for that of UfSPs-3, indicating certain structural unregularity among the extracts enzimatically obtained from the *U. fasciata* wall-matrix, but showing the main functional groups and high content of sulfated peaks as shown in figure 5. In order to evaluate the impact of the chemical differences, UfSPs-1→3 were further examined as antioxidants *in vitro*.

Analysis of the UfSPs as antioxidants *in vitro*

As there were some conflicting features among the UfSPs extracts (Figure 5) and when compared to other studies, extracts UfSPs-1 / -3 were *in vitro* tested as natural inhibitors of oxidant processes (Table 1). The reducing role of both polysaccharidic samples were examined on the initiation and propagation stages (Silva et al., 2012).

DPPH-scavenging method

Initially, the preparations of UfSPs-1 / -3 (from 0.125 to 4 mg mL^{-1}) were assayed by the DPPH method (Table 1). Both samples had no important scavenging actions and were unrelated to increasing concentration of UfSPs ($p > 0.05$), whose antioxidant profiles were inverses among the concentrations, showing only from 4.23 ± 0.10 and 4.00±0.44% at 0.12 mg mL^{-1} to 3.28 ± 0.14 and 4.16 ± 0.14% at 4 mg mL^{-1} for UfSPs-1 / -3, respectively. Theses actions represented, therefore, < 5% of the antioxidant abilities of the UfSPs since that the BHT synthetic product reduced the process by 94.15 ± 0.25% at concentration of 4 mg mL^{-1} ($p < 0.05$).

On comparative bases, the *in vitro* effects of UfSPs in the DPPH assay was, at least, ~28.70-fold less potent than BHT used in the same concentration (4 mg mL^{-1}). SPs isolated 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) were more biologically actives than UfSPs on DPPH method, suggesting that they showed as a limited substract to donate electrons and produce scavenging responses (Jridi et al., 2019).

Table 1. Effects of the *U. fasciata* SPs on DPPH, FIC and TAC methods.

UfSPs mg mL^{-1}	UfSPs-1			UfSPs-3		
	DPPH (%)	FIC (%)	TAC (%)	DPPH (%)	FIC (%)	TAC (%)
0.12	4.23±0.10^{a}	4.88±0.00^{a}	0.58±0.28^{a}	4.00±0.44^{a}	2.01±0.07^{a}	0.57±0.43^{a}
0.25	4.01±0.14^{a}	13.36±0.19^{b}	2.02±0.09^{b}	3.67±0.22^{a}	2.95±0.07^{a}	0.46±0.20^{a}
0,50	4.01±0.00^{a}	18.20±0.46^{b}	3.93±0.20^{b}	3.77±0.08^{a}	5.20±0.31^{b}	0.69±0.17^{a}
1.00	3.87±0.29^{a}	20.90±0.20^{b}	6.72±0.29^{c}	3.87±0.38^{a}	11.00±0.28^{c}	1.67±0.25^{b}
2.00	3.61±0.08^{a}	20.85±0.20^{b}	13.90±0.39^{d}	4.07±0.00^{a}	19.10±0.28^{d}	3.12±0.29^{c}
4.00	3.28±0.14^{a}	18.20±0.19^{b}	25.92±0.00^{e}	4.16±0.14^{a}	23.22±0.15^{e}	5.05±0.40^{d}
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, $p < 0.05$).

In fact, not only the high sulfation, but also the high molecular size of the UfSPs had no impact on the the antioxidant effect by DPPH assay, as also observed by Alves et al. (2012) for *H. musciformis* (Rhodophyta) SPs.

TAC method

In the TAC method (Table 2), UfSPs were able to discretaly reduce Mo to form a green phosphate/Mo complex total (Prieto et al., 1999). Both extracts (UfSPs -1 / -3) exhibited TAC with different antioxidant profiles of concentration-dependent effects (from 0.125 to 4 mg mL^{-1}), which showed reduction rates of up to ~25% for UfSPs-1 and ~ 5% for UfSPs-3, respectively, reaching 20% at 4 mg mL^{-1} *vs*. the standard ascorbic acid (99.77% inhibition, at 0.4 mg mL^{-1}, $p < 0.05$). These compared results of UfSPs significantly differed between both extracts sequentially obtained from the algal wall- matrix.

SPs 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 Ochophyta *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) showed to be antioxidants using TAC method. According to Cardozo et al. (2007) and Ai et al. (2023), the antioxidant action of SPs by TAC method may be influenced on the species, origin and extraction conditions due to the structural complexity of these compounds.

Considerating both DPPH and TAC methods, it was noted that the UfSPs modestly inhibited the initiation phase during the *in vitro* oxidant process (Silva et al., 2012). Possibly, such effetcs would be unrelated to charge/mass ratio of UfSPs-1 / -3 as previouly estimated by electrophoresis and FT-IR (Figures 4 and 5). It was speculated that the preponderant antioxidant effect of UfSPs-1 on TAC could involve specific sulfation sites in the reducing potential (Alencar et al., 2019).

In fact, the increased antioxidant property of UfSPs-1 was ~ 5.13-fold greater than the action found in UfSPs-3 and this drastical variation in the bioactivity between the extracts of UfSPs would reflect those particular features for each material (UfSPs-1 / -3), as already reported in figure 5. Therefore, they unlike to be a mere consequence of charge density effect, but thus on the different spatial pattern of the SPs (Costa et al., 2012), such as sterospecific features, monossacaridic composition, glycosylation sites, aromericity, and spartial conformation (Pomin, 2012).

FIC method

As shown in table 2, UfSPs also manifested chelantin effect on ferrous ions. Thus, both extracts (UfSPs-1 / -3) showed *in vitro* antioxidant actions by FIC method. However, this property was almost similar between themselves to block the propagation phase, since at concentrations of 4 mg mL^{-1}, the maximum inhibitory effects were dose-dependent leading to only 18.20 ± 0.19 and 23.22 ± 0.15%, respectively, for UfSPs-1 / -3 ($p > 0.05$) against the standard EDTA (99.56 ± 0.00% at 4 mg mL^{-1}, $p < 0.05$), which inhibited the oxidation by method, at least, 5.47-fold higher *vs*. the examined polymer samples.

As the chelating power to $Fe2^{+}$ by UfSPs was low very (< 25% inhibition), it was presumed to the weak property of substitution of hydroxyl group with ester group present on the algal polysaccharides. Like TAC method, the antioxidant profile varied between extractions as a controversial response where the influence of sulfated degree did not determine the UfSPs that were previously estimated to be high molecular sizes (> 100 kDa) to donate protons more effectively to process (Wang et al., 2008, 2014). SPs 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). The antioxidant actions could greatly depend on the composition and chemical structure of SPs, difficulting the development of medicine from natural product (Wang et al., 2008, 2014). It has been believed that the uronic acid:sulfate ratio would influence on the bioactivites of SPs and structure-function relationship lacks of investigation (Ai et al., 2023).

In summary, sequentially isolated SPs in papain digestions would characterize the occurence of distinct molecules in the *U. fasciata* matrix with bioactive properties like other SPs already described (Rodrigues et al., 2009, 2011, 2016). Marine plants frequently occur in intertidal areas which are daily sujected to climatic disturbances (*e.g.*, irradiation and water turbidity) and dehydration in litoral zone they also biosynsthetize antioxidant SPs as a mechanism of protection against cell injury and other physiological factors (Silva et al., 2012). Utilization of *Ulva* biomass would bring advantage to discovery of novel biological agents of human health-related interest (Cardozo et al., 2007; Wang et al., 2014).

Conclusion

Brazilian samples of *Ulva fasciata* (Chlorophyta) biomass collected from an eutrophicated area of Brazil showed as an alternative source to obtain large amount of sulfated polysaccharides. The system of ulvan-structure of high molecular mass and highly charged produced by this species was found to be heterogeneous varying its chemical complexity along the algal cell-wall matrix. Highest yield in antioxidant polysaccharides was obtained within three sequential extractions, but their intrinsic properties varied with the optimized process, when also analyzed by *in vitro* classical methods that revealed polysaccharides with significantly lower effects than the conventional synthetic agents.

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