

Characterization of polysaccharides and their contribution to antioxidant properties in *Bryothamnion triquetrum* and *Halimeda opuntia* seaweeds

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ABSTRACT

Seaweeds are renowned for their significant antioxidant properties, largely attributed to their polyphenolic compounds and polysaccharides. This study was aimed to characterize the polysaccharide-rich fractions from the seaweeds *Bryothamnion triquetrum* and *Halimeda opuntia* using reverse-phase high-performance liquid chromatography (RP-HPLC) and to assess their antioxidant activities through *in vitro* assays. Polysaccharide yields from *B. triquetrum* (1.6 % of algal dry weight) were notably higher compared to those from *H. opuntia* (0.3 %). RP-HPLC analysis indicated a diverse monosaccharide composition in both seaweeds, suggesting the presence of heteropolysaccharides with substantial amounts of galactose and sulfated polysaccharides. Differences in sulfation patterns were also observed. Antioxidant assays revealed that the polysaccharide fraction from *B. triquetrum* contributed partially to the antioxidant activity of its aqueous extract, particularly in reducing power and DPPH scavenging tests. Instead, *H. opuntia* demonstrated significant DPPH scavenging activity, indicating that its polysaccharides, despite being present in lower amounts, play a crucial role in its antioxidant properties, potentially due to a higher sulfate content. These results underscore the role of polysaccharides in seaweed antioxidant activity and suggest that both polyphenols and polysaccharides are important contributors to these properties. This study provides valuable insights for further research into seaweed-derived antioxidants.

Keywords: Sulfated polysaccharides, antioxidant activity, red seaweed, green seaweed

RESUMEN

Las algas marinas son reconocidas por sus importantes propiedades antioxidantes, atribuidas en gran medida a sus compuestos polifenólicos y polisacáridicos. Este estudio tuvo como objetivos caracterizar las fracciones ricas en polisacáridos de las algas marinas *Bryothamnion triquetrum* y *Halimeda opuntia*, mediante RP-HPLC, y evaluar la actividad antioxidante mediante ensayos *in vitro*. El contenido de polisacáridos de *B. triquetrum* (1,6 % del peso seco) fueron notablemente superior a los de *H. opuntia* (0,3 %). El análisis mediante RP-HPLC indicó una composición diversa de monosacáridos en ambas algas marinas, lo que sugiere la presencia de heteropolisacáridos con cantidades sustanciales de galactosa y polisacáridos sulfatados. También se observó diferencias en los patrones de sulfatación. Los ensayos antioxidantes revelaron que la fracción de polisacáridos de *B. triquetrum* contribuyó parcialmente a la actividad antioxidante de su extracto acuoso, particularmente en las pruebas de poder reductor y eliminación de 2,2-difenil-1-picrilhidracilo (DPPH). En cambio, las fracciones de *H. opuntia* mostraron una importante actividad de eliminación de DPPH, lo que indica que sus polisacáridos, a pesar de estar presentes en cantidades menores, desempeñan un papel crucial en sus propiedades antioxidantes, posiblemente debido a un mayor contenido de sulfato. Estos resultados subrayan el papel de los polisacáridos en la actividad antioxidante de las algas y sugieren que los polifenoles y los polisacáridos aportan de manera significativa importantes a estas propiedades. Este trabajo proporciona información valiosa para futuras investigaciones sobre los antioxidantes derivados de ambas especies de algas.

Palabras clave: polisacáridos sulfatados, actividad antioxidante, algas rojas, algas verdes

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Introduction

Oxidative stress and its clinical implications can be considered relatively innovative areas of research. This process is closely related to a variety of pathologies, including AIDS, atherosclerosis, diabetes, cancer, neurodegenerative diseases, aging, and apoptosis, among others [1].

At present, seaweeds have received greater attention, due to their potent pharmacological effects demonstrated both *in vitro* and *in vivo* [2, 3]. These effects could be explained by their rich diversity of bioactive compounds [4, 5]. The cell wall of seaweeds presents

polysaccharides that do not exist in terrestrial plants [6]. These macromolecules display various pharmacological properties as anticoagulants, antivirals, anticancer, immunomodulatory and antioxidants [7-10], with relatively low toxicity [11].

Several studies indicated that the antioxidant properties of seaweeds are related to their monosaccharide composition, molecular weight, and configuration [11, 12]. The sulfated content of the polysaccharides significantly influences the radical scavenging capacity of polysaccharides [11, 13, 14]. In this sense, red

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seaweed *Bryothamnion triquetrum* (S. G. Gmelin) M. Howe and the green seaweed *Halimeda opuntia* (Linnaeus) J. V. Lamouroux have gained attention. The aqueous extract from *B. triquetrum* showed high antioxidant potential on different *in vitro* assays of oxidative stress. This seaweed has shown antiradical activity, reducing power, chelating activity, and inhibition of lipid peroxidation related with polyphenols content [15, 16]. Moreover, it has demonstrated neuroprotective and hepatoprotective properties [17-19]. Regarding *H. opuntia*, it has demonstrated the ability to capture free radicals, exhibit reducing power, also inhibiting lipid peroxidation, and hemolysis due to its polyphenolic composition [16, 20, 21]. It has also been shown to have hepatoprotective effects in animal models [21]. Both seaweeds have also shown antioxidant activity associated with their polyphenolic content. However, their crude polysaccharides composition has not been characterized, nor its contribution to the antioxidant properties. Previous research identified the presence of agar in *B. triquetrum* [22] and sulfated galactans in *Halimedineae* [23], by NMR. Besides, chromatographic techniques such as RP-HPLC supports the characterization of monosaccharides' composition and polysaccharides homogeneity [24] in crude polysaccharidic fractions.

Therefore, this work was aimed to characterize the polysaccharide-rich fractions of *B. triquetrum* and *H. opuntia*, using RP-HPLC. The contribution of such fractions to their *in vitro* antioxidant activities was also assessed, to explore their potential application as phytopharmaceuticals and/or nutraceuticals.

Materials and methods

Collection of seaweeds

Samples of seaweeds *B. triquetrum* (S. G. Gmelin) M. Howe, and *H. opuntia* (Linnaeus) J. V. Lamouroux, were collected on April, 2020, off the coast of the Cuban Institute of Oceanology, located in Flores, Playa, Havana City (23.092167 North and -82.471725 West). The specimens were authenticated by the Algae Laboratory of the Marine Research Center. (*B. triquetrum* voucher CIM 069 and *H. opuntia* voucher CIM 183).

Preparation of fractions

Freshly collected seaweeds were washed with distilled water and dried at room temperature ($\pm 26^\circ\text{C}$) for 30 d. After grinding and sieving, the dry powder was extracted with distilled water (1:5 w/v) at room temperature for 24 h, and centrifuged at 800 g and 4°C , for 20 min. The supernatant (aqueous extract) was collected and lyophilized. Polysaccharides-rich fractions were generated by precipitation of the aqueous extract with 95 % ethanol (2:1). Next, it was centrifuged at 800 g and -4°C , for 20 min. Both, aqueous extract and the polysaccharides-rich fractions were kept at -20°C until use.

Determination of total sugars and phenolic compounds content of the aqueous extracts and polysaccharides-rich fractions

The total sugar content was determined according to the spectrophotometric method of Saha and Brewer [25], using calibration curves (10-100 μg) for glucose,

xylose and galactose. The results were expressed in micrograms of sugar equivalents/mg of sample analyzed. The phenolic compounds of the different fractions were determined using the Folin-Ciocalteu assay as described by Silva et al. [26], and expressed in milligrams of gallic acid equivalents per gram of sample. A calibration curve in the 0.020-50 μg range of gallic acid/mL was used for estimation of samples' phenolic content.

Analysis of the polysaccharide-rich fraction

Hydrolysis

The polysaccharide-rich fraction were subjected to acid hydrolysis according to Hapfi Emaga et al. [27]. For the analysis of the neutral monosaccharides (galactose, mannose, glucose, and fucose), 20 μL of 2 M trifluoroacetic acid (TFA) was added to an aliquot of the PRF, while for N-acetyl-glucosamine and N-acetyl-galactosamine determinations, 20 μL of 6 M HCl were added. The reactions were run for 3 h at 100°C in a thermocycler (Eppendorf, Germany). Subsequently, the samples were cooled and dried in a vacuum evaporator centrifuge. Finally, free monosaccharides were labeled with the commercial set of 2-AA Monosaccharide Release and Labeling reagents from Ludger Tag [28]. The samples were resuspended in 5 μL of sodium acetate and 10 μL of a solution (1:1 v/v) of 0.06 g/mL 2-aminobenzoic acid (2-AA) and 0.04 g/mL sodium cyanoborohydride. The reaction was run for 45 min at 80°C .

Identification of monosaccharides by RP-HPLC

The 2-AA-labeled monosaccharides fractions were analyzed by RP-HPLC with a GC Shimadzu 17 A equipment, by using a fluorescence detector at $\lambda_{\text{exc}} = 360\text{ nm}$; $\lambda_{\text{em}} = 425\text{ nm}$, and a LudgerSep R1 column (150 mm $\times$ 4.6 mm; Ludger, UK) at a constant temperature of 30°C .

Quantification of monosaccharides by 2-AA labeling

The 2-AA labeled monosaccharides were labeled by the RP-HPLC technique. The quantification was performed from a standard curve (5-150 pmol) prepared with a mixture of standards of the monosaccharide's N-acetyl-glucosamine, N-acetyl-galactosamine, mannose, galactose and fucose (2-AA monosaccharide release and labeling Ludger Tag; Ludger, UK), at a concentration of 2 nmol. Additionally, xylose and arabinose standards were applied under the same conditions. From this curve, the percentage composition for each monosaccharide within all the polysaccharide fractions was estimated.

Determination of sulfate groups in polysaccharides

Sulfate groups in the polysaccharides were assessed by RP-HPLC [29]. Briefly, portions of the polysaccharide-rich fraction were treated with 150 μL of a solution of anhydrous methanol plus 50 mM HCl, at 37°C for 3 h. The reaction mixture was taken to dryness. Then, hydrolysis with 2 M TFA was performed and the free monosaccharides were labeled as previously described. Then, samples were dissolved in phosphate-buffered saline and analyzed by RP-HPLC.

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Antioxidant activity *in vitro*

Antioxidant activity by the phospho-molybdenum assay

The ability of the different extracts to reduce ammonium molybdate to phospho-molybdenum was evaluated according to Prieto *et al.* [30]. The absorbance of the reagent solution was measured at 695 nm against a blank (H₂O). A standard curve of 0.03–0.4 µmol/mL ascorbic acid of was used, and the antioxidant activity calculated according to the formula:

$$\text{TAC} = \frac{\text{ICC} - \text{TVE}}{\text{W}}$$

where:

TAC: Total antioxidant capacity, expressed in mg of equivalents of ascorbic acid/g of dry extract;

ICC: interpolated reading on the calibration curve;

TVE: total volume of extract (1.1 mL); and

W: weight of the extract (g).

Reducing power

The reducing power was determined following the protocol of Oyaizu [31], as modified by Batista-González *et al.* [32]. Absorbance was measured at 700 nm on a VIS-723 G spectrophotometer (Rayleigh, China). An increased absorbance of the reaction mixture was indicated by the raise in the reducing power activity. Ascorbic acid (1 mg) was used as positive control.

Radical scavenging activity DPPH•

The DPPH• radical scavenging capacity was assessed following the protocol of Brand-Williams *et al.* [33] adapted to a microtechnique in a 96-well plate [23]. Optical density was measured at 517 nm against H₂O as blank. The reference antioxidant used was ascorbic acid (0.0625–1 mg/mL). The DPPH• radical scavenging activity was calculated according to the equation:

$$\text{PI DPPH}\cdot (\%) = \left[1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{ref}}} \right] \times 100$$

where:

PI DPPH• (%): percentage of entrapment of DPPH• radicals, and

ODref: OD of the vitamin C control.

Statistical analysis

The values of the different experiments are expressed as mean ± standard deviation (SD), and were performed in triplicate. The statistical significance of antioxidant activity was determined by ANOVA with a post Tukey test. Significant differences were set for $p < 0.05$. Data were processed using GraphPad Prism [34].

Results and discussion

Marine macroalgae have been widely investigated for their secondary metabolites, that possess a wide range of biological activities [7]. Seaweeds exhibit different pharmacological properties like antioxidant activity, which can be attributed to their diverse chemical structures such as phenolic compounds, and

polysaccharides as a key component of their cell walls [35, 36]. Several researchers have demonstrated the sulfated polysaccharides from algae possess antioxidant activities [7, 37–39]. Due to their limited toxicity, they have been used in cosmetic, food and pharmaceutical industries [7, 40].

Dry weight yields, quantification of total sugars and polyphenolic compounds of aqueous extract and polysaccharide-rich fractions

The dry weight yield in the aqueous extract was similar for both seaweeds, showing differences in the polysaccharides-rich fractions (Table 1). In fact, the dry weight of the polysaccharide-rich fraction of *B. triquetrum* showed five times more solute than that of *H. opuntia*. This last green seaweed has a high degree of calcification, its lower yields of solutes derived in part from its higher calcium content, as previously reported [23].

The highest content of sugars in aqueous extract and polysaccharide-rich fractions of *B. triquetrum*, indicated that most of the hydrophilic components of *B. triquetrum* are carbohydrates. The composition of polysaccharides in seaweeds varies among species, making it a widely used taxonomic marker for these species [41]. The same was reported by Shanura Fernando [7], with red algae of the *Gracilaria* genus, showing more carbohydrates that several green algae such as *H. discoidea* and *H. gracilis*.

A similar behavior was found regarding polyphenols in the aqueous extract of *B. triquetrum*, nearly 2.5-times that of *H. opuntia*, in agreement with previous works [26, 42]. No significant amounts of polyphenols were detected in the polysaccharide-rich fraction, as expected due to the isolation method applied.

Characterization of polysaccharides

Identification of monosaccharides in the polysaccharide-rich fraction

The first approach for the chemical characterization of polysaccharides in seaweeds fractions is the identification of monosaccharide constituents. Therefore, monosaccharides in *B. triquetrum* and *H. opuntia* fractions were identified and quantified by the RP-HPLC technique, their respective chromatograms shown in figures 1 and 2. They included the 2-amino-benzoic acid-labeled monosaccharides.

The polysaccharide-rich fraction from *B. triquetrum* contained major polysaccharide content of N-acetylglucosamine and N-acetylgalactosamine, with signals at retention times 8.811 and 9.752 min, respectively. The were signals for galactose (16.981 min), mannose (17.795 min), glucose (18.693 min), arabinose (20.030 min), xylose (21.277), and fucose (23.026 min). There were also two almost imperceptible peaks which signals appeared at 22.101 and 24.119 min, probably corresponding to modified monosaccharides.

The same fraction from *H. opuntia* (Figure 2) showed two major peaks of hexosamines, one for N-acetyl-glucosamine at 8.805 min, and another for N-acetylgalactosamine 9.750 min. Signals were also obtained for galactose (16.986 min),

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Table 1. Determination of yields, carbohydrates and polyphenolic compounds of aqueous extracts and polysaccharide-rich fractions of the seaweeds *Bryothamnion triquetrum* and *Halimeda opuntia**

Seaweed	Samples	Yield (g lyophilized/ g dry seaweed; %)	Polyphenolic compounds (µg GEA/ mg lyophilized extract)	Carbohydrates (µg Galactose equivalents/ mg sample)
<i>B. triquetrum</i>	Aqueous extract	3.9	51.21 ± 2.25	10.52 ± 1.06
	Polysaccharide-rich fraction	1.6	Not determined	24.93 ± 1.76
<i>H. opuntia</i>	Aqueous extract	3.4	19.99 ± 1.12	2.25 ± 0.25
	Polysaccharide-rich fraction	0.3	Not determined	13.09 ± 0.86

*Each assay was performed in triplicate

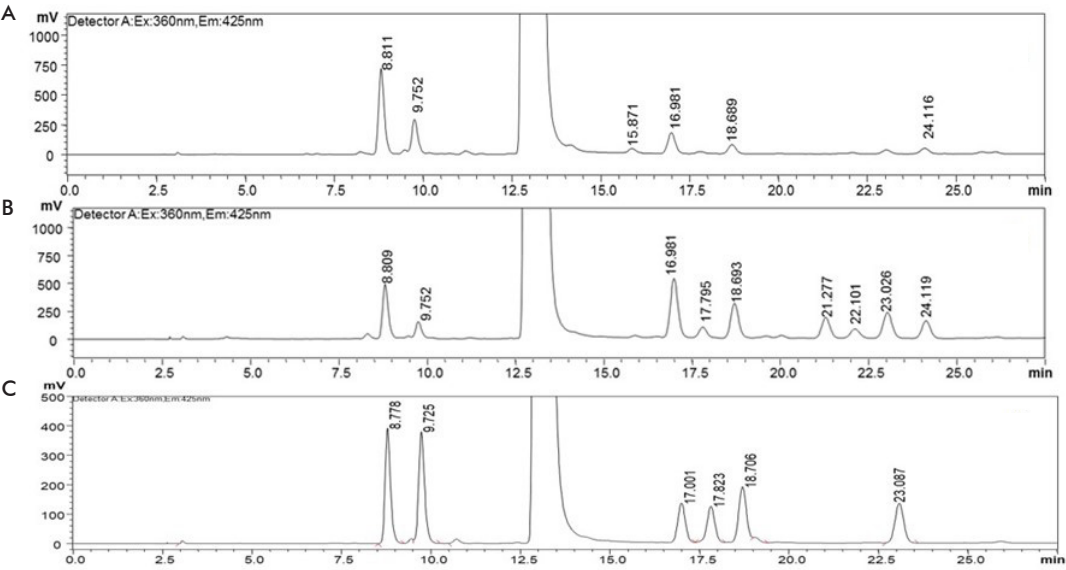


Figure 1. Identification of neutral monosaccharides for *Bryothamnion triquetrum* with different hydrolysis conditions. A) Profiles obtained for the fraction with 6 M HCl. B) Profiles with 2 M TFA. C) The ludger curve profile. Retention times were established using monosaccharide standards. The curve contains from left to right: N-acetylglucosamine (rt: 8.778 min); N-acetylgalactosamine (rt: 9.725 min); galactose (rt: 17.001 min); mannose (rt: 17.823 min); glucose (rt: 18.706 min); arabinose (rt: 20.030 min); xylose (rt: 21.280 min); fucose (rt: 23.087 min). The sampled were HPLC-fractionated with a LudgerSep R1 column. The excess of the 2-aminobenzoic acid reagent eluted after 12.5 min.

mannose (17.799 min), glucose (18.706), arabinose (20.030 min), xylose (21.280 min), and fucose (23.039). There were also two unidentified peaks at 15.852 and 22.091 min, probably of modified monosaccharides. Particularly, the unidentified peak at 22 min was common to both seaweeds, suggesting that it could be the same modified monosaccharide, since modified monosaccharides are abundant in marine algae cell walls [43, 44].

Other authors pointed out to these monosaccharides as the main components of marine algae [45]. In their case, rhamnose eluted at 23.010 min, and fucose at 23.026 min, but that time difference is not enough to definitely identify the type of monosaccharide. Another study reported the presence of rhamnose in green seaweed, whereas fucose, a characteristic monosaccharide of brown seaweed, was not found [46].

Quantification of monosaccharides in the polysaccharide-rich fraction

Monosaccharides were quantified and expressed as percentage (Table 2). Consistent with the relative size of the peaks seen in the identification profiles, galactose was

the most abundant monosaccharide in both seaweeds, roughly 30 %. It is known that green algae contain highly branched complex molecules of galactans, mannans, and xylans, composed of galactose, mannose and xylose units [47]. In some green seaweeds there has been identified the glucuronoxylorhamnan, composed of glucose and rhamnose units [7]. This contrasts with red algae, in which galactans composed by galactose provide the major fractions, the main monosaccharide found in agar and carrageenan [7, 24, 48-50]. Bastos Presa et al. [47] found galactose and glucose as the most abundant monosaccharides in the green seaweed *U. flabellum*. The presence of galactose in these two seaweeds may be attributed to the presence of sulfated galactans in the amorphous matrix, characteristic of green and red seaweeds [51, 52].

On the other hand, *H. opuntia* and *B. triquetrum* had similar amounts of glucose and fucose/rhamnose (Table 2).

The least abundant monosaccharide in *B. triquetrum* was arabinose, and N-acetylgalactosamine in *H. opuntia*. The most notable difference between the two seaweeds was the amount of mannose, which

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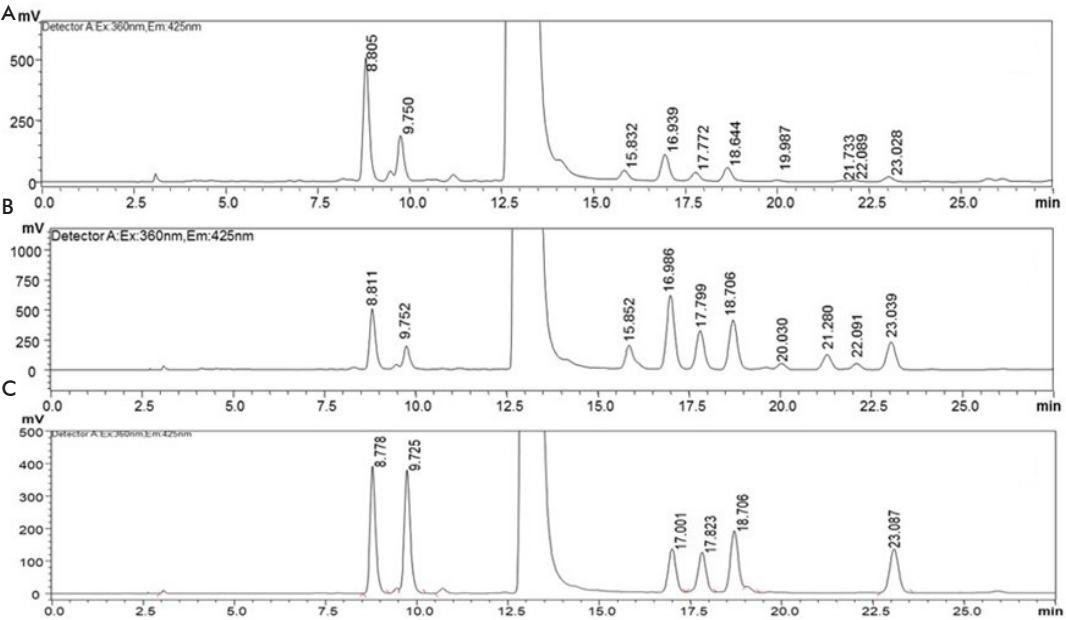


Figure 2. Identification of neutral monosaccharides for *Halimeda opuntia* with different hydrolysis conditions. A) Profiles obtained with 6 M HCl. B) Profiles with 2 M TFA. C) The ladder curve profile. The curves contain, from left to right: N-acetylglucosamine (rt: 8.778 min), N-acetylgalactosamine (rt: 9.725 min), galactose (rt: 17.001 min), mannose (rt: 17.823 min), glucose (rt: 18.706 min), arabinose (rt: 20.030 min), xylose (rt: 21.280 min), fucose (rt: 23.087 min). Retention times were established using monosaccharide standards. For HPLC separation, the LudgerSep R1 column was used. The excess reagent corresponding to 2-aminobenzoic acid elutes after 12.5 min.

was approximately three times higher in *H. opuntia* than in *B. triquetrum*. This coincides with reports by Fernández et al. [53], who found significant amounts of mannose in the *C. vermilara* green seaweed. *B. triquetrum* also had a higher amount of xylose than that of *H. opuntia*.

Noteworthy, the presence of N-acetyl glucosamine and N-acetyl galactosamine, monosaccharides which are minor constituents in polysaccharides, is mainly related to the glycoprotein domains of the cell wall [54]. The contribution of these glycoproteins or glycopeptides is more representative in red seaweeds such as *B. triquetrum* [55]. Despite, glycosaminoglycans are linear and heterogeneous sulfated glycans, that can be found not only in green but also in red algae [24]. It is relevant to point out that polysaccharides are not always present in their simple form, but are often conjugated with other components such as amino-acids, proteins, lipids, polyphenols, and nucleic acids. These conjugates often influence biological activities [55-57].

The large amounts of monosaccharides identified in the two seaweeds allow us to infer the heterosaccharidic character of the polysaccharides composing them. Unlike animal cells, polysaccharides in seaweeds are not usually homopolysaccharides, and form associations with other monosaccharidic units [58, 59]. This coincides with reports in the scientific literature, since there is a basic skeleton for each type of polysaccharide, and substitutions with neutral monosaccharides are common [60-63]. Previous analyses have found that the chemical compositions and structures of green seaweeds polysaccharides are very complex and vary among species. For instance, the polysaccharides from *H. tuna* are mainly a

Table 2. Monosaccharide composition of the polysaccharide-rich fractions of *Bryothamnion triquetrum* and *Halimeda opuntia* with hydrolysis with TFA*

Monosaccharide	<i>B. triquetrum</i> (%)	<i>H. opuntia</i> (%)
N-acetyl-glucosamine	14.4	9.5
N-acetyl-galactosamine	5.9	3.3
Galactose	30.1	30.6
Mannose	5.9	16.9
Glucose	11.7	13.8
Fucose/Rhamnose	13.3	11.3
Xylose	16.1	9.5
Arabinose	2.6	5.1

*Percentage was calculated against the total amount of identified monosaccharides (nM).

hetero-polysaccharide with monosaccharidic units, galactose, glucuronic acid, glucose, arabinose, xylose, fructose, and ribose [11]. Polysaccharides isolated from *Chaetomorpha linum*, a green algae, also revealed heterogeneous compositions, including arabinose, mannose, and glucuronic acid [12]. This variability in polysaccharide composition may be attributed to exogenous factors (temperature and protocol of extraction) and the endogenous changes in the organisms themselves (species, growth phase, morphological changes, and reproduction cycle, among others) [11].

Determination of sulfated monosaccharides

Sulfated sugars were determined by RP-HPLC chromatography (Figure 3). For this, the sample profile obtained under standard conditions was compared to that obtained with the sample subjected to desulfation by hypermethylation, resulting in changes in retention times and the area under the curve (AUC) for the modified (desulfated) monosaccharide, for both

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seaweeds. In fact, the analysis of changes in AUC is based on the modification of the chemical properties of monosaccharides. Sulfation modifies the charge of the monosaccharides, thereby changing its interaction with the chromatographic column, which ultimately leads to a shift in their retention times [49].

In *B. triquetrum*, under desulfation conditions, the peak obtained in the untreated sample at 22.101 min disappears, indicating that that monosaccharide was altered or removed by desulfation. Since this peak did not match any monosaccharide standard peak, it could be a sulfated monosaccharide. Additionally, the peak at 16.981 min, corresponding to galactose, shows both, increased signal intensity and AUC, suggesting it may have undergone sulfation modifications.

In the desulfated sample of *H. opuntia*, the signals at 15.852 and 22.091 min disappear, and a new peak was detected at 23.347 min. These peaks, atypical for monosaccharides, may correspond to modified monosaccharides, possibly in sulfated form. Additionally, the peaks of galactose at 16.986 and mannose at 17.799 min, show a slight increase in AUC, suggesting they might be sulfated monosaccharides. This analysis highlights the chromatographic changes due to the desulfation process, and is related to the finding of sulfate groups among the most common substituents of seaweed polysaccharides

and their involvement in biological activities [59]. In this sense, the differences shown in sulfation patterns between the two seaweeds indicated a higher number of modifications in the chromatographic profile under desulfation conditions for *H. opuntia*.

Analysis of the green alga aligns with previous reports indicating significant amounts of pyruvylated 3-linked β -D-galactose units, partially sulfated on C-2, 3-linked galactose 6-sulfate, or 3,6-linked galactose, or a mixture of these units. Such changes were detected in methylation analysis and NMR spectra in *H. opuntia* aqueous extracts at room temperature [23].

RP-HPLC desulfation results in *B. triquetrum*, supports the subsequent identification of sulfated monosaccharides. It coincides with the low sulfate content and NMR spectroscopy observations, which indicate that galactosyl residues in agar are either unsubstituted or substituted with methyl, carboxyethylidene, or sulfate groups [22]. Furthermore, it has been found that red seaweed shows a high content of sulfated sugars in polysaccharides, with 25.7 % in *B. triquetrum* and 20.4 % in *H. cornuta* [64]. In fact, carrageenans were found as the main source of sulfated polysaccharides in the red seaweed *C. crispus* [65], and the porphyran-type sulfated galactan is the main component in *Porphyra* species [66].

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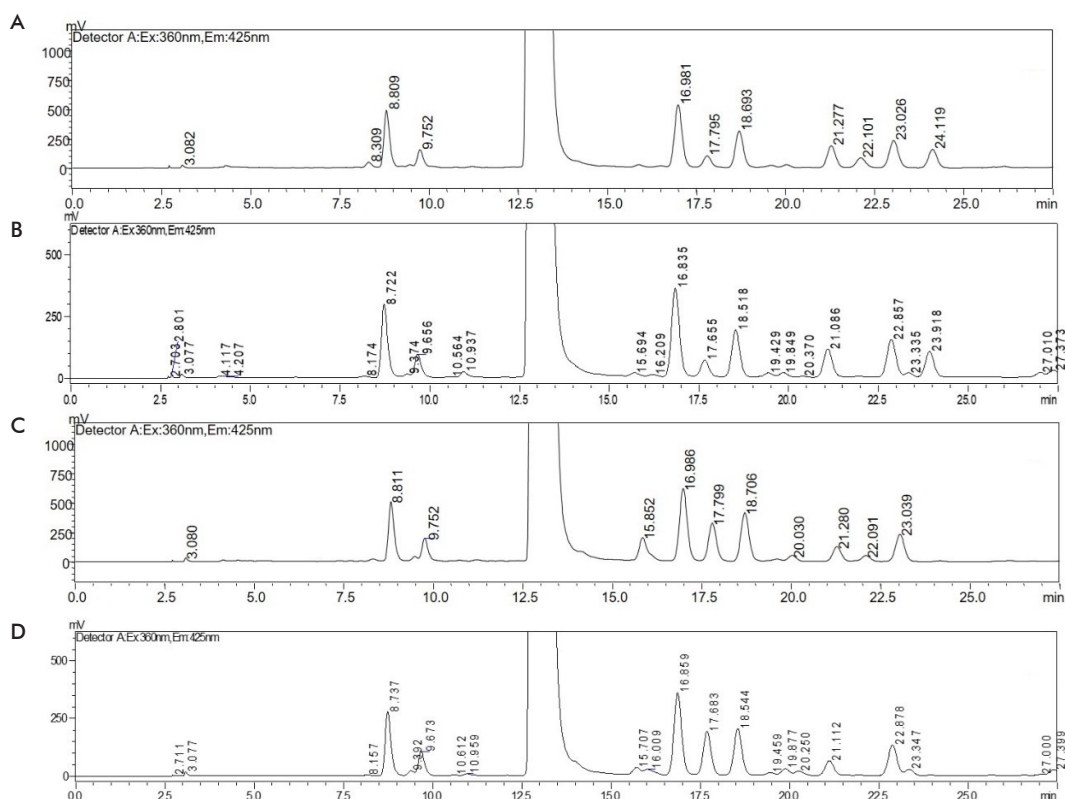


Figure 3. RP-HPLC chromatogram corresponding to hydrolysis with TFA under sulfation and desulfation conditions. The profiles obtained for the fraction with 2M TFA under sulfation conditions for *B. triquetrum* (A) and *H. opuntia* (C), and under desulfation conditions for *B. triquetrum* (B), and *H. opuntia* (D). The curve contains from left to right: N-acetylglucosamine (rt: 8.778 min), N-acetylgalactosamine (rt: 9.725 min), galactose (rt: 17.001 min), mannose (rt: 17.823 min), glucose (rt: 18.706 min), arabinose (rt: 20.030 min), xylose (rt: 21.280 min), fucose (rt: 23.087 min). Retention times were established using monosaccharide standards. For HPLC separation, the LudgerSep R1 column was used. The excess reagent corresponding to 2-aminobenzoic acid elutes after 12.5 min.

Regarding rhamnose, which has been established as a taxonomic marker in the green seaweed [65], there are two related modified sugars. Moreover, it was unable to accurately define among mannose, xylose, and arabinose in *H. opuntia*, due to the wide variety of sugars and high rate of sulfation in the polysaccharides present in green seaweed [47]. These results are in agreement with those by Lo *et al.* [67], in which five sulfated rhamnans were isolated from the green seaweed *M. latissimum* with anticoagulant activity. In this line, sulfated polysaccharides with diverse structure and monomeric composition have been found in chlorophytes, with some species of the *Codium* genus found to produce sulfated arabinans and arabinose polymers (including sulfated arabinogalactans), and sulfated rhamnans detected in *Monostroma* spp. [53].

Antioxidant activity of polysaccharides

The antioxidant activities of polysaccharides have become relevant, due to their role in counteracting the action of reactive oxygen species [7, 68], while the underlying chemical mechanisms remain unclear. There has been proposed that higher sulfation degrees in fucoidans may contribute to such an activity [69]. Additionally, polysaccharides-bound polyphenols may play a role. Therefore, there was studied the influence of total content of sugar and polyphenols, and the sulfation patterns of polysaccharides on the antioxidant activity of both algae.

Antioxidant activity by the phospho-molybdenum method

Similar antioxidant activities of 65.610 and 62.966 mg equivalents of ascorbic acid/g of dry extract were determined for the aqueous extracts of *B. triquetrum* and *H. opuntia*, respectively, by the phosphomolybdenum method. This contrasts with the higher values found in the protein rich fractions of *H. opuntia* (8.78 mg of equivalents of ascorbic acid/g of dry extract), as compared to the same fraction of *B. triquetrum* (5.27 mg of equivalents of ascorbic acid/g of dry extract). This points towards a greater reducing capacity in *B. triquetrum*, due to the highest polysaccharides and polyphenols in the aqueous extract, and, thereby, a greater antioxidant potential derived from the synergistic effect of both types of compounds. Since similar results were found in both aqueous extracts, differences

in the antioxidant activity could be attributed to the polysaccharide composition. In fact, there has been documented the significant impact of sulfate content in polysaccharides on their radical scavenging capacity [11, 13]. Additionally, the antioxidant properties of sulfated polysaccharides are attributed to their strong hydrogen donor ability [11, 14]. It is likely that the more promising antioxidant activity seen in the polysaccharides of the red alga derives from its magnitude rather than its sulfate pattern.

Antioxidant activity through reducing power

The antioxidant activity was determined by the reducing power method, as described by Oyaizu [31], which evaluates the ability to reduce Fe^{3+} species to Fe^{2+} [70] (Figure 4). Aqueous extracts of the green seaweed showed lower antioxidant activity than the red alga, in agreement with higher amounts of total polyphenols than the green one: 51.21 ± 2.25 vs 19.99 ± 1.12 μg galic acid equivalents/mg of lyophilized extract, respectively [16]. Nevertheless, other comparative evaluations of antioxidant activity between green and red algae, such as in He *et al.* [71], have reported higher antioxidant activity in green seaweed (*U. lactuca*) instead of the red seaweed (*S. ceylonensis* and *G. lemaneiformis*).

The increase in total sugars in *B. triquetrum* and *H. opuntia*, expressed as galactose, caused a rise in the absorbance and the resulting reducing power. Such a response was dose-dependent in terms of the capacity of the polysaccharides in the algal extract to reduce the species from Fe^{3+} to Fe^{2+} . The same have been described by Yang *et al.* [72] in the red seaweed *C. officinalis*.

The positive control (ascorbic acid, 1 mg/mL) showed an absorbance of 2.824, approximately 70-times higher than the aqueous extract of *B. triquetrum* and 225-times higher than that for *H. opuntia*. At the same time, the antioxidant activities of the polysaccharide-rich fractions were lower than that of the aqueous extracts, somewhat expected when comparing a purified molecule to crude extracts. The reduced antioxidant capacity of polysaccharides generally derives from the presence of bioactive compounds such as uronic acids, sulfates, and monosaccharides [13].

Previous reports by Wang *et al.* [73] described three extracted components from *Laminaria japonica*

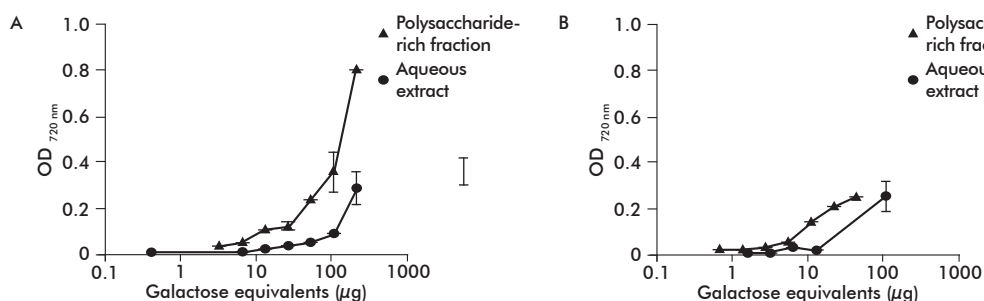


Figure 4. Antioxidant activity of *Bryothamnion triquetrum* (A) and *Halimeda opuntia* (B). The activity was determined as the reducing power, by the methodology described by Batista-González *et al.* (J Food Biochem. 2012;36:189-97). The antioxidant activity was expressed as μg of galactose equivalents as assessed in the aqueous extracts and the polysaccharide-rich fractions of both seaweeds. Data are represented as mean $\pm$ SD ($n = 3$).

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ca, with 23.3, 36.4, and 36.7 % of sulfate contents, and a proportionally inverse ability to scavenge superoxide anions, hydroxyl radicals, and their chelating and reducing powers, indicating that sulfate content is positively related to the bioactivities of the polysaccharides [73]. Despite, such positive relationship between antioxidant activity and sulfated pattern was absent in our samples of *B. triquetrum* and *H. opuntia*. This may suggest that the differing behavior between the two algae is defined by the total sugar content.

DPPH• radical scavenging activity

Inactivation of the DPPH• radical is among the most common techniques for the evaluation antioxidant properties in plant extracts [74]. This free radical is susceptible to react with antioxidants through the transfer of a hydrogen atom provided by the antioxidant. The aqueous extracts of the two seaweeds showed a dose-dependent response by this method. When comparing the IC_{50} values obtained for both algae, 0.625 ± 0.20 for *B. triquetrum* and 9.595 ± 0.36 for *H. opuntia*, the last was shown nearly ten-fold more potent, in agreement with reducing capacity findings. Díaz et al. [16] also demonstrated for both seaweeds that *B. triquetrum* has a greater free radical scavenging, supported by its higher content of polyphenolic compounds.

The aqueous extract of *B. triquetrum* caused the discoloration of the mixture, reaching the maximum DPPH• radical scavenging activity at 20 μ g of galactose equivalents, something not found in its polysaccharide-rich fraction (Figure 5A). This last rendered a five-fold IC_{50} values of 125 μ g galactose equivalents. Similar results were reported by Ma et al. when working with red seaweed [13]. As shown, all fractions exhibited a direct dependent relationship of the concentration vs. the detected free radical scavenging activity.

The IC_{50} values very close of both aqueous extract and protein rich fractions displayed IC_{50} values very close IC_{50} values (IC_{50} 9.595 ± 0.3 μ g galactose equivalents) (Figure 5 B), indicating similar antioxidant properties, although the polysaccharide fraction achieved total decolorization of the compound. This suggests that polysaccharides contribute significantly to the sequestering capacity of DPPH•.

These evidences point towards a direct link between polysaccharide content and the resulting reducing capacity and free radical scavenging activity.

Furthermore, greater potency was found for the aqueous extract fraction, when comparing equal amounts of galactose from both fractions of *B. triquetrum*. This is likely due to the presence of polyphenols, which significantly enhance antioxidant activity and may be absent or present in lower amounts in the green alga.

The same relative enhanced reducing potency was found in the *H. opuntia* aqueous extract, making evident that polyphenols enhance its antioxidant activity. On the contrary, the contribution of the polysaccharides was similar between both fractions, as determined by the DPPH• trapping method. Hence, sulfated polysaccharides may play a significant role in the antioxidant properties of *H. opuntia*. Comparatively, *B. triquetrum* shows a higher content of total polyphenols and polysaccharides than *H. opuntia*, so it is logical to assume its higher antioxidant activity.

It could then be argued that the polyphenols of *B. triquetrum*, together with the polysaccharides, are mainly responsible for the reducing power and the entrapment of the radical DPPH•. In the case of the green seaweed *H. opuntia*, it can be argued that the polysaccharides, together with polyphenols, contribute to the reducing power of the aqueous extract of this seaweed. However, for the trapping activity of DPPH•, the polysaccharides had as significant contribution as the polyphenols. In this sense, Flores et al. [75] pointed out that the trapping activity of DPPH• can be directly related to the sulfate content of the polysaccharides, consistent with the higher degree of sulfation we have found in *H. opuntia* than in *B. triquetrum*. Furthermore, Yang et al. [72] found less DPPH• trapping activity in desulfated polysaccharides in the red seaweed *C. officinalis*, demonstrating relevance of sulfate groups in antioxidant activity.

Conclusions

In summary, the composition and heterogeneity of monosaccharides in the polysaccharides of *B. triquetrum* and *H. opuntia* were effectively established by the P-HPLC methodology applied. It was possible to identify sulfate modifications on the monosaccharides, despite their specific polysaccharide structures remain to be described. As far as we know, this is the first study identifying and quantifying neutral monosaccharides, monosaccharides, hexosamines, and sulfate

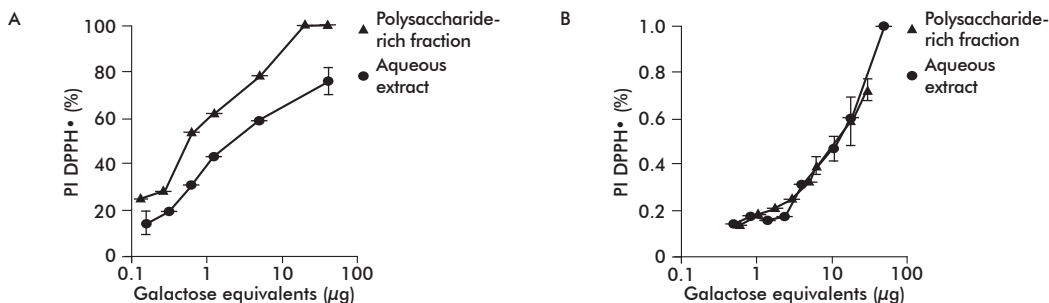


Figure 5. Antioxidant activity of *Bryothamnion triquetrum* (A) and *Halimeda opuntia* (B). The activity was determined as the reducing power, by the methodology described by Díaz Gutiérrez et al. (Ars Pharm;56(2):89-99). The antioxidant activity was expressed as μ g of galactose equivalents as assessed in the aqueous extracts and the polysaccharide-rich fractions of both seaweeds. Data are represented as mean $\pm$ SD ($n = 3$). PI DPPH• (%): percentage of entrapment of DPPH• radicals.

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groups by RP-HPLC. Both, *B. triquetrum* and *H. opuntia* seaweeds have galactose as predominant monosaccharide, possibly indicating the presence of sulfated galactans. Although their compositions were similar, their sulfation patterns differed, with *B. triquetrum* containing one sulfated monosaccharide, likely galactose, whereas *H. opuntia* exhibited a more complex sulfation pattern, with galactose potentially having one, to three sulfation substitutions.

Negatively charged ester-linked sulfate groups may contribute to the antioxidant activity. However, the sulfur in this case is on its maximum oxidative state, thus the reducing power cannot be attributed to the sulfate groups. This shows that their contribution to the oxidation/reduction system is partial in both seaweeds. Despite, the sulfate groups have the ability to trap free radicals, so the difference between the amounts of sulfated monosaccharides may influence antioxidant properties. For that, it is possible to understand the significant contribution on the potential antioxidant properties of *H. opuntia*. So far, this is the first comparative analysis of the polysaccharides content vs the antioxidant activity of this two algae, which may derived from the differences in their respective underlying antioxidant mechanisms.

Overall, here was demonstrated that polysaccharide-rich fractions influence the antioxidant properties of both examined seaweeds, higher impact for polyphenols in *B. triquetrum*. Instead, polysaccharides play a more critical role in the antioxidant properties of *H. opuntia*, despite their lower abundance as compared to the red alga. This variation is likely due to the higher sulfate content in *H. opuntia*, indicating a positive correlation between sulfate content and the bioactivities of polysaccharides. Consequently, owing to their outstanding biocompatibility and biodegradability, sulfate polysaccharides from *B. triquetrum* and *H. opuntia* can be considered promising candidates for pharmaceutical and biotechnological applications, remaining to be characterized in future attempts.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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