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Screening of the UV absorption capacity, proximal and chemical characterization of extracts, and polysaccharide fractions of the *Gracilariopsis tenuifrons* cultivated in Colombia

Yessica Monsalve-Bustamante[1], Stefanie Rincón-Valencia[1], Juan Mejía-Giraldo[1,2], Diego Moreno-Tirado[3], Miguel Puertas-Mejía[1*]

[1]Grupo de Investigación en Compuestos Funcionales, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Medellín, Colombia.
[2]Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Medellín, Colombia
[3]Fundación Terrazul, Centro de Desarrollo Tecnológico, Riohacha, Colombia.

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ABSTRACT

The sustainable use of marine resources is a competitive advantage of the most developed countries, which has a positive impact on the economic and social development of their populations. Currently, marine organisms such as macroalgae are an important source of functional compounds such as polysaccharides, proteins, unsaturated and polyunsaturated fatty acids, among others, with nutritional value and pharmacological properties. *Gracilariopsis tenuifrons* is a macroalga found in the Colombian Caribbean Sea, which has been neither chemically nor physicochemically studied; therefore, the aim of this study was to evaluate the polysaccharides composition and nutrients contents of crude extract and its UV radiation absorption capacity. The purification was carried out by ultrafiltration using membranes of molecular size exclusion 100, 50, 10, and 3 kDa. The chemical characterization was done by gel electrophoresis, Nuclear Magnetic Resonance, Fourier Transform Infrared Spectroscopy, and the particle size and potential zeta by Dynamic Light Scattering. The absorption coefficient (absorbance/g dry sample) was measured at 290, 310, 340, and 380 nm. Sulfated and non-sulfated polysaccharides were detected in the fractions and identified as polysaccharides type k, and β-carrageenan and alginate. The proximate analysis showed that the total content of protein, carbohydrates, fat, and calories is 15.58%, 69.81%, 0.15%, and 342.94% Kcal, respectively. The crude extracts showed an important absorption coefficient in UVB-UVA range. The findings suggest that *G. tenuifrons* seaweed propagated *in vitro* is a viable candidate of natural additives, such as phycocolloids and bioactive compounds, for designing new functional products in the pharmaceutical and cosmetic industry, in addition to its nutritional properties to be used in foods.

INTRODUCTION

In recent years, the demand for algae and their by-products are in constant growth, whether to produce different agro-industrial products as well as for human consumption. According to FAO reports, the world production of algae in 2012 reached 23.8 million tons and each year, it is estimated that about 25 million tons of seaweed for use in food, cosmetics, and fertilizers (Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2014).

Algae are rich in carbohydrates, proteins, minerals, fatty acids (e.g., eicosapentaenoic acid, docosahexaenoic acid, and γ-linoleic acid), as well as in polyphenols, terpenoids, carotenoids, and tocopherols (Michalak and Chojnacka, 2015). However, the chemical composition of the marine algae depends on the species, place of cultivation, atmospheric conditions, and harvest period. Moreover, nutritional facts of these marine organisms such as the content of dietary fiber (33%–50% dry weight), protein (brown 5%–24%, red and green 10%–47%), minerals (8%–40%), and low-lipid content (1%–2%) are interesting (Rupérez and Saura-Calixto, 2001). The polysaccharides are one of the most interesting

*Corresponding Author
Miguel Puertas-Mejia, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Medellín, Colombia.
E-mail: miguel.puertas @ udea.edu.co

components of the algae because of their biological activities (de Almeida *et al*., 2011; Kraan, 2012; Lee *et al*., 2010; Senni *et al*., 2011). Additionally, the marine algae are recognized as a rich source of sulfated polysaccharides. Most of them are easily transformed into nanoparticles, nanofibers, microparticles, scaffolds, membranes, gels, beads, and sponges (Jayakumar *et al*., 2010).

Colombia has two large coasts, with 565 species reported for the Caribbean coast and about 134 species on the Pacific coast (Delgadillo-Garzón and Newmark, 2008). However, Colombia does not have large commercial algae cultivation meadows. The exploitation of these products is deficient because of the scarce knowledge about the potential of seaweeds as a source of bioactive substances of medical, cosmetic, and food interest. Though there are several studies on marine algae, these are focused on the taxonomic nature, ecology, culture methods, and a minor extent on the chemical composition (Camacho and Montaña, 2012; Delgadillo-Garzón and Newmark, 2008). Like all organisms regularly exposed to sunlight, macroalgae from marine ecosystems are exposed to high UVR levels; therefore, they must produce a chemical adaptive response. In addition, some beneficial effects of seaweed are related to anti-carcinogenic effect, which is attributable to the presence of polysaccharides, such as carrageenan, alginate, etc (Newman and Cragg, 2007). Therefore, since *Gracilariopsis tenuifrons* is an abundant species in the Colombian Caribbean Sea, with no systematic reports on its chemical composition and/or UV absorbers, the objective of this research was to determine the UV absorption capacity, the content, and type of polysaccharides and propose it as a potential raw material for using in the food, pharmaceutical industry, and cosmetic industry.

MATERIALS AND METHODS

Biological material

Gracilariopsis tenuifrons was supplied by the Fundación Terrazul of the city of Riohacha, Guajira-Colombia, in June 2017 (Framework Contract for Access to Genetic Resources and their Derived Products N° 126 of 2016, register number RGE0156-5). The biological material was washed with seawater and then with distilled water, dried at 45°C, and protected from light. Next, the dried seaweed (DS) was coarsely powdered.

Extraction process of the crude extract

Briefly, 0.8 g of biological material was subjected to solvent extraction, using ethanol:methanol, ethanol:water (ratio 75:25 and 50:50), and methanol:water (ratio 75:25 and 50:50) solvent systems. All of them were mechanically stirred for 24 hours at room temperature. Afterward, the crude extracts were concentrated to dryness in a rotary evaporator (IKA, RV10 basic) at 40°C ± 2°C and the extraction yield was determined. All assays were done by triplicate as recommended by Jaros *et al*. (2018) and Li *et al*. (2019).

UVA-UVB absorption coefficient of the crude extract

Briefly, an aliquot of the extracts was transferred into a quartz cuvette and its absorption spectra (wavelengths 250–400 nm) were acquired using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer against a blank containing the respective solvent. The absorption coefficient (AC) was calculated at 290, 310, 340, and 380 nm according to (Mejía-Giraldo *et al*., 2016) and was given by

$$AC = \frac{\text{Absorption at each } \lambda}{\text{Weight of biological material}}$$

Isolation and purification of polysaccharides

25.0 g of the DS were submitted to reflux with 500 ml of ethanol (96%) at 65°C for 6 hours, to eliminate interferences, such as lipids, pigments, polyphenols, and other low-weight molecular substances. The resulting solution was filtered, and the solid material was dried at 40°C. The depigmented material was dissolved in 500 ml of deionized water and subjected to reflux extraction at 90°C for 10 hours. The crude polysaccharide extract (PSc) was stored at 4°C for further analysis. Afterward, the PSc was deproteinized by the Sevag reagent (chloroform-butanol 1:4) in 1:5 ratio (PSc:Sevag) for 20 minutes with mechanical stirring (this procedure was repeated five times). The residues were separated by centrifugation at 4,500 rpm for 10 minutes, and the supernatants were pooled and brought to 50 ml, obtaining the deproteinized polysaccharide (PSd). Then, the PSd was subjected to centrifugation at 4,500 rpm for 20 minutes (25°C) using separation membranes with a nominal limit of molecular weight of 100, 50, 10, and 3 kDa. The fractions were precipitated with ethanol (96%) at 1:3 v/v ratio and then lyophilized. Finally, the fractions were stored at 4°C (Jaros *et al*., 2018; Li *et al*., 2019; Pikulski and Brodbelt, 2003).

Gel electrophoresis

Purified polysaccharides were analyzed on polyacrylamide gel electrophoresis. The volume of sample to be applied on the gels was visually checked to observe the metachromacy (change from blue to pink) by adding small aliquots of samples and standards to the Dimethylmethylene blue (DMB) reagent (1,9 dimethylmethylene blue, 1 mg/ml). The molecular mass distribution was determined according to the electrophoretic mobility of the patterns. The samples and standards were prepared in a solution of cresol red in glycerol. The resulting solution was introduced to 7.5% polyacrylamide gel with 1.0 mm thickness in 0.02 M Tris-HCl (pH 8.6) and run for 30 minutes at 100 V. Thereafter, the gel was stained with 0.1% toluidine blue in 0.1% acetic acid and then washed with acetic acid (1.0 %) for 30 minutes (Turnbull and Gallagher, 1988). Dextran sulfate (> 500 kDa), chondroitin 6-sulfate (60 KDa), chondroitin 4-sulfate (40 KDa), heparin (15 kDa), and enoxaparin (4.5 kDa) were used as standards.

Proximate composition

The proximal analysis was done by evaluating the content of the moisture, organic matter and ash, according to AOAC methods (1990). The total protein content was determined by the Kjeldahl method adapted from where protein content is estimated by multiplying the nitrogen content by 6.25 (Mariotti *et al*., 2008), whereas the total fat content was determined by Soxhlet extraction. The total carbohydrate content was determined

by calculation, i.e., by subtracting protein content and fat content from total organic content.

Analysis of sugar content

Neutral sugars (phenol-sulfuric acid method): 15 µl of each fraction were added to 400 µl of deionized water. The standards and the samples were mixed with 2 ml of sulfuric acid and the mechanical stirring was done at 4,000 rpm for 15 seconds. Subsequently, 400 µl of phenol (5%) was added and the resulting solution was heated at 90°C for 5 minutes and cooled in a water bath (Li *et al*., 2007). The absorbance was read at 490 nm. The results were expressed based on the equation

$$\% \text{ Neutral Sugar} = \frac{A_{sample} - 0.0227}{0.0289} \times \frac{400}{V \times 10 \times W}$$

where
A_{sample}: Absorbance of the sample at 490 nm
V: Sample volume, µl
W: Mass of the sample to obtain 1 mg/ml

Acid sugars (carbazole-sulfuric acid method): 40 µl of each fraction were added to 400 µl of deionized water. The samples and the standards were mixed with 2 ml of sodium tetraborate (0.95 g/l sulfuric acid) and heated at 100°C for 12 minutes. Subsequently, 40 µl of carbazole (0.2% w/v, in ethanol) were added and heated at 100°C for 10 minutes. The absorbance was measured at 525 nm (Li *et al*., 2007). The results were expressed in terms of hexuronic acid, based on the following equation

$$\% \text{ Hexuronic acids} = \frac{A_{sample} + 0.0058}{0.0068} \times \frac{1}{W}$$

where
A_{sample}: Absorbance of the sample at 525 nm
W: Mass of the sample to obtain 1 mg/ml

Sulfated sugars: 50 µl (1 mg/ml) of the fraction were mixed with 500 µL of deionized water. The samples and the standard of chondroitin sulfate were mixed with 4 ml of the DMB solution (containing 11 mg of 1,9-DMB and 1 L of sodium acetate 0.05 M, pH 4.75). The mixture was mechanically stirred at 4,000 rpm for 15 seconds and left in darkness for 30 minutes. Afterward, the absorbance was read at 520 nm (Anand *et al*., 2018; Lin *et al*., 2018). The quantification of sulfated sugars was carried out by the equation;

$$\% \text{ Sulfated sugars} = \frac{A_{sample} + 0.0136}{0.0035} \times \frac{1}{W}$$

where
A_{sample}: Absorbance of the sample at 520 nm
W: Mass of the sample to obtain 1 mg/ml

FT-IR and NMR analysis

The Fourier Transform Infrared Spectroscopy (FT-IR) spectroscopy experiments were performed in a Bruker Tensor II FT-IR with a Diamond ATR (Bruker Optics, Germany). The spectrometer was equipped with a liquid N_2-cooled photovoltaic MCT detector. The samples were analyzed at a concentration of 20 mg/ml for 64 scans with a resolution of 4 cm^{-1} against the background subtraction. The spectra were collected at room temperature. The FT-IR spectra were baseline corrected and analyzed in the selected region using OPUS Software (Bruker Optics, Germany). The Nuclear Magnetic Resonance (NMR) analysis was performed in a Bruker Ascend III HD 600 MHz spectrometer with 5 mm TXI probe, equipment with suppression by HOD presaturation. The signals were expressed in ppm (δ) using deuterated acetone as an internal standard (2.23 ppm in ^{1}H).

Particle size, zeta potential, and viscosity

The particle size was determined by Dynamic Light Scattering using a HORIBA LB-550 equipment and the zeta potential in a Zetasizer nano Z particle analyzer. The viscosity tests were performed in a Brookfield viscometer (LVDV-I +) and a needle LV-1 at 100 rpm, using carboxymethylcellulose as a comparison standard.

Statistical analysis

The results were expressed as the means ± SD. All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey tests when appropriate, using R Development Core Team (2013). p values less than 0.05 ($p < 0.05$) were considered significant (R Development Core Team, 2015)

RESULTS

The yield for crude extract using a different solvent system ranged from 2.95 ± 0.28 to 4.52% ± 0.30%. According to these results, ethanol was selected as a solvent system, and the extraction of polysaccharides yielded 10.3% and 1.8% for the

Table 1. Yield extraction and sugar content in fractions of *G. tenuifrons*.

Fraction	Molecular weight distribution (kDa)	Yield (%)	Sugar content, %		
			Neutral	Acids	Sulfated
100 R	Ps* > 100	9.92 ± 0.55	6.41 ± 0.32	6.33 ± 0.56	31.56 ± 4.22
50 R	100 > Ps > 50	0.51 ± 0.22	-	-	-
10 R	50 > Ps > 10	2.25 ± 0.25	9.32 ± 0.85	53.56 ± 8.69	18.79 ± 2.23
3 R	10 > Ps > 3	10.85 ± 0.82	9.28 ± 1.87	5.83 ± 0.57	16.91 ± 2.38
3 E	3 > Ps	7.49 ± 0.49	7.52 ± 1.14	-	-
PSc	-	10.31 ± 0.55	-	-	-
PSd	-	1.81 ± 0.55	-	-	-

*Ps: polysaccharides. Results are expressed as the mean value ± standard deviation ($n = 3$).

PSc and PSd extracts, respectively. Subsequently, the yield of the fraction 3R was higher (10.8%) than other fractions (Table 1). The lower yield (<1% m/m) is obtained with the fraction 50R and was discarded. The higher content of sulfated sugar was exhibited in the fraction 100R, although, the fraction 10R showed the higher content of the neutral and acid sugars (Table 1).

The electrophoretic analysis was performed in polyacrylamide gel electrophoresis. This analysis allowed verifying effectively the electrophoretic mobility of the standards and its relationship with the molecular mass of electrically charged substances. Regarding the samples, the fraction 100R exhibited lower polydispersity than fraction 3E, with a molecular weight higher than chondroitin sulfate 6 (60 kDa) and lower than dextran sulfate (Mw> 500 kDa), whereas the fraction 3R showed a molecular weight distribution higher than enoxaparin (6 kDa) but lower than heparin (15 kDa) (Fig. 1).

The FTIR-ATR spectra of the fraction PSd (Fig. 2) showed an absorption band at 3,280 cm^{-1} corresponding to the O-H stretching band and a weaker signal at 2,930 cm^{-1} associated with the C-H alkyl stretching. Additionally, the absorption bands at 900, 970, 1,200, and 1,750 cm^{-1} were associated with the C-C and C-O bonds, typical absorption bands in pyranose ring. In addition, the double absorption band at 1,220 and 1,012 cm^{-1} corresponds to the sulfate ester groups S=O and the band at 1,150 cm^{-1} was associated with the C-O-C stretching of the glycosidic bond; the relatively strong band at 932 cm^{-1} is also present in carrageenan and agar and assigned to 3,6-anhydro-D-galactose. These results indicate the presence of sulfate groups in the fraction 100R. Also, characteristic absorption bands were observed at 1,640, 1,370, 1,250, 930, 900, 845, 805, and 705 cm^{-1}, present in polysaccharides type agar and carrageenan.

The H-NMR analysis of the fraction 100R (Fig. 3) showed a typical chemical shift of the non-anomeric protons between 3.4 and 4.0 ppm. The spectrum of the fraction 3E showed signals at 1.24 and 1.18 ppm of the methyl group. The signal at 8.37 ppm corresponds to the presence of the sulfate group, and the signals at 4.71 and 4.04 ppm are associated to the vicinal protons to the OH group, whereas the signal at 3.62 ppm corresponding with the vicinal proton at the sulfate group. On the other hand, the spectrum of fraction 3E showed signals at 1.24 and 1.18 ppm, which are associated with the methyl group; the signal at 8.37 ppm corresponds to the sulfate group, whereas the signals at 4.71 ppm and 4.04 ppm correspond to the OH group. The signal at 3.62 ppm is assigned to the proton next to the sulfate group. Finally, the spectra of the fractions 100R and 3R showed numerous signals in the range of 3.4–4.8 ppm, indicating a high degree of sulfation.

Concerning the proximate analysis, which showed a high content of carbohydrates, in addition to a significant quantity of protein, minerals, and a slight percentage of total fat (Table 2). Respecting the analysis of the size distribution, an average size of 349 ± 130 nm was obtained, with a zeta potential of −31 mV at pH 7.0 though the viscosity was 1.68 cP for the PSc extract.

Regarding the screening of the capacity of the UV absorption, the crude extracts showed an interesting broad-spectrum UVA/UVB. The relative absorption coefficient of the extracts was determined by comparison facts at 290, 310, 340, and 380 nm, typical wavelength for UVA and UVB solar spectrum (Fig. 4). All the six extracts using different solvent systems have shown broad-spectrum coverage but this was higher for the system ethanol:water (75:25 ratio) than ethanol itself ($p < 0.05$).

DISCUSSION

Colombia has a variety of aquatic environments such as the two large coasts, where a diversity of algae develops; however, the country does not have commercial strategy for industrial applications, partially to the lack of knowledge about the potential of these organisms as a source of bioactive substances (Márquez, 1996; Radulovich *et al*., 2013). However, to date, there are few studies on the chemical composition, especially about the

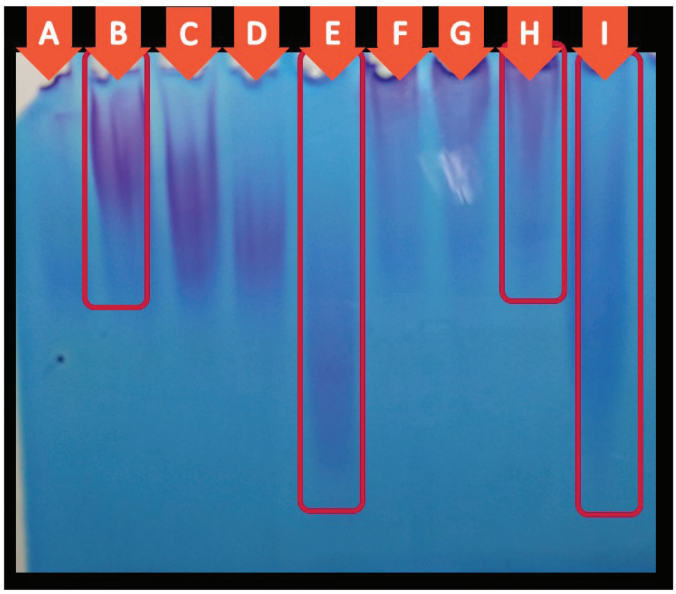

Figure 1. Polyacrylamide gel electrophoresis: a) Dextran sulfate> 500 kDa, b) Chondroitin 6-sulfate (60 kDa), c) Chondroitin 4-sulfate (40 kDa), d) Heparin (15 kDa), e) Enoxaparin (4.5 kDa), f) Fraction j, g) PSd, h) 100R, i) 3R.

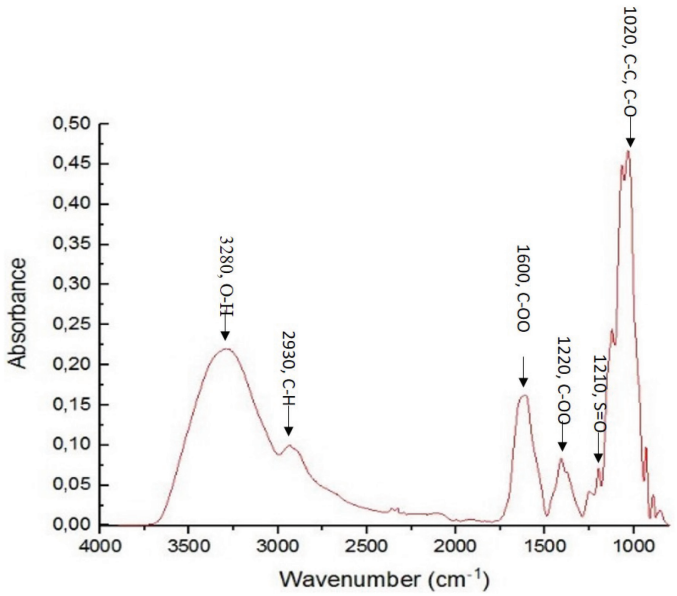

Figure 2. The FTIR-ATR spectrum of fraction PSc.

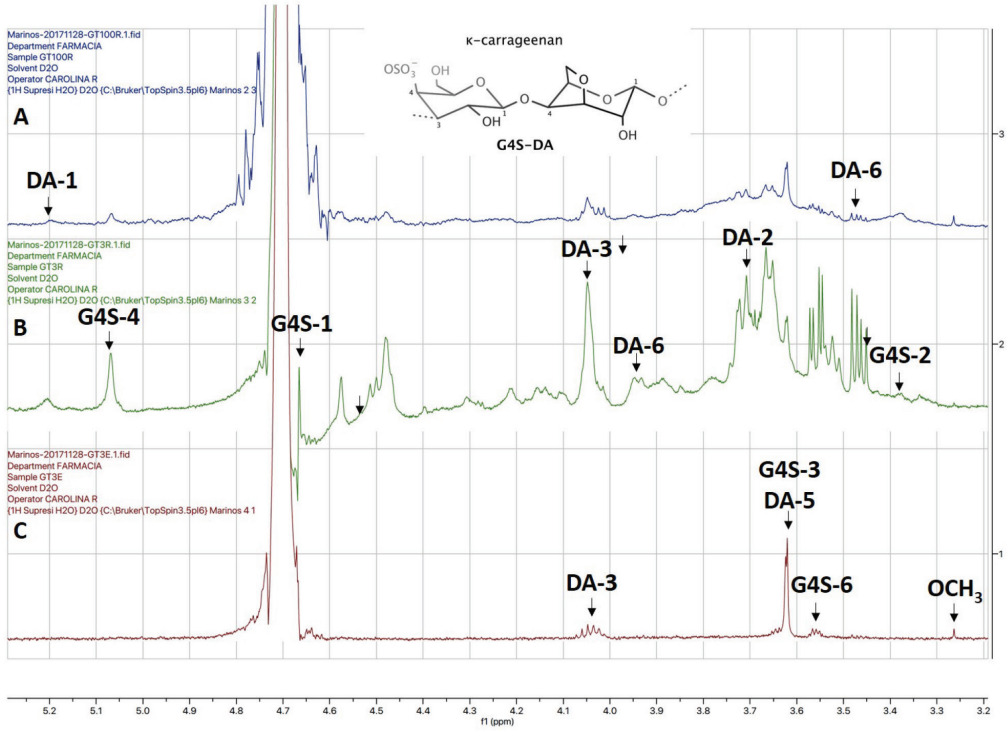

Figure 3. NMR 600 MHz spectra of *G. tenuifrons* fractions: A) 100R, B) 3R y, C) 3E.

Table 2. Proximate analysis of *G. tenuifrons*.

Parameters	*G. tenuifrons*	*G. gracilis*[3]	*G. turuturu*[3]
% Moisture*	8.70	7.99	11.68
% Total Fat*	0.15	0.60	2.2
% Total Protein*	15.58	20.2	22.5
% Ash*	5.75	24.8	20.52
% Total carbohydrate*	69.81	46.6	43.2
Calories**	342,94	-	-
% Fe	0.91	0.9	0.5

* g/100 g dried seaweed; **Kcal/100 g dried seaweed.

polymer distribution of *G. tenuifrons* (Zecchinel *et al*., 2000). The results showed that the proximate analysis of *G. tenuifrons* was comparable to those obtained by Rodrigues *et al*. (2015), who reported similar facts in *G. gracilis* and *G. turuturu*. These findings emphasize that *G. tenuifrons* could be a substrate with potential use as a supplementary food.

The spectroscopic analysis by ATR and NMR showed the evidence of highly sulfated polysaccharides (carrageenan type) and a lower degree of agar-type sulfate, which correlate with those reported by Rodrigues *et al*. (2015). Additionally, the NMR spectra of the fractions 100R and 3R showed a greater number of signals in the region of 3.4 and 4.8 ppm, confirming the degree of sulfation of the biopolymers obtained.

In general, the spectra for the three fractions were comparable in the chemical shift and the multiplicity of the protons (signals from 1.2 to 3.26 ppm) corresponding to the fractions 100R and 3E; whereas the spectra of 100R and 3R ranging from 3.47 to 8.37 ppm, were similar. The protons signal at 5–6 ppm was associated with α-anomeric protons and signals at 4–5 ppm to β-anomeric protons (Farhadi, 2017). As a result, β-D-GlcNAc and α-D-Glc residues were distinguished in fraction 100R (Pawan, 1992). Additional signals related to non-anomeric protons were detected at 3.4–4.0 ppm, which are in agreement to those reported in sulfated agarose (Liang *et al*., 2014), confirming data obtained through the infrared spectrum with respect to the presence of sulfate groups.

Regarding the particle size and the zeta potential, it demonstrates that polysaccharides could be used in the nanoencapsulation process because of their self-assembly capacity in aqueous medium forming nanoparticles, with negatively charged groups being more hydrophilic and produces a negative zeta potential on the surface. In addition, this isolated native polysaccharide fraction has not any chemical modification; therefore, it can be inferred that it has a high potential as an encapsulating agent. Furthermore, the viscosity was much lower (1.68 cP) compared to the standard (carboxymethylcellulose, 14.9 cP), however, it is noteworthy that carboxymethylcellulose is a structurally modified polysaccharide (Lopez *et al*., 2015; Rani *et al*., 2014), whereas the PSc does not present chemical modifications, with a good viscosity and could be a promising material as a rheological agent. Finally, according to analyses of particle size, zeta potential, and viscosity evaluated in this work, it can be presumed that fractions isolated from *G. tenuifrons* could be used as rheological modifiers, raw material for generating nanoparticles, as well as active ingredients for designing new functional product in the cosmetic, pharmaceutical, or food industry.

In the monitoring of the different polysaccharide solutions, a greater absorption around 280 nm can be observed,

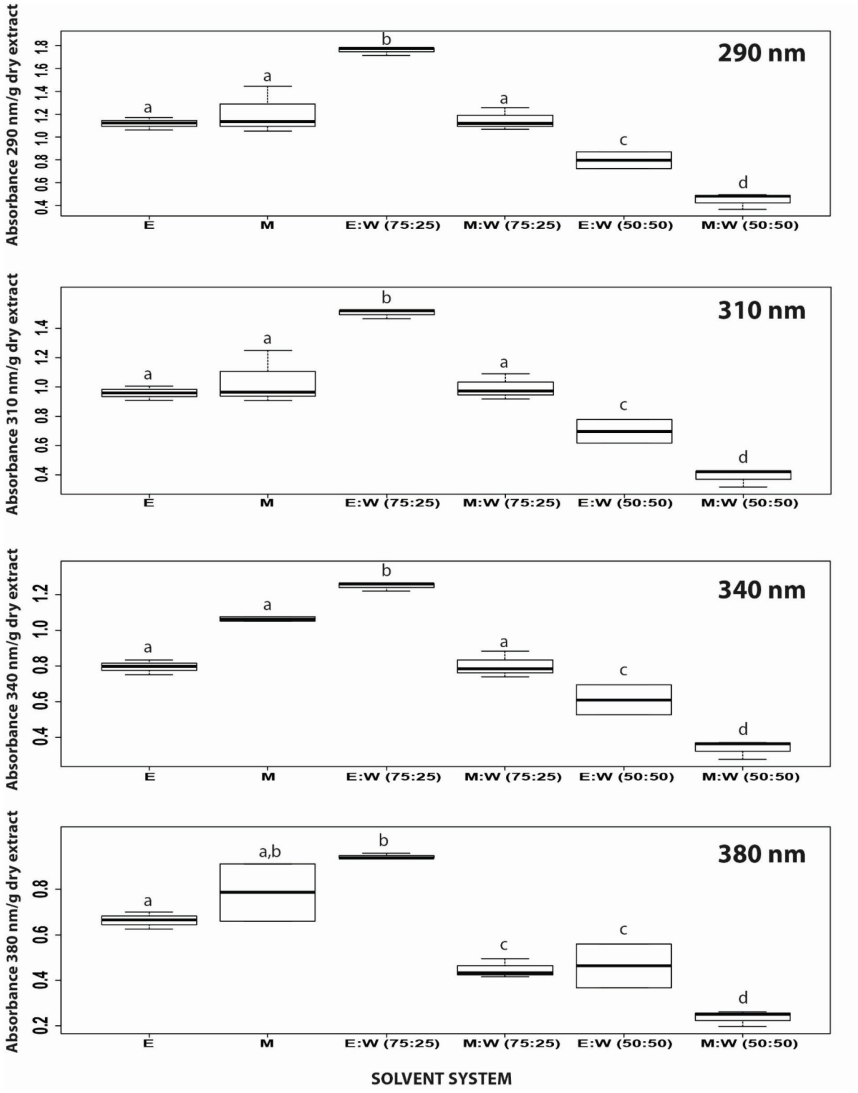

Figure 4. UVA–UVB absorption coefficient assays at 290, 310, 340, and 380 nm. The boxes with different letters have a statistically significant difference with a confidence level of 5%, according to the one-way ANOVA and Tukey tests.

which can be associated with the presence of polyphenols, tannins, or another type of chromophore present in the matrix that remains with the deproteinization. However, for different fractions, this absorption disappears significantly, so it is understood that these interfering molecules are no longer within the polysaccharide fraction, indicating a good degree of separation and purification of each polysaccharide. Furthermore, the absorption capacity of UVA-UVB radiation (290, 310, 340, and 380 nm) of the extracts obtained with the different solvent systems studied are shown in Figure 4. We could observe that the extracts obtained in the different solvent systems showed a similar profile with good absorption in the UVA-UVB range, with absorption maxima around 300–320 nm. However, from the ethanol:water solvent system (75:25), the greater broad-spectrum absorption capacity of the evaluated extracts was observed ($p < 0.05$); in this sense, a good protection was found in the UVA range, according to the parameters of COLIPA and FDA, which allows inferring that in the formulations where this extract is incorporated, it could be declared a broad-spectrum photoprotection (UVB-UVA) (COLIPA Guidelines, 2009; Department of Health and Human Service. Food and Drug Administration, 2011; ISO 24443:2012, 2012).

CONCLUSION

Polysaccharides type k, and β-carrageenan and alginate were identified in *G. tenuifrons*. The nutritional facts were comparable to that reported on the similar genus. The findings suggest that *G. tenuifrons* seaweed propagated *in vitro* culture is a feasible candidate for further study as a potential commercial cultivar for human consumption. In addition, it can be presumed its possible use as rheological modifiers, active ingredients for designing new functional foods or material to generate nanoparticles in the pharmaceutical industry.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest among them or with the parent institution.

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