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# Analysis of two precipitation methods on the yield, structural features and activity of sulfated polysaccharides from *Gracilaria cornea* (Rhodophyta)

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**ABSTRACT.** The global demand for natural products from seaweeds has increased worldwide; however, no description of the use of isoamly alcohol (IAA) for obtaining of sulfated polysaccharides (SPs) has been reported. We investigated the efficiency of two precipitation methods (M) in obtaining SPs from the red seaweed *Gracilaria cornea*. SPs enzymatically isolated were concentrated with cetylpyridinium chloride (M I) or IAA (M II) and extracts were examined with regard to their yield, structural features and *in vitro* effects on the activated partial thromboplastin time (APTT) using normal human plasma and standard heparin (193 IU $mg^{-1}$). Yield difference reached 12.99%. Quantitative determination of sulfate was similar between the two methods (~ 26%), but extracts revealed different pattern on charge density by agarose gel electrophoresis. Whereas both extracts revealed as agarocolloids, alternative M II was also efficient for lipids, proteins and nucleic acids according to the infrared analysis. Extracts had virtually no effect on APPT (1.95 and 2 IU $mg^{-1}$ for M I and M II, respectively). The results revealed IAA as an alternative solvent for obtaining SPs from the red seaweed *G. cornea*, depending on the industry' usage criterion.

**Keywords:** Rhodophyceae, polysulfated, precipitation methods, structural analysis, APTT test.

## Análise de duas metodologias de precipitação sobre o rendimento, características estruturais e atividade de polissacarídeos sulfatados de *Gracilaria cornea* (Rhodophyta)

**RESUMO.** A demanda global de produtos naturais de algas marinhas tem aumentado mundialmente. Entretanto, a obtenção de polissacarídeos sulfatados (PSs) com álcool isoamílico (AIA) não é relatada. Investigou-se a eficiência de dois métodos (M) de precipitação de PSs da alga marinha vermelha *Gracilaria cornea*. Os PSs isolados enzimaticamente foram concentrados com cloreto cetilpiridimínio (M I) ou AIA (M II). Os extratos foram examinados, segundo seu rendimento, características estruturais e efeitos *in vitro* sobre o tempo de tromboplastina parcial ativada (TTPA) usando plasma humano normal e heparina padrão (193 UI $mg^{-1}$). A diferença nos rendimentos foi 12,99% e semelhante determinação quantitativa de sulfato foi obtida entre os métodos (~ 26%). A eletroforese em gel de agarose revelou diferenças em termos de densidade de cargas entre os extratos. Enquanto ambos os extratos revelaram agarocoloides, o método M II também se mostrou alternativo para lipídios, proteínas e ácidos nucleicos de acordo com a análise de infravermelho. Os extratos praticamente não modificaram o TTPA (1,95 e 2 UI $mg^{-1}$ para M I e M II, respectivamente). Os resultados revelaram AIA como um solvente alternativo para obtenção de PSs da alga marinha vermelha *G. cornea*, dependendo do critério de utilização na indústria.

**Palavras-chave:** Rhodophyceae, polissulfatados, métodos de precipitação, análise estrutural, teste do TTPA.

## Introduction

The global demand for chemical metabolites derived from seaweeds has increased worldwide, especially sulfated polysaccharides (SPs). Agar and carrageenans are the most common sources of commercially important red seaweeds SPs (PEREIRA; COSTA-LOTUFO, 2012). These SPs differ on their stereochemistry, specifically galactans with 4-linked-α-galactose residues of the $_{L}$-series are termed agarans and those of the $_{D}$-series are termed carrageenans (Figure 1) (CARDOZO et al., 2007; CAMPO et al., 2009; PRAJAPATI et al., 2014).

An increasing demand based on their applications has been widely preferred over the synthetic polymers because seaweeds SPs are inert, safe, non-toxic, biocompatible, biodegradable and low cost (PRAJAPATI et al., 2014).

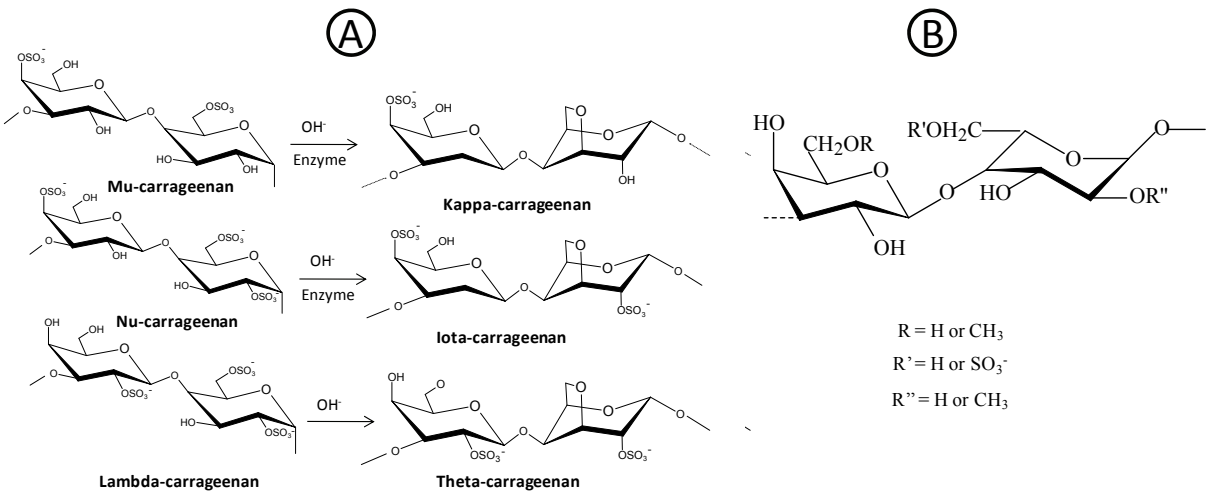


**Figure 1**. Chemical structures of Mu (μ)-, (Nu (ν)-, Lambda (λ)-, Kappa (κ)-, Iota (ι)- and Theta (θ)-carrageenans (A) and agar (B). Polysaccharides are formed by alternate units of D-galactose and 3,6-anhydro-D-galactose (A) or D-galactose and 3,6-anhydro-L-galactose (B) joined by α-1,3 and β-1,4-glycosidic linkage.
Source: Cardozo et al. (2007) and Campo et al. (2009).

Furthermore, their rheological (e.g., gelling, thickening and stabilizing) (CARDOZO et al., 2007; CAMPO et al., 2009) and pharmacological (e.g., anticoagulation, antiviral, antinociceptive and anti-inflammatory) (MAZUMDER et al., 2002; RODRIGUES et al., 2009; QUINDERÉ et al., 2013) applications have attracted more and more attention by pharmaceutical companies and scientists in recent times (CARDOZO et al., 2007; ARAÚJO et al., 2012; PRAJAPATI et al., 2014).

Rhodophyceae biosynthesize high amounts of SPs (RODRIGUES et al., 2009; ARAÚJO et al., 2012) compared with Chlorophyceae (RODRIGUES et al., 2011c, 2012) and Phaeophyceae (POMIN; MOURÃO, 2008; SIQUEIRA et al., 2011) and other natural sources (AQUINO et al., 2005; POMIN; MOURÃO, 2008; DANTAS-SANTOS et al., 2012; CHANG et al., 2013). The genera *Hypnea, Chondrus, Eucheuma, Gigartina*, *Kappaphycus* (carragenophytes) (CAMPO et al., 2009) and *Gracilaria* (agarophyte) (CARDOZO et al., 2007) of edible red seaweeds have been widely exploited in large scale to supply SPs to the industries. Their structures vary with algal species (MAZUMDER et al., 2002; POMIN, 2012; QUINDERÉ et al., 2014), but the quality and the specific physical and chemical and biological aspects can vary depending on harvest time and collection site of the algae, as well as the techniques used, such as extraction protocols and precipitation solvents (RODRIGUES et al., 2009, 2011a; YAICH et al., 2013). During the industrial processing, some factors, such as yield and purity, have also been considered as attributes of quantity and quality (CAMPO et al., 2009; PRAJAPATI et al., 2014; MORONEY et al., 2015), considering the difficulty in the standardization of a commercial product (PEREIRA; COSTA-LOTUFO, 2012).

Heparin (HEP) is a SP member of a family of glycosaminoglycans commercially extracted from pig or bovine tissue and is widely used in anticoagulant therapy, especially in extracorporeal circulation. By contrast, its prolonged administration cause several adverse consequences (e.g., hemorrhage and thrombocytopenia) (NADER et al., 2001), encouraging the search for new surrogates from different natural origins (POMIN; MOURÃO, 2008; RODRIGUES et al., 2009, 2011b; DANTAS-SANTOS et al., 2012; POMIN, 2012).

Isoamly alcohol (IAA) (3-methyl-1-butanol) is a clear and colorless liquid, very soluble in organic compounds (LIDE, 1998). In the food industry, it is commonly found as a final product in fermentation processes and milk beverages (MAMEDE; PASTORE, 2004). Furthermore, it is also usually employed in scientific studies to obtain cell components, such as DNA, lipids and proteins (YEH; CHEN, 2004; OLIVIERI et al., 2012; EL BAZ et al., 2013), and other industrial processes, including hydraulic and lube oil additives, frothing agent in mineral dressing applications and crude oil recovery processes (LIDE, 1998). To the best of our knowledge, there are no studies concerning the employment of this solvent to obtain seaweeds SPs.

*Gracilaria cornea* J. Agardh (Gracilariales, Gracilariaceae) is a red seaweed widely found along the Brazilian coast. The biotechnological importance

of this species has been recognized (LIMA et al., 2005; VALENTE et al., 2006; SOUZA et al., 2011). Melo et al. (2002) enzymatically extracted a crude polysaccharide fraction containing a agar consisting of 3,6-anhydro-α-L-galactose, with minor occurrence of 6-*O*-methyl-galactose, glucose, xylose and sulfated radicals. A subsequent study from Coura et al. (2012) revealed that this biopolymer had *in vivo* antinociceptive and anti-inflammatory effects, without toxicological significance in mice. This study analyzed two precipitation methods using cetylpyridinium chloride or IAA on the yield, structural features and the possible effect *in vitro* on coagulation of the crude SPs extracts. A structural analysis of lipids and other chemical components was also conducted.

## Material and methods

### Seaweed and isolation of SPs

Samples of *G. cornea* were collected on the seashore from the Flecheiras Beach, Trairí, Ceará State, Northeastern Brazil. After collection, specimens were taken to Carbohydrates and Lectins Laboratory (CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará, and then cleaned of epiphytes, washed with distilled water and stored at -20°C until use. A voucher specimen (#34739) was deposited in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil. The SPs were obtained through two different protocols, from dehydrated algal tissue (5 g, 25°C) cut into small pieces (COURA et al., 2012). This study did not involve endangered or protected species and was conducted in accordance with the law MP 2.186-16/2001, number resolution 29 of the Dispatch Component of Genetic Patrimony (CGEN).

### Method I (M I)

Crude SPs were extracted by papain digestion (6h, 60°C) (Vetec Química, Rio de Janeiro, Rio de Janeiro State, Brazil) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine (Sigma-Aldrich, St. Louis, Missouri State, USA) and EDTA (both 5 mM). Then, the material was filtered, centrifuged (2,725 × *g*, 4°C, 30 min.) and then 50 mL of 10% cetylpyridinium chloride (CPC) (Sigma-Aldrich, St. Louis, MO, USA) were added to the supernatant for precipitation of the SPs (24h, 25°C) as previously described by Coura et al. (2012).

### Method II (M II)

Crude SPs were extracted by papain digestion (6h, 60°C) (Vetec Química, Rio de Janeiro, Rio de Janeiro State, Brazil) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine (Sigma-Aldrich, St. Louis, Missouri State, USA) and EDTA (both 5 mM) (COURA et al., 2012). Then, the material was filtered, centrifuged (2,725 × *g*, 4°C, 30 min.) and 50 mL of IAA (Vetec Química, Rio do Janeiro, Rio de Janeiro State, Brazil) were added to the supernatant for precipitation of the SPs (24h, 25°C).

Crude SPs extracts obtained by the two methods were centrifuged, redissolved in distilled water, dialyzed against distilled water for 24 h (47 × 27 mm cellulose membrane, Sigma-Aldrich, St. Louis, Missouri State, USA), and then freeze-dried (Labconco FreeZone 4.5 apparatus) (COURA et al., 2012). Yields (%) of crude SPs extracts were calculated as the percentage of dehydrated matter (ARAÚJO et al., 2012).

### Chemical analyses and physical and chemical characterization

Total sugars (TSs) content was estimated by phenol-sulfuric acid analysis using D-galactose as a standard (DUBOIS et al., 1956). After acid hydrolysis of soluble polysaccharides (1 mL of HCl for 5 h at 105°C), the sulfate (S) content was measured by the $BaCl_2$ gelatin$^{-1}$ method (DODGSON; PRICE, 1962). The content of contaminant proteins (CPs) was measured by the Bradford's method (BRADFORD, 1976), using bovine serum albumin as reference. The degree of polydispersion and the charge density of SPs of each extract were checked by agarose gel electrophoresis as described in (DIETRICH; DIETRICH, 1976).

### Infrared (IR) spectroscopy

To analyze the chemical structure, IR spectra of both algal crude SPs extracts were determined. Fourier transform IR spectrum (FT-IR) was recorded with a SHIMADZU IR spectrophotometer (model 8300) between 4000 and 400 cm$^{-1}$. Samples were evaluated as KBr pellets (MELO et al., 2002). The presence of lipids and other chemical components was also evaluated (MI-KYUNG; JEUNE, 2009).

### Activated partial thromboplastin time (APTT) assay

The APTT assay was performed using normal human plasma (obtained from 10 different donors at the Hematology and Hemotherapy Center of Ceará - HEMOCE) following manufacturer's specifications (CLOT, Bios diagnostic, Sorocaba, São Paulo State, Brazil). For this, 50 µL of citrated human plasma was

mixed with 10 µL of a solution of different polysaccharide amounts before adding 50 µL of APTT reagent. The mixture was then incubated at 37°C for 3 min. Then, 50 µL of 0.025 M calcium chloride reagent was added to the mixture to trigger the coagulation cascade. The clotting time was recorded in a coagulometer (Drake Quick Timer). The possible anticoagulant effect of crude SPs extracts was expressed as international units per mg of polysaccharide using standard heparin (193 IU $mg^{-1}$). All the tests were performed in triplicate.

## Results and discussion

### Effect of precipitation methods on the yield and chemical composition

The result of adding CPC (M I) or IAA (M II) after papain incubation (6h, 60°C) to obtain crude SPs extracts from the red seaweed *G. cornea* is illustrated in Figure 2. The use of M II led us to obtain at least three different components present at two phases in the supernatant compared with M I. Possibly, IAA, a nonpolar organic solvent, would be capable of 'separating' metabolites according to physical and chemical characteristics (Figure 2B), such as hydrophobicity and molecular weight (LIDE, 1998). These hypotheses suggested the presence of nucleic acids (phase I) (YEH; CHEN, 2004), lipids (EL BAZ et al., 2013) and SPs (phase II) (MELO et al., 2002; COURA et al., 2012). As expected, only one phase was observed for M I (Figure 2A) because CPC, due to its cationic nature (LEWIS, 1996), had ability to more specifically interact with the hydrophilic (anionic) regions (ester sulfate groups) (CARDOZO et al., 2007; CAMPO et al., 2009) of the SPs extracted from the red seaweed *G. cornea* (COURA et al., 2012).

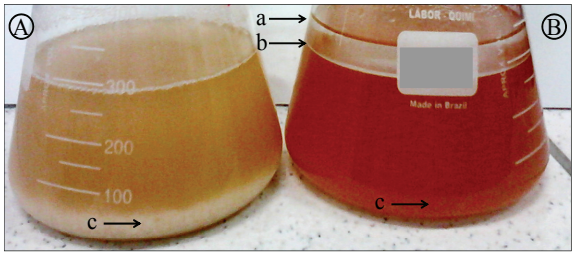


**Figure 2**. General aspects of CPC (M I-A) or IAA (M II-B) precipitate solution after papain incubation (6h, 60°C) to obtain crude SPs extracts from the red seaweed *Gracilaria cornea*. Different phases are observed. Arrows indicate the presence of nucleic acids (a), lipids (b) and pigments and SPs (c), respectively.

Another interesting observation was the presence of other useful components of the algal biomass, such as pigments, by M II (phase II) (Figure 2B). Literature describes that in obtaining lipids from seaweeds in the presence of nonpolar organic solvents (e.g., hexane and acetone), they could be accompanied with pigments, such as fucoxanthin in *Laminaria gurjanovae* (Phaeophyceae) (SHEVCHENKO et al., 2007). In our case, possibly IAA separated pigments by hydrophobic interaction with N-H groups of the proteins and by low relatively density of this solvent compared with water (LIDE, 1998) (Figure 2), especially phycoerythrin, a red protein-pigment complex from the light-harvesting phycobiliprotein family, which is easily extracted from Rhodophyceae, in which it play an important protective role against oxidative damage provoked by excess light exposure, and have several biotechnological applications for various fields, including food, medicine and aquaculture (CARDOZO et al., 2007). It is also believed that sources of antioxidants would stimulate coastal communities to produce high added-value products (SOUZA et al., 2011). Further studies concerning the structural conformation of these algal pigments are needed (CARDOZO et al., 2007; CAMARA-ARTIGAS et al., 2012).

Table 1 summarizes the yield and the S and TSs contents of the lyophilized polysaccharide precipitates obtained from both methods. Depending on the organic solvent, the higher yield was obtained by M I (21.17%), similarly to that (21.4%) previously found for *G. cornea* SPs by Melo et al. (2002), Brazil. However, this contrasts with the result obtained by M II (8.18%), which yielded about 2.6-fold lower compared with M I. Although revealing difference on the yield, interestingly, the S content (25-27%) was similar between the methods based on quantitative analysis (DODGSON; PRICE, 1962). These values were not in accordance with those previously obtained (4-15%) for the same algal species (MELO et al., 2002; COURA et al., 2012), but were in conformity with those found for other investigated agarophyte species (14-26%); thus, studies on the seasonal, regional and species variations of SPs compositions are needed (CARDOZO et al., 2007). The TSs content showed a difference between the protocols as measured by Dubois' method. This could be explained by the selectivity of M I for obtaining seaweed carbohydrates (ARAÚJO et al., 2012), confirming thus its higher yield. The presence of pigments did not alter the quality of lyophilized raw precipitate (Figure 2c) since CPs were not detected in both crude SPs extracts based on the Bradford' method (COURA et al., 2012). Melo et al. (2002) found a higher protein content (2.8%) in the crude SPs (M I) from Brazilian samples of *G. cornea*, when over

dried (60°C, 24h). Therefore, in the present study, the precipitation yields and the TSs contents of crude SPs extracts from *G. cornea* varied according to the method (CARDOZO et al., 2007; RODRIGUES et al., 2011a; YAICH et al., 2013).

**Table 1.** Yield and chemical analyses of crude SPs extracts from the red seaweed *Gracilaria cornea* from both precipitation methods.

| Method | Yield (%) [a] | TSs (%) [b] | S (%) [c] | CPs (%) [d] |
|---|---|---|---|---|
| M I | 21.17 | 69.00 | 25.97 | - |
| M II | 8.18 | 9.17 | 26.99 | - |

a – Yield was calculated as the percentage of dehydrated matter; b – Dosage by Dubois et al. method using D-galactose as standard; c – Dosage by Dodgson and Price method using $NaSO_3$ as standard; d – Dosage by Bradford method using bovine serum albumin (- not detected).

## Agarose gel electrophoresis

To evaluate the physical and chemical characteristics of the crude SPs extracts (M I and M II), agarose gel electrophoresis was performed (Figure 3). This technique revealed marked differences on the charge density and polydispersion of the crude SPs extracts between the analyzed methods. The electrophoretic profile also showed similar mobility of both polysulfated on gel, in which M I exhibited a single band; therefore, it was a homogeneous crude SPs extract in charge density than that obtained from the M II which revealed a polydisperse crude SPs extract and weak metachromasia, suggesting a low sulfate content in the sample (RODRIGUES et al., 2011a and c; QUINDERÉ et al., 2013). These observations could be supported by the differences in the crude SPs extract yields (Table 1), with better concentration capacity by M I for *G. cornea* SPs (Figure 2B).

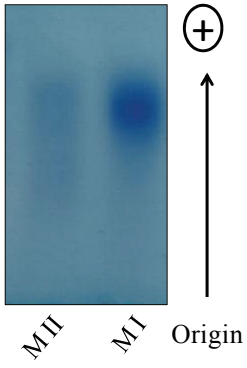


**Figure 3.** Agarose gel electrophoresis of SPs extracted from the red seaweed *Gracilaria cornea*. Crude SPs extracts (CPC-M I and IAA-M II) present on gel were stained with 0.1% toluidine blue.

From these data, our studies were also extended to investigate the presence of different molecular groups in *G. cornea* (M I and M II) by IR spectroscopy, a qualitative technique that provides structural information regarding sulfate (CAMPO et al., 2009) and other chemical components (MI-KYUNG; JEUNE, 2009).

## Spectral analysis of SPs

Both IR spectra of SPs (M I and M II), as seen in Figure 4, were very similar. Characteristic absorption signals (at 1370, 1250, 1153, 1070, 930, 890 and 740 $cm^{-1}$) related to the presence of polysaccharide agar were found and were in accordance with a previous study performed by Melo et al. (2002) who revealed the occurrence of this group of SP from the red seaweed *G. cornea*. As another result, biogenetic precursor (at 820 $cm^{-1}$, galactose-6-sulfate) of 3,6-anhydro-α-L-galactose (at 930 $cm^{-1}$) was also found in the spectra, confirming a degree of substitution on C-6 of the analyzed polymer (MELO et al., 2002). By contrast, the intensity of the peaks was not correlated with the S content of the studied raw material (M II) (Table 1), while M I showed higher absorption bands, especially for ester sulfate (at 1250 $cm^{-1}$), corroborating thus its metachromasia, when verified by electrophoresis (Figure 3). These data confirmed our previous observations (Figure 2 and Table 1) and showed CPC (M I) as a more appropriate solvent to obtain seaweeds SPs (ARAÚJO et al., 2012; RODRIGUES et al., 2011a).

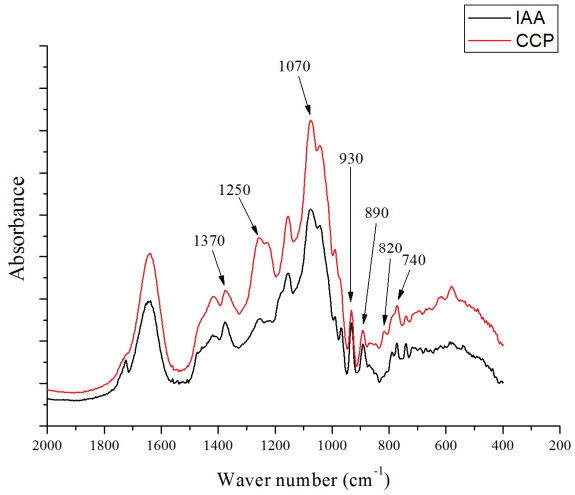


**Figure 4.** IR spectra (at 2000 and 200 $cm^{-1}$, kBr pellets) of crude SPs extracts from the red seaweed *Gracilaria cornea* in both precipitation methods (CPC-M I and IAA-M II).

## Spectral analysis of lipids and other chemical components (M II)

The spectral analysis of a lyophilized lipid sample and other chemical components is shown in Figure

5. Based on our main findings, it was proposed the occurrence of $^{-}$OH stretching at 3432 $cm^{-1}$ corresponding to some hydroxyl groups (EL BAZ et al., 2013), which is indicative of the presence of proteins (ESTEVEZ et al., 2009) and lipids (MI-KYUNG; JEUNE, 2009). The detection of this signal also leads to postulate a polysaccharide-protein complex (MELO et al., 2002).

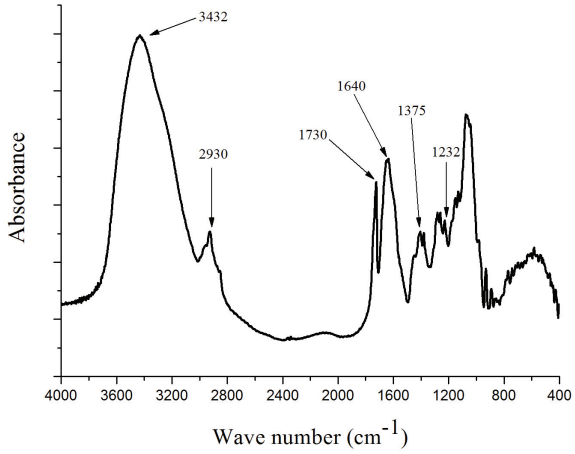


**Figure 5**. IR spectrum reveal lipids, proteins and nucleic acids of the raw material (AIA-M II) from the red seaweed *Gracilaria cornea* at 4000 and 400 $cm^{-1}$ in kBr pellets.
Source: The authors.

Symmetric $CH_3$ bending at around 1375 $cm^{-1}$ and C-H stretching at 2930 $cm^{-1}$ were attributed to the fatty acids and/or indicated the presence of $SO_3$ (sulfolipids) (EL BAZ et al., 2013). Possibly, the presence of this component could perhaps explain the sulfate content (Table 1) and the weak metachromasia (Figure 3) due to the solubility of lipids in benzine, a nonpolar organic solvent obtained from oil refineries and mostly used by pharmaceutical companies and in the manufacturing process (WILLIAMSON, 2004), during electrophoresis procedure (M II) (DIETRICH; DIETRICH, 1976). 1640 (C=O elongation), 1232 ($^{-}$OH) and 1730 $cm^{-1}$ suggested proteins and ester carbonyl derived from membrane lipids, such as glyceroglycolipids, cholesterols and some phospholipids (polyesters), respectively (SHEVCHENKO et al., 2007), postulating different class composition of *G. cornea* lipids (MIYASHITA et al., 2013). It was also speculated the presence of algaenans, a highly resistant and non-hydrolysable bioproduct naturally found in some microalgae (ALLARD et al., 2002), but additional studies on this hypothesis are required because seaweed lipid content varies along the year (MIYASHITA et al., 2013) and tropical species have significantly lower lipid content (CARNEIRO et al., 2014) than those collected from cold-water zones (MIYASHITA et al., 2013). Spectral analysis would also lead to a suggestion of nucleic acids in the analyzed raw material (MI-KYUNG; JEUNE, 2009) since AIA is usually used to DNA isolation from algae (YEH; CHEN, 2004).

### *In vitro* anticoagulant assay

In order to evaluate the *in vitro* anticoagulant potential of both crude SPs extracts (M I and M II) from the red seaweed *G. cornea*, the APTT test was carried out using normal human plasma, as shown in Table 2. According to our findings, testing samples containing SPs from both precipitation methods, no effect *in vitro* was found (1.95 and 2 IU $mg^{-1}$ for M I and M II, respectively), as measured by APTT assay, in comparison with HEP (193 IU $mg^{-1}$). This meant that crude SPs extracts isolated from this algal species had no inhibitory effect on the intrinsic and/or common pathways of the coagulation system (ARAÚJO et al., 2012).

**Table 2.** *In vitro* anticoagulant effect of the crude SPs extract obtained by M I or M II from the red seaweed *Gracilaria cornea* in comparison to HEP.

| Method | APTT test* | |
|---|---|---|
| | 1.00 mg $mL^{-1}$** | IU $mg^{-1}$*** |
| M I | 46.80 ± 0.56 s | 1.95 |
| M II | 48.30 ± 0.30 s | 2.00 |

M I – cetylpyridinium chloride or M II – isoamly alcohol; *Activated partial thromboplastin time (APTT); **SP concentration to prolong the APTT in seconds; ***Anticoagulant activity expressed in international units (IU) per mg of SPs (IU $mg^{-1}$); HEP (193.00 IU $mg^{-1}$; 0.01 mg $mL^{-1}$; APTT: 46.10 ± 0.81 s); Control: 44.20 ± 0.52 s (n = 3).
Source: The authors.

Some seaweed SPs have *in vitro* anticoagulant effect, and their actions have been naturally attributed to the molecular size, type of sugar, charge density and sulfate content (POMIN; MOURÃO, 2008; RODRIGUES et al., 2009, 2011c; POMIN, 2012). Structural requirements, such as the occurrence of dissulfated units in the same sugar residue, sulfate position, type of linkage and molecular geometry, have also been reported for their anticoagulant effects (POMIN; MOURÃO, 2008; CAMPO et al., 2009; POMIN, 2012). Furthermore, literature reports that the high galactose-6-sulfate content had been suggested as an important structural feature for anticoagulation of SPs (DANTAS-SANTOS et al., 2012). Serpin-independent anticoagulation had also been recently reported for a SPs fraction from the red alga *Acanthophora muscoides* (QUINDERÉ et al., 2014).

It has been reported that the agar polysaccharide from *Gracilaria* species is mainly composed of alternating 3-linked β-D-galactopyranosyl residues

(A-units) and 4-linked α-L-galactopyranosyl (or 3,6-anhydrogalactopyranosyl) residues (B-units). This backbone is further modified by different substitutions. Agar has low sulfate content when compared to other seaweeds SPs (Figure 1) (CARDOZO et al., 2007; CAMPO et al., 2009).

Previous study demonstrated that the crude SPs extract (M I) from the red seaweed *G. cornea* acted as an antinociceptive and anti-inflammatory agent (COURA et al., 2012). However, the SPs dose administrated in rodents was 27-fold higher than the concentration used on APTT assay in the present study (Table 2). Therefore, treatment of blood with both extracts at high concentration of SPs (1 mg $mL^{-1}$) did not modify the normal coagulation time (Table 2) and could be not correlated to other physiological interactions due to a lack of structure-function relationship data (MAZUMDER et al., 2002; POMIN, 2012). *Gracilaria* species could reveal differences in the relative proportions of sulfate and composition with possible impacts on their bioactivities (CARDOZO et al., 2007).

Another important observation was the addition of calcium chloride reagent to display the APTT assay which did not alter the evaluation by *in vitro* testing according to Araújo et al. (2012) who also verified a lack of anticoagulant action for the SPs from the red seaweed *Solieria filiformis*. It is described that the water solubility of seaweeds SPs depends essentially on the levels of sulfate groups and associated cations, like calcium, which contribute to the viscosity of solutions or strength of gels formed by SPs (CAMPO et al., 2009). Melo et al. (2002), investigating some chemical aspects of a crude SPs fraction from *G. cornea*, revealed that the structure deviation and the high sulfate content could explain the absence of a gelling behavior in comparison with the agar obtained from Mexican species. High values of calcium and aluminum present in SPs could determine a cross-linking property, which are also favorable for gelling formation. A more detailed study concerning these observations is needed using animals (RODRIGUES et al., 2011b; QUINDERÉ et al., 2014).

Overall, our study represents a good approach for identifying different functional groups of chemical metabolites from seaweeds (Figure 2). Results could open new frontiers for different areas, including chemotaxonomy due to the environmental plasticity and temporal variation in the morphology of some algae, which make species identification difficult at the biochemical and molecular level (YEH; CHEN, 2004; POMIN; MOURÃO, 2008; RODRIGUES et al., 2012), and identification of algae species as sources of biofuels and pigments for oil, food and pharmaceutical industries, respectively (CARDOZO et al., 2007). Additional studies on the effects of the different isolated metabolites from *G. cornea* focusing on their pharmacological effects should also be conducted inferring the use of AIA (M II), including *in vitro* and *in vivo* assays (EL BAZ et al., 2013; QUINDERÉ et al., 2013).

## Conclusion

The precipitation method using cetylpyridinium chloride is more appropriated for obtaining sulfated polysaccharides from the red seaweed *Gracilaria cornea* in comparison with isoamyl alcohol. Infrared and agarose gel electrophoresis revealed agar and marked differences on their physical and chemical characteristics between examined methods, respectively. The clotting assay (APTT) did not have significantly change after polysaccharide-treated plasma. By contrast, isoamyl alcohol reveals a good approach to obtain lipids, nucleic acids and proteins in order to further explore new biotechnological applications.

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