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Extraction process optimization of sulfated galactan-rich fractions from *Hypnea musciformis* in order to obtain antioxidant, anticoagulant, or immunomodulatory polysaccharides

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Abstract The seaweed *Hypnea musciformis* synthesizes sulfated galactans and these galactans have been reported to have antioxidant, anticoagulant, and immunostimulatory properties. However, efficient extraction of these polysaccharides depends on the particular extraction method used, some of these polysaccharides are not extracted. Previously, we obtained galactans with different properties by using a simple extraction method. Here, we performed slight modifications to the extraction method, which led to variation in the type of polysaccharides obtained and their biological activity. We obtained four sulfated galactan-rich fractions named as FT4v, FT6v, FT8v, and FT10v. The chemical composition data showed that the fractions had high sugar and sulfate contents. Fraction FT4v exhibited the highest sulfate/sugar ratio (0.47), while FT6v (0.37), FT8v (0.39), and FT10v (0.39) presented lower sulfate/sugar ratios. Fractions FT4v and FT6v showed the highest antioxidant activity. Fraction FT4v also exhibited the greatest anticoagulant potential; however, other fractions presented marked immunomodulatory action. FT10v stimulated the highest levels of NO production whereas FT6v stimulated the highest expression of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in RAW cells. Fraction FT10v exhibited the greatest cytotoxic action of the obtained fractions against HeLa and 786 cell lines. In vitro tests showed that the sulfated galactan-rich fractions isolated from *H. musciformis* with low sulfate/sugar ratios, i.e., the fractions containing fewer sulfated polysaccharides (FT6v, FT8v, and FT10v), showed marked immunostimulatory action while the fraction with the high sulfate/sugar ratio (FT4v) exhibited improved anticoagulant activity. Therefore, our results suggest that different extraction conditions promote the acquisition of sulfated galactan-rich fractions from *H. musciformis* with different biological activities.

Keywords Galactans · Sulfated polysaccharides · Red seaweed · Biological activity

Introduction

Marine seaweeds contain sulfated polysaccharides in their extracellular matrix. These polymers are able to protect seaweed from dehydration when exposed to the sun during low tide. Sulfated polysaccharides from seaweed also give flexibility to algae structure, which facilitates light and nutrient capture (Michel et al. 2010).

The red alga *Hypnea musciformis* is a recognized source of sulfated polysaccharides with significant industrial and pharmacological importance (Campo et al. 2009). These sulfated polysaccharides are known as sulfated galactans (Percival and McDowell 1967). All sulfated galactans synthesized by algae are anionic, and most galactans have a high molecular mass. These two features are mainly responsible for the different pharmacological properties of the galactans produced by seaweed (Pomin 2010), including their antiproliferative (Costa et al. 2010), antioxidant (Costa et al. 2010; Alves et al. 2012a), antitumor (Lins et al. 2009), gastroprotective (Damasceno et al. 2013), anticoagulant (Farias et al. 2000; Alves et al. 2012b), antiviral (Carlucci et al. 1997), and immunostimulatory (Zhou et al. 2004) activity.

Sulfated galactans from seaweed, such as all polysaccharides, are not synthesized based on models, unlike proteins

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and nucleic acids. Their synthesis is driven by factors such as concentrations of synthesis and degradation enzymes, availability and concentrations of enzyme substrates (monosaccharides) and cofactors, and cellular compartmentalization. Moreover, sulfated galactans may differ structurally depending on the type of seaweed from which the galactans are extracted, the collection period (different seasons), and the characteristics of the marine environment of the seaweed (Reis et al. 2008).

In addition to the structural characteristics, the extraction yield can also be affected by the aforementioned factors. For example, the yield or type of galactans from *Grateloupia doryphora* and *Gymnogongrus griffithsiae* (Perfecto 1998), as well as from *Gracilaria bursapastoris* (Marinho-soriano and Bourret 2003), are influenced by seasonal factors. Compounding these problems, different extraction methods can yield sulfated galactans with different characteristics from the same seaweed; thus the yield, physicochemical properties, and biological activities of the sulfated galactans are dependent on the extraction method used (Rodrigues et al. 2009).

It is possible that some seaweeds synthesize galactans with different pharmacological properties, but some of these polysaccharides are lost depending on the particular extraction method used. Thus, to avoid this loss during the first steps of galactan extraction, it is important to evaluate the pharmacological activities of these polymers in order to obtain a bioguided galactan purification process.

Hypnea musciformis is a red alga with a cosmopolitan distribution and synthesizes sulfated galactans (Geraldino et al. 2009). Previous studies by our group using an extraction methodology based on proteolytic digestion of seaweed and two volumes of aqueous solution of sodium chloride (NaCl) incubated at 60 °C for 18 h yielded sulfated galactans from the red seaweed *H. musciformis* with antioxidant (Alves et al. 2012a) and anticoagulant activities (Alves et al. 2012b). Thus, sulfated polysaccharides with different biological activities were obtained with the same extraction methodology. For this reason, we wanted to investigate whether slight modifications to the extraction method, such as the incubation time and the volume of solvent used in the proteolytic digestion, could promote variation in the types of polysaccharides obtained, and consequently variations in the biological activities exhibited by these polymers.

In this paper, we suggest a simple method of acquiring sulfated galactan-rich fractions from *H. musciformis*. We evaluated whether slight modifications to the incubation time and the amount of solvent used in the initial extraction step favored the acquisition of galactans with particular antioxidant, anticoagulant, and/or immunomodulatory activities from *H. musciformis*.

Materials and methods

Extraction of sulfated galactan-rich fractions

Hypnea musciformis was collected in the summer (December 2011, January 2012, December 2012, and January 2013) from the same location (05° 58′ 23″ S 35° 04′ 97′ W) at Búzios Beach, Nísia Floresta, RN, Brazil. Immediately after collection, the seaweed was identified by Dr. Hugo Alexandre Oliveira Rocha from Centro de Biociências/UFRN, Natal, RN, Brazil. The seaweed that was collected at each time point was kept separate and dried at 50 °C for 24 h under ventilation in an oven, then ground in a blender and stored. The ground seaweed was incubated with acetone at room temperature to eliminate lipids and pigments.

First, in order to determine the extraction conditions required to obtain an improved yield, about 10 g of powdered seaweed from each time point was suspended with 300 mL (six volumes,) of 0.25 M NaCl and the pH was adjusted to 8.0 with NaOH. Prolav 750 (150 mg; Prozyn Biosolutions, São Paulo, Brazil), a mixture of alkaline proteases, was added to the mixture for proteolytic digestion. After extraction/ proteolytic digestion for different lengths of time (8, 12, 18, and 24 h) at 60 °C under agitation, the mixture was filtered through cheesecloth and precipitated with two volumes of ice-cold methanol with gentle agitation at 4 °C. After 12 h, the precipitate was collected by centrifugation at $10,000 \times g$ for 20 min and dried under vacuum. The precipitated samples were named FT8h, FT12h, FT18h, and FT24h according to the extraction/proteolytic digestion time, and the yield of the sulfated galactan-rich fractions was evaluated.

The fraction with the greatest yield was chosen to determine the best mass/volume ratio of defatted powder/0.25 M NaCl. Hence, 10 g of defatted powder from each time point was placed in four separate vessels. To each vessel, a different volume of 0.25 M NaCl was added (4 vol, 200 mL; 6 vol, 300 mL; 8 vol, 400 mL; and 10 vol, 500 mL). After the proteolysis, the mixture was centrifuged and soluble polysaccharides were precipitated with 2 vol of methanol, obtaining the sulfated galactan-rich fractions named FT4v, FT6v, FT8v, and FT10v. The yields of the fractions were evaluated to determine the best conditions for the extraction of polysaccharides from *H. musciformis*.

Chemical composition

Total sugars were estimated by using the phenol–H_2SO_4 reaction (Dubois et al. 1956) using D-galactose as the standard. The sulfate content was measured after acid hydrolysis of the polysaccharides (6 N HCl, 100 °C, 4 h) by turbidimetric method (Dodgson and Price 1962). Phenolic compounds were measured by using the Folin–Ciocalteau method as described by Costa et al. (2010) with gallic acid as the standard. The

protein content was determined using the Bradford (1976) method, using Coomassie Brilliant Blue reagent and bovine serum albumin as the standard. The sulfate/sugar ratio was determined from the sulfate and sugar percentage contents of each sulfated galactan-rich fraction (FT4v, FT6v, FT8v, and FT10v). All assays were performed three times in triplicate ($n=3$) and were carried out with the samples from each time point.

Agarose gel electrophoresis

Agarose gel electrophoresis of the sulfated galactan-rich fractions was performed in 0.8 % agarose gel glass (7.5×10 cm, 0.2 cm thickness) prepared with 0.05 M 1,3 diaminopropane-acetate buffer at pH 9.0 (Dietrich and Dietrich 1976). Aliquots of each of the fractions (50 μg of each fraction) were applied to the gel and subjected to electrophoresis (100 V) for 45 min. The agarose gel glass was incubated with 0.1 % cetyltrimethylammonium bromide for 4 h. Afterwards, the gel was dried and stained for 15 min with 0.1 % toluidine blue prepared in a solution of 1 % acetic acid, 50 % ethanol, and 49 % water. The gel was incubated with a solution of 1 % acetic acid, 50 % ethanol, and 49 % water. This technique was repeated thrice for all fractions obtained from each time point.

Infrared spectroscopy

The infrared spectra of the sulfated galactan-rich fractions from each time point were obtained by using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany) equipped with OPUS 3.1 software. The fractions were ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4,000–500 cm^{-1}. All spectra were obtained for each fraction from each time point in triplicate.

Determination of total antioxidant capacity

The antioxidant capacity assay is based on the reduction of molybdenum(VI) to molybdenum(V) by the sulfated galactan-rich fractions and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH (Silva et al. 2012). Tubes containing sulfated galactan-rich fractions and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalents. All assays were performed three times in triplicate ($n=3$).

Anticoagulant activity

The activated partial thromboplastin time (APTT) coagulation assay was performed with a coagulometer and measured using citrate-treated normal human plasma incubated with the sulfated galactan-rich fractions. The APTT test was carried out according to the manufacturer's specifications (Labtest, São Paulo, Brazil). Briefly, normal human citrated plasma (90 μL) was mixed with 10 μL of a solution of the sulfated galactan-rich fractions (100 μg) or clexane (10 μg) and incubated for 3 min at 37 °C. Then, cephalin (100 μL) was added to the mixture and incubated for 3 min at 37 °C. Subsequently, 20 mM CaCl$_2$ (100 μL) was added and the clotting time was recorded. Citrated plasma (100 μL) was used as the control. All assays were performed three times in triplicate ($n=3$).

NO production on RAW cells

The RAW cells were incubated (3×10^5 cells/well) in 24-well culture plates at 37 °C in 5 % CO$_2$ with or without LPS in the presence of different concentrations of sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) from *H. musciformis*. After 24 h, the supernatants were collected and submitted to spectrophotometric analysis after Griess reaction for the detection of nitrite, which indicates NO production by RAW cells. The absorbance was measured at 540 nm (Jung et al. 2010). All assays were performed three times in triplicate ($n=3$).

Cytokine assays

The RAW cells were incubated (3×10^5 cells well^{-1}) in 24-well culture plates at 37 °C in 5 % CO$_2$ with different concentrations of sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) from *H. musciformis*. After 24 h, the supernatants were collected and submitted to detection of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels using an ENZO Life Sciences ELISA kit. The assays were performed according to the manufacturer's recommended procedures and the absorbance was measured at 405 nm by using microplate reader. All assays were performed three times in triplicate ($n=3$).

MTT assay

The MTT assay, according to Mosmann (1983), evaluates the capacity of cellular enzymes to reduce MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) to formazan in cells with active metabolism using HeLA, 786, and RAW cells. The cultures were exposed to the sulfated galactan-rich fractions (0.01, 0.1, 1.0, and 2.0 mg mL^{-1}) and incubated at 37 °C for 24 h and 48 h. After incubation, 100 μL of either DMEM or RPMI medium, depending on the cell type

containing MTT (1 mg mL^{-1} final concentration), was added to each well and the plates were incubated at 37 °C for 4 h. Subsequently, the supernatant was removed and 100 μL of 96 % ethanol was added to solubilize the formazan crystals. The plates were then homogenized and the absorbance was measured by using a microplate reader (Epoch da Biotek Instruments Inc) at 570 nm. For the control, the cells were incubated only with culture medium. All assays were performed three times in triplicate ($n=3$). The percentage reduction of MTT to formazan was calculated as follows:

% Reduction of MTT to formazan

$$= \text{Abs}_{sample}(570 \text{ nm}) \times 100/\text{Abs}_{control}(570 \text{ nm})$$

where Abs$_{sample}$ is the absorbance of the sample and Abs$_{control}$ is the absorbance of the control, which is considered as 100 % reduction of MTT to formazan.

Statistical analysis

The Pearson correlation coefficient was calculated for the sulfate/sugar ratio and the results of the biological assays to evaluate the sulfated galactan-rich fractions from *H. musciformis*.

All data are expressed as mean ($n=3$) ± standard deviation. Statistical analysis was performed by one-way ANOVA followed by Tukey–Kramer test ($P<0.05$) using GraphPad Prism 5.01.

Results

Acquisition of sulfated galactan-rich fractions from *H. musciformis*

The methodology chosen to obtain sulfated galactans from algae consists of extraction/proteolytic digestion with a proteolytic enzyme that will degrade proteins and promote galactan release. Therefore, to determine the best conditions for galactan extraction, the volume of 0.25 M NaCl solution was initially kept constant and the proteolytic digestion incubation time was varied. After incubation, the soluble polysaccharides were precipitated and the obtained fractions were named FT8h, FT12h, FT18h, and FT24h.

When we compared the yields of each of the fractions from each time point, there were no significant differences in the quantity of material obtained. On the other hand, as shown in Table 1, independent of the time point, the sulfated galactan-rich fraction with the highest yield was FT18h (486.3± 5.9 mg) ($P<0.001$), followed by FT24h, FT8h, and FT12h. For this reason, an extraction/proteolytic digestion time of 18 h was chosen to determine the best mass/volume (m/v) ratio

Table 1 Yield of sulfated galactan-rich fractions (FT) from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion with 6 volumes of NaCl 0.25 M for 8 h (FT8h), 12 h (FT12h), 18 h (FT18h), and 24 h (FT24h); $n=4$

Sulfated galactan-rich fractions	Yield (mg)				
	Extraction				Mean ± SD
	1°	2°	3°	4°	
FT8h	307	311	310	305	308±2.8
FT12h	281	279	284	285	282±2.8
FT18h	484	492	478	487	485±5.9
FT24h	466	470	462	460	465±4.4

of seaweed/0.25 M NaCl to be used for the extraction methodology.

After 18 h of extraction/proteolytic digestion, the samples were centrifuged and the soluble polysaccharides were precipitated. The extraction results are shown in Table 2. We did not did find any significant differences in the extraction yields between the time points. However, when we increased the volume of 0.25 M NaCl in the extraction process we observed that more sample was obtained. Thus, the yield of FT10v conditions was significantly ($P<0.001$) higher than the yields of FT8v and FT6v.

Chemical composition

The chemical composition of the sulfated galactan-rich fractions is depicted in Table 3. Sulfated galactan-rich fractions from all time points showed no protein contamination and exhibited a low percentage of phenolic compounds. The fractions exhibited high total sugar contents ranging from 67.84 to 72.99 % and high sulfate contents ranging from 26.77 to 31.77 %. From this data, we determined the sulfate/sugar ratio of the fractions. Fraction FT6v exhibited the lowest sulfate/sugar ratio (0.37) followed by fractions FT8v and FT10v (0.39), while fraction FT4v showed the highest sulfate/sugar ratio (0.47). These results allowed us to conclude that the sulfated galactan-rich fractions obtained with the lower volume of 0.25 M NaCl (four volumes) exhibited a greater sulfate/sugar ratio, showing that FT4v is composed of more sulfated polysaccharides.

Agarose gel electrophoresis

The agarose gel electrophoresis results are shown in Fig. 1. As shown, all fractions exhibited sulfated polysaccharides with similar mobility profiles, indicating that physicochemically similar sulfated galactans were obtained under all the different extraction conditions. Metachromasia with toluidine blue was

Table 2 Yield of sulfated galactan-rich fractions (FT) from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion for 18 h with 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), or 10 vol (FT10v) of NaCl 0.25 M; $n=4$

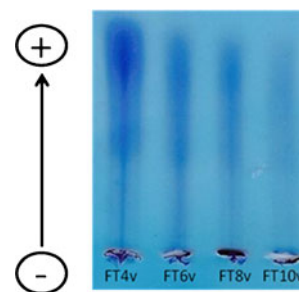| Sulfated galactan-rich fractions	Yield (mg)				
	Extraction				Mean ± SD
	1°	2°	3°	4°	
FT4v	20.4	28	17.4	23.8	22.4±4.6
FT6v	476	470	488	486	480±8.5
FT8v	516	508	525	524	518±7.9
FT10v	738	730	746	743	739±7.0

Fig. 1 Agarose gel electrophoresis of sulfated galactan-rich fractions from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion for 18 h with NaCl 0.25 M 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), and 10 vol (FT10v). *Arrow direction* refers to electrophoretic migration direction. One representative electrophoresis assay of three independent experiments is presented

more apparent in the FT4v fraction, probably owing to this fraction having a higher sulfate/sugar ratio (Table 3).

Infrared spectroscopy

The sulfated galactan-rich fractions from *H. musciformis* from each time point exhibited similar infrared spectra with characteristic signals of carbohydrate regions, as shown in Table 4. Signals at 3300–3500 cm^{-1} are related to O–H stretching vibrations and signals at 2929–2989 cm^{-1} are associated with C–H stretching vibrations (Wu et al. 2012). Signals around 1230–1275 cm^{-1} correspond to the asymmetric stretching vibrations of O=S=O (Fernández et al. 2013). The signal at 1150 cm^{-1} is assigned to the asymmetric vibrations of C–O–C glycosidic bonds, indicating the presence of a pyranosidic ring (Mohacek-Gresev et al. 2001). Signals around 930–940 cm^{-1} indicate the presence of 3,6-anhydrogalactose, and signals at 840–850 cm^{-1} are attributed to the C–O–SO$_4$ bond in the galactose C4 (Harding 1974; Jackson and McCandless 1979). The signal at 705 cm^{-1} is assigned to the sulfate group in galactose C4 (Sekkal and Legrand 1993).

The data from infrared spectroscopy, gel electrophoresis, and chemical analysis showed that FT4v, FT6v, FT8v, and FT10v obtained from December 2011 are similar to their counterparts obtained from other time points. Accordingly,

similar fractions were pooled and their activities were evaluated.

Total antioxidant capacity

There was no statistical difference in the total antioxidant capacities of FT4v and FT6v, as well as those of FT8v and FT10v. Sulfated galactan-rich fractions FT4v, FT6v, FT8v, and FT10v showed total antioxidant capacities of 60.1, 57.8, 41.1, and 45.3 mg equivalents of ascorbic acid g^{-1} of fraction, respectively (Fig. 2).

Anticoagulant activity

The activated thromboplastin time test evaluates the action of compounds on factors of the intrinsic and common coagulation pathways. Fractions FT4v, FT6v, FT8v, and FT10v prolonged the clot formation time by 4.1, 1.5, 1.7, and 1.4 times compared to the reaction control (Fig. 3), respectively. Fraction FT4v showed the greatest anticoagulant potential (124±3.3 s) compared to the other fractions, probably because of the greater sulfate/sugar ratio of the FT4v compared to the other fractions. The Pearson correlation coefficient (Table 5) for the sulfate/sugar ratio and the anticoagulant activity showed a strong positive correlation ($P=0.9811$), suggesting

Table 3 Chemical composition of sulfated galactan-rich fractions (FT) from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion for 18 h with 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), and 10 vol (FT10v) of NaCl 0.25 M; nd not detected; $n=4$

Sulfated galactan-rich fractions	Total sugar (%)	Sulfate (%)	Sulfate/ total sugar (%)	Protein (%)	Phenolic compounds (%)
FT4v	67.8±1.0	31.8±1.7	0.47	nd	0.4±0.0
FT6v	72.9±0.2	26.8±0.4	0.37	nd	0.2±0.0
FT8v	71.6±0.9	28.2±0.6	0.39	nd	0.2±0.0
FT10v	71.6±1.0	27.7±0.9	0.39	nd	0.7±0.0

Table 4 Signs of infrared spectroscopy evaluation of sulfated galactan-rich fractions from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion for 18 h with 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), and 10 vol (FT10v) of NaCl 0.25 M; *n*=4

Sulfated galactan-rich fractions	Signs (cm^{-1})
FT4v	3346, 2950, 1639, 1418, 1228, 1154, 1037, 924, 841, 699
FT6v	3378, 2963, 1633, 1428, 1231, 1154, 1030, 924, 849, 702
FT8v	3378, 2959, 1637, 1418, 1226, 1156, 1037, 923, 849, 703
FT10v	3388, 2952, 1641, 1419, 1224, 1156, 1037, 924, 841, 702

that galactan sulfation is an important factor in the anticoagulant activity of these polymers.

NO production

The sulfated galactan-rich fractions stimulated NO production in a dose-dependent manner in the presence of LPS (Fig. 4a). This result suggests that in some tested concentrations the fractions function synergistically with LPS stimulating NO production; greater NO levels were observed in RAW cells treated with fractions FT4v, FT6v, FT8v, and FT10v at 2 mg mL^{-1} together with LPS when compared to the positive control (macrophages treated with just LPS). Fractions FT4v, FT6v, FT8v, and FT10v at 2 mg mL^{-1} promoted production of 17.72, 34.58, 15.52, and 36.22 μM of sodium nitrite (10^5 cells)$^{-1}$, respectively. The fractions with a lower sulfate/sugar ratio exhibited greater NO stimulatory action, as confirmed by the high negative Pearson correlation coefficient between
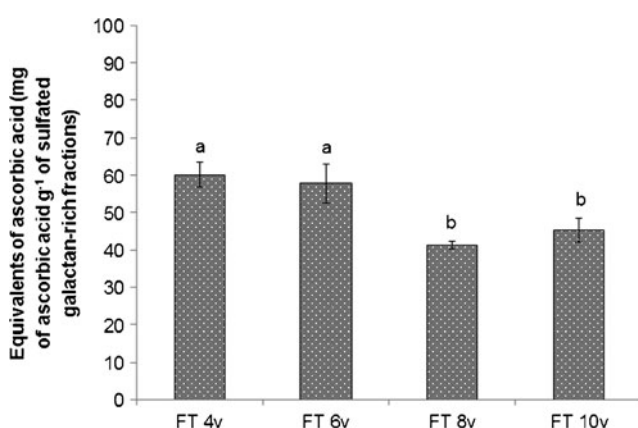
the sulfate/sugar ratio and NO levels in the presence of LPS ($P=-0.9667$).

The sulfated galactan-rich fractions promoted NO production by RAW cells in a dose-dependent manner in the absence of LPS (Fig. 4b). Thus, the fractions were able to positively modulate the macrophage response related to NO production even in the absence of LPS. The control cells treated with LPS promoted the production of 11.97 μM of sodium nitrite (10^5 cells)$^{-1}$. Fractions FT4v and FT8v showed stimulatory action equivalent to LPS whereas fractions FT6v and FT10v promoted the production of 28.43 and 41.81 μM of sodium nitrite (10^5 cells)$^{-1}$, respectively. The results allow us to determine the high stimulatory action on NO production promoted by some *H. musciformis* fractions. The fractions with lower sulfate/sugar ratios exhibited greater stimulation of NO production, which was confirmed by the negative Pearson correlation coefficient between the sulfate/sugar ratio and NO levels in the absence of LPS ($P=-0.7915$). The findings allow us to conclude that slight variations in the extraction method of *H. musciformis* fractions leads to the extraction of polysaccharides with different biological activities.

Cytokine assay

The cytokine assay showed that sulfated galactan-rich fractions promoted the production of TNF-α and IL-6 by RAW cells at all tested concentrations (Fig. 5).

Macrophages treated with the *H. musciformis* fractions exhibited greater or similar concentrations of these cytokines compared to the positive control in which cells were treated with LPS, a recognized inducer of macrophage activation. Fractions FT4v, FT6v, FT8v, and FT10v at 1 mg mL^{-1}
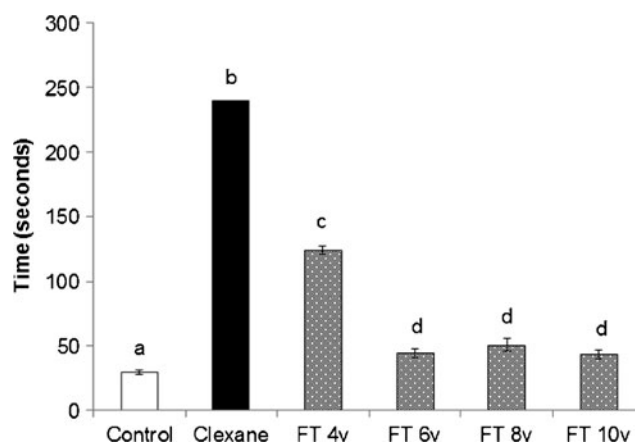
Fig. 2 Determination of total antioxidant capacity of sulfated galactan-rich fractions (100 μg) from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion with 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), and 10 vol (FT10v) of NaCl 0.25 M. *Different letters* indicate a significant difference between FT4v, FT6v, FT8v, and FT10v from *H. musciformis* by one-way ANOVA followed by Tukey–Kramer test (*P*<0.05), *n*=3

Fig. 3 Activated partial thromboplastin time (APTT) of sulfated galactan-rich fractions (100 μg) from *H. musciformis* precipitated with methanol and submitted to extraction/proteolytic digestion with 4 vol (FT4v), 6 vol (FT6v), 8 vol (FT8v), and 10 vol (FT10v) of NaCl 0.25 M. *Different letters* indicate a significant difference between control, clexane, FT4v, FT6v, FT8v, and FT10v by one-way ANOVA followed by Tukey–Kramer test (*P*<0.05), *n*=3

Table 5 Pearson correlation coefficient between sulfate/sugar ratio and biological assays evaluated in *H. musciformis* sulfated galactan-rich fractions

Assays	Pearson correlation coefficient (P)
Activated partial thromboblastin time	0.9811
Total antioxidant activity	0.5368
NO (with LPS)	−0.9667
NO (without LPS)	−0.7915
IL-6	−0.9275
TNF-α	−0.7193
HeLa 24 h	0.7893
HeLa 48 h	−0.1053
786-O 24 h	0.6642
786-O 48 h	0.7525
RAW 24 h	0.8937
RAW 48 h	0.7580

showed high immunostimulatory potential in RAW cells promoting IL-6 production of 48.36, 79.77, 56.38, and 67.75 pg $(10^5 \text{ cells})^{-1}$, respectively. Fractions FT4v, FT6v, FT8v, and FT10v at 1 mg mL^{-1} promoted TNF-α production of 58.44, 106.47, 81.12, and 94.17 pg $(10^5 \text{ cells})^{-1}$, respectively. Fractions with a lower sulfate/sugar ratio showed the highest macrophage immunostimulatory capacity, which was confirmed by the high negative Pearson correlation coefficient between the sulfate/sugar ratio and TNF-α levels ($P = -0.7193$) as well as the sulfate/sugar ratio and IL-6 levels ($P = -0.9275$) when the cells were treated with *H. musciformis* fractions at 0.1 mg mL^{-1}.

MTT reduction capacity assay

Sulfated galactan-rich fractions from *H. musciformis* promoted the reduction of MTT to formazan at all tested concentrations,

Fig. 4 Influence of sulfated galactan-rich fractions from *H. musciformis* on NO production detected by Griess reaction in RAW cells after 24 h of incubation with (**a**) and without (**b**) LPS. *Different letters x, y, z* indicate significant difference between the positive control and the negative control with tested concentrations of fractions. The *different letters a, b, c, d* indicate significant difference between the same concentrations (0.01; 0.1; 1; and 2 mg mL^{-1}) of different sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) by one-way ANOVA followed by Tukey–Kramer test ($P < 0.05$). *CP* positive control—cells incubated with LPS; *CN* negative control—only cells, $n = 3$
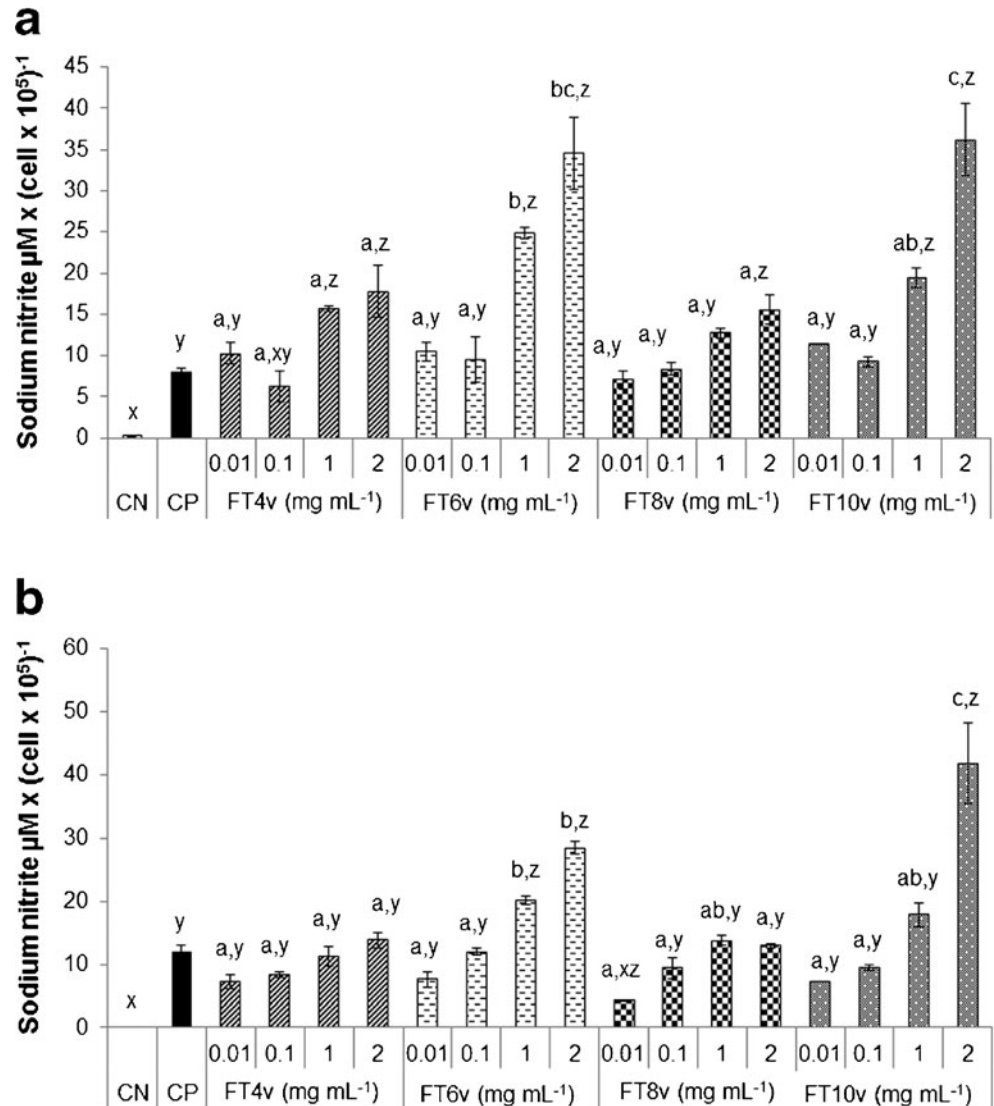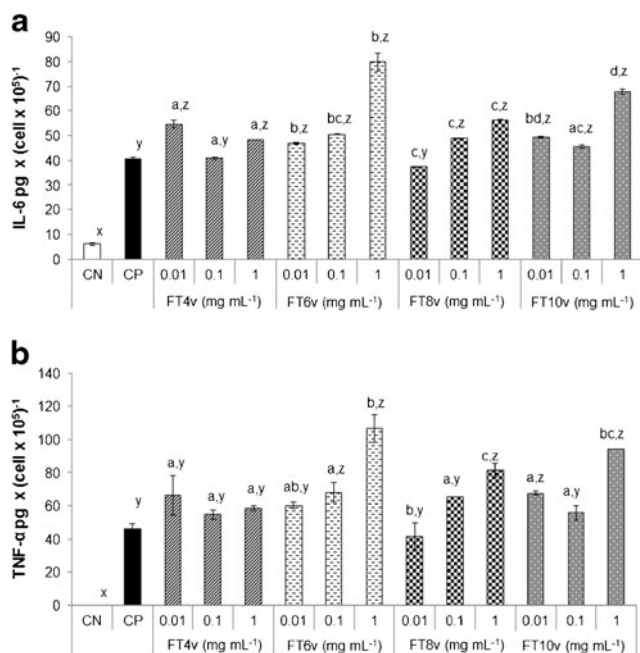
Fig. 5 Influence of sulfated galactan-rich fractions from *H. musciformis* on IL-6 (**a**) and TNF-α (**b**) production detected by enzyme immunoassay (EIA) in RAW cells after 24 h of incubation without LPS. The *different letters x, y, z* indicate a significant difference between the positive control and the negative control with tested concentrations fractions. The *different letters a, b, c, d* indicate a significant difference between the same concentrations (0.01, 0.1, 1, and 2 mg mL^{-1}) of different sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) by one-way ANOVA followed by Tukey–Kramer test (*P*<0.05). *CP* positive control—cells incubated with LPS; *CN* negative control—only cells, *n*=3

which indicates the lower reduction activity of the cells, and consequently fewer metabolically active cells when the cells were treated with fractions for 24 and 48 h. Fractions FT6v and FT10v exhibited marked cytotoxic potential in RAW cells treated for 24 h while fractions FT6v, FT8v, and FT10v showed marked cytotoxic potential in RAW cells treated for 48 h. On the other hand, FT4v exhibited lower cytotoxic potential than other fractions in RAW cells (Fig. 6).

Regarding tumor cells, at some tested concentrations, the *H. musciformis* fractions led to a decrease in the reduction of MTT to formazan by HeLa (Fig. 7) and 786 (Fig. 8) cells when the cells were treated for 24 and 48 h, with no statistically significant difference. A statistical comparison was performed between each tested concentration (0.01, 0.1, 1, and 2 mg mL^{-1}) of the evaluated fractions (FT4v, FT6v, FT8v, and FT10v). Fraction FT10v was more effective at decreasing the reduction of MTT to formazan in the two cell lines (HeLa and 786) and both evaluated incubation times (24 and 48 h), suggesting that FT10v has a greater cytotoxic action on these cell lines.

Discussion

The red alga *H. musciformis* is a recognized source of sulfated polysaccharides with significant industrial and pharmacological importance. Using an extraction methodology based on proteolytic digestion with a proteolytic enzyme and two volumes of NaCl for 18 h at 60 °C, sulfated galactans were obtained from *H. musciformis* with antioxidant (Alves et al. 2012a) and anticoagulant activities (Alves et al. 2012b). It was possible to obtain sulfated polysaccharides with different activities and we were interested in determining the optimal method of extraction to obtain polysaccharides with particular biological activities, such as anticoagulant, antioxidant, and immunomodulatory activities.

Initially, we varied the extraction/proteolytic digestion time to assess its influence on the levels of polysaccharides obtained. It was found that 18 h was the optimum extraction time as a greater yield of fractions was obtained; therefore, this incubation time was used for subsequent assays.

Subsequently, we evaluated the influence of the mass/volume ratio of delipidated algae powder/solvent used in the extraction of sulfated galactan-rich fractions. By varying the mass/volume ratio of algae delipidated powder/0.25 M NaCl (4, 6, 8, and 10 vol) and an extraction time/proteolytic digestion of 18 h, fractions FT4v, FT6v, FT8v, and FT10v were obtained. These fractions showed different yields. The results showed that the yield of the sulfated galactan-rich fractions was greater when larger volumes of solvent were used.

The sulfated galactan-rich fractions showed similar infrared spectra with characteristic signals of sulfated polysaccharides such as sulfated galactans. The similarity of the fractions was also confirmed by agarose gel electrophoresis. The fractions showed similar electrophoretic profiles with equivalent electrophoretic mobility; i.e., the fractions interact similarly through their sulfate groups with diaminopropane, as observed by Dietrich and Dietrich (1976) with glycosaminoglycans. The pattern of staining with toluidine blue is consistent with the presence of sulfated polysaccharides owing to the characteristic metachromasia presented by sulfated compounds. Fraction FT4v exhibited more apparent metachromasia in an electrophoresis blade, which can be explained by the fact that this fraction is the most sulfated among the evaluated sulfated fractions.

The determination of the chemical composition of the sulfated galactan-rich fractions allowed the confirmation of the presence of sulfates. These results showed that the fractions exhibited polysaccharides with different sulfation patterns depending on the *m/v* ratio of algae delipidated powder/solvent used for the extraction process. Fraction FT4v showed the greatest sulfate/sugar ratio followed by FT8v, FT10v, and FT6v, showing that FT4v is composed of more sulfated polysaccharides, corroborating the agarose gel electrophoresis results. Extraction with four volumes of solvent enabled the

Fig. 6 Influence of sulfated galactan-rich fractions (FT4v, FT6v, FT8v, FT10v) from *H. musciformis* on the reduction of MTT to formazan on RAW 267.4 cells after 24 h (**a**) and 48 h (**b**) of incubation. *Different letters* indicate a significant difference between the same concentrations (0.01, 0.1, 1, and 2 mg mL^{-1}) of different sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) obtained from *H. musciformis* by one-way ANOVA, followed by Tukey–Kramer test ($P<0.05$), $n=3$
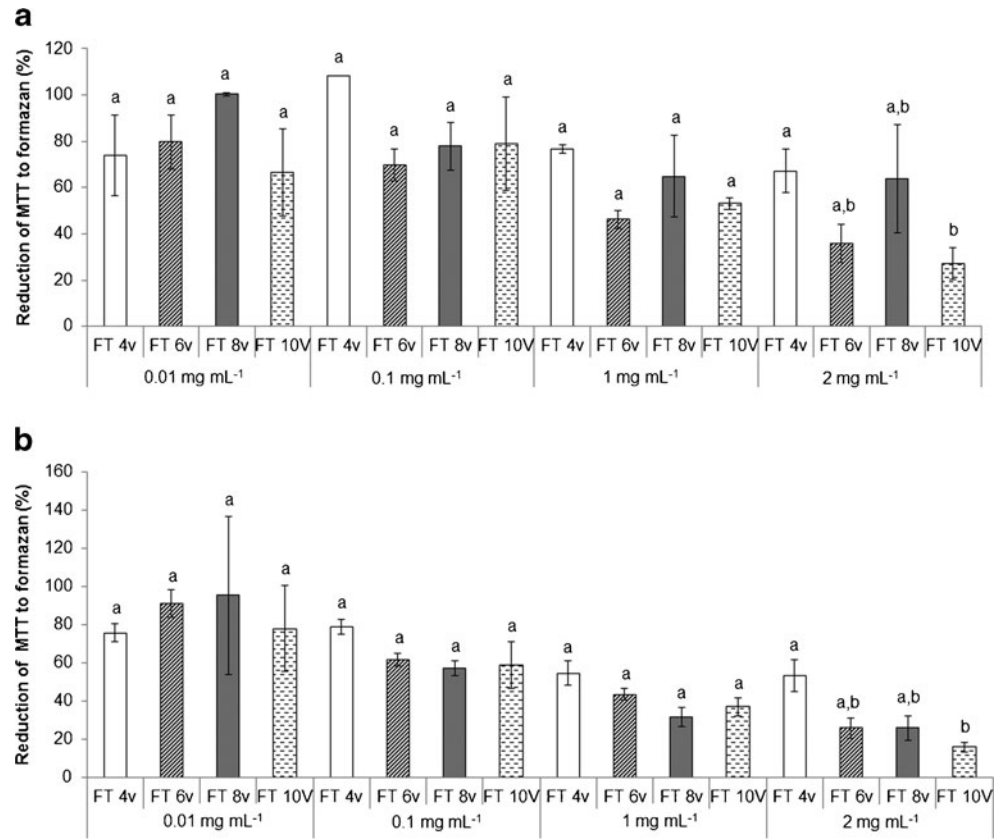

Fig. 7 Influence of sulfated galactan-rich fractions (FT4v, FT6v, FT8v, FT10v) from *H. musciformis* on the reduction of MTT to formazan on HeLa cells after 24 h (**a**) and 48 h (**b**) of incubation. *Different letters* indicate a significant difference between the same concentrations (0.01, 0.1, 1, and 2 mg mL^{-1}) of different sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) obtained from *H. musciformis* by one-way ANOVA, followed by Tukey–Kramer test ($P<0.05$), $n=3$
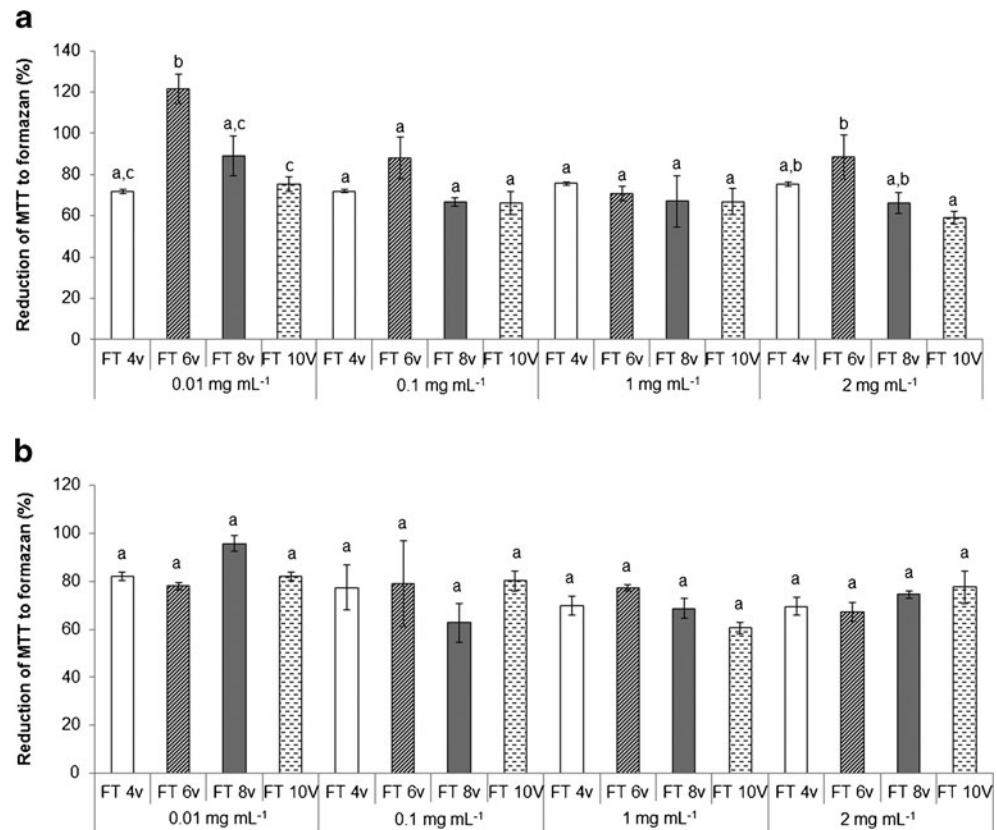
Fig. 8 Influence of sulfated polysaccharide-rich fractions (FT4v, FT6v, FT8v, FT10v) from *H. musciformis* on the reduction of MTT to formazan on 786-O cells after 24 h (**a**) and 48 h (**b**) of incubation. *Different letters* indicate a significant difference between the same concentrations (0.01, 0.1, 1, and 2 mg.mL^{-1}) of different sulfated galactan-rich fractions (FT4v, FT6v, FT8v, and FT10v) obtained from *H. musciformis* by one-way ANOVA, followed by Tukey–Kramer test (*P*<0.05), *n*=3
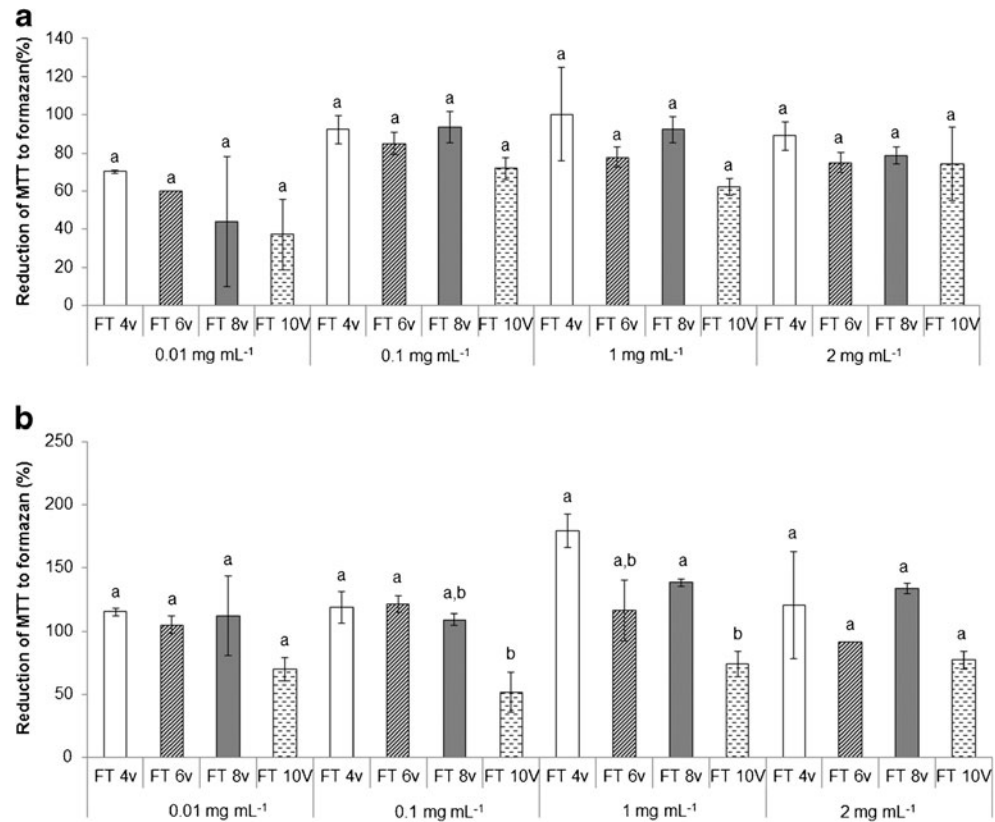
acquisition of polysaccharides with a greater degree of sulfation, which may be owing to the different aqueous solubilities of the polysaccharides present in the extracellular matrix of *H. musciformis*. The polysaccharides in fraction FT4v are more water-soluble than the other polysaccharides in the extracellular matrix and, for this reason, are present in greater concentrations in the fractions extracted with a smaller volume of sodium chloride solvent. Thus, it can be seen that slight modifications to the extraction process lead to the acquisition of different types of polysaccharides with different characteristics.

Many studies report the anticoagulant action of sulfated polysaccharides from marine organisms based on the APTT test. Compounds that promote prolonged APTT are associated with the inhibition of intrinsic coagulation pathway factors (Wijesekara et al. 2011). The fractions from *H. musciformis* with higher sulfate/sugar ratios, which indicates greater polysaccharide sulfation, have greater anticoagulant potentials, as was seen for fraction FT4v by the prolongation of APTT.

Thus, our results showed that the greater the degree of sulfation, the greater the anticoagulant activity of the sulfated polysaccharides obtained from *H. musciformis* (*P*=0.9811), suggesting a positive relationship between the sulfation and the anticoagulant activity of the sulfated galactan-rich fractions obtained in the study. The utilization of smaller volumes

of extraction solvent allows the obtainment of more sulfated polysaccharides with greater anticoagulant activity.

A relationship between the antioxidant activity, molecular weight, and degree of sulfation of sulfated polysaccharides from algae has been proposed (Zhao et al. 2004; Qi et al. 2005). Our results showed a weak positive relation between the degree of sulfation and the antioxidant action (*P*=0.5368). Regarding the volume of solvent used in the extraction method, we observed that the greater the solvent volume used for the acquisition of sulfated galactan-rich fractions, the lower the antioxidant activity.

The immunomodulatory activity of the compounds is related to their ability to modulate the production of molecules such as nitric oxide (NO) and inflammatory cytokines IL-6 and TNF-α. Sulfated polysaccharides can lead to the production of nitric oxide by inducing inducible nitric oxide synthase (iNOS) and proinflammatory cytokines (Leiro et al. 2007). NO is associated with several biological processes, such as immune defense, inflammation, and neurotransmission (Sharma et al. 2007). Cytokine secretion by activated macrophages is central to macrophages immunoregulatory role. TNF-α plays an essential role in host defense and can promote the induction of other immunoregulatory and inflammatory mediators (Baugh and Bucala 2001). Proinflammatory interleukin-6 (IL-6) is one of the most important immune and inflammatory mediators involved in the regulation of

several cellular functions, such as B and T cell proliferation and differentiation (Sobota et al. 2008).

Thus, we evaluated whether variation of the solvent volume used in the extraction process would lead to the obtainment of immunomodulatory polysaccharides. The Pearson correlation coefficient showed a negative relation between the immunomodulatory action of sulfated galactan-rich fractions of *H. musciformis* and the sulfate/sugar ratio of these polysaccharides. Therefore, fractions with the lowest sulfate/ sugar ratio containing less sulfated polysaccharides (FT6v, FT8v, and FT10v) showed greater immunostimulatory action owing to the increased levels of production of NO, TNF-α, and IL-6 cytokines by macrophages, while the fraction with the greatest sulfate/sugar ratio (FT4v) showed less stimulatory potential on the same cell line. These results suggest that the sulfate content is inversely proportional to the immunostimulatory potential of the sulfated galactan-rich fractions of *H. musciformis* and that the extraction process allowed the acquisition of fractions with different immuno-modulatory potentials.

The MTT test assesses the ability of cellular dehydrogenase enzymes to reduce tetrazolium salt to formazan in cells with active metabolism. As the tetrazolium ring is cleaved in active mitochondria, the reaction only occurs in living cells (Mosmann 1983). The Pearson correlation coefficients showed the existence of a directly proportional relationship between the sulfate/sugar ratio of sulfated galactan-rich fractions of *H. musciformis* and the percentage reduction of MTT to formazan in RAW cells treated for 24 ($P=0.8937$) and 48 h ($P=0.7580$). This suggests that the sulfate content of these fractions is important in maintaining the viability of these cells, as a greater reduction of MTT to formazan was observed in RAW cells treated with more sulfated polysaccharides (FT4v).

Costa et al. (2010) found no significant relationship when the correlation coefficient was evaluated for the sulfate content of polysaccharides from the red algae *Gracilaria caudata* and the inhibition of HeLa tumor cell proliferation. However, our results with sulfated galactan-rich fractions from *H. musciformis* do not agree with those of the study by Costa et al. (2010). The Pearson correlation coefficient showed a strong directly proportional correlation between the sulfate/ sugar ratio and the percentage reduction of MTT to formazan in HeLa cells treated for 24 h ($P=0.7893$) and 786 cells treated for 48 h ($P=0.7525$) with fractions from *H. musciformis*. These results suggest that the degree of sulfation of the sulfated galactan-rich fractions influences the viability of these cells; thus, sulfated galactan-rich fractions with a lower degree of sulfation promote less reduction of MTT to formazan and reduced viability, and consequently exhibit greater cytotoxic action on cancer cells.

Based on the Pearson correlation coefficient results, the sulfated galactan-rich fractions from *H. musciformis* with lower sulfate/sugar ratios, i.e., those with less sulfated poly-saccharides (FT6v, FT8v, and FT10v), showed greater immunostimulatory action while the fraction with the greatest sulfate/sugar ratio (FT4v) showed greater anticoagulant activity. Therefore, our results suggest that different extraction conditions can lead to the acquisition of sulfated galactan-rich fractions from *H. musciformis* with different biological activities. Thus, we believe that researchers in this area should be aware that slight modifications to the initial steps of any polysaccharide extraction method could yield polysaccharides with the desired biological activity.

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