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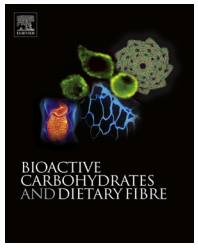

Bioactive polysaccharides from marine algae

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ABSTRACT

Laminaran, fucoidan and sulfated galactan were extracted from the seaweeds *Desmarestia distans*, *Lessonia vadosa*, and *Gigartina skottsbergii*, respectively. Modified polysaccharides were prepared by desulfation, depolymerization, and sulfation. Antioxidant, anticoagulant and immunostimulating activities of native and modified polysaccharides were assayed *in vitro*. The oversulfated derivatives showed high antioxidant capacity towards oxygen radical assay; however, no direct relation between sulfate content and antioxidant capacity was found. Oversulfated polysaccharides presented higher antioxidant capacity towards hydroxyl radicals than the native polysaccharide. Regarding the $ABTS^{\bullet+}$radical cation assay moderate inhibition values (35.1–3.4%) were observed. The anticoagulant activity of native and modified polysaccharides was measured using the activated partial thromboplastin time assay; the native sulfated galactan from *G. skottsbergii* presented the highest value, close to that shown by heparin at similar concentrations. The immunostimulating activity of polysaccharides was measured through their effects on bone marrow-derived mice dendritic cell maturation. The native sulfated galactan from *G. skottsbergii* presented good dose-dependent activity inducing increased levels of MHC class II in dendritic cells.

1. Introduction

In the last decades the study of polysaccharides from marine algae has gained renewed interest for their many valuable biological properties (Pomin & Mourão, 2010; Wijesekara, Pangestuti, & Kim, 2011). Brown seaweeds (Phaeophyceae) mainly produce alginic acid, and to lesser extent fucans and laminarans. Fucans, the polymers of L-fucose, may also contain galactose, mannose, xylose and uronic acids and sulfate groups; the term fucoidan is restricted to sulfated homo-L-fucans (Patankar, Oehninger, Barnett, Williams, & Clark, 1993; Chevolot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001; Bilan et al., 2002). Fucoidans have received much attention due to their many biological activities including, anticoagulant, antithrombotic, antitumor, antioxidant, antiviral and anti-inflammatory properties (Berteau & Mulloy, 2003; Li, Lu, Wei, & Zhao, 2008). Recently, commercial fucoidan had shown immunomodulatory properties on human and mice dendritic cells (Kim & Joo, 2008; Yang et al., 2008b). Laminarans are neutral 1→3-beta-D-glucans

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with beta-1,6 branching (Painter, 1983; Rioux, Turgeon, & Beaulieu, 2010). Lai et al. (2010) studied the immunomodulatory and adjuvant activities of a high molecular weight 1→3-beta-D-glucan fraction isolated from *Ganoderma lucidum*. They found that in mice this glucan showed an increase in the number of dendritic cells and displayed potent adjuvant activity while *in vitro* it induced the maturation of dendritic cells and stimulated the production of cytokines and chemokines. Agarans and carrageenans are the most common sulfated galactans from red seaweeds; they differ in the configuration of alfa-galactopyranosyl residues and in the pattern of sulfation (Lahaye, 2001; Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002; Campo, Kawano, Braz da Silva, & Carvalho, 2009). Various sulfated galactans have been studied in search of a relation between sulfate presence and anticoagulant activity (Farías, Valente, Pereira, & Mourão, 2000; Pereira, Melo, & Mourão, 2002; Pereira et al., 2005; Fonseca et al., 2008). It seems that there is no correlation between coagulation with sulfate content. However, Opoku, Qiu, and Doctor (2006) found that oversulfation of kappa-carrageenan gave a derivative with 30 times higher anticoagulant activity than the native carrageenan. Moreover, it has been reported that sulfated polysaccharides from seaweeds showed *in vitro* antioxidant capacity (Ruperez, Ahrazem, & Leal, 2002; Kim et al., 2007; Rocha de Souza et al., 2007; Barahona, Chandía, Encinas, Matsuhiro, & Zúñiga, 2011). Recently, Gómez-Ordóñez, Jiménez-Escrig, and Rupérez (2014) reported the antioxidant and anticoagulant activities of kappa-iota hybrid carrageenans from *Mastocarpus stellatus* (Rhodophyta). The brown seaweeds *Desmarestia distans* and *Lessonia vadosa*, and the green variant of tetrasporic *Gigartina skottsbergii* (Rhodophyta) grow abundantly in southern Chile, but so far they are not exploited for industrial purposes. The aim of this work is the study of antioxidant, anticoagulant, and immunostimulating properties of polysaccharides extracted from these polysaccharides. In order to study the relationship among sulfate content, molecular weight and biological activity, native polysaccharides were modified by oversulfation, desulfation and partial hydrolysis.

2. Materials and methods

2.1. Materials and general methods

D. distans (C. Agardh) J. Agardh, *L. vadosa* Searles, and the green variant of tetrasporic *G. skottsbergii* Setchellet Gardner were collected in Fuerte Bulnes (53°37′55 6″S, 70°55′17 9″W), Magellan region. Specimens were deposited in Sala de Colecciones, Departamento de Ciencias y Recursos Naturales, Universidad de Magallanes, Punta Arenas, Chile. Preparation of fucoidan from *L. vadosa* was performed according to Chandía and Matsuhiro (2008). Isolation of sulfated galactans from the green variant of tetrasporic *G. skottsbergii* and their chemical modifications were previously described (Barahona, Encinas, Mansilla, Matsuhiro, & Zúñiga, 2012). FT-IR spectra in KBr pellets were registered in the 4000–400 cm^{-1} region using a Bruker IFS 66*v* instrument (Bruker, Coventry, UK) according to Leal, Matsuhiro, Rossi, and Caruso (2008). Absorbance was measured in a Genesys 5 Thermospectronic spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). ^{1}H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra of the polysaccharides were recorded in D_2O, after isotopic exchange (3 × 0.75 mL) at 70 °C on a Bruker Avance DRX 400 spectrometer (Bruker, Coventry, UK) using the sodium salt of 3-(trimethylsilyl)-1-propane-d_4-sulfonic acid) as internal reference. Gas–liquid chromatography (GC) was carried out on a Shimadzu GC-14B chromatograph (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector using a SP 2330 column (0.25 mm × 30 m) and performed with an initial 5 min hold at 150 °C and then at 5 °C/min to 210 °C for 10 min. The helium flow was 20 mL/min. Molecular weight of polysaccharides was determined by the reducing end method (Park & Johnson, 1949; Cáceres, Carlucci, Damonte, Matsuhiro, & Zúñiga, 2000). Reagent grade solvents were purchased from Merck (Darmstadt, Germany); DEAE-Sephadex, Sephadex 100 and reagent grade chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Extraction of Desmarestia distans

Blades of *D. distans* were oven dried at 45 °C for 48 h. The dry seaweed (120 g) was milled and stirred for 30 min with 900 mL of *n*-hexane. The supernatant was concentrated *in vacuo* and the treatment was repeated four times. The treated seaweed was dried at room temperature for 48 h and soaked into 2000 mL of a solution containing 96% ethanol and 36% of formaldehyde in a 4:1 v/v ratio. After 72 h the seaweed was decanted and air dried. One hundred grams of the dried alga were stirred with 3% $CaCl_2$ solution at 85 °C for 4 h, and the mixture was cooled and centrifuged at 3000 × *g* for 20 min. The solid was recovered and the extraction process was repeated three more times. The supernatants were dialyzed using Spectra/Por membrane (MWCO 3500) (Spectrum Laboratories, Rancho Domínguez, CA, USA) against tap water, followed by distilled water, concentrated *in vacuo* and freeze-dried (Christ Alpha 1–2 Freeze Dryer, Osterode am Harz, Germany). The resulting solid was dissolved in 75 mL of distilled water, stirred for 2 h with 1 M HCl (50 mL) and centrifuged. The supernatant was neutralized with 1 M NaOH solution, dialyzed against distilled water, concentrated and freeze-dried.

2.3. Ion-exchange chromatography

The $CaCl_2$ extract from *D. distans* was dissolved in water (0.2 g/mL solution) and was deposited on a DEAE-Sephadex A-50 column (30 cm × 3 cm). Elution was carried out with a gradient increasing concentrations of NaCl solutions (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 1.6, 1.8, 2.0, and 2.5 M) and 3 mL fractions were collected. Elution was monitored by the Dubois method (Chaplin, 1986). Fraction 1 was submitted to total acid hydrolysis as previously described and the constituent monosaccharides were analyzed by GLC as alditol acetates (Chandía and Matsuhiro, 2008).

2.4. Partial hydrolysis of fucoidan from Lessonia vadosa

2.4.1. Partial acid hydrolysis

Native polysaccharide from *L. vadosa* (0.500 g) was stirred with 50 mL of a 0.5 M HCl solution at 60 °C. Aliquots were taken at

half an hour and then every hour until six hours. The reaction was stopped by addition of 2 M NaOH until neutrality. Each of the aliquots was dialyzed against distilled water for 9 h, exchanging water every hour, concentrated and freeze-dried. Each fraction (3 mg in 1 mL of distilled water) was chromatographed on a Sephadex G-100 column (30 × 3 cm) using 0.4 M NaCl solution as eluant. The column was calibrated with Blue Dextran 2000, dextran sulfates (500 and 8 kDa, Sigma) and glucose, and 3 mL fractions were collected. Elution was monitored with the phenol-H_2SO_4 acid reagent (Chaplin, 1986). The hydrolysis process was repeated with one gram of fucoidan during 4 h.

2.4.2. *Free radical depolymerization*

Partial free radical depolymerization of native polysaccharide from Li et al. (2008). After 6 h, the reaction was stopped by addition of EDTA (500 mg), and the solution was dialyzed using Spectra/Por membranes (MWCO 1000) against distilled water for 12 h, exchanging water every one hour. The resulting solution was concentrated *in vacuo* and freeze-dried (44.2% yield). The resulting solid was chromatographed on a Sephadex G 100 column as in Section 2.4.1.

2.5. *Desulfation of the sulfated polysaccharide from Gigartina skottsbergii*

Desulfation of the polysaccharide was performed as previously described (Barahona et al., 2012). In brief, the pyridinium salt of the polysaccharide was prepared by successive dialysis against pyridinium hydrochloride and then freeze-dried. The product obtained (0.1 g) was dissolved in 40 mL of a mixture of anhydrous DMSO–MeOH–pyridine (89:10:1 v/v/v) and maintained at 100 °C for 4 h. After the solution was cooled, distilled water (5 mL) was added and the mixture was dialyzed against distilled water and then freeze-dried (yield 50.3%).

2.6. *Sulfation of polysaccharides*

Sulfation was conducted according to Mähner, Lechner, and Nordmeier (2001). Briefly, a solution of the polysaccharide (0.200 g) in 20 mL of formamide was added to a solution of chlorosulfonic acid (0.8 mL) in dry pyridine (4 mL) and refluxed at 65 °C for 4 h, cooled and poured over ice-water with stirring. The resulting solution was added to 150 mL of cold methanol and the precipitate was separated by centrifugation, dissolved in distilled water and neutralized with 1 M NaOH, dialyzed against distilled water and freeze-dried.

2.7. *Antioxidant capacity assays*

2.7.1. *Oxygen radical absorbance capacity (ORAC) assay*

The consumption of fluorescein associated with its incubation with AAPH [2,2′-azo-bis(2-amidinopropane) dihydrochloride] was estimated from fluorescence measurements (Alarcón, Campos, Edwards, Lissi, & López-Alarcón, 2008). A reaction mixture containing 10 mM AAPH with and without the polysaccharide with different concentrations (0.1–1.0 mg/mL) in distilled water was incubated in a phosphate buffer (10 mM, pH 7.0) at 37 °C. Fluorescein (1.5 μM) consumption was evaluated from the decrease in the sample fluorescence intensity (excitation 491 nm, emission 512 nm). Fluorescence measurements were conducted using a Fluorolog-Spex 1681/0.22 m spectrofluorimeter (Spex, Metuchen, NJ, USA). Bandwidths of 1.25 nm were used for excitation and emission slits. Values of the fluorescence intensity I_F in relation to initial value I_F^0 (I_F/I_{F^0}) were plotted as time function. Integration of the area under the curve (AUC) was performed up to a time such that (I_F/I_{F^0}) reached a value close to zero. ORAC values were obtained from the ratio of the slope of the plots of (AUC–AUC^0) *versus* concentrations of polysaccharide and vitamin C.

2.7.2. *Ferrous ion chelating ability*

The method reported by Decker and Welch (1990) was used to investigate the ferrous ion chelating ability. The polysaccharides in the concentration range of 0.1–2.0 mg/mL were mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. The absorbance as time function was measured at 562 nm where the complex of ferrozine- Fe^{2+} showed strong absorbance. All absorbance measurements were done using an HP8453 diode array spectrophotometer (Hewlett Packard, Waldbronn, Germany).

2.7.3. *Hydroxyl radical scavenging activity assay (HRS)*

Hydroxyl radicals were generated from $FeSO_4$ and H_2O_2 (Fenton reaction) at 20 °C and their antioxidant capacity was studied using salicylate as probe (Smirnoff & Cumbes, 1989). The absorbance at 520 nm of aqueous solutions of 20 mM sodium salicylate, 1.5 mM $FeSO_4$, and varying concentrations of polysaccharide (0–1.5 mg/mL) were measured as time function immediately after the addition of H_2O_2.

2.7.4. *ABTS assay*

The ABTS radical cation ($ABTS^{\bullet+}$) was produced by the reaction of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) with 2.45 mM potassium persulfate at room temperature for 16 h. The ABTS radical cation solution was diluted with PBS (pH 7.0) to an absorbance of 0.70 at 734 nm. To polysaccharide solutions (0.3 to 20 mg/mL), 500 μL of $ABTS^{\bullet+}$ was added. Absorbance spectra and the absorbance at 734 nm were registered in order to measure the kinetics of the reaction. The inhibition of $ABTS^{\bullet+}$ was calculated as follows: Inhibition rate (%) = $100 \times (Abs_0 - Abs_{45})/Abs_0$ where Abs_0 was absorbance of $ABTS^{\bullet+}$ and Abs_{45} was the absorbance of the test group at 45 min.

2.8. *Anticoagulant activity by APTT assay*

Human plasma (0.4 mL) from healthy donors was mixed with 0.1 mL of polysaccharide samples ($1–7 \times 10^{-3}$ mg/mL), and heparin ($0.2–6.0 \times 10^{-3}$ mg/mL) (Claris Lifescience, Ahmedabad, India). All samples were incubated with 0.005 mL of STA-Cephascreen solution (DiagnosticaStago Inc., Parsippany, NJ, USA) at 37 °C for 4 min. Then, 0.005 mL of 0.025 M $CaCl_2$ solution was added and the clotting time was measured in triplicate in a STA Compact (DiagnosticaStago, Inc., Parsippany, NJ, USA) equipment and repeated on three different days. Distilled water was used as negative control. The anticoagulant activity was expressed as sample APTT/negative control APTT ($APTT_s/APTT_{NC}$).

2.9. Immunostimulating assay

2.9.1. Dendritic cells

Bone marrow-derived dendritic cells were obtained as previously described by Inaba et al. (1992). Briefly, female mice BALB/C of 8 weeks old were sacrificed by cervical dislocation and femurs and tibias were removed. Bones were placed in 70% alcohol, washed with phosphate buffered saline (PBS) and transferred into RPMI-1640 without supplement for bone marrow extraction. Cells were collected by centrifugation and erythrocytes were lyzed with ACK buffer pH 7.2 (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Bone marrow cells (1×10^6) were placed in RPMI-1640 medium supplemented with 10% SFB, 4 mM de L-Glutamine and 10 μg/mL of GM-CSF (granulocyte/macrophage colony-stimulating factor). The immature dendritic cells were obtained after 6 days of culture at 37 °C with 5% CO_2. The medium was changed on day 2 (with cytokine) and day 4 (without cytokine) aspirating 75% of the volume.

2.9.2. Cytotoxicity assay

Dendritic cells were incubated with the native and modified polysaccharides samples (10–1000 μg/mL) for 6 h at 37 °C and 5% CO_2. After incubation, treated cells were collected using a cell scraper and washed and recovered by centrifugation. Cells were suspended in 0.4 mL of IF (PBS pH7.2 and 2% fetal calf serum) and 2 μL of 1 mg/mL propidium iodide (IP) were added to each sample. Viability was then examined using a BD FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed using FacsDiva. Analysis was done in triplicate and a sample of dendritic cells incubated with 30% ethanol was used as positive control.

2.9.3. Immunostimulating activity assay

The assay was conducted after Spisek et al. (2004). Briefly, the native polysaccharides samples and the modified polysaccharides (10–1000 μg/mL) were incubated with dendritic cells for 6 h at 37 °C and 5% CO_2. After incubation, cells were removed and centrifuged at $1800 \times g$ during 8 min. The cells were suspended in 0.3 mL of IF containing PE conjugated anti-mouse CD11c (clone HL3, Pharmingen, 1:300) and FITC conjugated anti-mouse MHC II antibodies (IA^d clone AMS-32.1, Pharmingen, 1:300). The samples were incubated at 4 °C for 45 min and then 0.5 mL of IF was added. The resulting mixtures were centrifuged at $1800 \times g$ during 8 min at room temperature and the pellets were suspended in 0.4 mL of IF and 1–2 μL of IP was added. Samples were analyzed by flow cytometry and data were expressed as Mean Fluorescence Intensity (MFI). Analyses were performed in triplicate.

2.10. Statistical analysis

The data obtained were means ± S.D. of three determinations, and followed by the Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

3. Results and discussion

3.1. Extraction, characterization and chemical modifications of polysaccharides

Blades of *D. distans* were pretreated with *n*-hexane, followed by formaldehyde-ethanol to eliminate lipids and polymerize phenolic compounds, respectively. Extraction of *D. distans* with calcium chloride afforded in 5.5% yield, a white powder which was separated into two fractions by ion-exchange chromatography. Fraction I (32.3% yield) that eluted with distilled water showed to contain glucose as a sole monosaccharide by total hydrolysis and GLC analysis. Its IR spectrum presented a characteristic band at 890 cm^{-1} assigned to beta-linked glucopyranosyl residues; this result was confirmed by NMR spectroscopy. The ^1H NMR spectrum showed a doublet centered at 4.80 ppm ($J = 8.0$ Hz), while the ^{13}C NMR spectrum showed six signals at 105.37 (C1), 76.1 (C2), 87.05 (C3), 70.95 (C4), 78.45 (C5), and 63.53 ppm (C6) (figures not shown) which are in good agreement with data published for 1→3 beta-glucan (Yang & Zhang, 2009). These results confirmed that the structure of fraction I corresponds to a laminaran. This neutral polysaccharide is not often found in brown seaweeds. Fraction II (7.2% yield) was eluted with a 0.2 M NaCl aqueous solution showed to contain 19.8% of sulfate group and 7.5% of uronic acids. Total acid hydrolysis of fraction II and GLC analysis of alditol acetates indicated that it contained 96.4% of glucose and 3.6% of fucose. These results indicate that this fraction is a mixture of neutral glucan and a fucan. Due to the low yield obtained, fraction II was not further studied. Fraction I of $CaCl_2$ extract from *D. distans* herein after named laminaran, was sulfated with chlorosulfonic acid affording in 92.3% yield, a sulfated polysaccharide with a glucose:sulfate molar ratio of 1.0:2.1. Its IR spectrum showed the characteristic bands of sulfate groups at 1232.9 cm^{-1}, 854 cm^{-1}, 821.7 cm^{-1}, and 582.5 cm^{-1}. Moreover, the ^{13}C NMR spectrum of the sulfated polysaccharide showed new peaks (figure not shown), in relation to the native polysaccharide, at 72.65 and 72.34 ppm, which could be assigned to substitution by sulfate group at *O*-6 position, and a signal at 76.15 ppm which might be due to sulfation at *O*-4 position.

Extraction of the brown seaweed *L. vadosa* with $CaCl_2$ solution gave a polysaccharide in 15.4% yield. Total acid hydrolysis of the purified extract and GLC analysis of the reduced and acetylated hydrolyzate showed the sole presence of per-*O*-acetyl-fucitol. The ^1H NMR spectrum of the extracted polysaccharide was almost superimposable with the previously published spectrum of the extract from *L. vadosa*, indicating the presence of a fucoidan; spectroscopic analyses by IR and 2D NMR confirmed that its structure corresponded to alfa 1→3 fucoidan, mainly sulfated at position *O*-4 and partially sulfated at position *O*-2, as previously reported by Chandía and Matsuhiro (2008). This polysaccharide was also sulfated affording in 95.2% yield a derivative which contained 36.0% of sulfate groups.

The native polysaccharide from *L. vadosa* was partially depolymerized by two methods, an acidic depolymerization with 0.5 M HCl solution and a free radical depolymerization using Cu^{2+} as promoter of hydroxyl radicals. Proton NMR spectra of the polysaccharide and both depolymerized

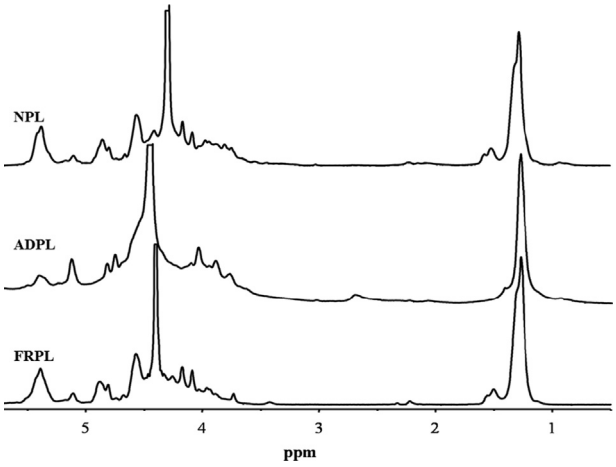

Fig. 1 – ^{1}H NMR (100.62 MHz) spectra in D_2O of fucoidan of *Lessonia vadosa* and its depolymerized fractions. NPL: native polysaccharide from *Lessonia vadosa*, ADPL: acid depolymerized polysaccharide from *L. vadosa*, FRPL: free radical depolymerized polysaccharide from *L. vadosa*.

fractions were very similar which indicated that the main structural features of native fucoidan was maintained during the depolymerization (Fig. 1). The acid partial hydrolysis afforded a homogeneous fraction (MW 6000) after 4 h of reaction (39.5%, yield), which contained fucose and sulfate group in the molar ratio 1:0.9. On the other hand, free radical depolymerization gave a homogeneous fraction (MW 8000) in 44.2% yield; it contained fucose and sulfate group in the molar ratio 1.0:1.2. The higher sulfate content of this fraction in relation to the one obtained by acid hydrolysis may be explained considering that in the free radical depolymerization reaction a complex is formed by interaction of Cu^{2+} with the anionic sulfate groups of the fucoidan which may prevent their hydrolysis; moreover, a selective depolymerization of 4-*O*-sulfate fucopyranosyl residues may take place (Torres, Ferraudi, Chandía, & Matsuhiro, 2011).

We recently reported the preparation and chemical properties of the sulfated galactan from the green variant of tetrasporic *G. skottsbergii* and its partial acid depolymerization (Barahona et al., 2012). It was shown that this sulfated galactan, although it had a lambda-carrageenan type backbone, contained lower amount of sulfate groups (25.9%) and a different sulfation pattern compared with commercial lambda-carrageenan. Sulfation with chlorosulfonic acid increased the sulfate content to 40.1%; however, the IR spectrum is very similar to that of the native polysaccharide, indicating that sulfate groups were not incorporated at new positions, but rather completed the sulfation at positions *O*-2 and *O*-6 of the galactopyranosyl residues. Besides, the native sulfated galactan from the green variant of tetrasporic *G. skottsbergii* was desulfated by solvolysis, giving a 50.3% yield of a polysaccharide which by elemental analysis was shown to contain no sulfur.

3.2. *Antioxidant capacity assays*

Several methods for evaluating the *in vitro* antioxidant capacity are known; generally, they are based on scavenging capacity assays against specific reactive oxygen and nitrogen species or scavenging capacity assays against non-biological radicals. According to Niki (2011, 2012) peroxyl radicals ($ROO^{\bullet}$) are the most suitable radicals for the study of antioxidants since they are involved in lipid peroxidation as chain-carrying radicals. The oxygen radical absorbance capacity (ORAC) was applied to assess the antioxidant capacity of hydrophilic samples, such as beverages, tea, and biological samples (Cao, Sofic, & Prior, 1996; López-Alarcón, & Lissi, 2006; Omata, Saito, Yoshida, & Niki, 2008; Alarcón et al., 2008; Atala, Vásquez, Speisky, Lissi, & López-Alarcón, 2009; Poblete, López-Alarcón, Lissi, & Campos, 2009; Zulueta, Esteve, & Frígola, 2009). Recently, the ORAC method has been used in our group for evaluating the antioxidant capacity of seaweed polysaccharides; and in order to establish a comparison, hydroxyl radical scavenging capacity assay (HRS) was also evaluated (Barahona et al., 2011, 2012). The hydroxyl radicals produced in HRS assay was assessed by the competitive reaction with Brillant Green dye, kinetics of the reaction with seaweed polysaccharides showed complex behavior, indicating a fast consumption of Brillant Green followed by formation of new products. It was suggested that two mechanisms may be involved in HRS capacity, the suppression against hydroxyl radical generation by chelation of Fe^{+2} with sulfate groups of the polysaccharide, and the chemical reaction of radicals with the polysaccharide. Salicylate was also used as a probe (Smirnoff & Cumbes, 1989). Salicylate in the presence of Fe^{2+} forms a complex that absorbs with a maximum at 520 nm; absorption decreases in the presence of hydroxyl radicals due to the salicylate hydroxylation. In the last decades many studies on the antioxidant capacity *in vitro* of polysaccharides using free radical scavenging assays, such as DPPH free radical, have been published (Ruperez et al., 2002; Chen, Xie, Nie, Li, & Wang, 2008; Yang, Zhao, Shi, Yang, & Jiang,2008a; Kong et al., 2010; Zhang, Li, Wu, & Kuang, 2012; Chen et al., 2013). However, DPPH free radical is soluble in organic solvents and the DPPH assay is not suitable for hydrophilic compounds (Niki, 2011). The long-lived 2,2′-azinonobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation, which has to be generated from ABTS is soluble in water and organic solvents and can be used for the determination of antioxidant capacity of hydrophilic and lipophilic compounds and biological samples (Niki, 2011).

3.2.1. *Oxygen radical absorbance capacity (ORAC) assay*

The ORAC method is based in hydrogen atom abstraction. It has the advantage to produce oxygen centered radicals through the decomposition of an azo-compound (Alarcón et al., 2008; Pereira et al., 2005). The peroxyl radicals scavenging was measured using fluorescein as fluorescent probe that presents an intensive emission band in the region 480–580 nm. In the presence of peroxyl radicals the fluorescence decreased and showed a total consumption at 15 min reaction with a negligible inhibition time. The addition of polysaccharides, measured by the integrated area under the fluorescence decay, decreased the reaction rate and then increased the consumption time. In all cases the area under the kinetic plots was linearly related to the amount of the polysaccharide addition. The protective effects of the different polysaccharides, reported respect to vitamin C are depicted in Table 1. The ORAC values

Table 1 – Molecular weight and sulfate content of polysaccharides and ORAC values.

Polysaccharide	MW ($\times 10^3$)[a]	Sulfate content (%)	ORAC ($\times 10^{-3}$)
NPL	300	35.2±3.4	2.0±0.3
FRPL	8	41.4±4.2	1.0±0.1
OPL	200.8	36.0±6.9	19.4±0.4
LPD	305	0	19.1±0.6
OPD	368.1	43.4±4.3	18.6±1.5
NPG	1700	25.9±2.0	2.9±0.4
HPG	90	24.7±2.4	2.8±0.2
DPG	1200	0	2.0±0.1
OPG	1000	40.1±3.1	3.8±0.3

[a] Estimated average MW by the method of Park and Johnson. NPL: native fucoidan from *Lessonia vadosa*, FRPL: free radical depolymerized polysaccharide from *L. vadosa*, OPL: oversulfated polysaccharide from *L. vadosa*, LPD: laminaran from *Desmarestia distans*, OPD: oversulfated laminaran from *D. distans*, NPG: native sulfated galactan from the green variant of tetrasporic *Gigartina skottsbergii*, HPG: partially depolymerized polysaccharide from the green variant of tetrasporic *G. skottsbergii*, DPG: desulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPG: oversulfated polysaccharide from green variant of tetrasporic *G. skottsbergii*.

do not show a clear correlation with the sulfate content. The value for oversulfated fucoidan (OPL) from *L. vadosa* is one order of magnitude higher than that for the native fucoidan (NPL), even both polysaccharides have similar sulfate content. Considerable degradation of the native fucoidan was found by sulfation, and a preferential sulfation at position *O*-2 in fucopyranosyl residue in the modified polysaccharide may explained its high antioxidant capacity. It was previously found that the ORAC values for sulfated polysaccharides, including commercial kappa-, iota- and lambda-carrageenans did not correlate with the sulfate content; moreover, it was observed that the sulfated polysaccharides that presented the highest ORAC values were the sulfated galactan from *Schizymenia binderi* and the fucoidan from *L. vadosa*, both with sulfation at position *O*-2 (Barahona et al., 2011). If it is considered that the hydrogen abstraction occurs from the anomeric hydrogen of the internal monosaccharide units, the sulfate group in the *O*-2 position should decrease the hydrogen bond energy, and then increases the H atom abstraction reaction rate (Chen, Tsai, Huang, & Chen 2009). No relation between molecular weight of compounds and ORAC activity was observed, it can be seen on Table 1 that fucoidan (NPL) and the sulfated galactan (NPG) from *G. skottsbergii* present similar activity in relation to their respective depolymerized polysaccharides. It is noteworthy the high ORAC value for laminaran (NPD), a neutral polysaccharide extracted from *D. distans*. The antioxidant capacity of neutral polysaccharide was previously described, for example Luo and Fang (2008) reported superoxide radical and hydroxyl radical scavenging activities of neutral polysaccharides from ginseng, mainly composed of glucose linked beta 1→3; besides, Liu et al. (2013) extracted from the fern *Athyrium multidentatum* neutral heteropolysaccharides, in which glucans were major components, with antioxidant activity against superoxide and hydroxyl radicals. Recently, Du and Xu (2014) reported the ORAC activity of oat beta-glucans with various molecular weight, they found that the sample of highest molecular weight presented the highest antioxidant capacity. Furthermore, it can be seen in Table 1 that desulfation of the native sulfated galactan from *Gigartina skkotsbergi* afforded a neutral polysaccharide (DPG) with still significant ORAC value. In these cases, the antioxidant activity probably proceeds through a mechanism which involves the direct abstraction of H atoms from the OH groups of the sugar residues (Chen et al., 2009). All these facts indicate that structural features, such as the sulfate position, type of monosaccharide units or glycosidic linkages are involved in the antioxidant activity.

3.2.2. *Chelating activity and hydroxyl radicals antioxidant capacity*

The chelating activity of polysaccharides was followed by the competitive formation of the ferrozine-Fe^{2+} complex, which presents an intense band in the region of 400 to 650 nm with a maximum absorption at 562 nm (Berker, Güclü, Demirata, & Apak, 2010). The complex formation follows a rapid kinetics (<2 min) but in the presence of sulfated polysaccharides a notable decrease of the ferrozine-Fe^{2+} formation rate was found. This effect is linearly related to the polysaccharide concentration as depicted for the fucoidan from *L. vadosa* (NPL) in Fig. 2A. Further evidence of this activity is the fact that native laminaran and desulfated galactan, that do not contain sulfate groups, did not inhibit the ferrozine-Fe^{2+} complex. It was established that the kinetics of complex formation is related to the sulfate content and the molecular weight of the polysaccharide. Compounds with higher molecular weight showed a higher competition with ferrozine, as shown by the lowest rate formation for the Fe^{2+}-ferrozine complex. Fig. 2B presents the chelating effect of native and modified galactans from the green variant of tetrasporic *G. skottsbergii*. The oversulfated galactan (OPG) showed the highest activity as expected from its sulfate content. On the other hand, depolymerized galactan presented smaller chelating activity than the native polysaccharide, both of similar sulfate content, which agrees with its lower molecular weight. The molecular weight effect can also be observed from comparison of the chelating activity of the oversulfated polysaccharides. The chelating activity, for 1 mg/mL of polysaccharide, was 34%, 25% and 21% for oversulfated galactan, oversulfated laminaran and oversulfated fucoidan, respectively. This chelating effect is not expected only from the content of sulfate groups, and point to an important influence of the molecular weight in the Fe^{2+}-polysaccharide

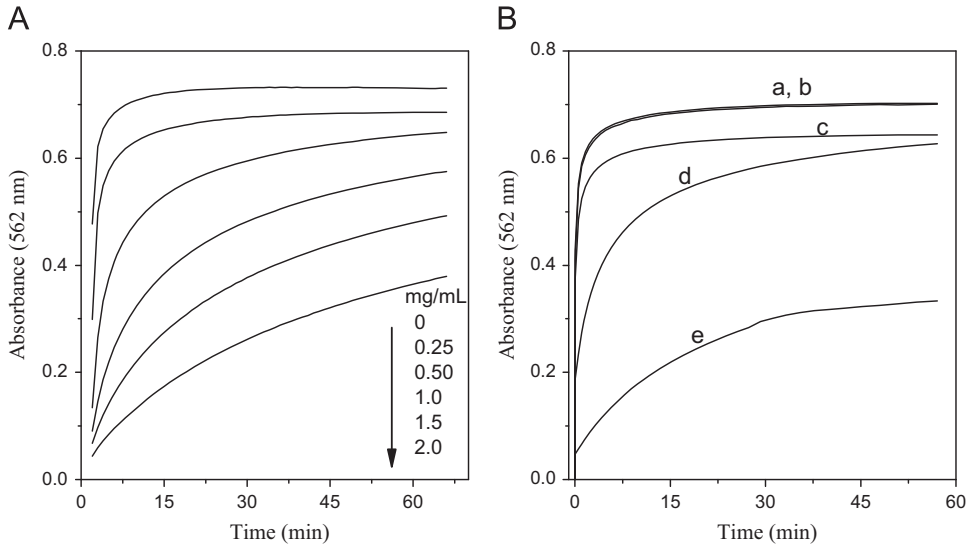

Fig. 2 – Effect of polysaccharide addition on the kinetic formation of ferrozine-Fe^{2+} complex. (A) Effect of fucoidan from *Lessonia vadosa* concentration. (B) Effect of polysaccharides from the green variant of tetrasporic *Gigartina skottsbergii* at 0.5 mg/mL: (a) in the absence of polysaccharide, and in the presence of (b) desulfated (DPG), (c) partially acid depolymerized (HPG), (d) native (NPG), (e) oversulfated (OPG). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chelating activity being more favorable at higher molecular weight.

It is well known that hydroxyl radicals are potent oxidants that react and produce damage to biomacromolecules, including polysaccharides. The high reactivity of the hydroxyl radical leads to its reaction with different functional groups of a molecule, consequently the damage to the target substrate will be dependent on several parameters. The reactivity of the hydroxyl radicals with the polysaccharides by the competitive reaction with salicylate hydroxylation was measured. Furthermore, the radical scavenging by polysaccharides shows a linear dependence with the concentration. This is contrary to results found with the widely used Brillant Green probe that forms new reactive species, and frequently shows a complex behavior with the polysaccharide concentration (Barahona et al., 2011). The hydroxylation of salicylate was inhibited in the presence of polysaccharides following a complex kinetics. It shows a very fast initial reaction followed by a slow reaction (Fig. 3). Further evidence of this complex kinetics is deduced from the absorption spectra at different reaction times (inset Fig. 3). At short times a new band in the region of 325–440 nm appeared, which decreased at longer reaction times. This result indicates a fast reaction of the hydroxyl radicals with the polysaccharide to give secondary products that react at slower rate. In general, it has been reported that the antioxidant activity of polysaccharides from marine algae increases with the increase of sulfate content, as well as by the suppression of the hydroxyl radical generation by chelating effect (Qi et al., 2005; Wang, Zhang, Zhang, & Li, 2008; Hu, Liu, Chen, Wu, & Wang, 2010). In order to have a comparison of the antioxidant capacity of polysaccharides here studied, the antioxidant capacity was expressed as the inhibition of the salicylate-Fe^{2+} complex absorbance at 10 min of reaction and at a constant polysaccharide concentration as presented in Table 2. It is interesting to note

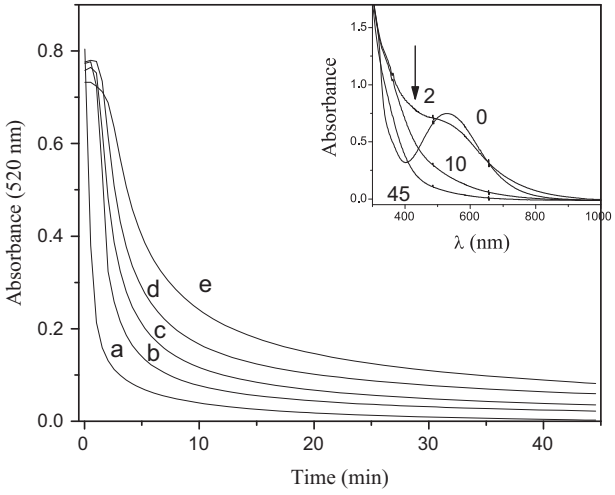

Fig. 3 – Decrease of the salicylate-Fe^{2+} complex absorbance in (a) absence of polysaccharide and in the presence of oversulfated laminaran from *D. distans* (OPD): (b) 0.125 mg/mL, (c) 0.25 mg/mL, (d) 0.5 mg/mL, and (e) 1.0 mg/mL. Inset: spectra at different reaction times in the presence of 1 mg/mL of OPD.

that hydroxyl radical antioxidant capacity is related to the chelating activity of these polysaccharides. The formation of polysaccharide-Fe^{2+}complex decreased the generation of the hydroxyl radicals through the Fenton reaction (H_2O_2 and Fe^{2+}). A clear relation between sulfate content and inhibition value was found, oversulfated polysaccharides presented the highest activity. The effect of the molecular weight is noticeable in the partial acid depolymerized sulfated galactan from *G. skottsbergii* that showed lower inhibition value than the native galactan; however, this result is not in accordance with Gómez-Ordóñez et al. (2014) who reported that low molecular

Table 2 – Antioxidant capacity of polysaccharides towards hydroxyl radicals at 10 min reaction. Polysaccharide concentration 1 mg/mL.

Sample	Inhibition (%)
NPG	58.6±6.3
OPG	86.0±4.5
HPG	38.0±1.8
OPD	63.0±3.4
NPL	62.8±2.2
OPL	79.1±2.9
FRPL	60.9±3.2

NPG: native sulfated galactan from the green variant of tetrasporic *Gigartina skottsbergii*, OPG: oversulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, HPG: partially acid depolymerized polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPD: oversulfated laminaran from *Desmarestia distans*, NPL: native fucoidan from *Lessonia vadosa*, OPL: oversulfated polysaccharide from *L. vadosa*, FRPL: free radical depolymerized polysaccharide from *L. vadosa*.

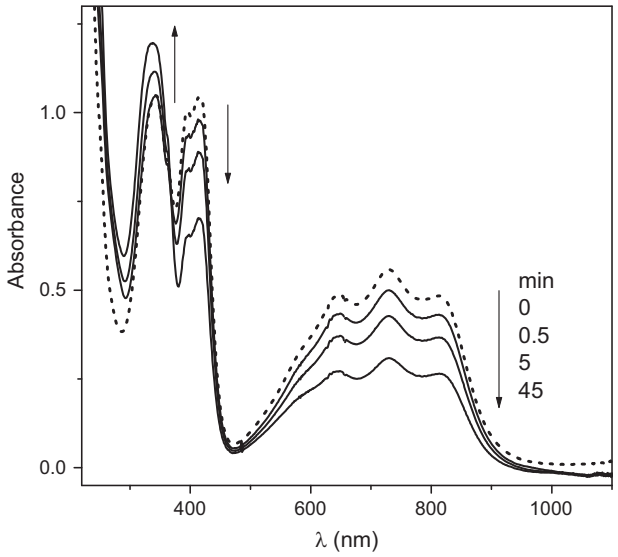

Fig. 4 – Decrease of ABTS$^{\bullet+}$ radical cation absorbance in the presence of free radical depolymerized polysaccharide from *Lessonia vadosa* (FRPL) (2 mg/mL) at different reaction times. Absorption spectra of ABTS$^{\bullet+}$: (–) in the absence, and (—) in the presence of polysaccharide.

weight polysaccharides, due to their conformation in solution present more potentially available hydroxyl groups reacting with free radicals. On the contrary, fraction obtained by free radical depolymerization of fucoidan did not present an increase in the antioxidant capacity towards hydroxyl radicals; similar results for the native polysaccharide from *Lesonia vadosa* and its free radical depolymerized fraction against hydroxyl radical using Brillant Green as a probe was found (Barahona et al., 2011). These results indicated that other structural features such as position of sulfate groups in the fucopyranosyl residues may have an effect on the antioxidant capacity. In addition, Yuan, Zhang, Li, Li, and Gao (2005) reported no effect of molecular weight on the antioxidant capacity towards hydroxyl radical of sulfated and acetylated fractions obtained by mild acid hydrolysis of κ-carrageenan. Comparison of the activity of the different polysaccharides indicates that the chemical structure of the sugar units also plays a role in the antioxidant capacity. It was previously found that the antioxidant capacity of commercial λ-carrageenan towards hydroxyl radicals also showed a complex kinetics, as measured with Brillant Green as probe. Its activity was higher than that observed for the sulfated galactan from the green variant of tetrasporic *G. skottsbergii* with lower sulfate content (Barahona et al., 2012). However, to understand the complex reaction of the highly reactive hydroxyl radicals it is not enough to carry out the measurements at a fixed point, it is necessary to know its time dependence. Also, more direct measurements of the hydroxyl radical reaction would be helpful.

3.2.3. *Scavenging of ABTS radical cation*

The antioxidant capacity of the modified polysaccharides respect to an electron transfer reaction was assayed with the ABTS$^{\bullet+}$ radical cation that presents a strong absorbance in the visible region. The effects of native polysaccharides were previously studied, it was reported that sulfated galactan and fucoidan displayed a fast reaction with the radical cation followed by a slow reaction to form secondary products (Barahona et al., 2011, 2012). Similar results were obtained in this work, it can be seen in Fig. 4 the absorption spectra of ABTS$^{\bullet+}$ monitored at different reaction times in the presence of free radical depolymerized polysaccharide from *L. vadosa* (FRPL) The absorption bands of the radical cation with maxima at 734 nm and 415 nm decreased and the band at 340 nm increased. This last absorption is attributed to the second reaction, since its enhancement is maintained at long reaction times. The fitting of the absorption decrease showed a second order decay with lifetimes lower than 1 min for the short living component and in the range of 10 to 50 min for the long component, depending on the polysaccharide concentration. Interestingly the short decay involved most of the absorption decay. This biphasic kinetics of ABTS$^{\bullet+}$ bleaching suggests a very fast reaction of the polysaccharide with the radical cation to form a product that reacts with slow rate to form other products. These findings indicate that, to obtain comparative values of the antioxidant capacity of different compounds it is necessary to know the kinetic pattern involved in the reaction with ABTS$^{\bullet+}$. As an approximation, the bleaching at 45 min of reaction of a 2 mg/mL polysaccharide solution was calculated (Table 3). Even it is difficult to obtain a comparison due to the complex reaction behavior, these results show that all the polysaccharides presented inhibition activity towards ABTS$^{\bullet+}$. It is shown that fucoidan from *L. vadosa* presented the highest inhibition value, like the carrageenan from the red seaweed *Schizymenia binderi* with similar antioxidant capacity which is sulfated at position O-2 of glycosyl residues (Barahona et al., 2011). In all cases the oversulfated polysaccharides presented the lower activity; results are in accordance with those reported for the ABTS$^{\bullet+}$ scavenging capacity of polysaccharides from *Auricularic auricular* mushroom whereas the sulfated derivatives with degree of substitution ~1 failed to exhibit stronger activity than the native neutral polysaccharides (Zhang et al., 2011). In addition, it was previously observed a decrease of the activity towards the radical cation

Table 3 – Inhibition (%) at 45 min reaction time of $ABTS^{\bullet+}$ by polysaccharides (2 mg/mL).

Samples	Inhibition at 45 min (%)
NPG	20.7 ± 2.3
DPG	13.8 ± 1.4
OPG	3.4 ± 0.6
HPG	22.1 ± 2.2
OPD	16.7 ± 2.2
NPL	35.1 ± 3.9
FRPL	24.0 ± 0.8
OPL	19.5 ± 1.5

NPG: native sulfated galactan from the green variant of tetrasporic *Gigartina skottsbergii*, DPG: desulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPG: oversulfated polysaccharide from green variant of tetrasporic *G. skottsbergii*, HPG: partially acid hydrolyzed polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPD: oversulfated laminaran from *Desmarestia distans*, NPL: native fucoidan from *Lessonia vadosa*, FRPL: free radical depolymerized polysaccharide from *L. vadosa*, OPL: oversulfated polysaccharide from *L. vadosa*.

with the increase in the sulfate content for commercial κ-, ι-, and λ-carrageenans (Barahona et al., 2011). Desulfated galactan from tetrasporic *Gigartina skottsbergi* presented significant inhibition of $ABTS^{\bullet+}$, with similar values to some of the oversulfated derivatives. It has been reported that neutral polysaccharides from *Dendrobium* plants showed high antioxidant capacity towards $ABTS^{\bullet+}$. Luo, He, Zhou, Fan, and Chun (2010) isolated from *D. nobile* four neutral mannogluco-galactans of MW between 136 and 11.4 kDa, although all the polysaccharides presented antioxidant capacity, the polysaccharide with MW 11.4 kDa presented high antioxidant capacity close to vitamin C. It is noteworthy that the authors also assayed the scavenging capacity of the polysaccharides towards DPPH radical, and found that only the polysaccharide with the lowest MW showed significant capacity. Luo and Fan (2011) reported the antioxidant capacity of *D. fimhriatum* polysaccharide (MW 209 kDa) and also found $ABTS^{\bullet+}$ scavenging capacity close to vitamin C, and insufficient antioxidant capacity towards DPPH radical. On the other hand, Shao, Chen, and Sun (2014) reported the antioxidant capacity of three polysaccharides with sulfate content between (19.4–11.4%) isolated from the brown seaweed *Sargassum horneri*, they found that the fraction with lowest MW (11.2 kDa) and with lowest content of sulfate groups presented the highest antioxidant capacity against $ABTS^{\bullet+}$. However, Gómez-Ordóñez et al. (2014), reported that fractions with the highest MW (1248–1425 kDa) and lowest MW (8–10 kDa) of galactans with low content of sulfate groups (3.9–1.9%) showed significant scavenging activity towards $ABTS^{\bullet+}$. Altogether, results found in this work and literature data indicate that the reaction of polysaccharides with $ABTS^{\bullet+}$ is complex, no correlation between sulfate content and antioxidant capacity was found, structural features such as monosaccharide composition, molecular weight, position of sulfate groups, and glycosidic linkage are involved.

3.3.3. *Anticoagulant activity*

The anticoagulant activity of native and modified polysaccharides was measured using the activated partial thromboplastin time (APTT) assay. Results are shown in Table 4. It was found that the native polysaccharide from the green variant of tetrasporic *G. skottsbergii* presented the highest value, among all the samples, close to those shown by heparin at similar concentrations. This polysaccharide presented sulfation at position *O*-2 of 4-linked alfa-D-galactopyranosyl residue and at position *O*-2 of 3-linked beta-D-galactopyranosyl residue, and it showed a molecular weight of $\sim 1 \times 10^6$ (Barahona et al., 2012). Partial acid depolymerization of the native polysaccharide decreased the anticoagulant activity probably due to the decrease in molecular weight. On the other hand, oversulfation of the native polysaccharide decreased the anticoagulant activity which may indicate that sulfation at other positions in the galactopyranosyl residues did not play a rol in the interaction with coagulation cofactors, and produced a deleterious effect. Recently, De Araujo et al. (2013) reported that regioselective oversulfation of kappa-, iota- and theta-carrageenan increased their anticoagulant activity determined by the APPT assay; they found that sulfation at position *O*-6 in beta-D-galactopyranosyl residues increased the activity. Pereira et al. (2002) studied the anticoagulant properties of sulfated galactans and fucans from invertebrates, they found that sulfation at position *O*-2 in alfa-L-galactans was responsible for the anticoagulant properties whereas sulfation at position *O*-4 in alfa-L-fucans was necessary for anticoagulant activity. In relation to anticoagulant properties of fucoidans, it was previously found that the native fucoidan from *L. vadosa* showed good anticoagulant activity measured by thrombin time (TT) while the free radical depolymerized fraction (MW 32,000) presented Li et al. (2008). However, it is shown in Table 4 that the free radical depolymerized fucoidan (FRPL) presented good anticoagulant activity whereas the partial acid depolymerized fucoidan (ADPL) had lower anticoagulant activity; the latter contained lower amount of sulfate groups. It is noteworthy that FRPL despite low molecular weight presented anticoagulant activity; its FT-IR spectrum (not shown) was very similar to the native polysaccharide and suggested mainly the presence of sulfate groups at position *O*-4 and partially at position *O*-2 in 3-linked alfa-L-fucopyranosyl residues. Wang, Zhang, Zhang, Song, and Li (2010) reported good anticoagulant activity for low molecular weight sulfated polysaccharides (MW 4800–7.800) isolated from *Laminaria japonica*; according to chemicals analysis, the polysaccharides were galactofucans which contained sulfate groups in high amount (30–42%), unfortunately the authors did not report the sulfate positions in the glycosyl residues. On the other hand, the desulfated polysaccharide from *G. skottsbergii* did not show anticoagulant activity in the concentration range of 0.01 to 1.44 mg/mL; moreover, oversulfation of sulfated polysaccharides from *G. skottsbergii* and *L. vadosa* did not increased their anticoagulant activity. These results agree with those published by Wang et al. (2010), and De Araujo et al. (2013) and indicate that the anticoagulant effect of sulfated polysaccharides was stereo-specific and independent of the sulfate content.

3.4. *Immunostimulating activity*

The immunostimulating activity of the native and modified polysaccharides was measured *in vitro* through their effects on dendritic cell (DC) maturation (Costa et al., 2010). DCs are antigen presenting cells which play a critical role initiating

Table 4 – Anticoagulant activity of native and modified polysaccharides[a]

NPG	Concentration (μg/mL)	24	15.2	7.6	4.6	3.0			
	APTT[a,b]	Máx.[c]	4.55±0.19	3.23±0.40	2.41±0.24	1.67±0.11			
NPD	Concentration (μg/mL)	640	64						
	APTT	0.97±0.03	1.04±0.06						
HPG	Concentration (μg/mL)	92.4	74	55.4	37	28	4.6		
	APTT	Max.	3.85±0.36	2.75±0.09	2.21±0.04	1.94±0.05	1.27±0.03		
FRPL	Concentration (μg/mL)	35	20	10	5	2.5	1	0.5	
	APTT	3.93±0.37	2.31±0.50	1.60±0.16	1.27±0.13	1.13±0.04	1.09±0.06	1.04±0.05	
ADPL	Concentration (μg/mL)	1430	720	520	260	100	50	10	
	APTT	2.68±0.33	1.85±0.09	1.75±0.14	1.46±0.13	1.24±0.01	1.10±0.06	1.03±0.02	
OPG	Concentration (μg/mL)	240	102	77	51.2	25.6	12.8		
	APTT	Máx.	3.97±0.41	3.30±0.46	2.47±0.17	1.89±0.05	1.58±0.02		
OPL	Concentration (μg/mL)	584	464	290	174	58	46	29	17
	APTT	1.52±0.24	1.41±0.18	1.26±0.14	1.17±0.07	1.08±0.07	1.03±0.07	1.02±0.04	1.01±0.03
OPD	Concentration (μg/mL)	680	544	340	204	68	54	34	20
	APTT	1.08±0.06	1.07±0.04	1.02±0.05	1.00±0.04	1.01±0.10	1.02±0.04	1.00±0.03	1.00±0.05
DPG	Concentration (μg/mL)	1440							
	APTT	1.06±0.07							
Hep	Concentration (μg/mL)	6.0	4.9	2.1	0.7	0.4	0.2		
	APTT	4.25±0.66	4.00±1.09	1.87±0.22	1.31±0.10	1.12±0.11	1.02±0.02		

NPG: native polysaccharide from the green variant of tetrasporic *G. skottsbergii*, NPD: native polysaccharide from *D. distans*, HPG: partially acid hydrolyzed polysaccharide from the green variant of tetrasporic *G. skottsbergii*, FRPL: partially free radical depolymerized polysaccharide from *L. vadosa*, ADPL: partially acid depolymerized polysaccharide from *L. vadosa*, OPG:oversulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPD: oversulfated polysaccharide from *D. distans*, OPL: oversulfated polysaccharide from *L. vadosa*, DPG: desulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, Hep:heparin.

[a] APTT is expressed as the quotient between sample APTT and negative control APTT.

[b] Error value is expressed using a $P=0.05$.

[c] Clotting time exceeded the equipment maximum value.

primary immune responses. These cells capture and process protein antigens derived from pathogens in the peripheral tissues and derived peptides are loaded in the major histocompatibility complex (MHC) class I or class II molecules on the DC cell surface. DCs thus undergo maturation and migrate to lymphoid organs where they activate resting T lymphocytes to initiate antigen-specific immune responses (Bauchereau et al., 2000). Mature dendritic cells have high levels of major histocompatibility complex (MHC) class molecules therefore analysis of expression level of the MHC class II is a good indicator of the induction of DC maturation.

Before determining the immunostimulating activity, the cytotoxicity effects of the polysaccharide samples were measured. All polysaccharide samples presented no cytotoxic effects in the concentration range of 10–100 μg/mL; moreover, it was remarkable that the native fucoidan from *L. vadosa* did not induce cytotoxicity in the 10–1000 μg/mL range. Fig. 5 shows the MHC class II levels on the surface of control and treated dendritic cells examined by flow cytometry. Values are expressed as mean fluorescence intensity (MFI). The sulfated galactan from tetrasporic *G. skottsbergii* presented good dose-dependent activity inducing increased levels of MHC class II in dendritic cells (NPG in Fig. 5). Activity was lost after increasing sulfate content but also after desulfation (DPG and OPG in Fig. 5) which indicate that it is not the content of sulfate groups but the native pattern of sulfation the structural feature needed for the immunostimulating activity. Sulfation recognition patterns may function as molecular recognition elements for dendritic cells as it has been demonstrated with glycosaminoglycans (Tully et al., 2006). For sulfated galactans, the complex and heterogeneous structures have precluded a better understanding of structural motifs which are required to disclose the mechanisms of recognition and biological activities. Native fucoidan from *L. vadosa* also induced dendritic cell maturation but difference did not reach statistical significance (NPL in Fig. 5). The immunostimulating effect of fucoidan in mouse spleen lymphocytes was previously studied by Choi, Kim, Kim, and

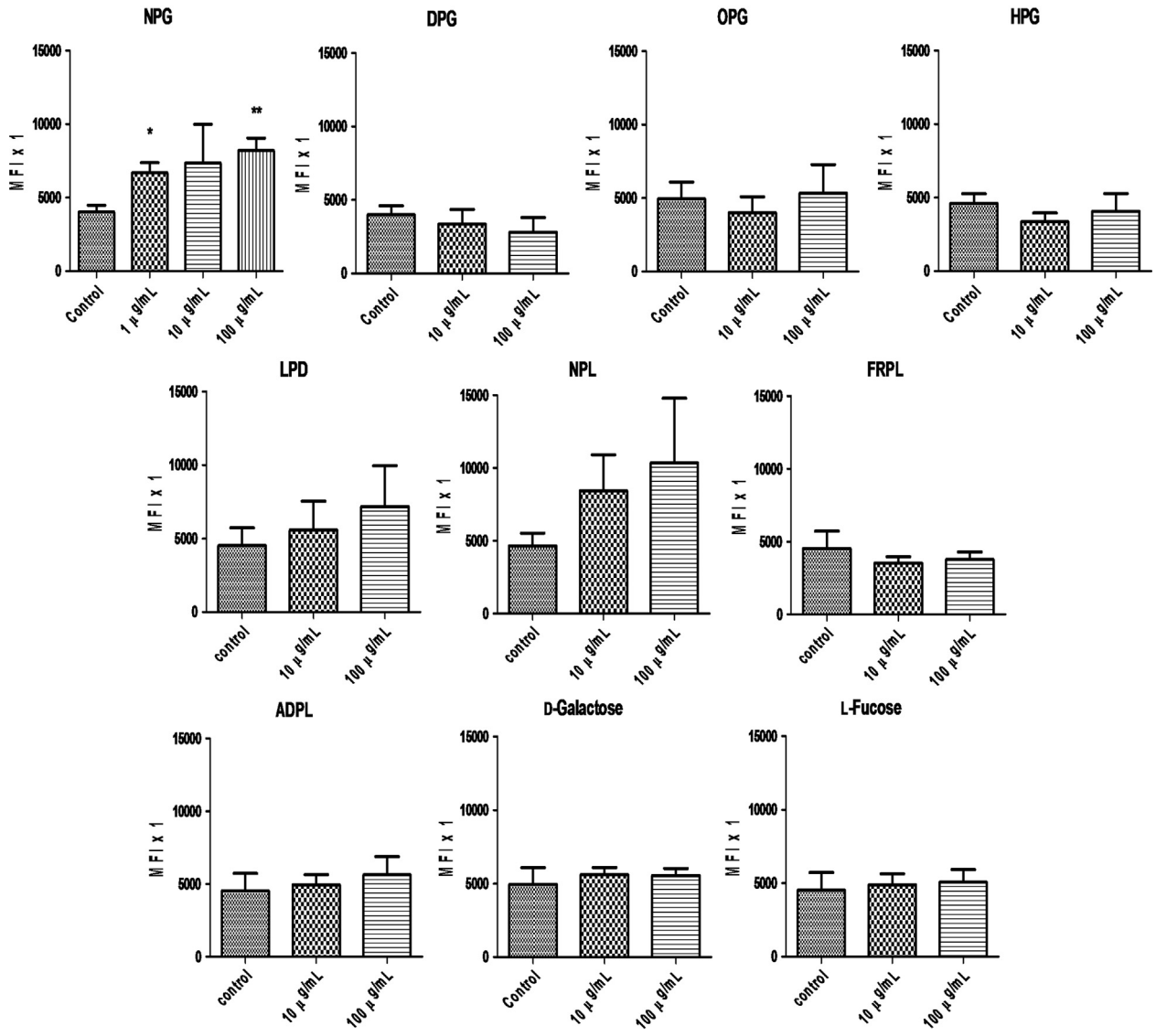

Fig. 5 – Mean Fluorescence Intensity (MFI) of mono- and polysaccharides samples. NPG: native sulfated galactan from the green variant of tetrasporic *Gigartina skottsbergii*, DPG: desulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, OPG: oversulfated polysaccharide from the green variant of tetrasporic *G. skottsbergii*, HPG: partially acid depolymerized polysaccharide from the green variant of tetrasporic *G. skottsbergii*, LPD: laminaran from *Desmarestia distans*, NPL: native fucoidan from *Lessonia vadosa*, FRPL: free radical depolymerized polysaccharide from *L. vadosa*, ADPL: acid depolymerized polysaccharide from *L. vadosa*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Hwang (2005) and in human dendritic cells by Yang et al. (2008b); however, both investigations employed commercial fucoidan, with unknown sulfate content and molecular weight. Depolymerization of fucoidan (FRPL and ADPL in Fig. 5) from *L. vadosa* and sulfated galactan from tetrasporic *G. skottsbergii* (HPG in Fig. 5) achieved lower molecular weight polysaccharides devoid of activity; furthermore, neither the constituent monosaccharides of the sulfated polysaccharides, L-fucose and D-galactose, showed activity. Results are in agreement with the model where the complex structure of polysaccharides, the structural motifs of sulfation and high molecular weights are important for immunostimulant activity tested on dendritic cells. Mechanisms of stimulation may involve C-type lectins carbohydrate recognition, a signal that potentiate cell endocytosis and microbicidal activity and contribute to innate and adaptive immunity (Sancho & de Sousa, 2012).

4. Conclusions

A simple correlation between sulfate content of polysaccharides and ORAC antioxidant capacity could not be found; structural features, such as the sulfate position, type of monosaccharide units or glycosidic linkages, could be involved. The chelating effect of sulfated polysaccharides agrees not only with the content of sulfate groups, and point to an important influence of the molecular weight in the

Fe^{2+}-polysaccharide complex formation being more favorable at higher molecular weight. The antioxidant activity of polysaccharides towards HO· radicals is related to the chelating activity of these compounds.

No relation was found between sulfate content of polysaccharides and the anticoagulant activity; results indicated that it depends on the position of sulfate groups, the structure of monosaccharide residues and the glycosidic linkages in the polysaccharides.

Results found in the immunomodulating effect of native and modified polysaccharides towards dendritic cells indicate a molecular weight effect over the sulfate content.

Based on the results found in this work, it can be concluded that native sulfated galactan from the green variant of tetrasporic *G. skottsbergii* is the most suitable polysaccharide among the native and modified polysaccharides here studied, for potential applications in food and pharmaceutical industries.

Acknowledgements

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