Salt pan brine water as a sustainable source of sulphated polysaccharides with immunostimulatory activity

Cláudia Nunes, Ana Rocha, Pedro Quitério, Sónia S. Ferreira, Alexandra Correia, Manuel Vilanova, Manuel A. Coimbra

PII: S0141-8130(19)30208-9
DOI: https://doi.org/10.1016/j.ijbiomac.2019.04.021
Reference: BIOMAC 12097

To appear in: *International Journal of Biological Macromolecules*

Received date: 8 January 2019
Revised date: 29 March 2019
Accepted date: 3 April 2019

Please cite this article as: C. Nunes, A. Rocha, P. Quitério, et al., Salt pan brine water as a sustainable source of sulphated polysaccharides with immunostimulatory activity, International Journal of Biological Macromolecules, https://doi.org/10.1016/j.ijbiomac.2019.04.021

Salt pan brine water as a sustainable source of sulphated polysaccharides with immunostimulatory activity

Cláudia Nunes[a,b*], Ana Rocha[b], Pedro Quitério[b], Sónia S. Ferreira[b], Alexandra Correia[c,d,e], Manuel Vilanova[c,d,f], Manuel A. Coimbra[b]

[a]*CICECO – Aveiro Institute of Materials, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal*

[b]*QOPNA/LAQV, Departamento de Química, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal*

[c]*i3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal*

[d]*IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal*

[e]*DGAOT, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, 4169-007, Porto, Portugal*

[f]*ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, 4050-313 Porto, Portugal*

* Author to whom correspondence should be addressed.
E-mail address: claudianunes@ua.pt (Cláudia Nunes)
Complexo de Laboratórios Tecnológicos
Campus Universitário de Santiago
3810-193 Aveiro

Abstract

Marine environments are an enormous source of materials with biological interest, such as sulphated polysaccharides, which have relevant biological activities. In this study the potential of salt pan brine water as an easily accessible source of sulphated polysaccharides was evaluated. This water revealed to have a high quantity of polymeric material, five times more than sea water, mainly composed by highly sulphated polysaccharides. Structural analysis identified a diversity of polysaccharides, namely rhamnans, fucans, mannans, xylomannans, glucuronomannans, galactans, and glucans. All these structures seem form complexes that are resistant to the salt pan conditions along salt production. These polysaccharides showed *in vitro* stimulatory activity for B cells, suggesting their potential application in nutraceutical and biomedical fields. Salt pan brine water is a valuable source of environmentally friendly and low-cost available bioactive compounds prone to be exploited.

1. Introduction

Marine environments, which cover more than 70% of the earth surface, are rich in a wide variety of organisms that have been extensively explored as potential sources of novel bioactive compounds. Most of the marine organisms can excrete polysaccharides for the environment. Polysaccharides, included in the extracellular polymeric substances present in sea water and estuaries, are among the most abundant materials [1], namely sulphated ones. The sources of these sulphated polysaccharides include a variety of microalgae, seaweeds, and animals, widely distributed in the ocean. Depending on their origin, polysaccharides may have different structures: I) sulphated galactans, carrageenan and agarans from red seaweeds; II) fucoidans from brown seaweeds; III) ulvans from green seaweeds; IV) chondroitin sulphates from sea cucumber and cartilaginous fish tissue; VI) sulphated glucan and heteropolysaccharides (containing galactose, fucose, arabinose and uronic acid) from sponges, and V) a wide range of heteropolysaccharides from marine microalgae [2-5].

The highly water soluble sulphated polysaccharides have been described to have important biological activities such as anticoagulant, antiviral, antioxidant, antitumoral, prebiotic, immunomodulatory, and anti-inflammatory [3-8]. In addition, these polysaccharides are generally considered biocompatible, having little or no toxicity. Besides their use as natural drugs (anticoagulant, antiviral, and anticancer), they are receiving growing interest for application in other health-related fields, namely for tissue engineering, immobilization of biomolecules, and coating of biomedical devices [9, 10]. Their ability to respond to external stimuli, such as pH and temperature, has allowed their use in the development of drug delivery systems [11]. Furthermore, marine polysaccharides have been proposed for other applications in food production and cosmetic industry [10].

Among the different marine species, seaweeds are one of the most abundant sources of marine polysaccharides, with a content up to 75% of their dry weight [6]. However, polysaccharides are mainly in their cell walls, needing exhaustive extraction and purification procedures. These methodologies also require the use of solvents, which render them environmentally and economically unsustainable. More convenient extraction methods have been developed, which include microwave-assisted extraction, ultrasound-assisted extraction, and enzyme-assisted extraction, with advantages regarding extraction time, energy consumption, and solvent use, being considered as green techniques. However, these extraction techniques are limited to research, since most of them are not yet applied at industrial scale [3]. Beyond seaweeds, another important source of sulphated polysaccharides that has been exploited is the microalgae, especially those that are able to produce exopolysaccharides. These are excreted to the medium and thus, obtained at a minimal cost when compared with those extracted from the cell walls. To be a sustainable source of the bioactive compounds, these microalgae must be grown in closed bioreactors under controlled conditions, which may prevent the production of high quantity of polysaccharides with homogenous characteristics [12]. For a commercial exploitation of sulphated polysaccharides, they need to be obtained in high amounts with simple extraction and separation processes and, consequently, low cost.

Salt evaporation ponds are man-made systems where seawater gives rise to sea salt due to its evaporation by the combined effect of wind and sunlight. Marine salt has polymeric material (144 mg/kg) that is mainly constituted by highly sulphated polysaccharides (46% w/w), composed by 45 mol% of sulphate esters, 21 mol% of uronic acids, 18 mol% of glucose, 15 mol% of galactose, and 13 mol% of fucose residues [13]. These polysaccharides have origin in sea water marine organisms [14] entrapped in the crystalline matrix. As these highly sulphated polysaccharides are very soluble in aqueous

solutions and resistant to microbial degradation enzymes, it is expected their accumulation also in salt pan brine water. Therefore, in this study it was evaluated the potential of salt pan brine water, which remained as a by-product after the salt crystallization, as a source of sulphated polysaccharides. Furthermore, polysaccharides have been shown to activate immune cells and to enhance both innate and adaptive immune responses [15]. Their potential application in new health therapeutics and/or incorporation in functional foods is thus worth exploring, and the immunostimulatory activity of the polysaccharide-enriched fractions was also assessed.

2. Experimental

2.1.Samples

The salt pan brine water was collected in Aveiro, in the North Cost of Portugal (Atlantic Ocean), at two stages of salt production, at the beginning of September and end of October (end of production), named as Brine Sept and Brine Oct, respectively. The seawater, which was used for comparison purposes, was only collected in October, at the entrance of the salt pan system. In addition, the salt produced in the same salt pan was collected for analysis.

2.2.Polysaccharides fractionation

2.2.1. Isolation of polymeric material

All water samples were dialysed using membranes with a cut-off of 12-14 kDa to obtain the polymeric material. The salt dialysis was performed as described by Silva *et al.* [13]. All the dialysis occurred under stirring with 2-3 times daily water exchanges, after at least 6 h, ended when the conductivity of the dialysate water was similar to that of the distilled water. Some drops of toluene and chloroform were added to the dialysis

water to avoid microbial contamination. Then, retentate was pooled and concentrated by rotary evaporation at 30 ºC until a volume of 30 mL. To eliminate the presence of any salt, the concentrated solution was again dialysed, controlling the conductivity of the dialysate until no more salt was present. The retentate (polymeric material) was then frozen, freeze-dried, and stored in a desiccator until analysis.

2.2.2. Fractionation of polysaccharides

The polymeric material of salt pan waters was fractionated by an anion-exchange chromatography. The sample (100 mg) was dissolved in 2.7 mL of Tris-HCl buffer (0.05 M, pH 7.4) and applied to a DEAE-Trisacryl M (Sigma-Aldrich) column (2.5 x 1.0 cm) pre-equilibrated with the Tris-HCl buffer. The column was eluted with buffer obtaining a neutral/positively charged fraction (Buffer). The retained material was eluted with a stepwise elution with Tris-HCl buffers increasing concentrations of NaCl, giving rise to four fractions, 0.125, 0.250, and 0.500 M. The column was washed with three column volumes (80 mL) for each elution buffer at a flow rate of 0.4 mL/min. Each fraction was dialysed (cut-off 12-14 kDa) against distilled water and freeze-dried.

The fractions from anion-exchange chromatography (0.125, 0.250, and 0.500 M of NaCl) were further fractionated by size-exclusion chromatography [16] using Sephacryl S-300 (Sigma-Aldrich) column (100 cm x 1.6 cm). The samples (10 mg) were dissolved in 1 mL of 100 mM sodium phosphate buffer at pH 7.4 with 3 M urea and eluted at a flow rate of 0.5 mL/min with the same buffer. Fractions of 1 mL were collected in a total of 140 mL. All fractions were assayed for sugars with the phenol-H_2SO_4 method to detect the presence of sugars, measuring the absorbance at 490 nm. Exclusion and total volume of the column were determined with blue dextran and glucose, respectively. The appropriate fractions were pooled, dialysed (cut-off 12-14 kDa) against distilled water, and freeze-dried.

2.3.Fractions characterization

2.3.1. Monosaccharides composition

Neutral monosaccharide composition was determined by reductive hydrolysis [17]. This methodology allows to have simultaneously hydrolysis (with trifluoroacetic acid, TFA) and reduction (with 4-methylmorpholine-borane, MMB) followed by acetylation to obtain the alditol acetates. These hydrolysis conditions prevent the degradation of acid sensitive monosaccharides, such as 3,6-anhydrogalactose.

The reductive hydrolysis was performed with 0.2 mL of 3 M TFA and 0.1 mL of 80 mg/mL MMB for 1 h at 120 ºC. Afterwards, at room temperature, were added more 0.1 mL of MMB and 0.1 mL of 2-deoxyglucose (1 mg/mL), as internal standard. The samples were dried by centrifugal evaporation (Univapo 100 ECH, UniEquip) under vacuum. The alditols were acetylated with acetic anhydride in the presence of 1-methylimidazol at 30 ºC for 30 min. The alditol acetate derivatives were analyzed by gas chromatography (GC) with a flame ionization detector (Perkin-Elmer Clarus 400) equipped with a 30 m x 0.25 mm and a film thickness of 0.15 μm DB-225 capilar column (Agilent J&W GC columns, USA). The operating temperatures of the injector and the detector were 220 ºC and 230 ºC, respectively. The GC oven temperature program was set for an initial temperature of 200 ºC for 1 min, raised to 220 ºC at 40 ºC/min, holding for 7 min at this temperature, then raised to 230 ºC at 20 ºC/min, and held for 1 min. The flow rate of the carrier gas (H_2) was set to 1.7 mL/min. All fractions were analyzed in duplicate.

Uronic acids (UA) content was determined by an adaptation of the *m*-phenylphenol colorimetric method [16]. The samples were submitted to pre-hydrolysis with 0.2 mL of 72% (w/w) H_2SO_4 during 3 h at room temperature and a hydrolysis with 1 M H_2SO_4

during 1 h at 100 ºC. UA concentrations were estimated using a calibration curve with D-galacturonic acid as standard (5-100 mg/L).

2.3.2. Sulphate esters determination

Sulphate esters content was determined by $BaCl_2$ turbidimetric method [18]. The samples were submitted to hydrolysis with 1 M HCl at 110 ºC for 5 h. Then, 3% (w/v) trichloroacetic acid and barium chloride-gelatin (1:1) reagent were added to the mixture, which was kept at room temperature for 15-20 min. The solution was analyzed at 360 nm (Jenway 6405 UV/Vis) against reagent blank containing distilled water instead of sample. The concentration of sulphate esters was determined using a calibration curve with K_2SO_4 as standard (50-600 mg/L).

2.3.3. Protein content

Protein content was calculated using elemental analysis (EA) through multiplication of %N by the nitrogen-to-protein conversion factor of 6.25. The EA was performed in a Truspec 630-200-200 elemental analyzer using 2 mg of each sample in duplicate. The operating temperatures of combustion furnace was 1075 ºC and the afterburner temperature was 850 ºC. Thermal conductivity was used to detect the nitrogen.

2.3.4. Glycosidic linkage and substitution analysis

Glycosidic linkages were determined by methylation analysis as described by Nunes et al. [16]. The partially methylated alditol acetates (PMAA) were analyzed by gas chromatography connected to an Agilent 5973 quadrupole mass selective detector (GC-MS, QP2010 Ultra, Shimadzu). GC has a split injector equipped with a 30 m DB-1 column (Agilent J&W GC columns, USA) with diameter and film thickness of 0.25 mm and 0.10 μm, respectively. The samples were injected in split mode with the injector operating at 250 ºC. GC oven temperature program was set to an initial temperature of 80

ºC, raised to 140 ºC at 10 ºC/min, holding for 5 min, raised to 150 ºC at 0.20 ºC/min, then raised to 250 ºC at 60 ºC/min, holding for 2 min. The flow rate of the carrier gas (He) was set at 1.84 mL/min. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 50–700 m/z, in full scan acquisition mode. Chromatogram peaks were identified comparing all mass spectra with a laboratory made database of PMAA.

The position of sulphate esters in sugars residues was determined by analysis of PMAA before and after desulphation procedure [18]. The samples (3 mg) were dissolved in dried DMSO (1.8 mL) and 100 μL of pyridine, 6.5 mg of pyromellitic acid, 12 mg of flourine sodium, and additional 200 μL of pyridine were added. The mixture was incubated at 120 ºC for 3 h. Afterwards, 3 % (w/v) sodium bicarbonate solution (1 mL) was added and reaction mixture was dialysed (cut-off 12-14 kDa) against distilled water, being recovered by lyophilization. Then, desulphated polysaccharides were converted to PMAA and analyzed as described above.

2.4.Immunostimulatory activity assays

2.4.1. Mice

BALB/c mice were purchased from Charles River (Barcelona, Spain) and were kept at the animal facilities of the Institute for Biomedical Sciences Abel Salazar. Experiments were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of the animals used for scientific purposes, and Portuguese rules (DL 113/2013).

2.4.2. In vitro lymphocyte stimulating effect by flow cytometry analysis

Spleens were aseptically removed from BALB/c mice and splenocyte suspensions were obtained by gently teasing the organ in Hanks' balanced salt solution (HBSS) and filtered through a sterile Pasteur pipette with a glass fiber filter. Splenocytes were resuspended in ammonium-chloride-potassium (ACK) lysing buffer for 3 min, for lysing erythrocytes, washed with HBSS, and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES solution, 100 IU/mL penicillin, 50 mg/L streptomycin, and 50 mM 2-mercaptoethanol. Spleen cell suspensions were distributed on 96-well plates (106 cells/well) and cultured for 6 h at 37ºC, in 95% humidified atmosphere containing 5% CO_2. Plated cells were stimulated with RPMI medium alone (negative control), 2.5 mg/L of bacterial lipopolysaccharide (LPS) from *Escherichia coli* (B cells positive control), 2.5 mg/L of concanavalin A (T cells positive control), or with 25, 50, and 75 mg/L of the samples. Co-incubation with 100 µg/mL polymyxin B (PB) was done in parallel to assess possible endotoxin contamination.

After incubation, the cells supernatant was removed by centrifugation at 500 g for 5 min and cells were washed by centrifugation with 150 µL of PBS. The pelleted spleen cells were suspended in 25 µL of FACS buffer (1% BSA and 5 mM sodium azide in PBS) containing the following monoclonal antibodies (mAb) diluted 1:100: anti-CD19 (PE-conjugate; clone 1D3; Biolegend), anti-CD3 (PE/Cy7-conjugate; clone 145-2C11; BD Bioscience) and anti-CD69 (FITC-conjugate; clone H1.2F3; Biolegend). After incubation for 30 min at 4 ºC in the dark, cells were washed by centrifugation with 150 µL of FACS buffer to remove unbound antibodies. The washed cells were then suspended in FACS buffer and analysed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter). Non viable cells were excluded by propidium iodide (PI; Sigma). The collected data files were analysed using the FlowJo v10.3. software (Tree Star inc., Ashland, OR, USA).

2.5.Statistical analysis

Statistical analysis was performed through two statistical tests using Excel software (Microsoft office 2013). The F-test was used to analyse the variance and the Student's T-test was used to detect significant differences between samples. Statistical analysis of the B cell stimulatory activity was done using One-Way ANOVA in GraphPad prism, Version 7.0 (GraphPad Software, Inc. La Jolla, CA, USA) and Dunnett's or Tukey's multiple comparisons tests, as indicated. Differences were deemed significant at *p*-value<0.05.

3. Results and Discussion

3.1.Polymeric material composition of brine and seawater

Brine samples were collected in two periods, in the middle (Brine Sept) and in the end (Brine Oct) of salt production, to evaluate their composition along salt production season. For comparison purposes, seawater and salt produced in the same saltpan were also analysed.

Yield and overall composition of polymeric material are present in table 1. The amount of polymeric material obtained was higher for the brines, around 370 mg/L, whereas from seawater only 67 mg/L were recovered. Sea salt revealed a content of 161 mg of polymeric material per one kilogram of salt, which is in accordance with the content reported for Atlantic Ocean sea salt [13]. Carbohydrates are the main component for all water samples, ranging from 40 to 47%. Sulphate esters represent 7 to 10% of the polymeric material, which are reported to belong to polysaccharides structure present in a wide range of marine organisms [2, 3]. Sulphate content is higher in seawater (10%) in comparison with brine (7%), which allows to infer that some desulphation can occur

under the salt pan conditions [19]. The water/brine samples have an ash content ranging from 11 to 16%, possibly having a contribution of the cationic sulphate and uronic acids counter ions. Protein is the component present in lower amounts, only 5-6%. Sea salt has a slightly different composition comparing with the seawater and brine samples, with a higher content of ashes (36%) and protein (9%), and lower content of carbohydrates (28%) and sulphate esters (6%). This polymeric material composition is in agreement with described for the Atlantic Ocean salt, where 35% of carbohydrates, 14% of sulphate esters, and 4% of protein were reported [13].

Table 1 – Polymeric material yield and composition of seawater, salt pan water collected in September (Brine Sept) and October (Brine Oct), and salt.

	Yield	**Polymeric material (mg/g)**			
		Carbohydrates	**Sulphate esters**	**Protein**	**Ash**
Seawater	67[#]	465 ± 16[a]	103 ± 2[a]	57 ± 2[a]	112
Brine Sept	374[#]	396 ± 18[b]	74 ± 2[b]	53 ± 5[a]	163
Brine Oct	369[#]	450 ± 33[a,b]	78 ± 4[b]	64 ± 4[a]	150
Salt	161[§]	275 ± 2[c]	57 ± 1[c]	92 ± 2[b]	359

[#] mg/L; [§] mg/kg; . In each column, different letters indicate statistically different values according to Student's T-test ($p < 0.05$).

Total content of polysaccharides in polymeric material of brine and seawater is 470 mg/g for Brine Sept, 528 mg/g for Brine Oct, and 568 mg/g for seawater (Table 2), considering the sulphate esters content. As the yield of polymeric material is higher for brine than seawater (Table 1), it can be concluded that saltpan brine is a good source of these sulphated polysaccharides. From one litre of brine it is possible to find around 200 mg of sulphated polysaccharides, whereas from seawater only 38 mg can be recovered per litre (5 times less).

3.2.Polysaccharides characterization of brine

In order to obtain more information about the structure of the polysaccharides present in all samples, their monosaccharide composition was determined. As sulphate is a

component of the polysaccharides, the calculation of the molar percentage of each monosaccharide present in the polysaccharides takes into account also the content of sulphate esters (Table 2). These results showed that polysaccharides from brine and salt are mainly composed by uronic acids (UA, 35 and 30 mol%, respectively) and sulphate esters (16 - 22 mol%), whereas in seawater the sulphate is present in slightly higher proportion (31 mol%) than UA (27 mol%). All the other monosaccharides are present in samples in lower amounts (below 13 mol%), namely fucose, galactose, glucose, rhamnose, mannose, xylose, and arabinose. This diversity in monosaccharides composition was also observed in the carbohydrates present in seawater and estuaries [1]. The presence of low quantity of ribose (< 1 mol%) could be attributed to the presence of RNA, as already stated for sea salt [13].

Table 2 – Composition (mol%) and total content of polysaccharides (mg/g of polymeric material) of seawater, brine collected in September (Brine Sept) and October (Brine Oct), and salt.

	Seawater	**Brine Sept**	**Brine Oct**	**Salt**
Composition (mol%)				
Rha	6.9 ± 0.3	9.3 ± 0.2	5.9 ± 0.6	3.0 ± 0.8
Fuc	8.2 ± 0.1	10 ± 0.8	8.8 ± 0.5	7.1 ± 1.1
Rib	0.6 ± 0.2	1.1 ± 0.2	1.0 ± 0.5	0.4 ± 0.2
Ara	2.8 ± 0.2	4.2 ± 0.4	7.2 ± 0.9	3.0 ± 0.1
Xyl	3.9 ± 0.3	8.5 ± 0.4	7.8 ± 1.6	3.1 ± 0.2
Man	4.7 ± 0.3	8.3 ± 1.4	4.1 ± 0.8	13 ± 0.7
Gal	7.4 ± 0.1	13 ± 1.3	8.2 ± 0.8	7.6 ± 0.3
Glc	7.4 ± 0.2	11 ± 1.3	7.1 ± 0.9	11 ± 0.5
UA	27 ± 0.5	35 ± 3.9	35 ± 1.5	30 ± 1.8
Sulphate	31 ± 0.6	20 ± 0.9	16 ± 1.5	22 ± 0.6
Total content (mg/g)	***568 ± 15***	***470 ± 16***	***528 ± 28***	***332 ± 4***

The presence of UA and sulphate esters shows that these are highly negatively charged polysaccharides, which should render them more soluble in water and prevent their precipitation in saltpan.

To unravel more about the structure of polysaccharides from the brines, they were further analysed by methylation analysis for the identification of glycosidic linkages present. Furthermore, methylation analysis was carried out before and after desulphation to identify also sulphate location in monosaccharides (Supplementary material, Figure S1 and Table S1). Methylation analysis showed also that polysaccharides from both brine samples revealed a similar glycosidic linkage composition, characteristic of several sulphated polysaccharides structures, namely rhamnan, fucan, mannans, xylomannans, glucuronomannan, galactans, glucans, and heteroglucan. This kind of structures was identified in several marine organisms, such as red, green, and brown seaweed, microalgae, and invertebrates (Table 3). These polysaccharides could be released into the medium by cell lysis, apoptosis or exudation [14], which resist to the environmental conditions of saltpans during the salt production.

Table 3 – Glycosidic linkages and sulphate position of the carbohydrates component of the polymeric material from brine, type of polysaccharide and source described in literature.

Sugar residues	Glycosidic linkage and sulphate position	Type of polysaccharide	Source	Reference
T-Rha*p*; 2-Rha*p*; 3-Rha*p*; 2,4-Rha*p*; 2,3,4-Rha*p*	(1→2)- and (1→3)-Rha*p* SO_3 in *O*-2, *O*-3 and/or *O*-4	Sulphated rhamnan	• green seaweed • marine microalgae	[3, 20, 21]
T-Fuc*p*; 3-Fuc*p*; 3,4-Fuc*p*; 2,3-Fuc*p*; 2,3,4-Fuc*p*	(1→3)- and (1→3,4)-Fuc*p* SO_3 in *O*-2 and/or *O*-4	Sulphated fucan	• brown seaweed • sea urchins • sea cucumber	[22-24]
3-Man*p*; 2,3-Man*p*; 3,4-Man*p*; 2,3,4,6-Man*p*; 2,3,4-Man*p*	(1→3)-Man*p* SO_3 in *O*-2 and *O*-6	Sulphated mannans	• red seaweed	[3, 25]
T-Xyl*p*; 3-Xyl*p*; 4-Xyl*p*; 3-Man*p*; 2,3-Man*p*; 3,4-Man*p*; 2,3,4,6-Man*p*; 2,3,4-Man*p*	(1→3)- and (1→2,3)-Man*p* SO_3 in *O*-2 and *O*-6 Branch: (1→3)- and (1→4)-Xyl*p*	Sulphated xylomannans	• red seaweed	[25, 26]
3-Man*p*; 2,3-Man*p*; 2,3,4,6-Man*p*; 2,3,4-Man*p*; 3,4-Man*p*	(1→3)- and (1→2,3)-Man*p* SO_3 in *O*-2 and *O*-6	Glucuronomannan	• marine microalgae	[27, 28]
T-Gal*p*; 3-Gal*p*; 4-Gal*p*; 2,3-Gal*p*; 2,3,4-Gal*p*; 2,3,4,6-Gal*p*	(1→3)- and/or (1→4)-Gal*p* SO_3 in *O*-2, *O*-3, or *O*-6	Sulphated galactans	• red seaweed • marine invertebrates • sea urchins	[22, 29-31]
T-Glc*p*; 4-Glc*p*; 4,6-Glc*p*; 2,3,4,6-Glc*p*	(1→4)-Glc*p* SO_3 in *O*-2, *O*-3, or *O*-6	Sulphated glucans	• red seaweed	[32]
T-Rha*p*; 2,4-Rha*p*; T-Xyl*p*; 4-Xyl*p*; 4-Glc*p*; 2,3,4,6-Glc*p*	(1→4)-Glc*p* SO_3 in *O*-2 or *O*-3 Branch: (1→4)-Xyl*p* and (1→2,4)-Rha*p*	Sulphated heteroglucan	• green seaweed	[33]

3.3.Fractionation of saltpan water polysaccharides

As the polymeric material analysis revealed a mixture of different polysaccharide structures, they were submitted to an anion-exchange chromatography to evaluate their charge properties. Four fractions were obtained (Supplementary material, Figure S2) with a yield of 5-14% for the elution with buffer without NaCl addition (neutral fraction), 18% for 0.125 M NaCl (slightly negatively charged), 18-27% for 0.250 M NaCl, and 7-10% for 0.500 M NaCl (strongly negatively charged, Table 4).

Table 4 – Yield and composition of the fractions from anion-e hy of polymeric material from brine collected in September (Brine Sept) and October (Brine Oct).

	Yield (%)	Monosaccharides (%mol)									Total Polysaccharides (mg/g)	Protein (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	$-SO_3^{-2}$		
Brine Sept												
Buffer	4.9	8.2	13.3	6.2	11.4	10.5	15.9	14.7	19.8	-	207	248
0.125 M	17.5	7.4	10.5	4.3	8.1	5.1	9.9	9.4	43.9	1.5	244	231
0.250 M	18.2	5.3	11.5	2.4	5.6	5.0	11.0	8.2	37.3	13.5	347	180
0.500 M	7.4	5.4	12.4	1.6	4.1	7.4	10.0	9.1	24.8	25.2	450	138
Brine Oct												
Buffer	13.7	4.8	11.5	10.5	11.4	4.3	17.3	10.8	29.6	-	284	201
0.125 M	18.0	7.1	13.1	6.1	10.6	8.2	10.7	11.1	33.1	-	385	164
0.250 M	26.8	6.7	16.4	5.5	7.6	4.2	13.1	7.8	29.6	9.2	437	129
0.500 M	9.8	6.0	17.1	4.2	5.0	4.3	10.7	7.6	22.6	22.6	512	109

nd – not determined

Total content of polysaccharides in the fractions increase from 21 to 51% (w/w), with a concomitant decrease in protein content (from 23 to 11%), along with the increase of NaCl concentration used in eluent buffer. The polysaccharides were eluted according to their higher content of negatively charged sulphate groups, from 0 to 25 mol%. Therefore, fractions eluted with 0.5 M NaCl were the richest in sulphated polysaccharides, besides their lower mass yield (7-10%). Nevertheless, only small differences in their sugars composition were observed, namely, the Buffer had higher content of Ara, Xyl, and Gal when compared with negatively charged fractions (Table 4). Uronic acids were the major residues present in the samples (20 to 44 mol%), followed by fucose (11 to 17%),

galactose (10 to 17%), glucose (8 to 15%), xylose (4 to 11%), mannose (4 to 11%), rhamnose (5 to 8%), and arabinose (2 to 11%). The glycosidic linkage and substitution analysis performed to the four fractions revealed a similar proportion between the residues identified (Supplementary material, Table S1). In addition, the residues identified were also the same identified in the polymeric material of brine. These results allowed to conclude that although having different sulphate content, glycosidic linkages and sugar residues components of the polysaccharides are identical in all fractions. This suggests that polysaccharides present in the saltpan brine water form a complex mixture of several polysaccharides, as has been reported in polysaccharide from processed matrices that prevent the isolation of characteristic native polysaccharides, as occurs in roasted coffee with the formation of the high molecular weight nitrogenous brown compounds known as melanoidins [34]. In melanoidins, the sugars and glycosidic-linkage composition characteristic of arabinogalactans and galactomannans are present in fractions with different properties. The polysaccharides obtained from salt pan brines, although from different sources and not submitted to any processing, resemble those derived from the thermal-treated matrices where new glycosidic-linkages appear to be formed interlinking the polysaccharides [35]. These complex structures are hypothesized to be resistant to the extreme conditions of the salt pan.

3.4.Molecular weight profile of the polysaccharides

The fractions enriched in polysaccharides and eluted with NaCl by anion-exchange chromatography were submitted to a size-exclusion chromatography (SEC) to determine the molecular weight profile of the polysaccharides. As Buffer fraction had a low sugar content, it was not evaluated. Since both brine samples revealed the same content in polysaccharides, only the three fractions recovered from Brine Sept sample (0.125 M;

0.250 M; and 0.500 M) were used. This semi-preparative analysis allows to separate each fraction in four groups with different apparent molecular weights (Mw): fraction A, Mw > 400 kDa; fraction B, 400 kDa < Mw < 120 kDa; fraction C, Mw < 120 kDa; and fraction D, Mw = 2 kDa (Figure 1). For fractions 0.125 M and 0.250 M, the yield was identical for the four groups obtained by SEC (8-13 % for 0.125 M and 18-21% for 0.250 M) and 0.500 M fraction revealed a higher yield (28-39%) for fractions A and B, the higher molecular weight polymers (Table 5). These results showing that polysaccharides were spread through a wide range of molecular weight without a clear separation allow to infer they are very heterogeneous concerning also their size.

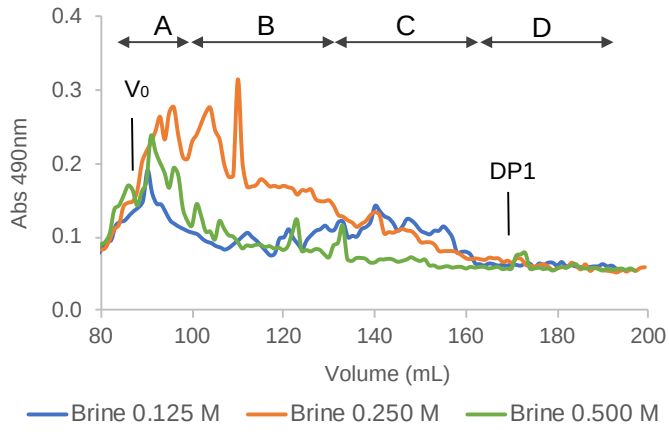


Figure 1 – Size exclusion chromatography of fractions from anion-exchange chromatography (0.125 M; 0.250 M, and 0.500 M) of brine. V0 – exclusion volume; DP1 – volume of elution of monosaccharide.

The analyses of sugar content of the different groups obtained after SEC showed that total content in polysaccharides was higher (30% of their total mass) for fractions with higher molecular weight, mainly A and B (Table 5). As observed in the anion-exchange chromatography fractions, monosaccharide composition of the SEC fractions was very similar among them and also when compared with the initial brine samples polymeric material. Uronic acids were the major monosaccharide present in all the fractions (29 to 61 mol%), followed by fucose, galactose, glucose, xylose, mannose, rhamnose, and arabinose (Table 5). These results reinforce the heterogeneity of the polysaccharides from

brine, not separated by SEC, and having an apparent molecular weight of more than 120 kDa. The different profiles and more defined peaks observed in Figure 1 should be related more to differences in molecular weight than on different polysaccharides composition.

Table 5 – Yield and monosaccharides composition of the fractions from size-exclusion chromatography of anion-exchange chromatography of brine.

	Yield (%)	%mol								Total sugars (µg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Brine Sept 0.125 M										
Fraction A	13.1	7	12	6	12	5	7	16	35	216
Fraction B	9.0	8	8	2	8	8	10	11	45	322
Fraction C	12.3	8	6	1	6	4	3	10	61	154
Fraction D	8.0	0	0	0	11	8	4	23	54	63
Brine Sept 0.250 M										
Fraction A	18.5	8	17	3	13	6	12	12	29	201
Fraction B	18.2	7	14	3	11	6	12	12	37	216
Fraction C	21.0	7	11	3	8	6	11	16	40	229
Fraction D	17.7	9	10	2	6	8	14	14	37	158
Brine Sept 0.500 M										
Fraction A	38.7	8	18	2	8	6	12	11	35	288
Fraction B	27.6	9	12	2	8	8	12	16	34	279
Fraction C	15.3	8	6	1	10	8	10	27	30	162
Fraction D	16.0	0	0	0	7	5	6	21	61	116

3.5.Evaluation of *in vitro* lymphocyte stimulatory activity

The fractions from the anionic-exchange chromatography (0.125 M, 0.250 M, and 0.500 M), having different proportions of sulphated groups (from 2 to 25 mol%), a relevant structural feature for the immunomodulatory activity [15], were used to assess their ability to stimulate *in vitro* BALB/c mouse lymphocytes. In addition, the initial polymeric material from both brine samples (Brine Sept and Brine Oct) was also evaluated concerning its immunomodulatory potential.

All the samples tested maintained their viability in the range of concentrations tested, between 25 to 75 mg/L (Supplementary material, Figure S3). The negligible expression of the early activation marker CD69 on the surface of $CD3^+$ cells showed that none of the samples tested extensively stimulated T cells (Supplementary Material, Figure S4). On

the other hand, polysaccharide fractions from brine stimulated $CD19^+$ cells (B cells), showing values significantly higher than the negative control (RPMI), with *p*-values ranging from 0.01 to > 0.0001 (One-Way ANOVA and Dunnet's multiple comparisons test). Only a few significant differences were observed among samples at the concentrations tested (Figure 2). Although a dose-response relationship was not seen in the range of concentrations tested, except for the buffer samples, the existence of such relationship at higher or lower concentrations cannot be ruled out. The percentage of CD69-expressing B cells ranged between 28 to 37% when cultures were stimulated with the initial polymer material (Brine Sept and Brine Oct) and no significant differences were observed between both samples. In general, the Buffer and 0.125 M NaCl eluted fractions had higher activity than the corresponding brine samples (Brine Oct), as the Buffer and 0.125 M fractions induced the expression of CD69 on 39 to 54% of B cells. In contrast, B cell-stimulatory ability was decreased in fractions from anion-exchange chromatography eluted at higher salt concentrations, since 0.250 M and 0.500 M fractions showed a slightly lower stimulatory effect (28 to 31%, Figure 2).

The activation of B cells by all fractions was also evaluated in the presence of polymyxin B to exclude that observed activation was due to possible contamination of the samples with bacterial lipopolysaccharides. Polymyxin B binds to LPS and reduces LPS-induced B cell activation to nearly basal levels [36]. A slight decrease or no difference in B cell activation was observed in the presence of polymyxin B. Such a reduction was only statistically significant ($p < 0.05$) in brine from September, indicating that this particular sample could contain non-negligible endotoxin amounts. The proportions of activated cells nevertheless remained significantly higher than that observed for the negative control (Figure 2). Therefore, these results confirm that polysaccharides from brine have *in vitro* stimulatory activity on B cells.

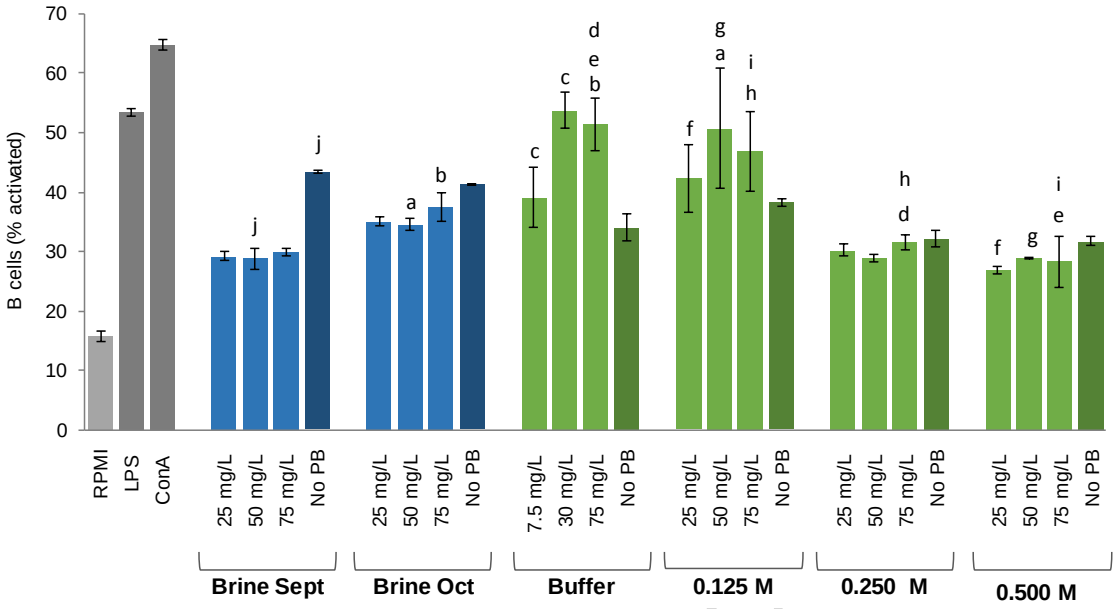


Figure 2 - Proportion of B-cells expressing the early activation marker CD69 (% activated) induced by polymeric material from brine (Brine Sept and Brine Oct) and fractions from anion-exchange chromatography (Buffer, 0.125 M, 0.250 M, and 0.500 M) at concentrations of 25, 50, and 75 mg/L in the presence of polymyxin B. All samples (50 mg/L) were analysed also in the absence of polymyxin B (No PB). Culture medium (RPMI) alone was used as negative control. Lipopolysaccharide (LPS) and concanavalin A (ConA) were used as positive controls. Similar letters above bars indicate statistical significant differences between compared groups ($p < 0.05$; One-Way ANOVA and Tukey's multiple comparisons test).

In order to confirm the relevance of sulphate groups for the observed lymphocyte stimulatory activity, polymeric material from brine was analysed after desulphation of the brine sample. The desulphated polysaccharides showed a significantly lower B cell-stimulatory effect (20% of $CD69^+$ cells) than the native samples that, on a repeated experiment, induced the expression of CD69 in near 50% of B cells (Figure 3) and were significantly different from the negative control ($p < 0.001$). In opposition, desulphated polysaccharides failed to induce B cell activation, since no statistically significant differences were observed in the percentage of CD69-expressing B cells from RPMI and DesBrine stimulated cultures. This decrease in B cell activation allowed to infer that sulphate esters are relevant for the detected immunostimulatory activity of polysaccharides from saltpan brine water, in accordance with a previous report on sulphated polysaccharides immunostimulatory activity [15].

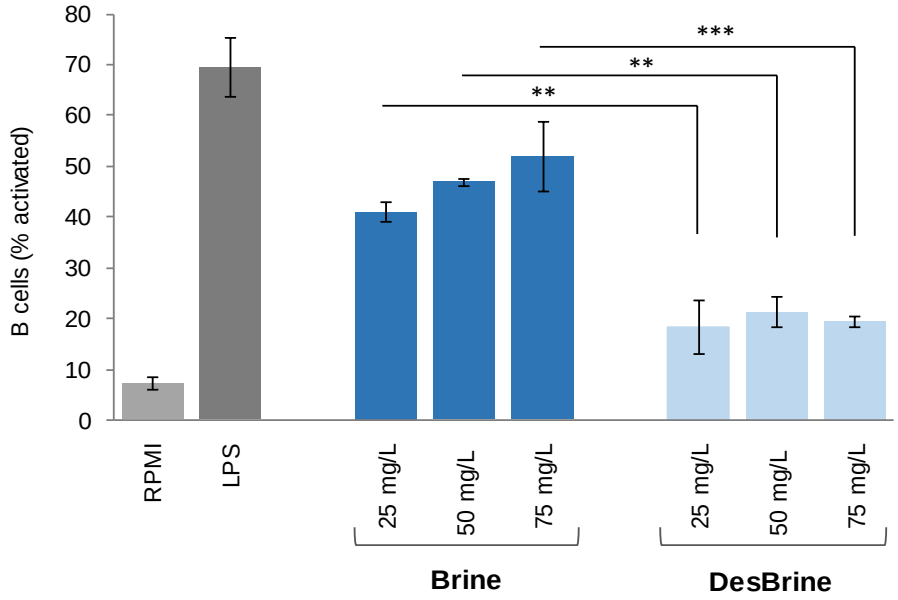


Figure 3 - Proportion of B-cells expressing the early activation marker CD69 (% activated) induced by polymeric material from brine before (Brine) and after desulphation (DesBrine) at concentrations of 25, 50, and 75 mg/L in presence of polymyxin B. All samples were analysed also in absence of polymyxin B (No PB). Culture medium (RPMI) alone was used as negative control. Lipopolysaccharide (LPS) was used as positive control. Statistically significant differences between samples are indicated above bars (** $p < 0.01$; (*** $p < 0.001$; One-Way ANOVA and Tukey's multiple comparisons test).

4. Concluding remarks

Saltpan brine water revealed to be composed by approximately 200 mg/L of highly sulphated polysaccharides (20 mol% of $-SO_3^{2-}$), which is five times higher than the values of seawater. These polysaccharides comprise a complex mixture of several monosaccharide residues and glycosidic linkages, resembling the sulphated polysaccharides found in marine environments, namely seaweed, microalgae, and invertebrates. These polysaccharides are very soluble in aqueous solutions and very resistant to the extreme environmental conditions of saltpans. The *in vitro* immunostimulatory assays show that this polymeric material is able to stimulate B lymphocytes.

These results show the potential of saltpan brine water as a sustainable source of sulphated polysaccharides with potential application as nutraceuticals or in the biomedical field. The methodology proposed for obtaining them is easy and

environmentally friendly, since it is only necessary to remove the low molecular weight material, for example by membrane separation. Furthermore, it is not necessary any purification procedure to have *in vitro* immunomodulatory activity, which is an advantage for scaling-up processes.

Acknowledgements

Thanks are due to FCT/MEC for the financial support to the QOPNA research Unit (FCT UID/QUI/00062/2013) and CICECO-Aveiro Institute of Materials POCI-01-0145-FEDER-007679 (Ref. FCT UID/CTM/50011/2013), through national founds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement. CN, SSF, and AC thanks FCT for the grant (SFRH/BPD/100627/2014, SFRH/BD/103003/2014, and SFRH/BPD/91623/2012, respectively).

References

[1] Guillaume, P. Zhao, J.-M. Orvain, F. Dupuy, C. Klein, G.L. Graber, M. Maugard, T., Seasonal dynamics of extracellular polymeric substances (EPS) in surface sediments of a diatom-dominated intertidal mudflat (Marennes–Oléron, France), J. Sea Res. 92 (2014) 26-35. https://doi.org/10.1016/j.seares.2013.07.018.

[2] Zierer, M.S. Mourão, P.A.S., A wide diversity of sulfated polysaccharides are synthesized by different species of marine sponges. Carbohydr. Res. 328 (2000) 209-216. https://doi.org/10.1016/S0008-6215(00)00076-8.

[3] Xu, S.-Y. Huang, X. Cheong, K.-L., Recent Advances in Marine Algae Polysaccharides: Isolation, Structure, and Activities. Mar. Drugs 15 (2017). https://doi.org/10.3390/md15120388.

[4] de Jesus Raposo, M.F. de Morais, R.M.S.C. de Morais, A.M.M.B., Bioactivity and Applications of Sulphated Polysaccharides from Marine Microalgae. Mar. Drugs 11 (2013) 233-252. https://doi.org/10.3390/md11010233.

[5] de Jesus Raposo, F.M. de Morais, M.A. de Morais, M.R., Marine Polysaccharides from Algae with Potential Biomedical Applications. Mar. Drugs 13 (2015) 2967-3028. https://doi.org/10.3390/md13052967.

[6] Lee, Y.-E. Kim, H. Seo, C. Park, T. Lee, K.B. Yoo, S.-Y. Hong, S.-C. Kim, J.T. Lee, J., Marine polysaccharides: therapeutic efficacy and biomedical applications. Arch. Pharm. Res. 40 (2017) 1006-1020. https://doi.org/10.1007/s12272-017-0958-2

[7] Valcarcel, J. Novoa-Carballal, R. Pérez-Martín, R.I. Reis, R.L. Vázquez, J.A., Glycosaminoglycans from marine sources as therapeutic agents. Biotechnol. Adv. 35 (2017) 711-725. https://doi.org/10.1016/j.biotechadv.2017.07.008

[8] Oh, G.-W. Ko, S.-C. Lee, D.H. Heo, S.-J. Jung, W.-K., Biological activities and biomedical potential of sea cucumber (Stichopus japonicus): a review. Fish. Aquatic Sci. 20 (2017) 28. https://doi.org/10.1186/s41240-017-0071-y

[9] Cunha, L. Grenha, A., Sulfated Seaweed Polysaccharides as Multifunctional Materials in Drug Delivery Applications. Mar. Drugs 14 (2016) 42. https://doi.org/10.3390/md14030042.

[10] Ruocco, N. Costantini, S. Guariniello, S. Costantini, M., Polysaccharides from the Marine Environment with Pharmacological, Cosmeceutical and Nutraceutical Potential. Molecules 21 (2016) 551. https://doi.org/10.3390/molecules21050551.

[11] Cardoso, J.M. Costa, R.R. Mano, F.J., Marine Origin Polysaccharides in Drug Delivery Systems. Mar. Drugs 14 (2016) 1-27. https://doi.org/10.3390/md14020034.

[12] Koller, M. Muhr, A. Braunegg, G., Microalgae as versatile cellular factories for valued products. Algal Res. 6 (2014) 52-63. https://doi.org/10.1016/j.algal.2014.09.002.

[13] Silva, I. Nunes, A. Rocha, S.M. Coimbra, M.A., Composition of food grade Atlantic salts regarding triacylglycerides, polysaccharides and protein. J. Food Compos. Anal. 41 (2015) 21-29. https://doi.org/10.1016/j.jfca.2014.12.023.

[14] Engel, A. Händel, N., A novel protocol for determining the concentration and composition of sugars in particulate and in high molecular weight dissolved organic matter (HMW-DOM) in seawater. Mar. Chem. 127 (2011) 180-191. https://doi.org/10.1016/j.marchem.2011.09.004.

[15] Ferreira, S.S. Passos, C.P. Madureira, P. Vilanova, M. Coimbra, M.A., Structure–function relationships of immunostimulatory polysaccharides: A review. Carbohydr. Polym. 132 (2015) 378-396. https://doi.org/10.1016/j.carbpol.2015.05.079.

[16] Nunes, C. Silva, L.;Fernandes, A.P. Guiné, R.P.F. Domingues, M.R.M. Coimbra, M.A., Occurrence of cellobiose residues directly linked to galacturonic acid in pectic

polysaccharides. Carbohydr. Polym. 87 (2012) 620-626. https://doi.org/10.1016/j.carbpol.2011.08.027.

[17] Stevenson, T.T. Furneaux, R.H., Chemical methods for the analysis of sulphated galactans from red algae. Carbohydr. Res. 210 (1991) 277-298. https://doi.org/10.1016/0008-6215(91)80129-B.

[18] Oliveira, C. Ferreira, A.S. Novoa-Carballal, R. Nunes, C. Pashkuleva, I. Neves, N.M. Coimbra, M.A. Reis, R.L. Martins, A. Silva, T.H., The Key Role of Sulfation and Branching on Fucoidan Antitumor Activity. Macromol. Biosci. 17 (2017) 1600340. https://doi.org/10.1002/mabi.201600340.

[19] Bedini, E. Laezza, A. Parrilli, M. Iadonisi, A., A review of chemical methods for the selective sulfation and desulfation of polysaccharides. Carbohydr. Polym. 174 (2017) 1224-1239. https://doi.org/10.1016/j.carbpol.2017.07.017.

[20] Li, H. Mao, W. Chen, Y. Ren, S. Qi, X. Chen, Y. Zhao, C. Li, N. Wang, C. Lin, C. Yan, M. Shan, J., Sequence analysis of the sulfated rhamno-oligosaccharides derived from a sulfated rhamnan. Carbohydr. Polym. 90 (2012) 1299-1304. https://doi.org/10.1016/j.carbpol.2012.06.076.

[21] Ropellato, J. Carvalho, M.M. Ferreira, L.G. Noseda, M.D. Zuconelli, C.R. Gonçalves, A.G. Ducatti, D.R.B. Kenski, J.C.N. Nasato, P.L. Winnischofer, S.M.B. Duarte, M.E.R., Sulfated heterorhamnans from the green seaweed Gayralia oxysperma: partial depolymerization, chemical structure and antitumor activity. Carbohydr. Polym. 117 (2015) 476-485. https://doi.org/10.1016/j.carbpol.2014.09.089.

[22] Mourão, P.A.S. Pereira, M.S., Searching for Alternatives to Heparin: Sulfated Fucans from Marine Invertebrates. Trends Cardiovasc Med. 9 (1999) 225-232. https://doi.org/10.1016/S1050-1738(00)00032-3.

[23] Ale, M.T. Meyer, A.S., Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation. RSC Adv. 3 (2013) 8131-8141. https://doi.org/10.1039/C3RA23373A.

[24] Deniaud-Bouët, E. Hardouin, K. Potin, P. Kloareg, B. Hervé, C., A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges. Carbohydr. Polym. 175 (2017) 395-408. https://doi.org/10.1016/j.carbpol.2017.07.082.

[25] Recalde, M.P. Noseda, M.D. Pujol, C.A. Carlucci, M.J. Matulewicz, M.C., Sulfated mannans from the red seaweed Nemalion helminthoides of the South Atlantic. Phytochem. 70 (2009) 1062-1068. https://doi.org/10.1016/j.phytochem.2009.05.001.

[26] Mandal, P. Pujol, C.A. Carlucci, M.J. Chattopadhyay, K. Damonte, E.B. Ray, B., Anti-herpetic activity of a sulfated xylomannan from Scinaia hatei. Phytochem. 69 (2008) 2193-2199. https://doi.org/10.1016/j.phytochem.2008.05.004.

[27] Chiovitti, A. Harper, R.E. Willis, A. Bacic, A. Mulvaney, P. Wetherbee, R., Variations in the substituted 3-linked mannans closely associated with the silicified walls of diatoms. J. Phycol. 41 (2005) 1154-1161. https://doi.org/10.1111/j.1529-8817.2005.00140.x.

[28] Le Costaouëc, T. Unamunzaga, C. Mantecon, L. Helbert, W., New structural insights into the cell-wall polysaccharide of the diatom Phaeodactylum tricornutum. Algal Res. 26 (2017) 172-179. https://doi.org/10.1016/j.algal.2017.07.021.

[29] Recalde, M.P. Canelón, D.J. Compagnone, R.S. Matulewicz, M.C. Cerezo, A.S. Ciancia, M., Carrageenan and agaran structures from the red seaweed Gymnogongrus tenuis. Carbohydr. Polym. 136 (2016) 1370-1378. https://doi.org/10.1016/j.carbpol.2015.10.007.

[30] Wang, S.C. Bligh, S.W.A. Shi, S.S. Wang, Z.T. Hu, Z.B. Crowder, J. Branford-White, C. Vella, C., Structural features and anti-HIV-1 activity of novel polysaccharides from red algae Grateloupia longifolia and Grateloupia filicina. Int. J. Biol. Macromol. 41 (2007) 369-375. https://doi.org/10.1016/j.ijbiomac.2007.05.008.

[31] Restrepo-Espinosa, D.C. Román, Y. Colorado-Ríos, J. de Santana-Filho, A.P. Sassaki, G.L. Cipriani, T.R. Martínez, A. Iacomini, M. Pavão, M.S.G., Structural analysis of a sulfated galactan from the tunic of the ascidian Microcosmus exasperatus and its inhibitory effect of the intrinsic coagulation pathway. Int. J. Biol. Macromol. 105 (2017) 1391-1400. https://doi.org/1391-1400. 10.1016/j.ijbiomac.2017.08.166.

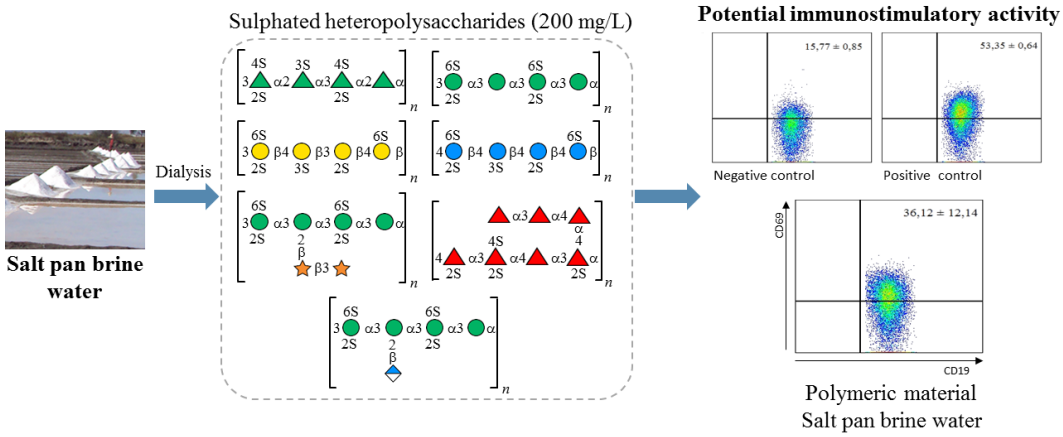
[32] Lechat, H. Amat, M. Mazoyer, J. Buléon, A. Lahaye, M., Structure and distribution of glucomannan and sulfated glucagon in the cell walls of the red alga Kappaphycus alvarezii (Gigartinales, Rhodophyta). J. Phycol. 36 (2001) 891-902. https://doi.org/10.1046/j.1529-8817.2000.00056.x.

[33] Ray, B., Polysaccharides from Enteromorpha compressa: Isolation, purification and structural features. Carbohydr. Polym. 66 (2006) 408-416. https://doi.org/10.1016/j.carbpol.2006.03.027.

[34] Nunes, F.M. Coimbra, M.A., Melanoidins from Coffee Infusions. Fractionation, Chemical Characterization, and Effect of the Degree of Roast. J. Agric. Food Chem. 55 (2007) 3967-3977. https://doi.org/10.1021/jf063735h.

[35] Moreira, A.S.P. Nunes, F.M. Simões, C. Maciel, E. Domingues, P. Domingues, M.R.M. Coimbra, M.A., Transglycosylation reactions, a main mechanism of phenolics incorporation in coffee melanoidins: Inhibition by Maillard reaction. Food Chem. 227 (2017) 422-431. https://doi.org/10.1016/j.foodchem.2017.01.107.

[36] Schepetkin, I.A. Quinn, M.T., Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. Int. Immunopharmacol. 6 (2006) 317-333. https://doi.org/10.1016/j.intimp.2005.10.005.



Graphical abstract

Highlights:

- Salt pan brine water contains five time more polymeric material than sea water**;**
- The polymeric material is mainly constituted by negatively charged polysaccharides;
- This polymeric material showed *in vitro* stimulatory activity for B cells;
- Salt pan water is an easily accessible and low-cost source of bioactive polysaccharides.

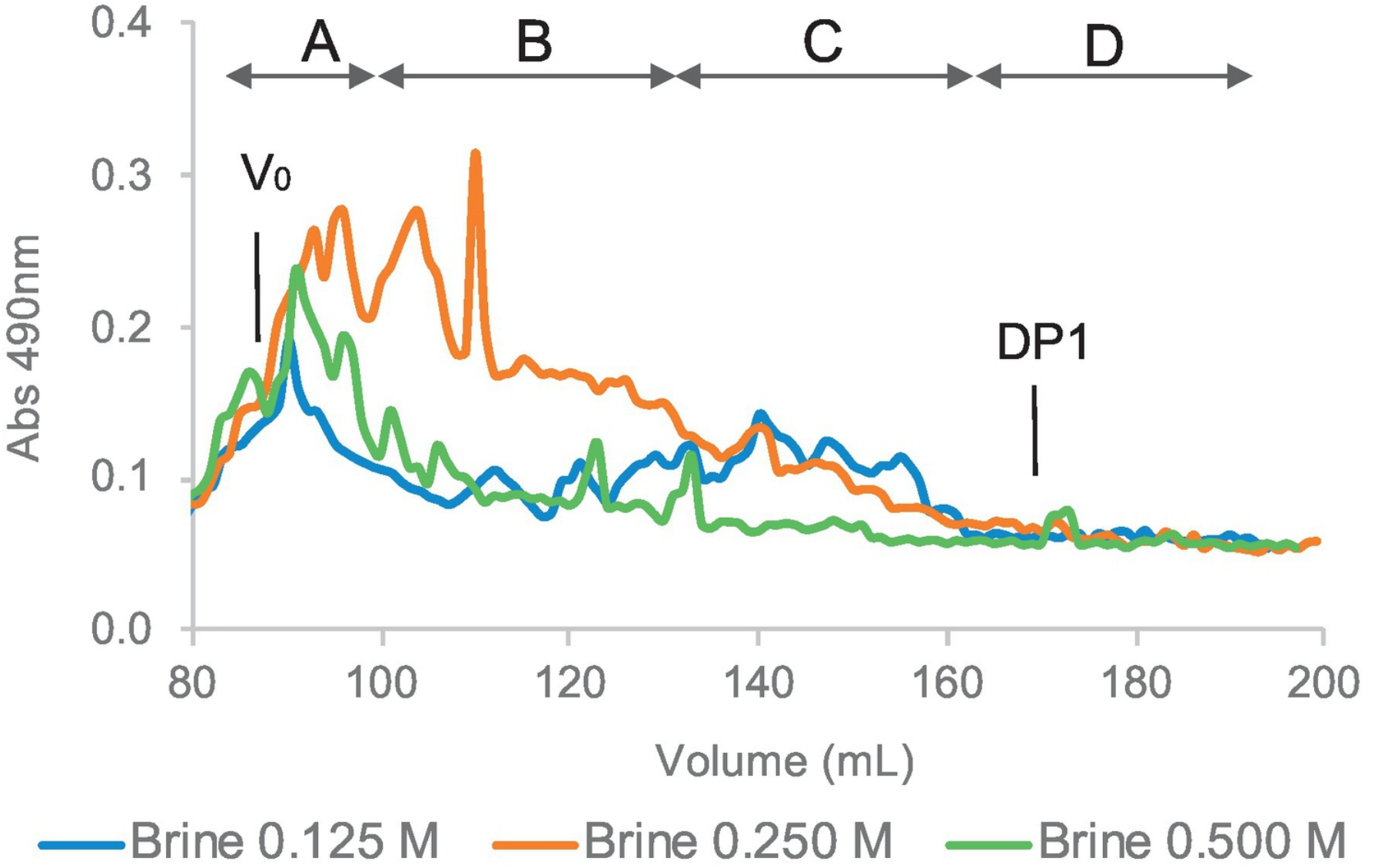

A
B
C
D
V0
DP1
0.4
0.3
0.2
0.1
0.0
Abs 490nm
80
100
120
140
160
180
200
Volume (mL)
Brine 0.125 M
Brine 0.250 M
Brine 0.500 M

Figure 1

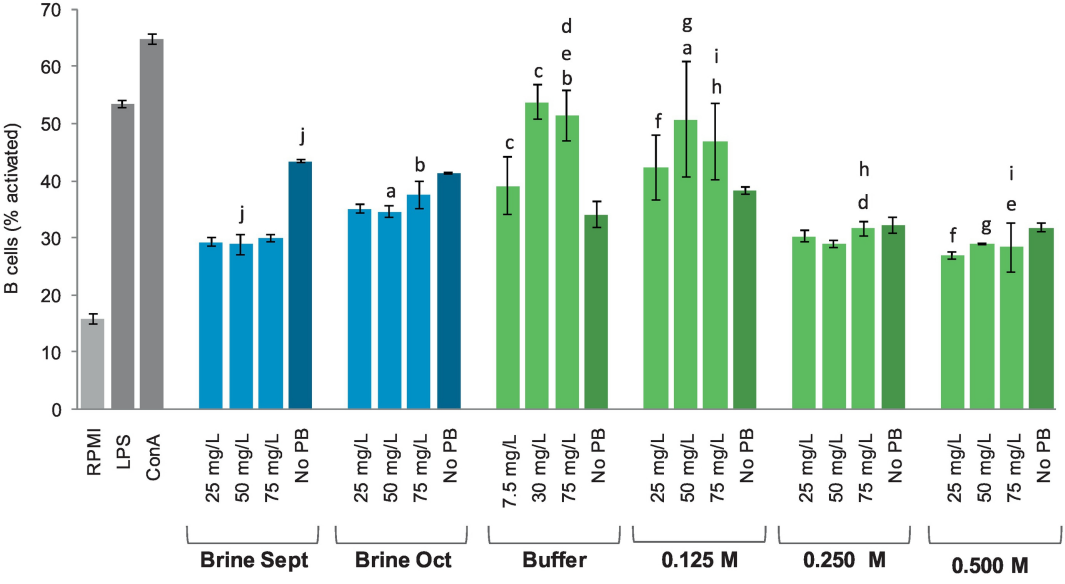

B cells (% activated)
70
60
50
40
30
20
10
0
RPMI
LPS
ConA
25 mg/L
50 mg/L
75 mg/L
No PB
25 mg/L
50 mg/L
75 mg/L
No PB
7.5 mg/L
30 mg/L
75 mg/L
No PB
25 mg/L
50 mg/L
75 mg/L
No PB
25 mg/L
50 mg/L
75 mg/L
No PB
25 mg/L
50 mg/L
75 mg/L
No PB
Brine Sept
Brine Oct
Buffer
0.125 M
0.250 M
0.500 M

Figure 2

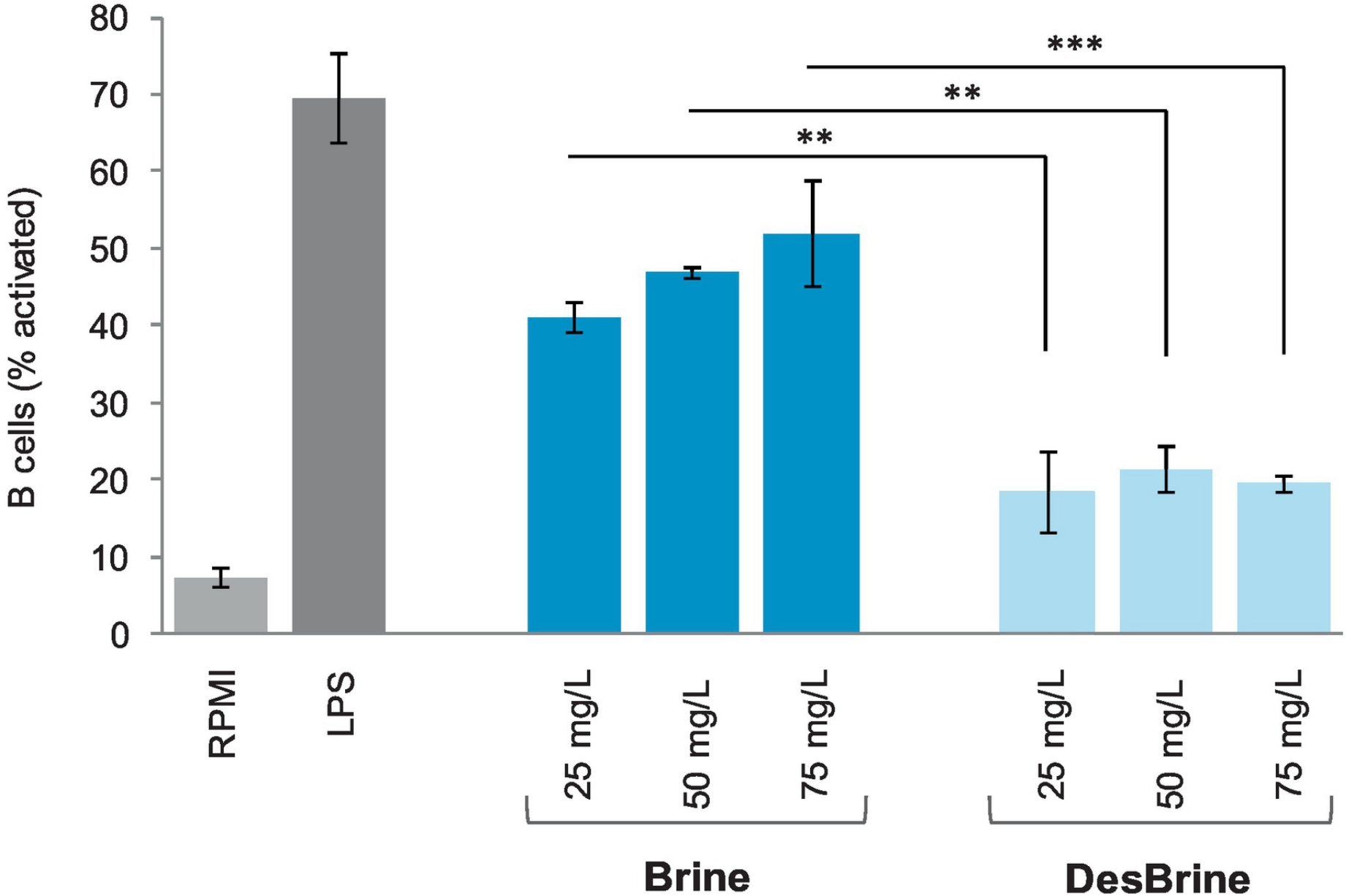

80
70
60
50
40
30
20
10
0
B cells (% activated)
***
**
**
RPMI
LPS
25 mg/L
50 mg/L
75 mg/L
25 mg/L
50 mg/L
75 mg/L
Brine
DesBrine

Figure 3