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Sulfated fucans extracted from algae *Padina gymnospora* have anti-inflammatory effect

Cybelle Teixeira Marques,[1] Tarciana C. G. de Azevedo,[1] Marília S. Nascimento,[1] Valquíria P. Medeiros,[1] Luciana G. Alves,[1] Norma M. B. Benevides,[2] Hugo A. O. Rocha,[1] Edda Lisboa Leite*[,1]

[1]Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Brazil,
[2]Departmento de Bioquímica, Universidade Federal do Ceará, Campus do Pici, Brazil.

Abstract: Sulfated polysaccharides were extracted with acetone from brown algae *Padina gymnospora*. The fraction precipitated with 1.5 volumes of acetone (F1.5) purified in Sephadex G-75 was characterized by infrared and nuclear magnetic resonance of ^{13}C and ^{1}H, through which the presence of sulfate groups on the C4 of α-L-fucose could be observed. This polysaccharide showed that an MW of 25,000 Da was effective in reducing leukocyte influx into the peritoneal cavity in mice at 10 mg/kg and 25 mg/kg body weight, causing a decrease of 60 and 39%, respectively. In the present study, it was observed that this fucan has anti-inflammatory properties but no cytotoxic action, indicating its potential use in the pharmaceutical industry.

Article

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Introduction

The key to developing an anti-inflammatory response, including autoimmune diseases such as rheumatoid arthritis, is the recruiting of leukocytes around the inflammation site (Tarrant & Patel, 2006). The migration of leukocytes to anti-inflammatory sites begins with cell capture, followed by transmigration and leukocytes rolling over the endothelium (Ulbrich et al., 2003). In inflammation, macrophages have three main functions: antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors (Fujiwara et al., 2005). Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation. They kill microorganisms and tumor cells and damage tissue by two separate oxidative pathways that are related (Morel et al., 1991). The initial capture of leukocytes by the endothelium is mediated by cell surface proteins belonging to the selectin family and its ligands (Tedder et al., 1995). However, the excess migration of leukocytes to the inflammation site and the uncontrolled production of nitric oxide, mainly by these cells, may cause tissue damage, resulting in a series of inflammatory diseases (Silva et al., 2006).

Fucoidan is a term used to define a polysaccharide based mainly on sulfated L-fucose, and less than 10% on other monosaccharides. The term sulfated fucan can be used to define heterofucans containing sulfated fucose and neutral sugars. However, fucans and fucoidans are often used interchangeably. These polymers occur in the intercellular tissues or mucilaginous matrix of brown. However, the structure of algal fucans varies among species and sometimes among different parts of the seaweed (Dietrich et al., 1995; Rocha et al., 2005a). Thus, each new purified sulfated fucan is a unique compound and thus a potential new drug. Many studies have shown the anti-inflammatory action of a fucoidan from the alga *Fucus vesiculosus*, called sulfated fucan (Cardoso et al., 2010). It is a potent inhibitor of leukocyte migration during the inflammatory response, owing to its interaction with P and L-selectin (Zang et al., 2001; Klintman et al., 2002). Recently, it was revealed that sulfated fucans from the Fucales and Laminariales orders inhibit leukocyte recruitment in an inflammation model in rats (Cardoso et al., 2010; Paiva et al., 2011). However, there are no reports of leukocyte migration inhibition by fucans from the Dictyotales order. In this study we report on the chemical characterization of a sulfated fucan from the alga *Padina gymnospora* (Dictyotales), as well as its effect on leukocyte migration to the inflammation site, their citotoxicity, and nitric oxide (NO) production.

Material and methods

Chemicals

Propylenediamine (1,3-diaminopropane) was purchased from Aldrich (Milwaukee, WI, USA). Xylose, fucose, glucuronic acid, sodium thioglicollate and galactose were obtained from Sigma (St. Louis, MO, USA). Agarose low-MR was purchased from Biorad (Richmond, CA, USA). Acetone and sulfuric acid were obtained from Merck (Darmstadt, Germany).

Animals

We used male albino Swiss mice (6 to 8 weeks old, 25-30 g) from the Department of Biochemistry of the Federal University of Rio Grande do Norte. The mice were housed in standard polypropylene cages, four per cage at 25±1 ºC on a 12 h light/dark cycle (lights on 6 am - 6 pm) with free access to food (Purina) and water. The experiments were carried out in accordance with research guidelines for the care of laboratory animals. After the experiments, the mice were killed by cervical dislocation. The protocol for these experiments was approved by the Committee of Ethics in Research of the Hospital Universitário Onofre Lopes (HUOL, UFRN) under approval number 082/07.

Algae

Padina gymnospora is a brown algae collected on the Natal state of Brazil and identified by Muttutamby Durairatnam, from Department of Oceanography and Limnology of UFRN, RN. A voucher specimen has been deposited in DOL-Center Bioscience (06-1994), UFRN (Universidade Federal do Rio Grande do Norte).

Polysaccharide extraction

The algae *Padina gymnospora*, brown algae, were collected on the Natal coast, Brazil. Sulfated polysaccharide extraction was carried out as described earlier (Silva et al., 2005, Rocha et al., 2005a). Briefly, the extraction involved proteolytic digestion for 24 h, after which the mixture was filtered through cheesecloth and the filtrate was fractionated by precipitation with acetone as follows: 0.3 volumes (v) of ice cold acetone were added under gentle agitation to the solution, which was stored at 4 ºC. The solution was kept in the cold for 24 h. The precipitate formed was collected by centrifugation (10000 x g at 20 min), dried under vacuum, resuspended in distilled water, and analyzed. We added 0.5, 1.1, 1.5, and 2.6 v of acetone to the supernatant, calculated from the initial solution, and then we repeated the procedures as above. Thus, we obtained five fractions denominated F0.3, F0.5, F1.1, F1.5, and F2.6 respectively. To visualize the fucans in these fractions, we submitted them to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer (PDA), pH 9.0. The gel was dried and stained for 15 min with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:4.9, v/v). The gel was then distained with the same solution without toluidine blue (Dietrich & Dietrich, 1976). The molecular weight was observed in Sephadex G-75 and eluted with 0.2 M acetic acid.

Chemical analyses and molecular weight determination

Total sugars were estimated by the phenol-H_2SO_4 reaction (Dubois et al., 1956). The monosaccharide content of the polymers was estimated as described earlier (Rocha et al., 2005). After acid hydrolysis of the polysaccharides (4N HCl, 100 ºC, 4 h), the sulfate content was measured by the toluidine blue method, as described by the turbidimetric method (Dodgson & Price, 1962). Protein content was measured as described by Spector, 1978. Molecular weight was determined by high-performance gel permeation chromatography.

Molecular weight

The samples (500 mg) were dissolved in H_2O (800 µL). The MW of the F1.5 fraction was eluted with Sephadex G-75 in fractions of 1 mL. Column calibration was performed with standard dextrans (MW: 22.8, 47.3, 112, 212, and 404 kDa, respectively, purchased from Fluka). The elution of polysaccharides was made by phenol-H_2SO_4 reaction (Dubois et al., 1956). The fractions were eluted with 0.2 M acetic acid.

Infrared spectroscopy

Fucan (5mg) was mixed thoroughly with dry potassium bromide. A pellet was prepared and the infrared spectrum was measured by a Perkin-Elmer instrument.

NMR experiments

^{1}H and ^{13}C NMR of the fucan were recorded using a Bruker DRX 400 apparatus with triple resonance probe. About 15 mg of each sample was dissolved in 0.7 mL of 99.9% D_2O (Cambridge Isotope Laboratory). All spectra were recorded at 60 ºC with H_2OD suppression by presaturation.

Peritoneal exudate cell (PEC) preparation

Thioglycollate-elicited PEC were obtained from mice following intraperitoneal injection of 3 mL of thioglycollate medium (3.0 g/100 mL) and collected by infusing their peritoneal cavity with 5 mL of ice-cold

sterile PBS, 3-4 days later. After centrifugation, the cell pellet was washed twice with cold, sterile PBS and then resuspended in RPMI 1640 medium. The cells were counted in a Neubauer chamber and were plated to adhere to a tissue culture plate. After 30 min at 37 °C under a 5% CO_2 atm, the cells were washed twice with warm sterile PBS to remove nonadherent cells. Adherent macrophages were then incubated in a standard medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 50 mg/mL penicillin and 100 U/mL gentamicin. Ninety percent of adherent cells were macrophages and the preparation was not purified further (Piemonte & Buchi, 2002).

Cell viability by MTT assay

Adherent macrophages were incubated (24 h) in the standard medium in the absence (control) or presence of fraction F1.5 (125-500 mg/mL) at 37 °C under 5% CO_2. Cell viability was then evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosmann, 1983).

Cell viability (Trypan blue)

Groups of five animals (n=7) were used for in vivo assessment, and cell viability was observed with trypan blue. All control groups received sterile saline. Control and test mice (10 or 25 mg/kg) were killed by ether anesthesia 3 h after they received a single intraperitoneal (*i.p.*) dose of fucan solution and after the PEC were obtained. Each mouse was injected with 5 mL of sterile PBS, and after centrifugation at 3200 x *g* for 5 min, the cell pellet was washed twice with cold PBS and resuspended in the same solution. The viable cells were counted in a Neubauer chamber by the trypan blue exclusion test and Griess's reaction (Wallum et al., 1990).

Nitric oxide (NO) production

NO synthesis was determined by measuring the accumulation of nitrite (NO_2^-), a stable metabolic product of NO, by using the method proposed (Green et al., 1982). Macrophages treated in vitro were plated into 96-well tissue culture plates. After 24 or 48 h, 100 mL aliquots of cell-free supernatant were mixed with an equal volume of Griess-reagent (0.5% sulfanilamide and 0.05% *N*-1-naphtyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min at 25 °C. The optical density of the samples was then measured at 540 nm on a microplate reader. Nitrite concentration was determined by referring to a standard curve using sodium nitrite (10-80 mM) diluted in culture medium.

Thioglycollate-induced peritonitis

Groups of five animals (n=7) were used for the thioglycollate-induced peritonitis. All control groups received sterile saline, which was the fucan solvent. The mice were treated with a subcutaneous injection of F1.5 fraction solution at different concentrations (10 or 25 mg/kg), as described (Xie et al., 2000). After 30 min, the animals received a 1 mL intraperitoneal injection of 3% thioglycollate medium or sterile pyrogen-free saline. The mice were killed 3 h later and the peritoneal leukocytes were harvested by peritoneal lavage with 5 mL of saline containing 2 mM EDTA. The leukocytes were counted after staining with Turck solution.

Statistical analysis

Results are expressed as mean±SD. Statistical analysis of the data was carried out using analysis of variance (ANOVA) and Tukey's test ($p<0.05$) for comparison of means.

Results and discussion

In this study, the brown marine algae *Padina gymnospora* was treated with acetone to remove lipids and pigments. Immediately after, the supernatant was submitted proteolytic digestion with maxatase resulted in low protein contamination (Silva et al., 2005; Rocha et al., 2005b; Queiroz et al., 2006). This step was important because the sulfated fucans were usually bound to a large number of proteins. Fractionation with acetone was used because polysaccharide molecules were precipitated through the action of organic solvents. From this methodology we obtained five polysaccharide fractions denominated F0.3, F0.5, F1.1, F1.5 and F2.6. The chemical composition of sugars obtained from acetone fractionation is shown in Table 1. All the fractions contained fucose and xylose; however, different proportions were observed in these sugars when the fractions obtained with different acetone volumes were compared. Only fractions F0.3, F0.5, and F1.1 contained uronic acid, which indicated the presence of alginic acids, thus explaining why these fractions had a larger amount of total sugars. The sulfate was observed in all the fractions. The F0.5 and F2.6 fractions showed a larger amount of this compound (31.2 and 17.5% respectively) when compared with the other fractions. The molar ratio of F1.5 for fucose:galactose:xylose:glucose was 1.0:0.4:0.2:0.1 respectively.

The confirmation of fucose in all the fractions, together with the presence of sulfate and of other monosaccharides, indicates the presence of fucans in the ketonic fractions obtained. Earlier studies indicate that the presence of sulfated fucans seems to be common in

brown algae of the Dictyotales order (Leite et al., 1998; Silva et al., 2005). The purity of the sulfated fucan preparation was checked by agarose gel electrophoresis and chemical analysis.

After preliminary chemical characterization, it was observed that fraction F1.5, despite its low yield compared with the other fractions, had a polysaccharide population free of contaminants, such as alginic acids. Accordingly, this fraction was chosen for the subsequent studies.

Previously, we were able to show that several Dictyotales contain three main sulfated fucans (Dietrich et al., 1995) These were clearly observed when the electrophoretic migration was investigated using 1,3-diamino propane buffer (PDA). This type of complex formed between sulfated polysaccharides and diamine is similar to that described for sulfated glycosaminoglycans from animal tissues (Queiroz et al., 2006). Thus, the fucans from brown seaweed display structural variability; nevertheless, they can be divided into three different classes, according to their electrophoretic behavior, which reflects the proportion and sequence of sugar residues in addition to the sulfation pattern (Leite et al., 1998). We have named the heterofucans from brown algae fucans A, B, and C, according to their relative migration in agarose gel electrophoresis in 1,3-diaminopropane acetate buffer (Rocha et al., 2005a). When fraction 1.5 was submitted to agarose gel electrophoresis (Figure 1), the electrophoretic profile showed the presence of a single band in F1.5, which has electrophoretic mobility similar to that of fucan C from other Dictyotales. Fraction 1.5 was applied to a Sephadex G-75 column (Pharmacia Biotech) and eluted with 0.1 M acetic acid. Fractions of approximately 1 mL were collected. One peak was obtained (fraction numbers 33-45), with 25,000 kDa.

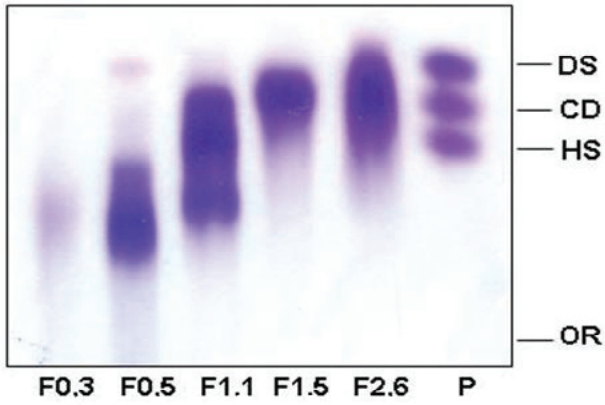

Figure 1. Agarose gel electrophoresis of heterofucans fractions from *Padina gymnospora.* Five-milliliter aliquots (10 mg/mL) of the fractions shown were applied to agarose gel prepared in 0.05 M 1,3-diaminopropane-acetate buffer (PDA), pH 9.0, and subjected to electrophoresis at 110 V/cm for 60 min. The gel was then kept in 0.1% cetyltrimethylammonium bromide for 4 h and dried, and the polysaccharides were stained with 0.1% toluidine blue in a solution containing 50% ethanol and 1% acetic acid in water for 15 min. The gels were then destained with the same solution without toluidine blue. Fucans obtained with several concentration of acetone were named (0.3, 0.5, 1.1, 1.5 and 2.6 v). P: standard of glycosaminoglycans:chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS), 5 µg each. OR: origin. The definition of acetone fractions is given in the legend.

Infrared spectrum and nmr

Infrared spectrum studies of F1.5 show bands with typical sulfate polysaccharide absorption (Chevolot et al., 1999; Cumashi et al., 2007). The signals at 3419 and 1255 cm^{-1} were from stretching vibration of O-H and C-O, respectively. Typical absorption bands were observed at 1255 cm^{-1}, corresponding to sulfate ester groupings (S=O), and at 847 cm-1, indicating that most of the sulfate is axially positioned at C4. The ^{1}H NMR spectrum had characteristics consistent with the presence of α-L-fucose units (Figure 2A). The anomeric protons appear in region 5.0 and signals of the fucose methyl group appear in the 1.35-1.40 region (Chevolot et al., 1999). The signal at 4.81 ppm was identified as β-D-xylose and 4.31 ppm was attributed to the C-4 protons in galactose or its methyl.

Several fucans had a very complex ^{13}C-NMR spectrum, which was difficult to interpret The ^{13}C-spectroscopy of F1.5 is shown in Figure 2B. A peak at 17.7 ppm (C-6) corresponded to $-CH_3$ of sulfated fucose and 77.5 ppm to the (C-3) of this sugar. The anomeric region showed signals at 105.0 and 101.0 ppm; for the C-1 of 4-α-L-fucose, a signal at 99 ppm corresponded to 3,6-di-substituted ß-D-galactose. Minor signals were also observed at 81.5 and 69.0 ppm confirmed ß-D-galactose units (Leite et al., 1998; Rocha et al., 2005a). The same spectrum, which also showed peaks at 84.1 ppm, may be attributed to 1,3 fucose. The results confirmed that F1.5 is a sulfated fucan from the fucose-branch.

A balance between the therapeutic and toxicological effects of a compound is an important parameter to verify the pharmacology applicability of a specific compound. Before initiating the tests to assess the anti-inflammatory activity of heterofucan F1.5, we assessed its toxicity. The method used is based on the different metabolic conditions of the cells, since MTT reduction (Mosmamm et al., 1983) provides information on their mitochondrial function. The results are shown in Figure 3 and indicate that fraction F1.5 does not cause cell death after 24 h in concentrations of up to 125 µg/mL. Viability was also observed with trypan blue (results not shown). This chromopore is negatively charged and does not interact with the cell, demonstrating that membranes were not damaged.

Table 1. Chemical composition (%) and molar ratio of the polysaccharides from brown algae *Padina gymnospora.*

Fraction	Yield (%)	Total sugar (%)	Protein (%)	Monosaccharides Molar ratio Fuc:Xyl:Man:Glc:Gal	Uronic acid (%)	Sulfate (%)
F0.3	12.4	30.0	0.7	1 : 0.3: 0.2: 0 : 0	9.2	10.7
F0.5	59.6	33.5	1.0	1 : 0.9: 0.2: 0.3: 0	5.8	31.2
F1.1	25.8	41.5	1.2	1 : 0.9: 0.2: 0.3:0.6	6.7	13.1
F1.5	1.8	21.0	0.5	1 : 0: 0.2 :0: 0.1: 0.4	¬-	8.9
F2.6	0.5	23.0	1.0	1 : 0.8: 0.1: 0.5: 0.3	¬-	17.5

Like heparin, fucoidans have been shown to affect many biological activities, such as inflammation, cell proliferation and adhesion, viral infection, and fertilization (Linnemann et al., 2000). However, relatively few studies have interpreted the biological activity of fucoidans in terms of molecular structure (Xie et al., 2000). The experimental inflammation models utilized revealed that fucans, like heparin, inhibit the leukocyte migration to the inflammation site (55-70%).

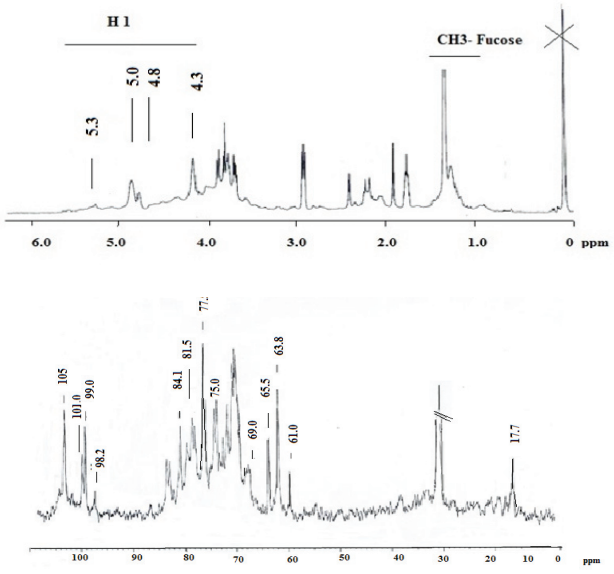

Figure 2. A. ^{1}H NMR spectrum of fucan (F1.5) from *Padina gymnospora.* The spectrum was recorded at 60 ºC in a D_2O solution of fraction. B. ^{13}C spectrum of fucan (F1.5) from *P. gymnospora.* The spectrum was recorded at 60ºC in a D_2O solution of fraction.

Earlier studies show that the fucoidan, or fucan, extracted from the brown alga *Fucus vesiculosus*, inhibits in a dose-dependent manner, but not specifically, the rolling and migration (Yang et al., 1996; Paiva et al., 2011) of leukocytes in processes mediated by molecules of the selectin family. Furthermore, this polymer is also capable of decreasing injury in models of inflammation caused by non-infectious agents (Ostergaard et al., 2000). Given that there are no reports on the action of a heterofucan from Dictyotales in blocking leukocyte migration to the inflammatory site, we carried out preliminary assays for this purpose. Acute peritonitis induced in the mice was characterized by an increase in the number of leukocytes in the peritoneal cavity (Figure 4). Intraperitoneal injection of 10 and 25 mg/kg concentrations of fucan reduced leukocyte extravasation by 60% and 39%, respectively. Our results show that the brown alga *Padina gymnospora* contains a sulfated fucan with anti-inflammatory action.

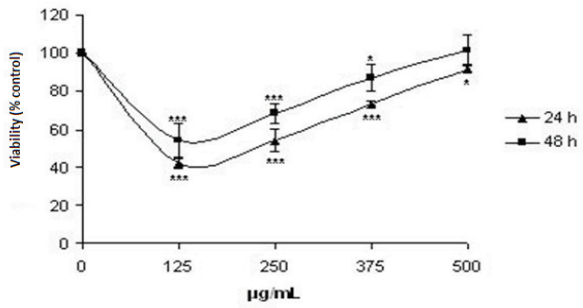

Figure 3. Cytotoxic assessment of the effect of the sulfated fucan from *Padina gymnospora* on peritoneal macrophages. Cellular viability was measured by the MTT colorimetric method. Adhering macrophages ($5x10^5$ cells/well) were incubated for 24 h with different fucans concentrations. The MTT reagent was then added and was followed by incubation for 3 h. The crystals were dissolved by adding isoproponol acid and cell viability was determined. Viability was established as A_{540nm} and expressed as % compared to control. The values represent the mean±SD of different samples (*p< 0.05; ***p<0.001 vs control).

Measurement of NO

Since NO is a molecule rapidly transformed into nitrite and nitrate, these parameters are currently used to monitor NO production; for this reason, the quantification of the amount of nitric oxide released by macrophages was determined by Griess's reaction (Green et al., 1982). We observed a significant reduction in nitric oxide (Machiavell et al., 2007) production in the presence of high fucan concentrations (above 375 µg/mL). However, we found a bifunctional effect (Yang et al., 2006; Di Rosa et al., 1996), given that low fucan concentrations

stimulate NO production (Figure 5).

Our data show that low fucan concentrations do not decrease cell viability and stimulate the release of NO. Several studies have reported that low concentrations of NO protect the macrophages from cell death (Ushakov et al., 2005), whereas excessive NO causes cell death (Li et al., 1999; Ostergaard et al., 2000). It was shown for the first time that a low concentration range of fucans from *Laminaria japonica* increased the level of NO in macrophages, whereas high concentrations inhibited the release of NO in macrophage cells (Ley et al., 1993). Further studies may help to determine which proteins are involved in the sulfated fucans effect of Dictyotales on NO release.

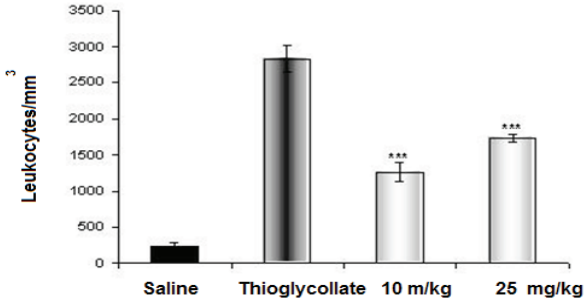

Figure 4. Anti-inflammatory action of the sulfated fucan from *Padina gymnospora.* Leukocyte inhibition was assessed by the induced peritonitis model using Swiss mice. The figure represents leukocyte migration 3 h after intraperitoneal administration of a single dose of 3% thioglycollate. The negative control received sterile saline solution and the positive control received no treatment. The polysaccharides were injected subcutaneously 30 min before thioglycollate administration. Each experimental group contained five animals and the results show the mean±SD (***p<0.001 vs control).

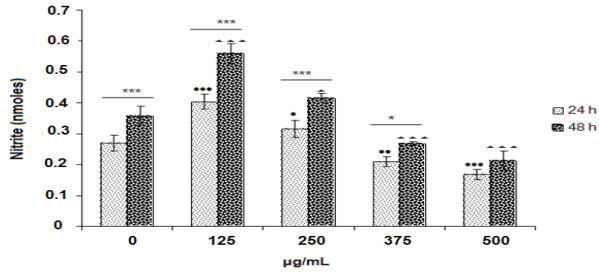

Figure 5. Effects of fucan from *Padina gymnospor*a on *in vitro* nitric oxide production. Adherent macrophages were incubated for 24 or 48 h with fucan (125-500 µg/mL) or control (medium alone). The NO accumulation was measured in the supernatant using Griess's reaction and calculated as nmol nitrite. Values are mean±SD of five different samples. •Significant compared to the control after 24 h (●p<0.05; ●●p<0.01; ●●●p<0.001); ▲Significant compared to the control after 48 h (▲p<0.05; ▲▲p<0.01; ▲▲▲p<0.001); *Significant compared to the same concentration at the same times (*p<0.05; **p<0.01; ***p<0.001).

Conclusion

In this work, the confirmation of fucose in all the fractions, together with the presence of sulfate and other monosaccharides, indicates the presence of sulfated fucans in the ketonic fractions. Infrared and NMR spectra showed that several F1.5 fraction is a heterofucan containing fucose, galactose, xylose and traces of glucose. Given that there are no reports on the action of a heterofucan from Dictyotales in blocking leukocyte migration to the inflammatory site, our findings show that *i.p*. administration with sodium thioglycollate resulted in a remarkable decrease of leukocyte recruitment in an experimental model of peritonitis in mice. This study demonstrated that this heterofucan has an anti-inflammatory effect. We observed a significant reduction in nitric oxide production in the presence of high fucan concentrations. These unexpected results suggest two different ways in which fucans act, depending on cell status.

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***Correspondence**

Edda Lisboa Leite
Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte
59082-970, Natal-RN, Brazil
eddaleite@cb.ufrn.br
Tel.: +55 84 3215 3416
Fax.: +55 84 211 9208