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Antioxidant and anti-inflammatory effect of polysaccharides from *Lobophora variegata* on zymosan-induced arthritis in rats

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ABSTRACT

This study analyzes the action of sulfated polysaccharides, fucans, from algae *Lobophora variegata* on zymosan-induced arthritis in rats. Groups of fucans, obtained after acetone fractionation (0.3–2.0 volumes), were denominated F0.3, F0.5, F0.8, F1, F1.5, and F2. The results that F1 contained a high yield in relation to other fractionated fucans. Chemical and structure analysis of F1 was performed by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopies. The *in vitro* antioxidant activities of the fraction F1 were also observed. Thus, 2 mg/mL of F1 inhibited the phosphomolybdate in the total antioxidant activity assay. The EC_{50} values were 0.3 mg/mL and 0.12 mg/mL for superoxide and hydroxyl radicals, respectively. Fucan F1 (25, 50, and 75 mg/kg by body weight), diclofenac sodium (10 mg/kg), and L-NAME (25 mg/kg) were administered intraperitoneally (i.p.) in rats, according to body weight of different groups of animals ($n=6$). After 6 h, analyses of cell influx and nitrite levels were conducted. Then after 96 h, analysis of edema and concentration of serum TNF-α was carried out along with histopathological analysis. F1 at 25, 50, and 75 mg/kg i.p. by body weight reduced cell influx in 52.1–96.7% and nitric oxide level in 27.2–39% compared with the control group. The reduction of edema and serum TNF-α was observed at 50 mg/kg i.p. ($p<0.001$). These results suggest that this heterofucan from the brown algae *L. variegata* has potential anti-inflammatory activity in acute zymosan-induced arthritis in rats and that antioxidant activity promotes modulation in the cellular redox state.

1. Introduction

Fucans are polysaccharides classified into homofucans, formed by fucose and sulfate, and heterofucans, which contain other sugars and uronic acids, in addition to sulfate and fucose (1,2). Fucans from brown algae are usually heterogeneous and branched, with irregular patterns of sulfatation (4). Fucoidans are considered by some researchers as sulfated homofucans, although they contain small amounts of other sugars. These compounds are involved in a number of pharmacological activities, such as anticoagulant, antithrombotic, antioxidant, and inflammatory activities (3), regulation of cytokine production, and P and L-selectin blocking (1).

Arthritis, an inflammatory disease of the joints, is caused by several factors, including chemical, biological and immunological factors. It is characterized according to the articular structure affected. Thus, affection of the synovial membrane after intra-articular administration of zymosan in rats promotes the emergence of inflammatory parameters similar to those of rheumatoid arthritis (RA), a systemic inflammatory disease of autoimmune etiology, characterized by chronic and erosive synovitis of peripheral joints (5,6). Recent studies point to imbalances in redox processes in the inflammatory response to RA, which would account for worsening of the pathology due to uncontrolled oxidative stress (7). This etiology may be explained by genetic defects, as well as immunoregulation and mechanism defects that lead to tissue damage, including the imbalance between oxidants and antioxidants, in favor of oxidants, which would result in disorder of both signaling and molecular damage control (8,9).

In synovitis, inflammatory cells respond with increased cytokine production and intense formation of free radicals, such as reactive oxygen species, leading to oxidative stress that amplifies the inflammatory process, causing articular destruction, edema, and pain (10). Studies demonstrated that, although inflammation is the persistent characteristic, the generation of high levels of free radicals in the inflamed joint and the deficit levels of antioxidant systems are involved in this pathology (11).

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In earlier studies we observed the action of fucoidans and homofucans in intra-articular inflammation. The present study focused on structural characterization, antioxidant activity, and the articular inflammation model in Wistar rats of a heterofucan from the brown seaweed *Lobophora variegata*.

2. Materials and methods

2.1. Materials

For this study, materials were obtained as follows: acetone and sodium chloride (Reagen Quimibrás Indústrias Químicas S.A., Rio de Janeiro, RJ, Brazil); sulfuric acid (Merck, Darmstadt, Germany); coomasie brilliant blue R 250, α-(L)-fucose, D-galactose, L-xylose, glucuronic acid, D-mannose, NADPH, nitroblue tetrazolium, phenazin metasulfate, nitrate reductase (Sigma Aldrich, St. Louis, USA); agarose (Bio Rad Laboratories); 2% xylazine chloridrate and 5% ketamine chloridrate (Köning do Brasil, Ltda).

2.2. Sulfated polysaccharide extraction from L. variegata

L. variegata in dried powder form was suspended in 2 volumes of 0.15 M NaCl and the pH of the mixture was adjusted to 8.0 with NaOH. Fifteen milligrams of maxatase per gram of seaweed was added for proteolytic digestion. The reaction mixture was then incubated for 24 h at 60 °C and filtered through cheesecloth. The polysaccharides of the filtrate were further purified and fractionated by increasing the acetone concentration (0.3, 0.5, 0.8 1.0, 1.5, and 2.0v).

2.3. Chemical analyses

Total sugars were estimated by phenol-sulfuric acid reaction (12) using a standard galactose curve. Sulfate was measured after hydrolysis (6 N HCl, 100 °C, 4 h) using the turbidimetric method (13) with sodium sulfate as standard. Proteins were estimated by the Bradford reagent (14).

Monosaccharidic compositions of the fractions were determined by high-performance liquid chromatography (HPLC) (15). The polymers were hydrolyzed (2 M HCl, 100 °C, 2 h) and their sugar content was determined by high performance liquid chromatography (HPLC) with a refractive index detector using LichroCART®250-4. As references, the following sugars were analyzed: arabinose, galactose, glucose, fucose, mannose, rhamnose, and xylose. The mobile phase consisted of a mixture of 0.1 mol/l KH_2PO_4 (pH 10)-acetonitrile (80:20). The flow-rate was 1.0 mL/min and column temperature was 30 °C. Sugar was identified by a comparison with reference sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, galacturonic acid, glucuronic acid, mannuronic acid, and *N*-acetyl-β-D-glucosamine).

2.4. Electrophoresis

Aliquots of the fractions (5 μg) were applied to 0.6% agarose gel prepared with 0.05 M 1,3 diaminopropane acetate buffer, pH 9.0. The compounds were then fixed in the gel with 0.01% N-cetyl-N-N-N trimethylamonium bromide for 4 h. The gel was dried and stained with 0.1% toluidine blue (16).

2.5. Infrared spectroscopy and NMR spectroscopy

The infrared spectra were measured with a Perkin–Elmer instrument in KBr pellets containing 5 mg of the sample dissolved in D_2O. The ^{1}H-nuclear magnetic resonance spectra were obtained in a Bruker AMX 500 MHz and the ^{13}C-NMR spectra were obtained in a Bruker AM-400WB 500 spectrometer at 60 °C.

2.6. Total antioxidant activity assay

Total antioxidant activity was determined using 1 mL of reagent solution, containing ammonium molybdate (4 mM), sulfuric acid (600 mM) and sodium phosphate (28 mM), and 0.1 mL of test solution at different concentrations (0.0156–5 mg/mL) in tubes, which were then sealed and incubated at 95 °C for 90 min. After cooling to ambient temperature, the activities were spectrophotometrically monitored for Mo^{+6} formation from Mo^{+5}, forming phosphomolybdenum, which shows maximum absorbance at 695 nm. Concentrations of ascorbic acid were used to obtain a standard curve and total antioxidant capacity was expressed in ascorbic acid equivalents (17).

2.7. Reducing power assay

The reducing power of fraction F1 was determined based on the method proposed by Yen and Chen (1995). One mL aliquots, with different concentrations of F1 (0.156–5 mg/mL), were mixed with 2.5 mL of 0.2 M of sodium phosphate buffer, pH 6.6, and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Next, 2.5 mL of 10% (w/v) trichloroacetic acid was added to the mixture, which was centrifuged at 1500 g for 10 min. The supernatant (2.5 mL) was diluted with 2.5 mL of distilled water and added together with 0.5 mL of 0.1% (w/v) ferric chloride. In this reaction, $K_3Fe(CN)_6$ was reduced by the sample and $K_4Fe(CN)_6$ was formed, which in turn reacted with Fe^{+3}, giving rise to Prussian blue, which showed maximum absorbance at 700 nm (18). Concentrations of ascorbic acid were used to obtain a standard curve and the reducing power of F1 was expressed as ascorbic acid equivalents.

2.8. Superoxide radical scavenging assay

Superoxide radicals were generated in 3 mL of tris–HCl (16 mM, pH 8.0), containing 78 μM of NADH (reduced form), 50 μM of nitroblue tetrazolium (NBT), 10 μM of metasulfate phenazine, and assorted concentrations of polysaccharides (0.078–5 mg/mL). The reaction was detected by monitoring absorbance at 560 nm. The white reaction was free of NADH and substituted by tris–HCl. The removal rate (RR) of superoxide radicals was calculated using the following equation: $RR\ (\%) = (1 - Aa/Ac)^*100$, where Aa is the mean of the absorbances obtained in readings of specific sample concentration and Ac is the mean of absorbances obtained in readings of control reaction mediums (19).

2.9. Hydroxyl radical scavenging assay

The deoxyribose colorimetric method was used to determine removal activity of hydroxyl radicals (•OH). The following compounds were used: 200 μL of KH_2PO_4–KOH (100 mM), 200 μL of deoxyribose (15 mM), 200 μL of $FeCl_3$ (500 mM), 100 μL of EDTA (1 mM), 100 μL of ascorbic acid (1 mM), 100 μL H_2O_2 (10 mM), and 100 μL of sample (0.025–2 mg/mL). The mixture was incubated at 37 °C for 1 h. After the incubation period, 1 mL of 1% (w/v) thiobarbituric acid (TBA) and 1 mL of 2.8% (w/v) trichloroacetic acid (TCA) were added. The solution was heated in a double-boiler at 80 °C for 20 min, developing a pink color, characteristic of the malondialdehyde bond, the product of deoxyribose oxidation, and TBA. Absorbance was determined at 532 nm. The removal rate (RR) of hydroxyl radicals was calculated using the following formula: $RR\ (\%) = [1 - (A_o/A)] \times 100$, where A_o is the mean absorbances of control readings (without deoxyribose) and A is the mean absorbances of sample readings (20).

2.10. Cytotoxic assessment of F1

Venous blood was collected in the presence of heparin and then processed. The blood was diluted with the same volume of sterile

saline solution and then added together with isopaque-Ficoll (HISTOPAQUE-1077) at a proportion of 3 mL for each 5 mL of heparinized blood. This mixture was then centrifuged at 1450 g for 30 min at 25 °C. Peripheral mononuclear blood cells were carefully aspirated and transferred to another tube. Twenty mL of the RPMI medium at 4 °C was added to the cells, and suspension was centrifuged at 1600 g for 15 min at 4 °C. This procedure was repeated twice. Next, the supernatant was removed and 2 mL of RPMI was added to the precipitate. A 10 μL aliquot was mixed with 40 μL of Turck solution for microscopic visualization of the cells, which were counted in a Neubauer chamber. After the cell count, an aliquot of the suspension was removed and the concentration was adjusted with RPMI, such that the final solution was 1×10^6 cells/mL.

The cells were cultured in 96-plate wells containing 1×10^6 cells/well. F1 concentrations were prepared from sterile stock solutions and serially diluted in RPMI-1640 medium, obtaining a final volume of 100 μL in each well. Thus, final concentrations were between 0.5 and 1.0 mg/mL. Next, MTT stain was added (10 μL of MTT per 100 μL of medium), then dissolved in PBS buffer at 5 mg/mL, filtered, and sterilized. The plates were kept in humid atmosphere containing 5% CO_2 at 37 °C for 24 and 48 h. Living cells are capable of converting the MTT into a blue compound called formazan. After the necessary reaction time, acid/isopropanol was added to each well to stabilize the formazan crystals. The readings were carried out on an ELISA reader at 540 nm (21).

2.11. Zymosan-induced joint inflammation

Wistar rats, aged 3–4 months (190–240 g), were housed in cages under controlled illumination (12–12 h light-dark cycle) at 22–23 °C and supplied food and water *ad libitum*. The negative control group (n = 6) received a 50 μL intra-articular (i.a.) administration of 0.9% saline solution, while the positive control group and the other groups received 50 μL of zymosan (20 μg/μL) diluted in 0.9% saline (22). Inflammation was induced with zymosan after the animals had been anesthetized with 1 mL of xylasine chloridrate (5 mg/kg) and ketamine chloridrate (10 mg/kg). After 1 h of induction, F1 (25, 50 and 75 mg/kg), sodium diclofenac (5 mg/kg), a nonsteroidal anti-inflammatory drug (NSAID), and NG-nitro-L-arginine methyl ester (L-NAME) (25 mg/kg) were intraperitoneally (i.p.) administered. In the first experiment, after 6 h of arthritis induction, the animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and then killed by decapitation. The synovial fluid was then collected to analyze cell influx and quantify nitric oxide. In a second experiment F1 (25, 50 and 75 mg/kg), sodium diclofenac (5 mg/kg) and L-NAME (25 mg/kg) were intraperitoneally administered, 1, 24, 48, and 72 h after induction, and at 96 h (5th day) the animals were anesthetized with chloral hydrate (400 mg/kg i.p.), exsanguinated, and killed. Joint edema was measured and blood was collected daily to analyze serum TNF-α. After the animals were killed, the synovial membrane was removed for histopathological analysis.

2.12. Analysis of cell influx and determination of nitric oxide (NO) production

Intra-articular lavage fluid was obtained by aspiration of the rat knee joint using sodium phosphate as buffer (0.15 M and pH 7.4) and EDTA (0.01 M). Total cell count was done in a Neubauer chamber using 20 μL of the lavage fluid diluted in 400 μL of Turk solution. After the articular lavage fluid was centrifuged at 600×*g* for 8 min, the supernatant was used to evaluate nitrite (NO_2^-) and nitrate (NO_3^-) production, indicators of NO synthesis. The NO_3^- of the exudate was reduced to NO_2^- by incubation with the nitrate reductase enzyme (670 μm/mL) in the presence of NADPH (160 μM) at room temperature for 3 h. Nitrite concentration was then measured by the Griess reaction, with the addition of 100 μL of this reagent to 100 μL of the sample. Absorbance at 540 nm was measured in an ELISA reader. Nitrite concentrations were calculated by comparing sample absorbance with standard solutions of sodium nitrite prepared in saline solution (23).

2.13. Edema evaluation

The edema was analyzed by measuring the thickness of injured knee joints using the caliper method (Lotus ABS digital caliper, REF: 5571, scale: 0–150 mm). The first measure was taken immediately before arthritis induction and the second immediately before administration of the treatment compounds. Edema thickness was always measured at the height of the kneecap on each of the 5 days of the experiment.

2.14. Determination of serum TNF-α

To determine serum TNF-α (BD OptEIA kit), 0.1 mL of animal serum was used. Identification of TNF-α was carried out using the ELISA method, at a wavelength of 450 nm. A standard curve with recombinant TNF-α was created to determine the effect of polysaccharides on this cytokine.

2.15. Histopathological analyses of the synovial membrane

The synovial membranes were collected and preserved in 10% formaldehyde. This was followed by routine processing until placement in paraffin blocks, which were subsequently sectioned with a 4-mm microtome steel razor for hematoxylin and eosine (H&E) staining (24). The histological cuts of the synovial membrane in rats were examined under optical microscope to analyze proliferation of intrascapsular fibrous tissue, the adipose tissue, cell infiltrate, villosities, and giant cells (25,26). The histological findings then underwent semiquantitative analysis.

2.16. Statistical analyses

Analysis of variance (ANOVA) with a significance level of $p<0.05$ and the Tukey–Kramer test were used to determine differences in the values obtained by the control and experimental groups.

3. Results

3.1. Extraction and chemical analyses

After collection, washing and drying at 37 °C under constant air current, the algal material, consisting of 40 g of dry seaweed, was weighed. Differential precipitation in acetone fractionated the polysaccharides of *L. variegata,* resulting in 6 fractions denominated as F0.3, F0.5, F0.8 F1, F1.5, and F2. Total fraction (TF) was considered "crude polysaccharide," and the other fractions were obtained from it. The resulting fractions showed differences in yield and in the amounts of proteins, total sugars, and sulfate (Table 1).

Sample yield was an important requirement for use in experimental models. Fraction F1, in addition to exhibiting greater yield than the others (51.9%), showed higher polysaccharide (46.6%) and sulfate (22.7%) content and lower amounts of protein (0.12%). Fractions F0.3, F0.5, and F0.8 exhibited a low yielding, proteins (0.2% and 0.42%, respectively) and alginic acids (not shown). For this reason, fraction F1 was chosen to study its effects in experimental models of arthritis and antioxidant activity.

The agarose gel electrophoresis and 1,3 diaminopropane acetate buffer, pH 9, of the above-mentioned fractions, demonstrated the polydisperse due several molecular weight. The metachromatic characteristics is common in sulfated polysaccharides from algal and animals as fucans and heparin respectively. Metachromasy, color reddish blue or pink, is a characteristic change in the color of staining carried out in biological tissues.

Table 1
Yield, amount of proteins assessed using the standard albumin curve, percentage of sugars using the standard galactose curve, and sulfate percentage with the standard sodium sulfate curve (1 mg/mL) of fractions extracted from *Lobophora variegata*, obtained from the differential precipitation with acetone. *SD (standard deviation).

Fraction	Yielding (%)	Protein (%)	Sugars (%)	Sulfate (%)
FT	–	0.13 ± 0.02	41.4 ± 0.63	21.5 ± 3.4
F0.3	2.1	0.2 ± 0.03	26.2 ± 0.09	26.4 ± 1.8
F0.5	2.7	0.42 ± 0.09	29.4 ± 0.09	36.6 ± 2.5
P0.8	16.5	0.18 ± 0.06	44.5 ± 0.12	29.5 ± 2.3
Fl	51.9	0.12 ± 0.08	46.6 ± 0.36	22.7 ± 2.1
F1.5	25.3	0.23 ± 0.02	52.6 ± 0.09	17.1 ± 2.6
F2	1	0.15 ± 0.04	40.8 ± 0.18	13.2 ± 1.6

3.2. Identification of F1 sugars by HPLC

Chromatographic analysis, using high performance liquid chromatography of acid hydrolysate from the polysaccharide fraction (F1), demonstrated the presence of fucose, galactose, and glucose sugars, with molar ratio of 1: 1.4: 1.9, respectively, containing traces of xylose.

3.3. Infrared spectroscopy of polysaccharide F1 from the seaweed L. variegata

In Fig. 1A, the 3734 and 3671 cm^{-1} bands showed axial deformation of O–H, corresponding to the intermolecular and intramolecular hydrogen bond. The bands at 3075 and 2885 cm^{-1} were attributed to axial deformations of the C–H for the secondary (–CH_2–) and primary (–CH_3) bonds, respectively. A band that was attributed to the angular deformations of the C–H Bond of the primary and secondary carbons was observed between 1630 and 1400 cm^{-1}. F1 showed an intense absorption band at 1238 cm^{-1} common to the sulfate ester. An additional sulfate absorption band at 850 cm^{-1} revealed the presence of sulfate in the axial position of C-4, common to fucans (2,27).

3.4. Nuclear magnetic resonance spectrum

The ^{1}H nuclear magnetic resonance (NMR) spectrum of the fucan was poorly resolved, but signals at 1.7 ppm corresponded to the methyl group of the fucose (not shown). The anomeric resonances at 5.26–5.28 and 4.08–4.41 confirmed the presence of anomeric signals (Fig. 1B). The analysis of ^{13}C-NMR spectroscopy is shown in Fig. 1C. Like other algal fucans (28), it has a very complex ^{13}C NMR spectrum, which was difficult to interpret thoroughly. The peaks at 103.5–104.0 ppm corresponded to β-D-galactose residues and at 97.2–97.8 ppm to α-L-fucose residues. The signal at 16.5 ppm corresponded to CH_3 of fucoses (29).

3.5. Antioxidants activities "in vitro"

Fraction F1 showed total antioxidant activity (Fig. 2A) in relation to ammonium molybdate. A concentration range between 0.039 and 10 mg/mL was used for F1, where maximum activity was observed above 5 mg/mL. The antioxidant activity of ascorbic acid was obtained and compared with the activity of fraction F1 from *L. variegata*. Reducing activity of ascorbic acid used as standard, reached a peak at 0.25 mg/mL. A comparison between the activity (absorbance) of 5 mg/mL in F1 and the maximum activity of ascorbic acid at 0.25 mg/mL showed a reduction rate of 75%.

The reducing power assay, which has ascorbic acid as standard, demonstrated that F1 was capable of reducing the reaction system (Fig. 2B). Thus, it promoted a reduction of Fe^{+3} to Fe^{+2}, forming the Prussian blue complex, indicating high reducing power (30). The maximum reducing power of F1 (2.5 mg/mL) was compared with the

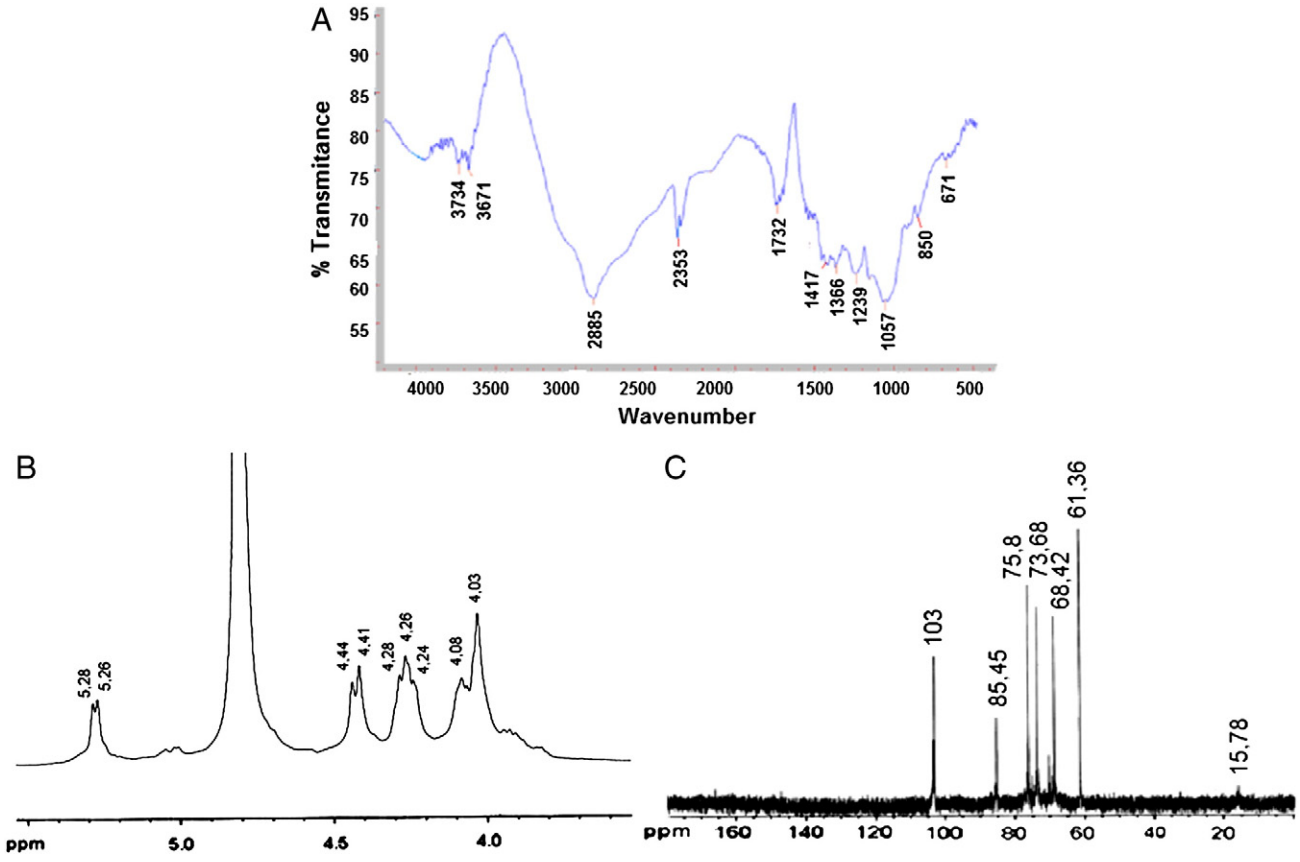

Fig. 1. A—Infra red spectrum of fraction F1 from *L. variegata*, B—H¹-NMR spectrum of fraction F1. C—C¹³-NMR spectrum of fraction F1.

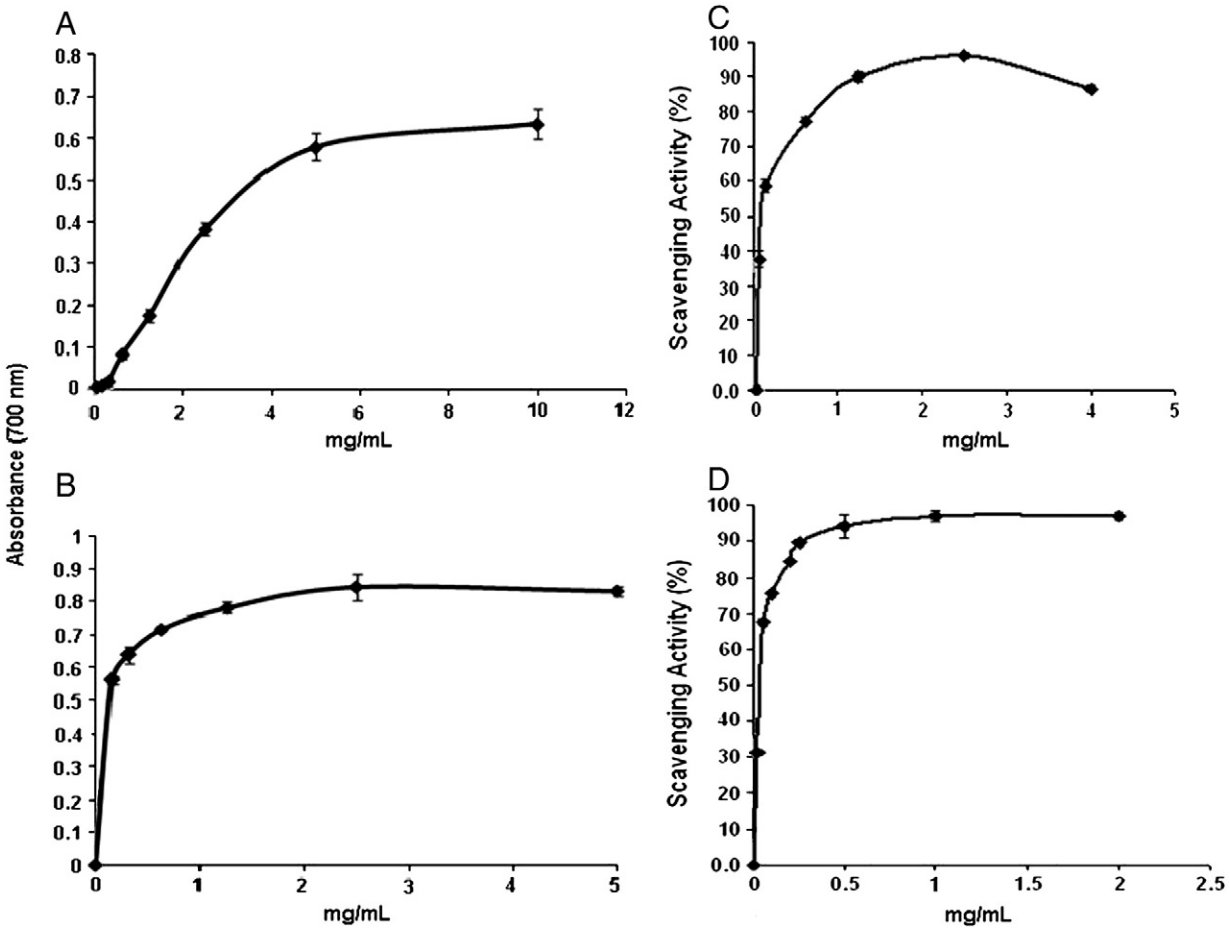

Fig. 2. A—Total antioxidant capacity of polysaccharides (0.039–10 mg/mL of F1) from the brown seaweed *L. variegata*. B—Reducing power activity of F1 in an oxidant system. C—Antioxidant activity curve of *L. variegata* in a superoxide-generating generating system with different concentrations of Fraction 1. D—Inhibitory activity of hydroxyl radical formation in a system generated from this free radical.

reducing activity of 0.25 mg/mL of ascorbic acid. Therefore, fraction 1, at a concentration of 2.5 mg/mL, showed a reducing rate of 85.7%. The reducing capacity of F1 served as a convincing indicator of its potential antioxidant potential.

The superoxide radical is a species produced in a phenazine metasulfate/NADH system with NBT reduction. Fig. 2C shows the percentage inhibition of superoxide radicals at different concentrations of F1. A maximum inhibition percentage of 94% of these radicals at 2.5 mg/mL of F1 was observed. Although the superoxide radical has weak oxidating action, its conversion to hydroxyl radicals has a very negative effect on living systems.

Fig. 2D shows that the hydroxyl radical was inhibited with the increase in F1 concentration, exhibiting inhibitory activity of around 98% when used at a concentration of 8 mg/mL.

The EC_{50} (50% of efficient concentration) values of the antioxidant assays were obtained for fraction F1 of *L. variegata*. The EC_{50} calculated represents the concentration of the sample required to provide 50% of the inhibitory activity of free radicals. Thus, 2 mg/mL of F1 inhibited 50% of phosphomolybdate in the total antioxidant activity assay, while in the reducing power assay, the EC_{50} of F1 was 0.675 mg/mL. In the removal assays of the superoxide and hydroxyl radicals, the EC_{50} values were 0.3 mg/mL and 0.12 mg/mL, respectively.

3.6. Cytotoxicity of F1

The formation of formazan by living cells can be detected after 24 h of incubation with F1 (0.5 and 1 mg/mL). The cytotoxicity assay revealed that the concentrations used (0.5 and 1 mg/mL) showed no statistically significant differences ($p>0.05$) compared with the control assay, with no cytotoxic capacity in relation to blood leukocytes (Fig. 3).

3.7. Effect of treatment with F1, sodium diclofenac, and L-NAME on leukocyte influx and on nitrite production in the articular lavage fluid

After 6 h of zymosan (Zy) induction, the treated groups were able to reduce leukocyte influx in the articular lavage fluid. Of the groups treated with F1, the group with 25 mg/kg showed less reduction (52.1%) in cell infiltrate ($p<0.05$). The groups at F1 at 50 mg/kg and 75 mg/kg had significant reductions ($p<0.001$) in the number of total leukocytes in the articular lavage fluid (83.5% and 96.7%, respectively), compared with the positive control (Fig. 4A). No significant differences were observed between the results of group F1 at 50 mg/kg and the groups treated with nonsteroidal anti-inflammatory drugs (NSAID) and with L-NAME ($p>0.05$). The two latter groups also demonstrated significant action against leukocyte influx, with diclofenac and L-NAME showing an 83.3% and 80.1% decrease in influx ($p<0.001$), respectively.

Treatment with F1 at the different concentrations (25, 50, and 75 mg/kg), sodium diclofenac (5 mg/kg), and L-NAME (25 mg/kg) (Fig. 4B) reduced ($p<0.001$) nitrite and nitrate production in the articular lavage fluid collected in the 6th h of induced inflammation compared with the group that only received intra-articular zymosan. The group treated with F1 (50 mg/kg) showed a greater effect (39%) on NO inhibition compared with the groups treated with 25 mg/kg (27.2%) and 75 mg/kg (33.1%) of F1. The treatment with F1 reduced

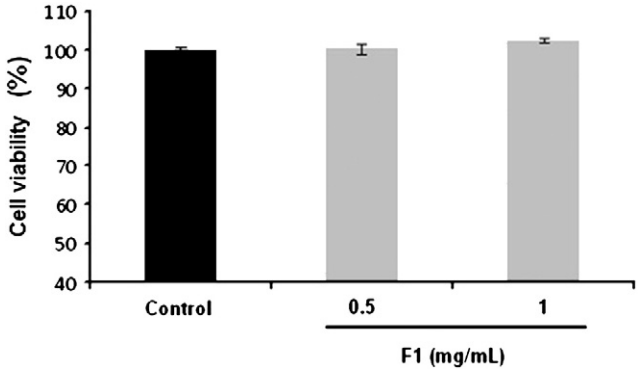

Fig. 3. The cytotoxicity assay of F1 sulfated fucan from *L. variegata*.

nitrite production in the articular lavage fluid in a non-dose-dependent manner. The action of the fucan was similar to that of sodium dicoflenac (37.5%). The action of L-NAME (50.7%) showed greater nitrite reduction.

3.8. Effect of treatment with F1, sodium diclofenac, and L-NAME on the articular edema of rats submitted to zymosan-induced arthritis(ZyA)

Animals from the negative control group (intra-articular saline) showed constant knee thickness. The positive group (50 μL of Zy 20 μg/μL i.a.) exhibited statistical differences from the negative control group, with $p<0.001$ between 1 and 96 h after induction (Fig. 5A).

The group treated with F1 at 25 mg/kg was not efficient in diminishing edema (Fig. 5B). The concentrations of 50 ($p<0.01$) and 75 mg/kg ($p<0.001$) of F1 reduced the edema on the 2nd and 3rd days of treatment (Fig. 5C and D).

The treatment with a 50 mg/kg dose of polysaccharide from *L. variegata* reduced the articular edema 24 h after induction compared with the positive control group under the same conditions ($p<0.001$). Compared with the edema 1 h after induction, the F1 group at 50 mg/kg diminished the edema by 63.4% in the 24th h and 94.5–100% after the 48th h, remaining so until the end of the experiment (5th day). The F1 group at 50 mg/kg showed a decrease in edema similar to that exhibited by the group of animals treated with 5 mg/kg of sodium diclofenac (Fig. 5E). The F1 group with 75 mg/kg diminished the edema by 11.8% in the 24th h and by 100% after the 48th h, remaining so until the end of the experiment (5th day), showing a decrease in edema on the 3rd day of treatment ($p<0.001$). The groups treated with sodium diclofenac (5 mg/kg) and L-NAME (25 mg/kg) (Fig. 5F) demonstrated significant effects ($p<0.001$) in the 24th h and 48th h, respectively.

Assessment of serum TNF-α cytokine was determined from the blood serum samples of animals from different experimental groups using the ELISA method. Comparison between the negative and positive control groups (Fig. 6A) demonstrated a statistically significant difference ($p<0.001$) on the 5th experimental day. A slight drop in TNF-α levels was observed in the group treated with F1 (Fig. 6B). The group treated with diclofenac showed no significant differences in TNF-α concentration (Fig. 6C), while the group treated with L-NAME had decreased serum TNF-α ($p<0.001$) after 72 h of experiment (Fig. 6D).

3.9. Hematoxylin and eosin staining (H&E) and immunohystochemistry

The histological findings then underwent semiquantitative analysis ($n=6$), where fibrous tissue, adipose tissue, cell infiltrate, capsular integrity, and giant cells were analyzed in terms of abundance and attributed scores as follows: 0 = absent, 1 = slight presence, 2 = moderate presence, and 3 = marked presence. The effect of F1 from *L. variegata* on histopathological parameters was determined in the synovial membranes of Wistar rats submitted to 96 h (5 days) of experiment and treated until the 4th day against zymosan-induced articular inflammation (Table 2). The semi-quantitative analyses of slides evaluated the following histopathological parameters of each experimental group: cellular infiltrate, number of adipocytes, synovial capsule degradation, fibrinoid deposits, and giant cells. A score was attributed to each parameter analyzed. Table 2 shows a marked presence of cellular infiltrate in the zymosan group (score 3) and F1 (score 1). Capsular integrity (score 1) was the same to L-NAME, F1 (50 mg/mL), diclofenac. This confirmed the pro-inflammatory effect of zymosan and anti-inflammatory action of F1.

Analysis of the negative control (synovial membranes of animals that received only intra-articular saline) (Fig. 7A), showed the presence of a large number of lipid-storing cells (adipocytes) (score 3), normal synovial capsule (1–4 cell layers and without villosities) (score 3), little fibrous tissue between the adipocytes (score 1), and no element that indicated a chronic immune reaction, such as giant cells (score 0). In the positive control (animals submitted to zymosan-induced inflammation), few or no adipocytes (score 0), abnormal synovial capsule (score 1), a large amount of fibrous tissue (score 3), and elements that characterize reaction of the local immunological system were observed, such as cell infiltrate and giant cells (Fig. 7B and C). Fig. 7D shows the F1-treated synovial membrane submitted to the zymosan-induced inflammatory process, clearly demonstrating the previously mentioned decrease in inflammatory parameters. This can also be observed in the histological analyses of animal membranes treated with sodium

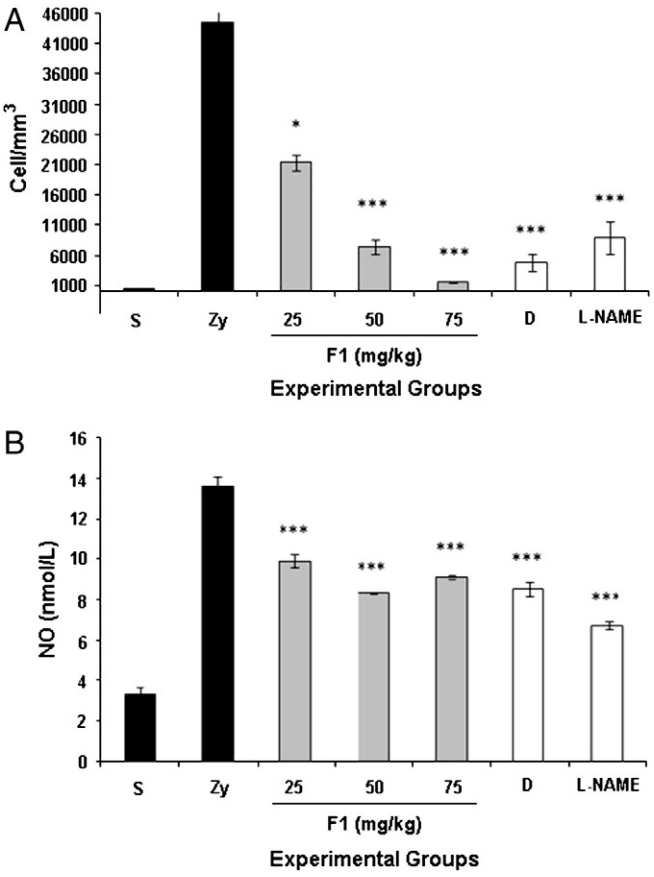

Fig. 4. A—Effect of F1 fucans from *L. variegata* on the number of leukocytes in samples of synovial fluid from animals submitted to zymosan-induced arthritis (20 μg/μL). ***$P<0.001$/**$P<0.01$ compared to the positive control group. B—Analysis of nitric oxide in the synovial fluid of animals. S represents the group that received saline i.a. (negative control). Zy represents the group that received zymosan i.a. (positive control). F1 (25, 50 and 75 mg/kg), D (5 mg/kg of sodium diclofenac), and 25 mg/kg of L-NAME *** = $P<0.001$.

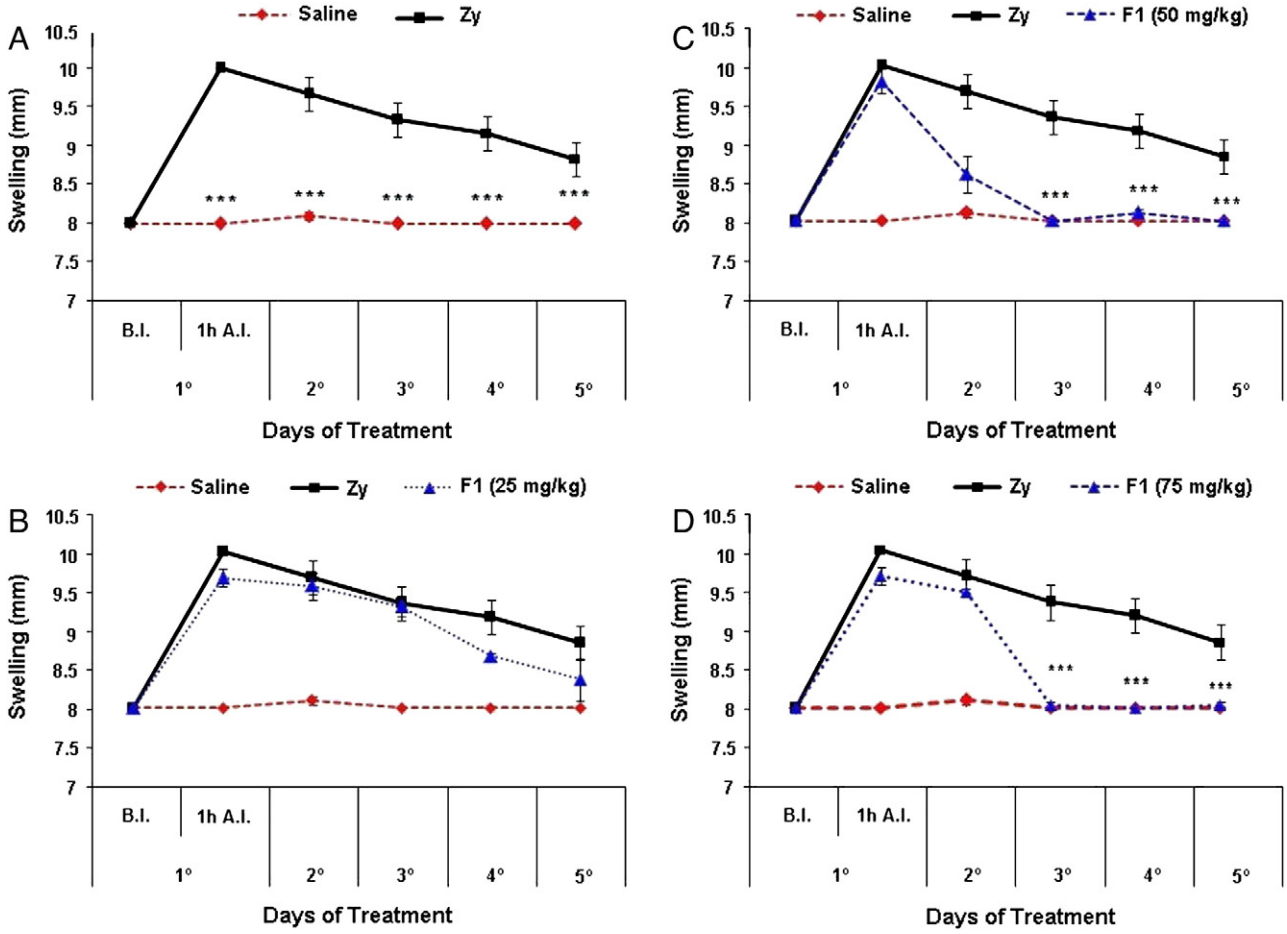

Fig. 5. A—Assessment of knee edema in Wistar rats submitted (Zy i.a.) and not submitted (saline i.a.) to induced arthritis. *** = P<0.001. B—Wistar rats submitted (Zy i.a. and treated with 25 mg/kg of F1) and not submitted (saline i.a.) to induced arthritis. *** = P<0.001. C—Assessment rats submitted (Zy i.a. and treated with 50 mg/kg of F1) and not submitted (saline i.a.) to induced arthritis. *** = P<0.001. D—rats submitted (Zy i.a. and treated with 75 mg/kg of F1) and not submitted (saline i.a.) to induced arthritis. *** = P<0.001.

diclofenac (Fig. 7E) and NG-nitro-L-arginine methyl ester (Fig. 7F). After the 1st h and until the 4th day of Zymosan-induced arthritis (ZyA), group 1 animals were treated with 50 mg/kg; after which they were killed on the 5th day. Histopathological analysis of the synovia showed an inflammatory exudate of polymorphonuclear (PMNs) cells (score 1) and a large amount of adipose tissue (score 2); however, fibrous tissue was found in few regions (score 2). Histopathological analysis of the membranes of sodium diclofenac-treated animals (5 mg/kg) showed little PMN infiltrate (score 1). The L-NAME-treated animals (25 mg/kg) also exhibited few PMNs (score 1). The efficacy of F1 is evidenced by the decrease in synovial inflammatory parameters, such as decreased leukocyte infiltrate, increased synovial capsule integrity, and increased number of adipocytes. The reduced inflammatory process was confirmed by histopathological and immunohistochemistry analyses, which showed a decrease in cell infiltrate in the synovial membrane and consequent reduction in TNF-α expression in saline, F1, AINES, and L-NAME (Fig. 8A, C, D, and E). Analyses of immunohystochemistry (TNF-α) of the negative control (synovial membranes of animals that received only intra-articular saline) (Fig. 8A) showed a low level of these cytokines. Although TNF-α had been detected in a high level in tissues with zymosan induced (Fig. 8B), we observed a low level in the rats with F1 fraction (Fig. 8C), dicofenac (Fig. 8D), and L-NAME (Fig. 8E). These profiles suggest an immunocellular response by F1 during the acute phase. TNF-α levels are associated with the severity of the inflammation. TNF-α is produced mainly by monocyte macrophages stimulated with zymosan and other agents such as lipopolysaccharide (LPS).

4. Discussion

Brown seaweeds have been extensively studied since the discovery of chemical components and immune response modulators involving inflammatory response mechanisms in experimental models. This study focused on the brown seaweed *L. variegata* and the therapeutic action of its fucans and their purified fractions with acetone on zymosan-induced arthritis.

The study of the brown seaweed *L. variegata* revealed the significant presence of carbohydrates and small amount of proteins. Fraction F1 showed a reasonable amount of sulfate and the presence of galactose, fucose, and glucose. NMR ^{13}C spectroscopic analysis revealed signals related to 1 → 3 galactose bonds (103.2 ppm) and fucose methyl groups (15.9 ppm). Infrared analysis demonstrated bands related to the sulfate clusters (1230 and 851 cm^{-1}) in addition to the characteristic bands of the hydrogen–oxygen bond (3210 cm^{-1}). Different analysis methods showed the antioxidant properties of fucans from F1, which are capable of reducing and removing reactive oxygen species, such as superoxide anions and hydroxyl radicals. We suggest that if possible the high results obtained could be overestimated because of high viscosity of polysaccharide solutions.

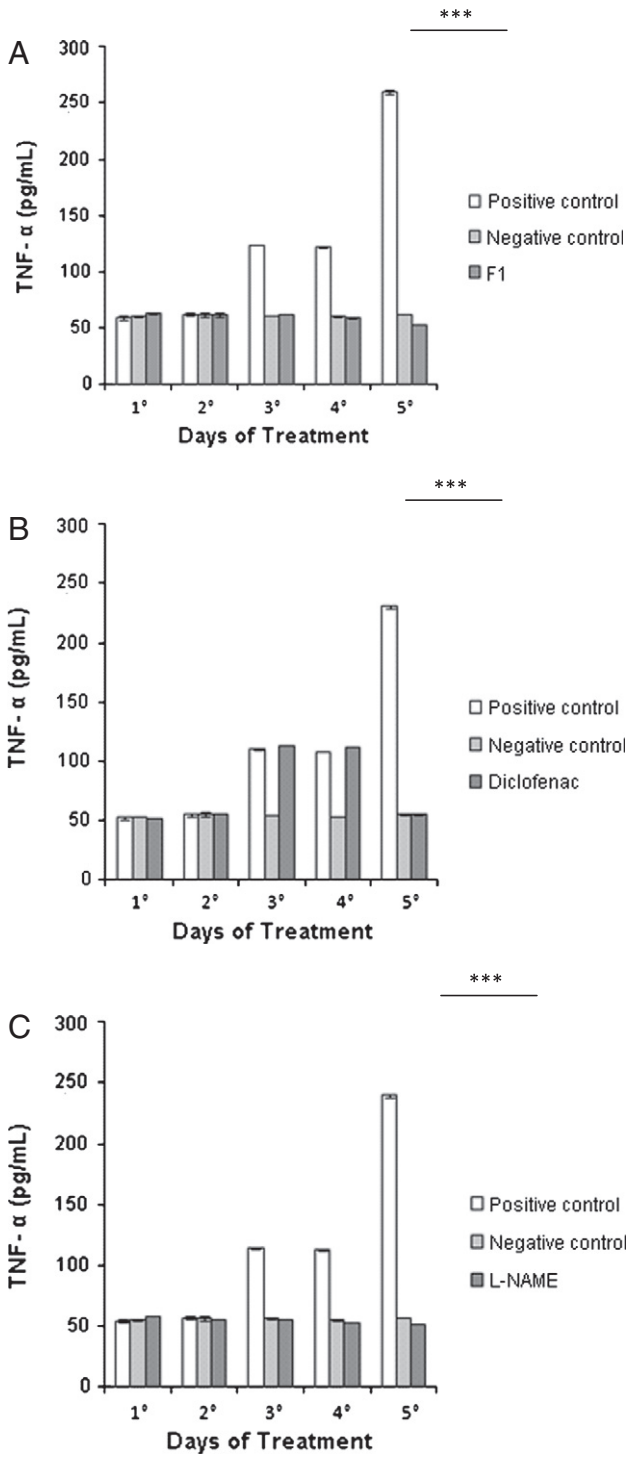

Fig. 6. A—Assessment of serum TNF-α during the 5-day experiment. The saline group was composed of the animals that only received intra-articular saline. The Zy group consisted of the animals that received intra-articular zymosan *** = P < 0.001. B—The F1 group consisted of the animals treated with 50 mg/kg of F1 from *L. variegata* and 5 mg/kg of sodium diclofenac. *** = P < 0.001. C—Group was composed of animals treated with 5 mg/kg of sodium diclofenac. *** = P<0.001. C–The L-NAME group consisted of animals treated with 25 mg/kg of this non-selective inhibitor of NOS. *** = P < 0.001.

Several studies have shown that Zy arthritis in its chronic phase displays a progressive synovitis that mimics the rheumatoid *pannus*. In arthritis, perpetuation of the inflammatory process is attributed to pro-inflammatory cytokines such as TNF-α and IL1-β, known initiators of the NF-kappa B activation pathway (31). In addition to promoting pro-inflammatory gene expression via NF-Kappa B, TNF-α also stimulates NADPH oxidase, the enzyme responsible for producing the superoxide in the respiratory chain (32). This free radical has been shown to be a mediator of tissue damage in rheumatoid arthritis along with pro-inflammatory cytokines. It was experimentally confirmed that the excessive production of reactive oxygen species can lead to accelerated articular cartilage damage and osteoclast activation (33). Oxide-reducer analysis of F1 revealed a possible blocking mechanism in the amplified inflammatory process and significant antioxidant activity.

Table 2
Scores for each parameter used in the histopathological analysis of the synovial fluids.

Groups	Cell infiltrate	Adipocytes	Fibrous tissue	Capsular integrity	Giant cells
Saline	0	3	1	3	0
Zy	3	0	3	1	1
F1(50 mg/kg)	1	2	2	2	0
Diclofenac	1	3	2	2	0
L-NAME	1	3	2	2	0

A number of studies have shown the capacity of fucosylated compounds to inhibit endothelial rolling of leukocytes, attributed to binding with P and L selectins. This property is due to the capacity of fucosylated compounds to compete with a natural ligand of selectin, Sialyl Lewis X (Sle^x), which is a fucosylate tetrasaccharide (34).

In a 6 h experiment, the decrease in cell influx showed a strong correlation with the amount of nitric oxide in the fluid. In this experimental model of zymosan-induced arthritis, the neutrophils are responsible, at least partially, for NO production, likely because of the increased expression of nitric oxide synthase (iNOS) in these cells (35). In this study it was demonstrated that post-treatment with F1, sodium diclofenac, and L-NAME significantly inhibited cell influx and NO release in ZyA. Comparing the activity of both, we observed similar action between 5 mg/kg of diclofenac and 50 mg/kg of F1. This result indicates that F1 has a significant anti-inflammatory effect in this experimental model, similar to that of the reference drug. The use of F1, diclofenac, and L-NAME also significantly decreased NO, showing no significant differences among these groups. Some studies report that low NO concentrations may protect immune system cells, such as macrophages, from death, whereas excessive NO production may cause cell death (36). Nitric oxide may not only cause inflammation but also stimulate the enzymatic action of cyclooxygenase, increasing prostaglandin production. Some prostaglandins (e.g., prostaglandin E_2) increase vascular permeability, thereby facilitating leukocyte diapedesis (37,38).

After the 96 h experiment, the edema and TNF-α concentration were evaluated in each of the different groups. The zymosan group promoted significant edema during the 96 h of the experiment. Of the F1 concentrations only 50 and 75 mg/kg F1 exhibited an anti-edematous effect. The use of 50 mg/kg of F1 and 5 mg/kg of sodium diclofenac significantly decreased the edema after 24 h, remaining so until the end of the experiment (5th day). It was observed that 75 mg/kg of F1 and L-NAME significantly reduced edema after 48 h. Analysis of serum TNF-α demonstrated an increase in this cytokine of the zymosan group and a decrease in the F1 and L-NAME groups. Administration of 5 mg/kg of sodium diclofenac did not significantly alter TNF-α concentration over the course of the experiment. One of the functions of TNF-α is to induce the secretion of other inflammatory cytokines with paracrine function, such as IL-1 and IL-6, in addition to strengthening its autocrine secretion (39–41).

Keffer et al., 1991 studied transgenic mouse lines with hyper-expression of one human TNF-α gene (with consequent increased production of TNF-α). The authors observed that the animals

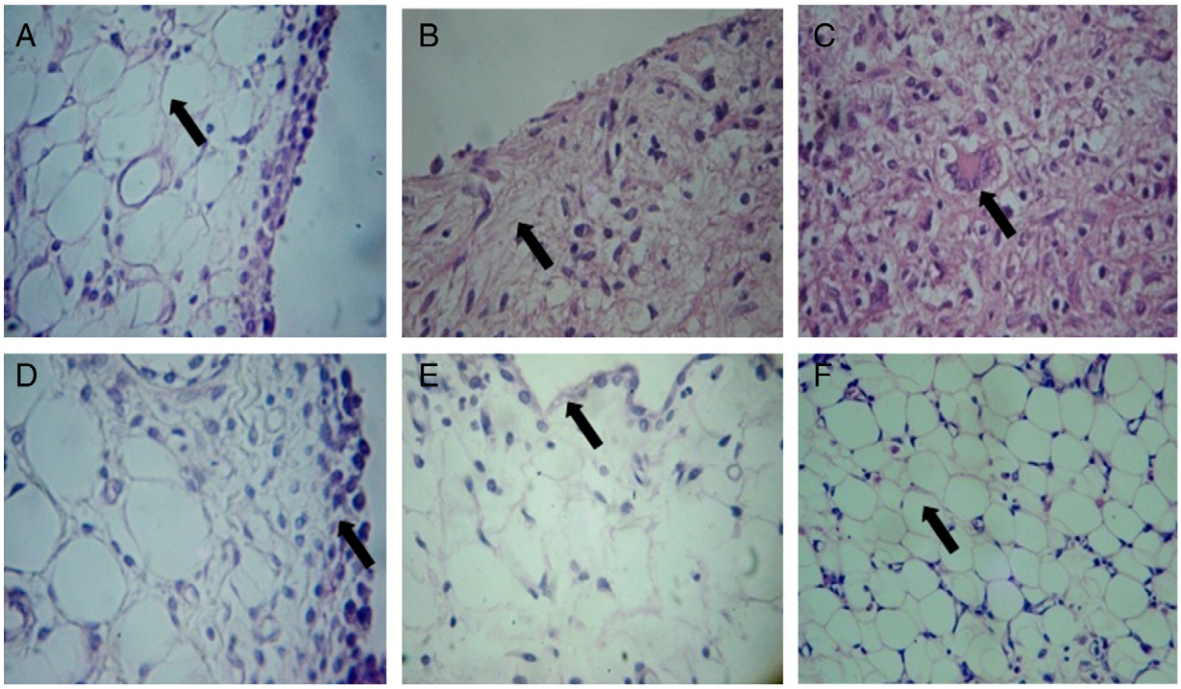

Fig. 7. Hystochemical of assay of F1 sulfated fucan from *L. variegata*. A—Analysis of the negative control (synovial membranes of animals that received only intra-articular saline). B—Synovial membranes cell infiltrate. C—Shows giant cells. D—Shows the F1-treated synovial membrane submitted to the zymosan-induced inflammatory process. E and F—-Histopathological cuts of the synovial membranes of animals submitted to Zy-induced arthritis and treated during the experiment with diclofenac and L-NAME (original magnification: ×40).

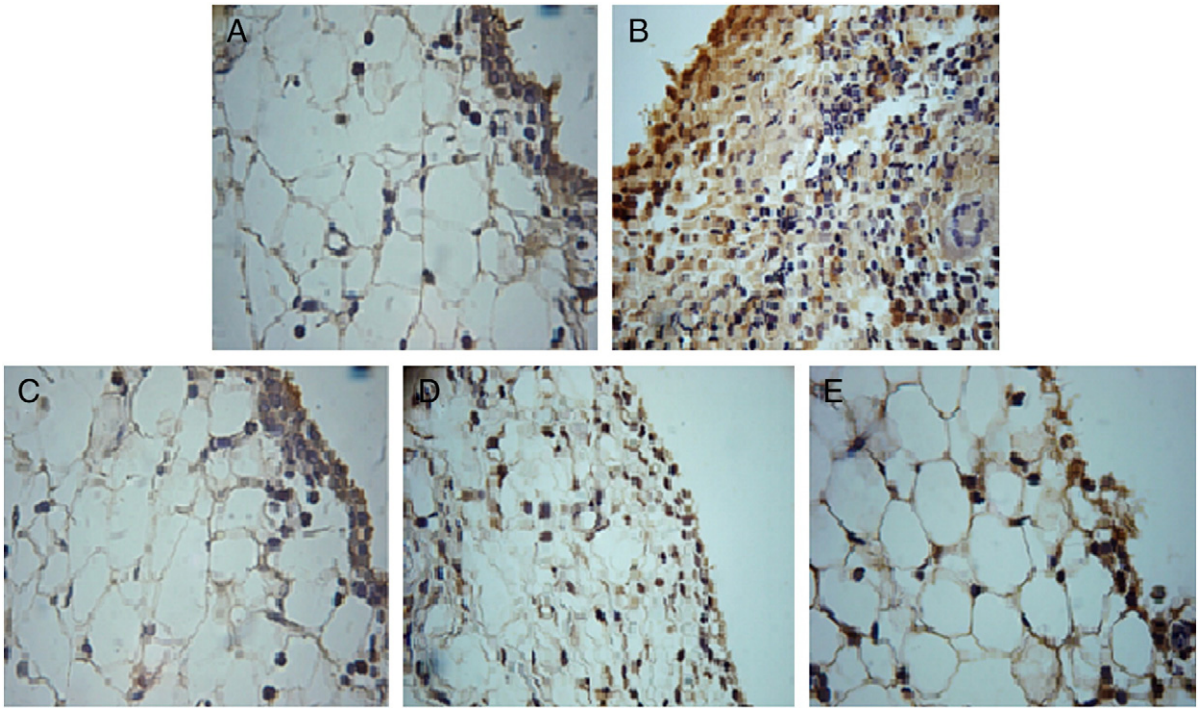

Fig. 8. Immunohystochemical TNF-α assay of F1 sulfated fucan from *L. variegata*. A—Negative control (intra-articular saline); B—Shows positive control (intra-articular zymosan). C—Shows the synovial membrane submitted to the zymosan-induced inflammatory process treated with F1. D—Shows synovial membranes of animals treated with diclofenac (original magnification: ×40). E–Synovial membranes of animals treated with L-NAME.

spontaneously developed destructive arthropathy and inflammation similar to that of rheumatoid arthritis (42).

The reduced inflammatory process was confirmed by histopathological and immunohistochemistry analyses, which showed a decrease in cell infiltrate in the synovial membrane and consequent reduction in TNF-α expression. In summary, these results suggest that the sulfated polysaccharides contained in fraction F1 of the seaweed *L. variegata* exhibit antioxidant power and anti-inflammatory activity

in animals with zymosan-induced arthritis, indicating a possible redox modulation mechanism exercised by fucosylated and sulfated saccharide compounds in this pathology.

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