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Sulfated Galactofucan from *Lobophora variegata*: Anticoagulant and Anti-inflammatory Properties

V. P. Medeiros[1], K. C. S. Queiroz[2], M. L. Cardoso[1], G. R. G. Monteiro[1], F. W. Oliveira[1], S. F. Chavante[1], L. A. Guimaraes[1], H. A. O. Rocha[1], and E. L. Leite[1]*

[1]*Departamento de Bioquimica, Centro de Biociencias, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil; fax: +55 (84) 32119208; E-mail: eddaleite@cb.ufrn.br*
[2]*Departamento de Biofisica e Farmacologia, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil*

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Abstract—Sulfated polysaccharides (fucans and fucoidans) from brown algae show several biological activities, including anticoagulant and anti-inflammatory activities. We have extracted a sulfated heterofucan from the brown seaweed *Lobophora variegata* by proteolytic digestion, followed by acetone fractionation, molecular sieving, and ion-exchange chromatography. Chemical analyses and ^{13}C-NMR and IR spectroscopy showed that this fucoidan is composed of fucose, galactose, and sulfate at molar ratios of 1 : 3 : 2. We compared the anticoagulant activity of *L. variegata* fucoidan with those of a commercial sulfated polysaccharide (also named fucoidan) from *Fucus vesiculosus* and heparin. The experimental inflammation models utilized in this work revealed that fucoidan from *L. variegata* inhibits leukocyte migration to the inflammation site. Ear swelling caused by croton oil was also inhibited when sulfated polysaccharides from *F. vesiculosus* and *L. variegata* were used. The precise mechanism of different action between homo- and heterofucans is not clear; nevertheless, the polysaccharides studied here may have therapeutic potential in inflammatory disorders.

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Key words: brown algae, *Lobophora variegata*, fucan, fucoidan, anti-clotting activity, anti-inflammatory activity

Sulfated polysaccharides containing fucose (fucans or fucoidans) are polymers that occur in marine brown algae and echinoderms [1, 2]. In seaweeds, these polymers occur in the extracellular matrix and have structural functions [3]. Their structures are complex and ramified, and they have been studied extensively [4-7]. These compounds are present in brown seaweeds and have led to new research into potentially bioactive compounds [8, 9]. Among the most widely studied properties of these compounds are inhibition of complement activation [10] and anti-thrombotic [11], anti-adhesive [12], antiviral [13], and anti-mitogenic properties [14]. The anti-clotting activity of fucans or fucoidans has been reported in several studies [15-17]. Furthermore, despite the widespread clinical use of heparin, a sulfated glycosaminoglycan produced by mammals, this sulfated polysaccharide has a number of major disadvantages—problems with animal pathogens, variable and extremely steep dose response curve, and poor bioavailability [18]. Some structural similarities between sulfated polysaccharides from marine brown algae and heparin have been reported by many authors [1]. These similarities are related to the presence of glucuronic acid, sulfate groups, and polymer heterogeneity [8, 18].

Several carbohydrates can interact closely with cell receptors. The L-selectins present in leukocytes are an example of that interaction [19-21]. Selectin adhesion molecules involved in the inflammatory process mediate leukocyte rolling along the vascular endothelium at sites of inflammation [22]. This is a multi-step process, which includes the rolling of leukocytes along the vascular endothelium, the subsequent adhesion of leukocyte proteins to integrins, and their emigration into inflammatory tissues [23]. Leukocytes circulating within the vascular system constitute an army patrolling the endothelial walls of the blood vessels in search of specific signals that dictate extravasation and mobilization within tissues.

Fucans have been reported to inhibit the rolling of leukocytes in mesentery venules and leukocyte influx into inflammation sites in meningitis and lung inflammation in rabbit models [21]. Many studies have demonstrated

Abbreviations: APTT) activated partial thromboplastin time; ICD) irritant contact dermatitis; PT) prothrombin time.

* To whom correspondence should be addressed.

that several polymers, such as heparin, can bind to L-selectin and inhibit its adhesion. This action is essential for adhesion and subsequent leukocyte migration. The molecular structures of sulfated polysaccharides seem to be close analogs of natural ligands recognized by selectins, such as sialyl Lewis X [20, 21] that inhibits leukocyte rolling on endothelial cells.

Fucans now attract attention in biochemical and biomedical fields because of their immunomodulatory, antitumor, and anticoagulant properties. The discovery and development of safe new drugs without serious side effects is an important goal. In this study, a fucoidan extracted from the brown alga *Lobophora variegata* has been investigated for its anticoagulant activity and potential capacity to block leukocytes by contact hypersensitivity, a form of hypersensitivity reaction, in the skin of animals. Also, the effect of this polysaccharide on peritonitis induced in rats with thioglycolate was measured in an acute inflammation model. Fucoidan from *Fucus vesiculosus* is described as an anticoagulant and anti-inflammatory polymer in several papers, and for this reason we have used it as a control [1].

MATERIALS AND METHODS

Reagents. Heparin from bovine mucosa was a gift from Dr. Elizeu Antunes Santos (Department of Biochemistry, UFRN, Natal, Brazil). Propylenediamine (1,3-diaminopropane) was purchased from Aldrich (USA). Glucose, glucuronic acid, xylose, fucose, galactose, mannose, arabinose, fructose, croton oil, and fucoidan from *F. vesiculosus* were obtained from Sigma (USA). Low-molecular-weight agarose was purchased from BioRad (USA).

Animals. Wistar rats weighing 150-200 g (eight weeks) were used. The animals were housed under standard environmental conditions in individual cages maintained on a 12-h light-dark cycle and provided with food and water *ad libitum*.

Extraction of polysaccharides. The marine alga *L. variegata* (Phaeophyceae, Dictyotales) was collected on the seashore of Natal, RN, Brazil and identified by Dr. Eliane M. Soriano (Department of Oceanography and Limnology, UFRN). Immediately after collection, the alga was dried at 50°C under ventilation and ground in a blender. The seaweed was then treated with acetone to eliminate lipids and pigments. The sulfated polysaccharides were extracted as described earlier [23]. Briefly, 100 g of defatted, dry, and powdered alga was suspended in 500 ml of 0.25 M NaCl and the pH adjusted to 8.0 with NaOH. Twenty milligrams of maxatase, an alkaline protease from *Sporobacillus* (Biobras, MG, Brazil) were added to the mixture for proteolytic digestion. After 18-h incubation at 60°C under agitation, the mixture was filtered through cheesecloth. The filtrate was fractionated by precipitation with acetone as follows: 0.3 volume of ice-cold acetone was added to the solution under gentle agitation, and the sample was maintained at 4°C for 24 h. The precipitate formed was collected by centrifugation (10,000*g* for 20 min), dried under vacuum, resuspended in distilled water, and analyzed. The operation was repeated adding 0.5, 0.8, 1.0, 1.5, and 2.0 volumes of acetone to the supernatant. The fraction precipitated with one volume of acetone (200 mg) contains the sulfated galactofucan used in the present work. This polysaccharide was purified by molecular sieving thru a Sepharose CL-4B column (120 × 1.8 cm). About 50 mg of the acetone fraction dissolved in 2 ml of water was applied to the column and eluted with a solution of 0.2 M acetic acid and 6 M urea, and fractions of 1 ml were collected and assayed by the phenol–H_2SO_4 reaction and by metachromatic assay using 1,9-dimethylmethylene blue. The fucan eluted from the resin was further purified by ion-exchange chromatography (Lewatit from Bayer, Brazil) eluted stepwise with increasing concentrations of NaCl (0.25 to 3.0 M). The eluates were precipitated with two volumes of methanol (18 h, 4°C). The precipitates were collected by centrifugation (10,000*g*, 15 min), dried, and resuspended in distilled water for subsequent analysis. The sulfated galactofucan eluted from the resin with 2 M NaCl.

Chemical analyses. The sulfated galactofucan was hydrolyzed with 5 M trifluoroacetic acid. The resulting monosaccharides were converted to their alditol acetate derivatives and analyzed by gas chromatography. Fucose, xylose, and uronic acid were also estimated by the methods described by Dische [24-26]. Total sugars were estimated by the phenol–H_2SO_4 reaction [27]. Sulfate was estimated after acid hydrolysis (6 N HCl, 100°C, 4 h) of the sulfated polysaccharides using the turbidimetric method [28]. The protein content was measured as described by Lowry [29]. IR spectra were recorded in KBr pellets on a Perkin-Elmer spectrometer. The D-configuration of galactose was confirmed by quantitative determination with D-galactose dehydrogenase as described by Bilan et al. [5].

NMR experiments. ^{13}C spectra of the fucan were recorded using a Bruker DRX 600 (USA) apparatus with triple resonance probe [12]. About 15 mg of each sample was dissolved in 0.7 ml of 99.9% D_2O (Cambridge Isotope Laboratory, USA). All spectra were recorded at 60°C with HOD suppression by pre-saturation. Chemical shifts are relative to external methanol for ^{13}C.

Anticoagulant activity. The activated partial thromboplastin time (APTT) was determined using citrated normal human plasma according to the producer's specifications (Labtest, Brazil). For the prothrombin time (PT) assay, 90 µl of citrated normal human plasma was mixed with 10 µl of a sulfated polysaccharide solution at different concentrations and incubated for 1 min at 37°C. The PT assay reagent (200 µl), preincubated for 10 min at 37°C, was then added, and the clotting time was recorded with a Quick

Times coagulometer (Drake Ltd., Brazil). Anticoagulant action was also measured for citrated human plasma according to the United States Pharmacopeia as described by Nader et al. [8]. All the experiments were performed in triplicate for each data point.

Sulfated polysaccharides in the contact hypersensitivity reaction. To evaluate the action of fucans by the contact hypersensitivity reaction in Wistar rats, groups of six animals per experimental set were sensitized by subcutaneous injection of 100 µl of 4% croton oil in acetone–olive oil (3 : 1 v/v) into the abdomen. Physiological saline was used in the control group. After six days, the experimental group was subjected to challenge by subcutaneous application of 50 µl of 2% croton oil to the underside of the right ear. Physiological saline was applied in the same manner to the control group. Ear thickness was measured with a thickness gauge before and 24 h after challenge. Varying concentrations of sulfated polysaccharides from *F. vesiculosus* and *L. variegata* (1-100 mg/kg rat body weight) dissolved in physiological saline were administered intravenously 24 h after challenge. The control group received the same volume of saline.

Effects of algal polysaccharides on sodium thioglycolate-induced peritonitis. Wistar rats (groups of seven individuals, eight weeks old, 150-200 g) anesthetized with metofane (Arovet AG, Switzerland) were subcutaneously administered with varying amounts of fucoidans from *F. vesiculosus* and *L. variegata* (20, 50, 70, and 100 mg/kg), heparin (1, 10, and 100 µg/kg), or sterile pyrogen-free saline. After 30 min, the animals were treated with a 1 ml intraperitoneal injection of 3% sodium thioglycolate broth (Sigma-Aldrich). Three hours after this injection, the rats were sacrificed and peritoneal leukocytes were harvested by peritoneal lavage with 10 ml of sterile saline containing 2 mM EDTA. The collected peritoneal liquid was placed in a glass tube containing EDTA. The numbers of leukocytes were counted in a Neabauer chamber, after red cell lyses, by staining with Turck solution.

Statistical analysis. Differences between groups were evaluated with the F-test and the Tukey multiple comparison test. The variance within the experimental and control groups was analyzed by the Levene test followed by the Kolmogorov–Smirnov procedure for normal distribution. Data are shown as mean ± SEM.

RESULTS

Chemical analysis. Samples of the alga *L. variegata* served as starting material for the isolation of fucoidan. Water-soluble polysaccharides were extracted from defatted biomass of the alga by proteolysis under heating. The isolated crude fucoidan preparation was fractionated by precipitation with different volumes of acetone.

The fucose-rich fraction (L4) was purified by molecular sieving (Fig. 1) and anion-exchange chromatography. The compositions of compounds are given in Table 1. The L4 fraction is composed of laminaran (homoglucan) and fucoidan, whereas the purified fucoidan from anion-exchange chromatography (eluted with 2.0 M NaCl) contains fucose, galactose, and sulfate at molar ratio 1 : 3 : 2.

The IR spectrum of fucoidan shows an intense absorption band at 1254 cm^{-1} (S=O) and a broad asymmetric absorption band with a maximum at 851 cm^{-1} (C–O–S, secondary axial sulfate) and 824 cm^{-1} (C–O–S, secondary equatorial sulfate) whose shape indicates the presence of both axial and equatorial sulfate groups in the polysaccharide. The presence of the O-acetyl group was confirmed by the small band at 1734 cm^{-1}. The ^{13}C-NMR spectrum of the polysaccharide, like the corresponding spectra of other algal fucoidans, was complex and could not be interpreted completely. The signals in the anomeric (93.3-93.5 ppm) and high field (16-18 ppm) regions of the carbon spectrum (Fig. 2) were typical of α-L-fucose. The signals in the region of 21-22 ppm confirmed the presence of acetyl groups in fucoidan. Several signals in the anomeric region (97-103.6 ppm) might correspond to β-D-galactose.

Anticoagulant activity. The anticoagulant activity analyzed by the Pharmacopeia method showed heparin from bovine lung with 179.2 IU/mg, while 120 and 98 IU/mg were found for *F. vesiculosus* and *L. variegata* fucoidans, respectively. Other anticoagulant tests, APTT and PT assays, are summarized in Table 2. The APTT test, which is associated with intrinsic coagulation pathways, revealed that both fucoidans prolong the coagula-

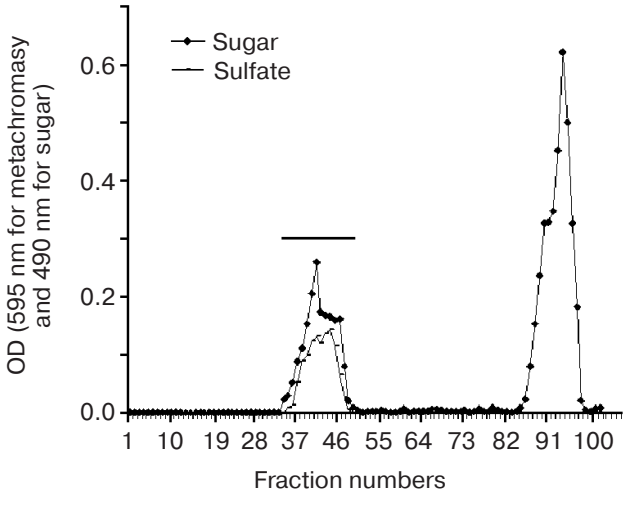

Fig. 1. Purification of fucoidan from *L. variegata*. The L4 fraction was applied to a Sepharose CL-4B as described in "Materials and Methods". Fractions were monitored by their metachromatic property and by the Dubois method, which indicated the presence of sulfated polymers and the presence of sugar, respectively. The fractions corresponding to fucoidan as indicated by the horizontal bar were pooled, dialyzed against distilled water, and lyophilized.

Table 1. Chemical composition of polymers from brown algae

Polymer	Carbohydrates, %	Protein, %	Molar ratio					
			Fuc	Xyl	Gal	Glu	UA	sulfate
Fucoidan Fv	64.0	0.1	1	–	–	–	0.1	1.7
Fraction L4	46.2	0.1	1	–	1.5	2	–	1
Fucoidan Lv	54.2	n.d.	1	n.d.	3	n.d.	n.d.	0.2

Note: Fuc, fucose; Xyl, xylose; Gal, galactose; Glu, glucose; UA, uronic acid; Fucoidan Fv, fucoidan from *F. vesiculosus*; Fucoidan Lv, fucoidan from *L. variegata*; n.d., not detectable; –, traces.

tion time. However, fucoidan from *L. variegata* was less potent than fucoidan from *F. vesiculosus*. The extrinsic coagulation pathway analyzed by the PT test showed no anticoagulant activity for fucoidan from *L. variegata*.

Effect of fucoidans on the acute inflammatory process. Because fucoidan from *F. vesiculosus* is known to inhibit P/L-selectin mediated leukocyte adhesion, we examined whether treatment with fucoidan from *L. variegata*, whose structure is heterogeneous and with low sulfation, attenuates the inflammatory process induced in rats. In this study, peritonitis was induced in Wistar rats with 3% sodium thioglycolate, and the ability of polysaccharides extracted from *F. vesiculosus* and *L. variegata* to reduce neutrophil migration to the inflammation sites was evaluated. Three hours after thioglycolate injection, neutrophils were collected from the inflamed peritoneal

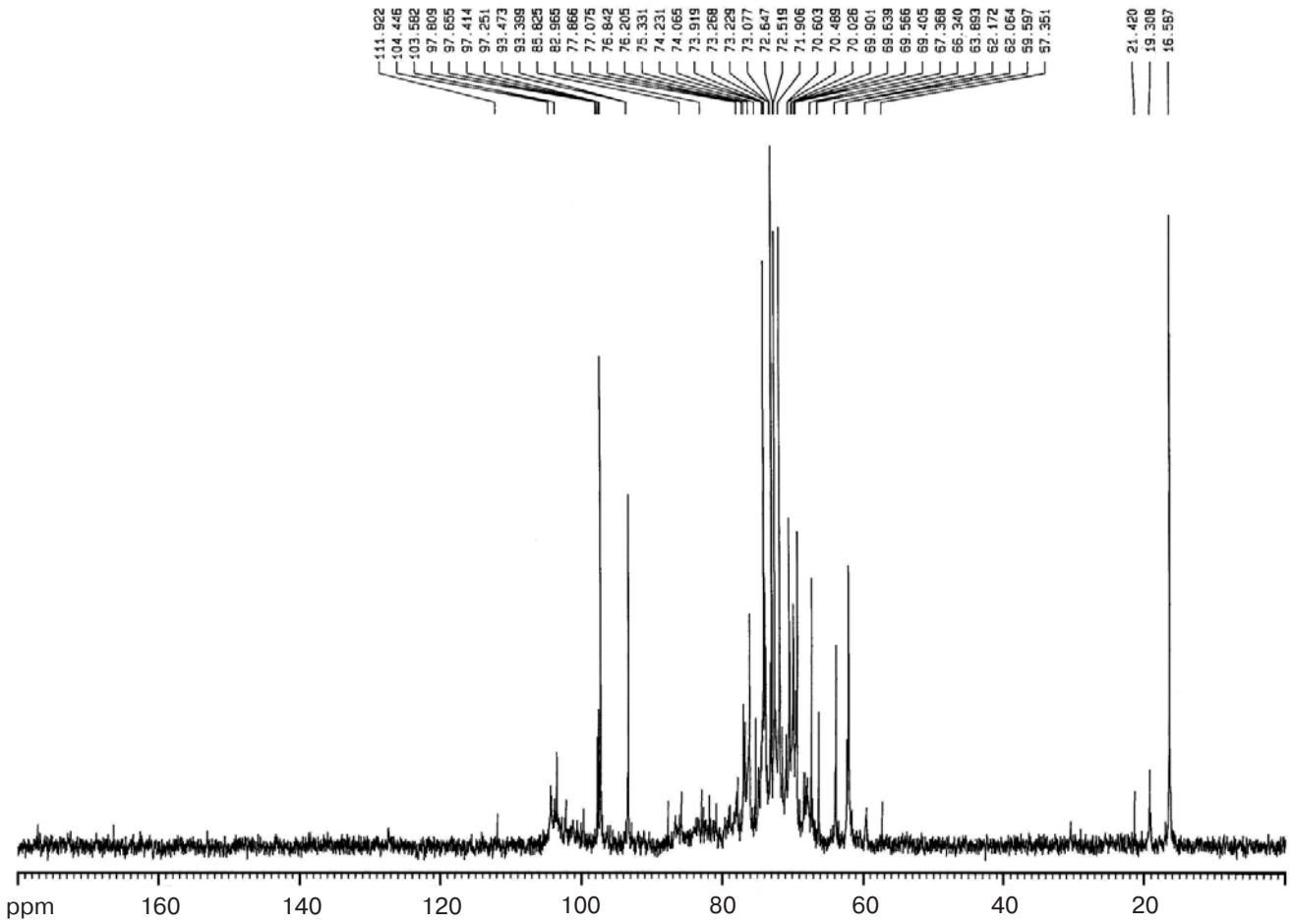

Fig. 2. ^{13}C-NMR spectrum of fucoidan from *L. variegata* at 600 MHz.

Table 2. Anticoagulant activity (APTT and PT tests) of sulfated polysaccharides

Polymer	APTT, sec			PT, sec		
	I	II	III	I	II	III
Fucoidan Fv	68	140	> 240	16	22	82
Fucoidan Lv	60	110	165	14	13	15
Heparin	188	> 240	> 240	40	>120	>120

Note: I-III, amount of polysaccharide (2, 10, and 50 μg, respectively). Standard deviation was 8-12% for three measurements. Fv, *F. vesiculosus*; Lv, *L. variegata*. Control of APTT = 28 sec; control of PT = 13 sec.

cavity and counted. According to Wang et al. [30], thioglycolate injected into the peritoneal cavity of rat induces acute inflammation and neutrophil infiltration dependent on both L- and P-selectin.

The results showed that in control experiments, thioglycolate induced an approximate 3-fold increase in neutrophils in the peritoneal cavity after 3 h compared with the saline-injected animals ($3.2 \cdot 10^6$ versus $1.1 \cdot 10^4$ per rat, respectively). Using *F. vesiculosus* (50 mg/kg) and *L. variegata* (50 mg/kg) polymers (Fig. 3) in this inflammation model, we found that fucoidan treatment significantly ($p < 0.05$) reduce the number of leukocytes in the peritoneal fluid. Our results showed 70% (*F. vesiculosus*) and 55% (*L. variegata*) leukocyte reduction in the peritoneal fluid. However, the differences between the effects of the two fucoidans were not statistically significant. To examine the physiological relevance of the results, we explored the effect of heparin *in vivo* in this inflammatory model and observed that heparin treatment does not significantly alter the number of leukocyte at any concentration used (Fig. 3). The data show normal distribution using the Kolmogorov–Smirnov test and homogeneity of variances for fucoidans, demonstrated by the Levene test, was 0.064 and 0.137, respectively, for *F. vesiculosus* and 0.089 and 0.381 for *L. variegata*. The results also show that the fucoidan effect is not dose-dependent.

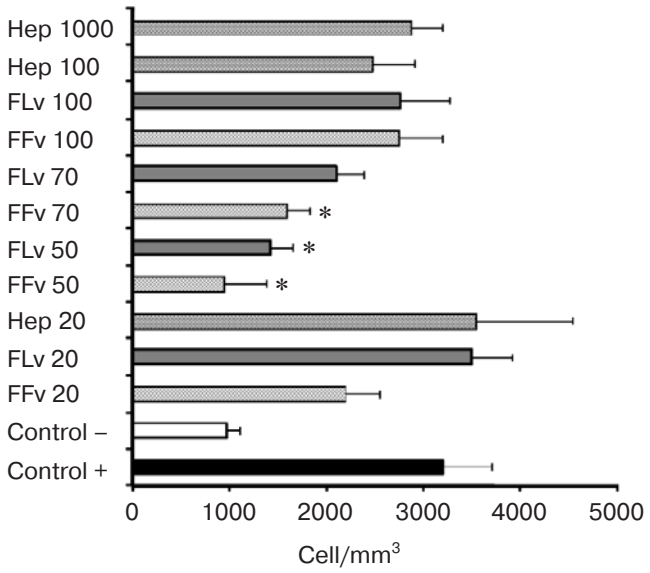

Fig. 3. Effect of polysaccharides on neutrophil migration in inflamed rat peritoneum. Control rats were injected with sterile, pyrogen-free saline alone (white column); neutrophil migration 3 h after injection of 3% thioglycolate (black column). Fucoidans and heparin were injected subcutaneously 30 min before thioglycolate. Hep, heparin; FLv, fucoidan from *L. variegata*; FFv, fucoidan from *F. vesiculosus*. Data represent mean ± SEM; asterisks indicate significant change ($p < 0.05$ vs. control; $n = 7$-9).

Effect of fucoidans on the contact hypersensitivity reaction. To determine whether the inhibitory effect of fucoidans in the peritonitis model could be generalized to other tests of inflammation, we examined their effects on acute contact dermatitis. We tested the hypothesis that the action of fucoidans results in an inhibited inflammatory response in the skin of tested animals. To address the possibility of this sulfated polysaccharide acting on the inflammation process, we measured the relative contribution of these compounds in an experimental ICD (irritant contact dermatitis) model using Wistar rats. Our results indicate that the fucoidan from *F. vesiculosus* and the fucoidan from *L. variegata* result in a decreased cutaneous inflammatory response to challenge by irritants. This model involves sensitization by croton oil through epicutaneous immunization followed by challenge with topically applied croton oil to the ear. The reaction is characterized by local ear swelling caused by the accumulation of lymphocytes, monocytes, and neutrophils. In this experimental inflammation model, Wistar rats were sensitized by the subcutaneous injection of 4% and subsequently by 2% croton oil. Ear thickness of the sensitized rats significantly increased after 24 h, whereas unsensitized rats did not. Treatment of rats with sulfated polysaccharide from *F. vesiculosus* reduced ear swelling by 98.4% (1 mg/kg) and 100% (100 mg/kg; $p = 0.05$) when compared with the control (see Fig. 4a). Animals treated by intravenous injection of polysaccharides from *L. variegata* (1 and 100 mg/kg; $p = 0.05$) showed reduced ear edema, as seen in Fig. 4b.

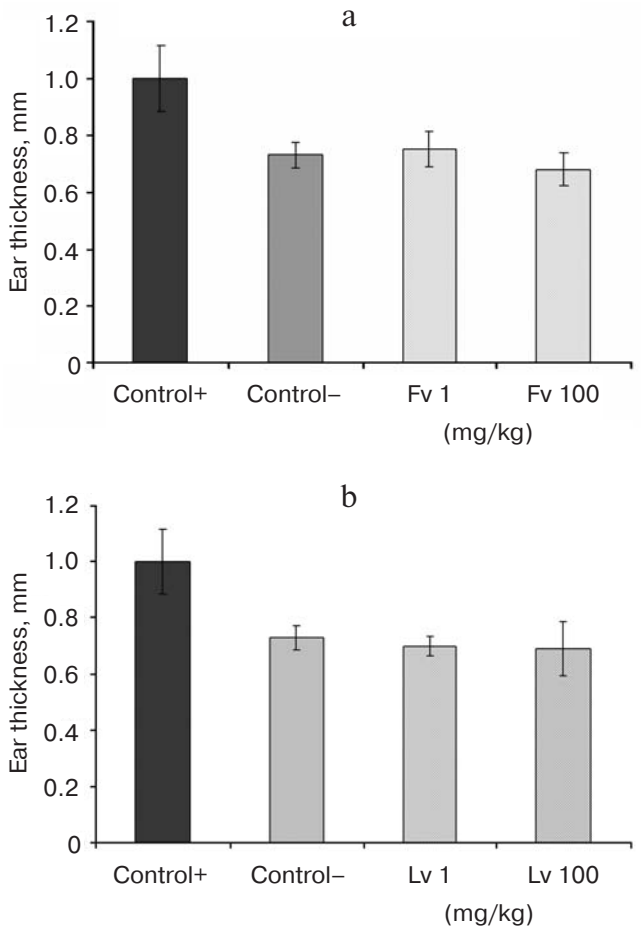

Fig. 4. Inhibition of croton oil-induced ear swelling in sensitized rats. Swelling was measured by ear thickness, and the values obtained from the vehicle-treated control ear were compared with those obtained from croton oil-treated animals. a) Animals received a single intravenous injection of fucoidan from *F. vesiculosus* (1 and 100 mg), or saline 24 h after antigen challenge. b) Animals received a single intravenous injection of fucoidan from *L. variegata* (1 and 100 mg), or saline 24 h after antigen challenge. The negative control is saline. Significant difference ($p < 0.05$) in ear swelling in the control rat injected with croton oil (positive control). Each bar represents mean value ± SD; $n = 7$.

DISCUSSION

Although fucans and fucoidans have been reported since 90 years ago, their precise structure is still debated. This can be partly explained by the difficulty of their extraction and isolation in pure form. Moreover, their heterogeneity and polydispersity limit their structural study. In this paper, we partially characterized a fucoidan from *L. variegata*. It is a sulfated galactofucan. Since the 1950s, several sulfated fucans containing galactose have been described [31], but only a few with galactose as the major component, and most of them were found in algae from the order Dictyotales [12, 17] like *L. variegata*. Because sulfated galactofucans have not been frequently described, we decided to analyze the anticoagulant and anti-inflammatory activities of the sulfated galactofucan from *L. variegata*. The monosaccharide analysis of fucoidan from *F. vesiculosus* indicated fucose (97%) with trace amounts of galactose, xylose, glucose, and uronic acid (⩽3%). When this fucoidan was analyzed by Patankar et al. [4], it showed fucose (93%) and trace amounts of galactose, xylose, and uronic acid (⩽7%). However, glucose was not detected. Recently, Cumashi et al. [32] found those sugars in fucoidan from *F. vesiculosus*. In addition, glucose and mannose were also detected. The differences observed could be due to sample handling or differences in the isolation procedure for the fucoidan.

Fucan preparations have been proposed as alternatives to the anticoagulant heparin [15], which is isolated from mammalian mucosa. Fucans are less likely to contain infectious agents, such as viruses or prions, since they are extracted from organisms of marine origin. The literature on the action mechanism of fucoidans or fucans is complex and somewhat contradictory. For example, some reports have suggested that fucans act on thrombin inhibition by antithrombin or heparin cofactor II, whereas other investigators have reported that this enhanced thrombin inhibition is caused by direct inactivation of thrombin. Several studies have demonstrated that the negative charges and molecular size of fucans rather than a specific carbohydrate structure are important in determining their biological activities [1, 15]. In this study, we found that fucoidan from *L. variegata* significantly altered APTT, and this feature may explain the potent anticoagulant effect of fucoidans observed here.

Coagulation and inflammation are two related pathways, and recent investigation has identified specialized cell surface receptors on various leukocyte subsets capable of interacting with proteins/proteases in the coagulation cascade. However, the molecular mechanism by which coagulation factors exert their pleiotropic effects in pathophysiology remains one of the major challenges in this field.

One of the earliest steps in the inflammation cascade takes place when certain leukocytes called neutrophils leave the circulation and move into diseased tissue. Leukocyte extravasation is one of the biological processes in which specific carbohydrates are known to play a critical role. In the inflammatory process, molecules called selectins are transiently expressed on the endothelial cell surface lining blood vessels. To address the possibility of these sulfated polysaccharides acting on the inflammation process, we measured the relative contribution of these compounds in an experimental ICD (irritant contact dermatitis) model using Wistar rats. Our results indicate that fucoidan from *F. vesiculosus* and fucoidan from *L. variegata* decrease the cutaneous inflammatory response to challenge by irritants.

In this paper, the effect of fucoidans in another inflammation model was assessed. We used thioglycolate injection into the rat peritoneal cavity, inducing acute

inflammation and L-selectin-dependent neutrophil infiltration [33]. In this study, we used sulfated polysaccharides heparin and fucoidans in peritonitis tests. Our results provide substantial evidence of a major anti-inflammatory role of sulfated polysaccharides. Earlier, administration of fucoidan was found to reduce, in a dose-dependent manner, neutrophil migration into peritoneum [34]. Cumashi et al. [32], working with different fucoidans from ten algae, showed that there is no direct relation between the anti-inflammatory activity of fucoidans and the content of monosaccharides and sulfate, as well as other parameters of their main chains, such as the presence of branching points. They suggested that there are some structural motifs present on the fucoidans, which could mimic sialyl Lewis X. In addition, heparin and heparin-like compounds can inhibit L-selectin or P-selectin binding to sialyl Lewis X-related compounds [30]. On the other hand, to the best of our knowledge there are no data about the mechanism of action of galactofucans. These points led us to suggest that reduction of neutrophil infiltration by sulfated galactofucan could be due to its interaction with selectins.

The precise mechanism of the different action of heterofucans is not clear. Our data suggest that fucoidans from brown seaweed can influence the physiology of inflammation, and their anticoagulant activity might be an important therapeutic strategy.

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