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Effects of a sulfated polysaccharide isolated from the red seaweed *Solieria filiformis* on models of nociception and inflammation

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ABSTRACT

This work reports the effects of a sulfated polysaccharide (SP-Sf), isolated from the seaweed *Solieria filiformis* and characterized by Fourier transformed infrared (FT-IR), on nociception and inflammation. Male Swiss mice were pretreated with SP-Sf 30 min before receiving an injection of 0.8% acetic acid, 1% formalin or 30 min prior to a thermal stimulus. We observed that SP-Sf (1, 3 or 9 mg/kg) significantly reduced the number of writhes. SP-Sf also reduced the second phase of the formalin test and did not cause a significant antinociceptive effect in the hot plate test, suggesting that its antinociceptive action occurs through a peripheral mechanism. SP-Sf (1, 3 or 9 mg/kg) did not show a significant anti-inflammatory effect in Wistar rats when administrated by the systemic route 1 h before testing using carrageenan or dextran. Finally, SP-Sf (9 mg/kg) did not show significant signs of toxicity when administrated in mice.

1. Introduction

Tissue injury, invasion of microorganisms or surgical trauma can all lead to the release of exogenous and endogenous chemical mediators that cause inflammation. The endogenous chemical mediators released by cells that infiltrate sites of damage include the eicosanoids and complement components. These factors are important for host defense, but they can also lead to additional tissue damage. The release of inflammatory mediators can be amplified by the activation and excessive recruitment of neutrophils to the site of injury. Neutrophils act to sustain the inflammatory response by secreting noxious granule contents and other chemical mediators (Sehan, Chiang, & Van Dike, 2008). Previous studies using animal models have investigated the effects of conditioning nerve injuries on subsequent nociceptive responses and inflammation evoked at distant sites (Kurihara, Nonaka, & Tanabe, 2003).

Marine organisms can serve as sources for structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potentials (Jiang et al., 2010; Yasuhara-Bell & Lu, 2010). Among the substances biosynthesized by algae, polysaccharides have intriguing potential as novel anti-inflammatory and analgesic drugs (Cardozo et al., 2007).

Sulfated polysaccharides are complex macromolecules that can interact with a wide variety of matrix and cellular proteins due to their chemical structure, which is rich in polyanions (Arfors & Ley, 1993). In red seaweeds, these compounds exist mainly as galactans (Fonseca et al., 2008; Shanmugam & Mody, 2000). The enantiomeric configuration of the α-galactose moiety classifies the various galactans into two major groups, the carrageenans and the agars (Stortz & Cerezo, 2000).

Carrageenan is a generic term that refers to a family of linear, sulfated galactans obtained from certain species of red seaweeds (Navarro & Stortz, 2005). The three main industrial types are *kappa* (κ), *iota* (ι) and *lambda* (λ) carrageenan. The κ and ι forms are gelling polymers, whereas λ is a non-gelling, thickening agent

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(Usov, 1998). The chemical structure of carrageenan from *Solieria filiformis* (Kützing) P.W. Gabrielson (Gigartinales, Solieraceae) was previously characterized with Fourier transformed infrared (FT-IR) and NMR spectroscopic analyses. In general, carrageenans from *S. filiformis* seem to contain a higher number of different structural elements. The main structural component of carrageenans is a 3,6-anhydrogalactose 2-sulfate-galactose 4-sulfate (DA2S-G4S)-type structure, which is characteristic of gelling carrageenans with a dominant ι repeating structure. Additionally, a resonance typical of κ-carrageenan was detected in *S. filiformis*, although with a very low intensity (Murano, Toffanin, Cecere, Rizzo, & Knutsen, 1997).

Sulfated polysaccharides have diverse biological activities, including anticoagulant (Pomin & Mourao, 2008), antioxidant (Costa et al., 2010), immunomodulatory (Ahn et al., 2008; Zhou et al., 2004), antiviral (Yasuhara-Bell & Lu, 2010), anti-inflammatory (Ananthi et al., 2010), antinociceptive (Assreuy et al., 2008; Viana et al., 2002), antitumor (Lins et al., 2009) and pro-inflammatory effects (Assreuy et al., 2008, 2010; Silva et al., 2010).

In the present study, we isolated a sulfated polysaccharide from the red seaweed *S. filiformis* and examined its nociceptive and inflammatory effects using experimental animal models.

2. Materials and methods

2.1. Animals

Male and female Swiss mice (20–25 g) and Wistar rats (180–240 g) from the Animal Care Unit of the Federal University of Ceará in Fortaleza, Brazil, were used for all experiments. They were housed in a temperature-controlled room with free access to water and food on a 12/12 h light/dark cycle. For each experiment, groups of six animals were segregated and handled separately. All procedures and animal treatments were performed at ambient temperature (20–22 °C) and special care was taken to avoid environmental disturbances that might influence animal responses. This study was conducted in accordance with the guidelines set forth by the U.S. Department of Health and Human Services and with the approval of the Ethics Committee of the Federal University of Ceará, Fortaleza, Brazil (CEPA no. 125/07).

2.2. Drugs and reagents

The following drugs and reagents were used: dextran sulfate, λ-carrageenan, cetylpyridinium chloride (CPC), 1,9-dimethylmethylene blue (DMB), indomethacin, L-*N*-nitro-arginine-methyl ester (L-NAME), DEAE-cellulose, o-dianisidine dihydrochloride, *N*-acetyl-*N*,*N*,*N*-trimethylammonium bromide, potassium phosphate monobasic, potassium phosphate dibasic, hexadecyltrimethylammonium bromide (HTAB), cysteine, papain and bovine serum albumin purchased from Sigma (St. Louis, MO, USA); dexamethasone purchased from Aché, Guarulhos, SP (Brazil); pentoxifylline purchased from EMS, São Bernardo do Campo, SP (Brazil); meclizine purchased from APSEN, Santo Amaro, SP (Brazil); morphine sulfate purchased from Dimorf®, Cristália, Itapira, SP (Brazil); gelatin purchased from Oxoid, Ltd., England; and ethylenediaminetetraacetic acid (EDTA), formaldehyde, glacial acetic acid and hydrate chloral purchased from VETEC Química Farm. LTDA, SP (Brazil). Drugs and SP-Sf were solubilized in 0.9% sterile NaCl (saline). The enzymatic kits used for evaluation of the SP-Sf systemic toxicity were from LABTEST (Diagnostic Tests – Brazil). All chemicals were of analytical grade.

2.3. Isolation of sulfated polysaccharides (SPs)

S. filiformis was obtained from the Atlantic coast of Brazil (Flecheiras Beach, Trairí-Ceará). After collection, specimens were taken to the Carbohydrates and Lectins Laboratory (CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará, and cleaned of epiphytes, washed with distilled water and stored at −20 °C until further use. A voucher specimen (no. 35682) was deposited in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil.

Dried tissue (5 g) was cut in small pieces; suspended in 250 ml 0.1 M sodium acetate buffer (pH 5.0) containing 510 mg papain, 5 mM EDTA and 5 mM cysteine; and incubated at 60 °C for 6 h according to Farias, Valente, Pereira, and Mourão (2000). The total sulfated polysaccharide (TSP) obtained from *S. filiformis* (50 mg) was dissolved in 25 ml 50 mM sodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose column (26 cm × 2.0 cm) equilibrated with the same solution. The column was developed by a step-wise gradient of 0–1.5 M NaCl at 0.25 M intervals in the same solution. The flow rate of the column was 2.3 ml/min. Fractions of 4.6 ml each were collected and analyzed for sulfated polysaccharides using the metachromatic assay (A_{525} nm) with DMB as described (Farndale, Buttle, & Barret, 1986) and for the total sugar content using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The TSP and fractions obtained were analyzed by 0.5% agarose gel electrophoresis (Dietrich & Dietrich, 1976). The biological protocols were performed with the fraction that showed the highest yield, called SP-Sf.

2.4. Chemical composition

The total sugar content was estimated by phenol–sulfuric acid analysis using D-galactose as the standard (Dubois et al., 1956). After acid hydrolysis of the soluble polysaccharides in 1 M HCl at 110 °C for 5 h, free sulfate was measured with the gelatin–barium method previously described, using Na_2SO_4 as the standard (Dodgson & Price, 1962). The protein content was measured using Coomassie Brilliant Blue G-250 method, using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.5. Infrared spectroscopy (FT-IR)

TSP and the F I and F II fractions, eluted with 0.5 and 0.75 M of NaCl, respectively, were also characterized by infrared spectroscopy. The Fourier transform IR (FT-IR) spectra were recorded with a Shimadzu IR spectrophotometer (model 8300) between 400 and 400 cm^{-1}. The samples were analyzed as a KBr pellet.

2.6. Antinociceptive activity

2.6.1. Writhing test

The writhing test was used to evaluate analgesic activity (Koster, Anderson, & De Beer, 1959). First, mice received an injection of either SP-Sf (1, 3 or 9 mg/kg; i.v.) or sterile saline (0.9%, w/v, NaCl). After 30 min, 0.8% (v/v) of acetic acid was injected intra-peritoneally (10 ml/kg). The number of writhes (abdominal muscle contractions and hind paw extensions) occurring between 0 and 30 min after acetic acid injection was recorded. Morphine (5 mg/kg; s.c.), a non-selective opioid agonist and indomethacin (5 mg/kg; s.c.), a non-specific inhibitor of cyclooxygenase, the enzyme responsible for prostaglandin synthesis (Hull, Gardner, & Hawcroft, 2003), were used as controls.

2.6.2. Formalin test

The formalin test, which causes a local tissue injury to the paw, has been previously used as a model for tonic pain and localized inflammatory pain (Hunskaar & Hole, 1987). Mice were injected with either SP-Sf (1, 3 or 9 mg/kg; i.v.) or sterile saline (0.9%, w/v, NaCl). After 30 min, 1% aqueous formalin (20 μl) was injected into

the right hind paw. The amount of time that the animal spent licking the injected paw was measured during the first 5 min (Phase 1, corresponding to the direct chemical stimulation of nociceptors) and 20–25 min after formalin injection (Phase 2, inflammatory). Morphine (5 mg/kg; s.c.) or indomethacin (5 mg/kg; s.c.) was used as controls.

2.6.3. Hot plate test

The hot plate test was performed according to Eddy and Leimbach (1953). Each mouse was placed onto the heated plate (51 ± 1 °C) two times, with a 30-min inter-trial interval. The first trial familiarized the animal with the test procedure and the second served as the control reaction time (licking the paw or jumping). Animals showing a reaction time greater than 10 s were not included in subsequent analyses. Immediately after the second trial (control reaction time), mice were divided into groups of six. Mice then received an injection of sterile saline (0.9%, w/v, NaCl), SP-Sf (1, 3 or 9 mg/kg; i.v.), morphine (5 mg/kg; s.c.) or indomethacin (5 mg/kg; s.c.) and reaction times were measured at time zero (0 time) and 30, 60 and 90 min after drug administration. A cut-off time of 40 s was used to avoid paw lesions.

2.7. Anti-inflammatory activity

2.7.1. Carrageenan-induced rat paw edema

One hour before injection with carrageenan (s.c. into the right paw; 500 μg/paw; 100 μl), rats were pretreated with SP-Sf at doses of 1, 3 or 9 mg/kg (0.1 ml/100 g body weight; s.c.). In a control experiment, dexamethasone (1 mg/kg; s.c.), a synthetic glucocorticoid with potent anti-inflammatory and immunosuppressant properties (Assreuy et al., 2008), was administered 1 h before carrageenan (Winter, Risley, & Nuss, 1962). Control animals received the same volume of sterile saline (0.9%, w/v, NaCl). Paw volume was measured immediately before (zero time) the stimulus and at selected time intervals (1, 2, 3 and 4 h) using a plethysmometer (Panlab, Spain). The results are expressed as the variation in paw volume (ml), calculated as the difference from the basal volume (zero time).

2.7.2. Dextran-induced rat paw edema

Dextran (400 μg/paw; 100 μl), a classical osmotic agent (Lo, Almeida, & Beaven, 1982), was injected s.c. into the right paws of rats. Animals were pretreated with SP-Sf at doses of 1, 3 or 9 mg/kg (0.1 ml/100 g body weight; s.c.) 1 h before stimuli. Control animals received the same volume of sterile saline (0.9%, w/v, NaCl).

Paw volume was measured immediately before the stimulus (zero time) and at selected time intervals following the stimulus (0.5, 1, 2, 3 and 4 h) using a plethysmometer (Panlab, Spain).

2.8. Determination of myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme found primarily in azurophilic granules within neutrophils and has been used extensively as a biochemical marker of granulocyte infiltration in various tissues. Neutrophil accumulation in paw tissue was measured using an MPO activity assay as previously described (Bradley, Christensen, & Rothstein, 1982). Briefly, 50–70 mg of paw tissue was homogenized in potassium phosphate buffer containing 0.5% HTAB (1 ml buffer per 50 mg of tissue). The homogenate was then centrifuged at 40,000 × *g* for 7 min at 4 °C. MPO activity was determined by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. One unit of MPO activity was defined as the activity required to convert 1 μmol of hydrogen peroxide to water in 1 min at 22 °C. Results are reported as MPO units/mg of tissue.

2.9. Pharmacological modulation of SP-Sf edematogenic activity

SP-Sf was injected (9 mg/kg; 100 μl; s.c.) into the right paws of rats 30 min or 1 h after treatment with one of the following compounds: indomethacin (5 mg/kg; s.c.; 1 h); dexamethasone (1 mg/kg; s.c.; 1 h); L-NAME, a non-specific inhibitor of nitric oxide synthase activity and nitric oxide (NO) production (30 mg/kg; i.p.; 30 min) (Assreuy et al., 2009); pentoxifylline, an inhibitor of interleukin (IL-1) and tumor necrosis factor-α (TNF-α) production (90 mg/kg; s.c.; 1 h) (Cunha et al., 2000); or meclizine, an inhibitor of histamine H_1 receptors (40 mg/kg; s.c.; 1 h) (Figueiredo et al., 2009). Control animals received equal volume injections of SP-Sf (9 mg/kg) or sterile saline (0.9%, w/v, NaCl). Edema was measured at 0.5, 1, 2, 3, 4 and 5 h after stimulus (SP-Sf) using a plethysmometer (Panlab, Spain).

2.10. Subchronic toxicity of SP-Sf

Body mass loss, organ weight alteration and the blood levels of the biochemical parameters alanine amino transferase (AST), aspartate amino transferase (ALT) and urea were evaluated after once-daily subchronic treatment of SP-Sf (9 mg/kg; i.p.) or sterile saline (0.9%, w/v, NaCl) for fourteen consecutive days. After treatment, mice were weighed and peripheral blood was collected for biochemical analysis (determined by enzymatic and colorimetric tests – LABTEST). After sacrificing the animal, the liver, kidney and heart were removed and weighed. Possible ulcerative lesions or hemorrhaging were quantified and macroscopically measured.

2.10.1. Histological analysis

After sacrifice, the liver, heart and right kidney were fixed with formalin. The material was then dehydrated using ethanol and processed for embedding in paraffin. The resulting blocks were sliced into 5-μm thick sections, stained with hematoxylin–eosin (HE) and observed under a light microscope.

2.11. Statistical analysis

The data are presented as the mean ± standard error (s.e.m.) for six animals per group. Variance analysis (ANOVA) was performed using Bonferroni's test and Student's *t*-test for unpaired values. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Isolation of sulfated polysaccharides

S. filiformis had a high TSP yield (19.14%), a high content of both total sugar (29.21%) and free sulfate (27.75%) and trace amounts of protein. Two different fractions of SP (F I and F II, eluted with 0.5 and 0.75 M of NaCl, respectively) were obtained from the DEAE-cellulose column and their metachromatic properties were monitored with DMB and by total sugar content (Fig. 1A). The fractions showed yields, total sugar and free sulfate contents of 6.80%, 30.09% and 12.69% (F I), respectively, and 4.80%, 23.92% and 22.35% (F II), respectively. No protein content was detected. The SPs obtained were analyzed by 0.5% agarose gel electrophoresis and had different charge densities. Each purified fraction was visible as a single band, whereas TSP appeared as two distinct bands on the gel (Fig. 1B). Because F I provided a higher yield than F II, biological experiments were performed with F I (SP-Sf).

3.2. FT-IR spectroscopy

The FT-IR spectra of TSP as well as F I and F II are shown in Fig. 2. Typical absorption bands corresponding to carrageenans

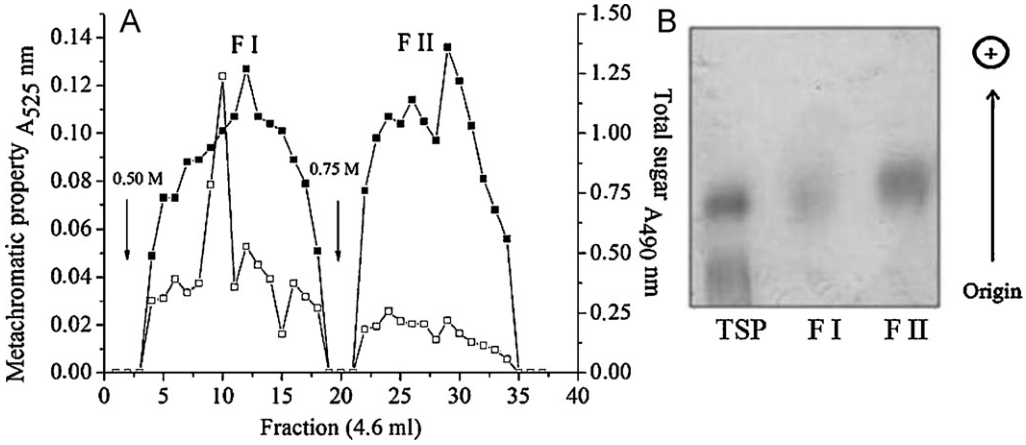

Fig. 1. (A) Separation of SP-Sf by DEAE-cellulose. Fractions were collected and checked by metachromasia using 1,9-dimethylmethylene blue (■—■). The total sugar was determined according to phenol–sulfuric acid method (□—□). Arrows represent the NaCl concentration (↓). (B) Agarose gel electrophoresis of isolated SP-Sf. Total sulfated polysaccharides (TSP), and fractions F I (0.5 M) and F II (0.75 M) within the gel were stained with 0.1% toluidine blue.

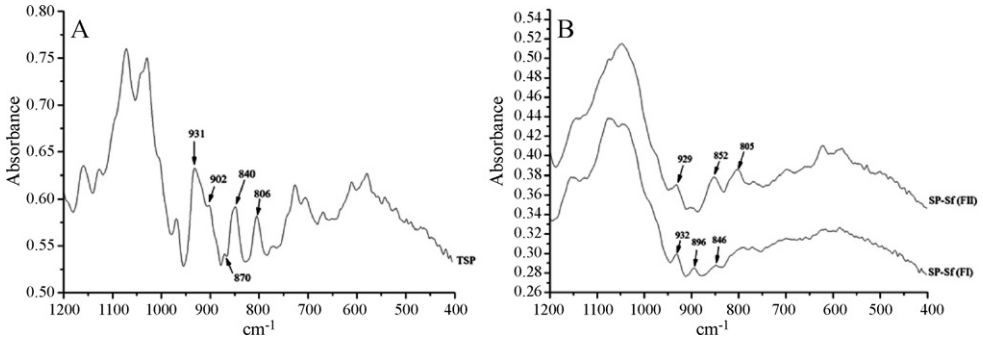

Fig. 2. Infra red spectra (400–1200 cm^{-1}) of *S. filiformis*. (A) Total sulfated polysaccharides (TSP). (B) Fractions SP-Sf (FI and FII).

were identified. For TSP, the FT-IR spectra showed absorption bands at 931, 902, 848 and 806 cm^{-1}, indicating the presence of 3,6-anhydrogalactose, β-D-galactose-6-carbon, β-D-galactose-4-sulfate and 3,6-anhydrogalactose-2-sulfate, respectively (Fig. 2A). Interestingly, band absorbance from F I showed the presence of 3,6-anhydrogalactose at 932 cm^{-1}, β-D-galactose-6-carbon at 896 cm^{-1} and galactose-4-sulfate at 845 cm^{-1}, characteristic of κ-carrageenan. However, in F II, 3,6-anhydrogalactose at 932 and 805 cm^{-1} and galactose-4-sulfate at 852 cm^{-1}, which are characteristics of ι-carrageenan, were also observed (Fig. 2B). Also, all of these spectra display an absorbance at approximately 1250 cm^{-1}, confirming the presence of ester sulfate groups (data not shown).

3.3. Antinociceptive activity

Pretreatment with SP-Sf (1, 3 or 9 mg/kg; i.v.) injected 30 min prior to acetic acid inhibited the writhing response of mice in a dose-dependent manner (40.60%, 56.60% and 70.20% for 1, 3 and 9 mg/kg, respectively). For this experiment, animals pretreated with either morphine or indomethacin (5 mg/kg; s.c.) were used as positive controls. Morphine inhibited 96% of writhing responses and indomethacin pretreatment resulted in 54% inhibition (Fig. 3A).

Intraplantar injection of a 1% formalin solution in mice induced a nociceptive response, characterized by an increase in licking time. No reduction of licking time was observed during the first phase (neurogenic) with any of the tested doses of SP-Sf (Fig. 3B). However, SP-Sf (1, 3 or 9 mg/kg; i.v.) injected 30 min prior to formalin caused a dose-dependent inhibition of the formalin response during the second phase (inflammatory) of 67.20%, 83.60% and 86.40%, respectively. Similarly, morphine (5 mg/kg; s.c.) and indomethacin (5 mg/kg; s.c.) inhibited the second phase by 90% and 51.60%, respectively (Fig. 3B).

In the hot plate test, neither SP-Sf (1, 3 or 9 mg/kg; i.v.) nor indomethacin (5 mg/kg; s.c.) induced a significant antinociceptive effect on reaction time during 90 min of observation. Morphine (5 mg/kg; s.c.), which was used as a positive control, induced analgesia, as shown by the delays in reaction time of 36.30 ± 0.80 and 32.10 ± 1.90 s at the 30 and 60 min time points, respectively (Fig. 3C).

3.4. Anti-inflammatory activity

Carrageenan (500 μg/paw; s.c.) caused intense paw edema, which reached a maximum level at 3 h (0.68 ± 0.03 ml). SP-Sf (1 mg/kg) significantly reduced the occurrence of edema 1 h after carrageenan administration by 46%. In contrast, a higher dose of SP-Sf (9 mg/kg) increased edema from 0.60 ± 0.06 ml to 0.83 ± 0.06 ml. Pre-treatment of animals with dexamethasone inhibited edema by 66.60% (Fig. 4A). In addition, SP-Sf (1, 3 or 9 mg/kg) caused a marked neutrophil accumulation in the paw, as demonstrated by MPO activity. Pre-treatment with dexamethasone also inhibited MPO activity (Fig. 4B).

Dextran (400 μg/paw; s.c.) also induced a significant increase in vascular permeability, with the maximum level occurring 30 min (0.70 ± 0.06 ml) after treatment. Administration of SP-Sf (1 mg/kg; s.c.) 1 h before dextran reduced the increase in vascular permeability by 38.57%, but pre-treatments with higher doses of SP-Sf (3 or 9 mg/kg; s.c.) did not alter dextran-induced edema (Fig. 5).

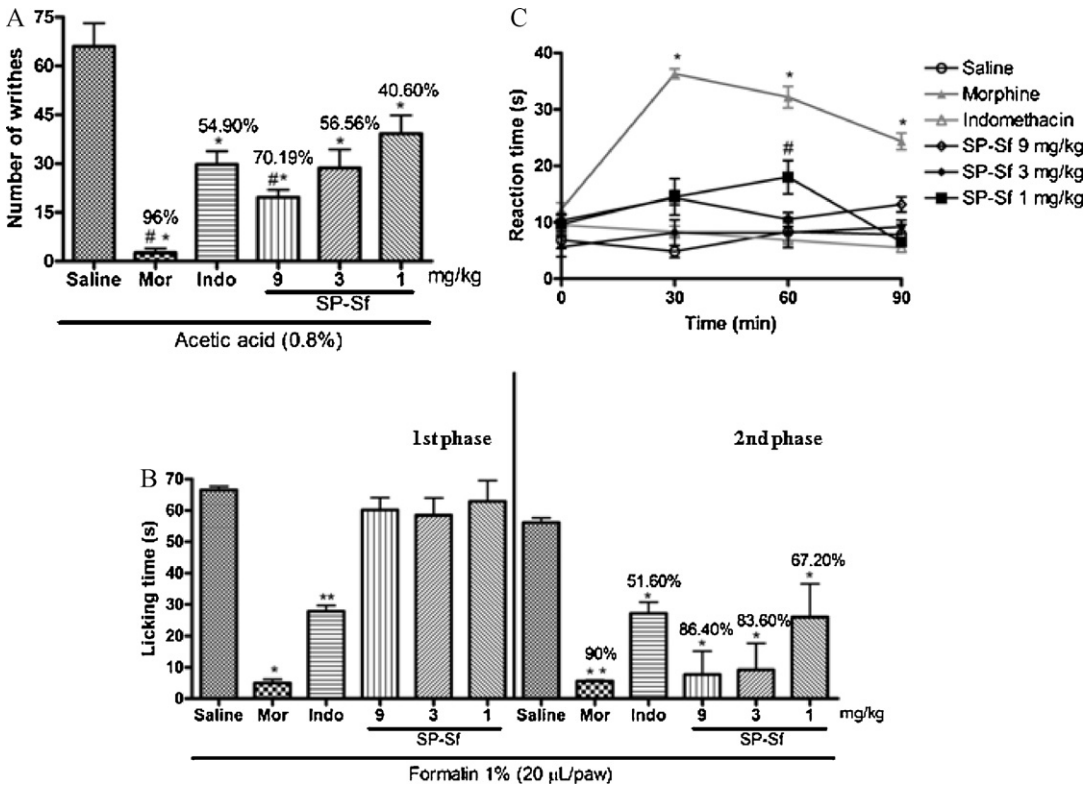

Fig. 3. Effect of SP-Sf in nociceptive models. Mice received i.v. sterile saline or SP-Sf (1, 3 and 9 mg/kg). Morphine (5 mg/kg) or indomethacin (5 mg/kg) was given s.c. 30 min before stimuli. Data are expressed as mean ± s.e.m. of six animals for each group (ANOVA; Bonferroni's test). (A) Effect of SP-Sf on the writhing response induced by acetic acid in mice. *Significant difference from the sterile saline group ($P < 0.05$), and #no significant difference between SP-Sf treated animals (9 mg/kg) and the morphine group ($P > 0.05$). (B) Effect of SP-Sf on the formalin test in mice. The time spent licking was determined during the first 5 min (1st phase) and during the period 20–25 min (2nd phase) after 1% formalin injection in mice. *Significant difference from the saline group ($P < 0.05$). (C) Effect of SP-Sf on reaction times to thermal stimuli in mice. *Significant difference from the saline group ($P < 0.05$), and #significant difference between SP-Sf (1 mg/kg) and indomethacin treatment ($P < 0.05$).

3.5. Modulation of the SP-Sf edematogenic effect

Due to the observed potentiating effect of SP-Sf on paw edema induced by classical inflammatory stimuli, we next examined whether SP-Sf exhibits edematogenic properties.

Local s.c. injection of SP-Sf (1, 3 or 9 mg/kg) into the paw induced intense paw edema at all doses tested, with maximal edema after the injection of the highest dose of SP-Sf (9 mg/kg). Injections of lower doses of SP-Sf (1 or 3 mg/kg) also induced edema (data not shown).

Intense paw edema was observed 2 h after local s.c. injections of SP-Sf at a dose of 9 mg/kg (0.83 ± 0.04 ml). This edematogenic effect was inhibited in animals pre-treated with indomethacin (5 mg/kg; s.c.), dexamethasone (1 mg/kg; s.c.) or L-NAME (30 mg/kg; i.p.) by 40.96, 46.98 and 32.53%, respectively; this effect was also inhibited in animal pretreated with pentoxifylline (90 mg/kg; s.c.) by 64.28 and 46.98% at 60 min and 120 min, respectively. However, administration of meclizine (40 mg/kg; s.c.) before administration of SP-Sf did not alter its edematogenic effect (Table 1).

3.6. Subchronic toxicity

Repeated injections of SP-Sf (9 mg/kg; i.p.) over fourteen consecutive days did not produce any signs of toxicity in mice. The overall body mass and the wet weights of the liver, kidney and heart were normal. Serum levels of the enzymatic markers of hepatic function, ALT and AST, did not differ from respective controls. The reduction in levels of blood urea did not indicate toxicity (Table 2).

3.6.1. Histopathology and morphological changes

Histopathological analyses of heart tissue removed from animals treated with SP-Sf (9 mg/kg) did not reveal damage to cardiac tissue. In the liver, cellular tumefaction and a slight subcapsular infiltration of mononuclear cells were observed. However, necrosis and interstitial fibrosis were not observed. In addition, the kidney presented slight degeneration in tubular and subcapsular areas; however, this degeneration was considered reversible and no changes in the renal capsule were observed (Fig. 6).

4. Discussion

There is a great deal of interest in identifying new natural compounds for a wide variety of pharmaceutical applications (Campo, Kawano, Silva Junior, & Carvalho, 2009; Yasuhara-Bell & Lu, 2010). In our study, F I, which was eluted with 0.5 M of NaCl (SP-Sf), had the highest total sugar content. Curiously, the physicochemical characteristics of SP-Sf observed in this study were distinct from those previously described (Assreuy et al., 2010). These data suggest that differential climatic conditions or alterations in the life cycle of seaweed species influence the biosynthesis of different molecules (Marinho-Soriano & Bourret, 2003). As expected, in the FT-IR spectra, we also observed that the TSP from *S. filiformis* contains both κ- and ι-carrageenans. The occurrence of these polysaccharides is in accordance with Murano et al. (1997). However, the separation of TSP by DEAE-cellulose columns into F I (SP-Sf) and F II revealed κ- and ι-carrageenans, respectively, upon FT-IR analysis. In fact, the previous determination of sulfate content and the charge density presented by agarose gel electrophoresis of these fractions suggest the presence of polysaccharides. According to Campo et al. (2009), the differences between κ- and ι-carrageenans are due to the num-

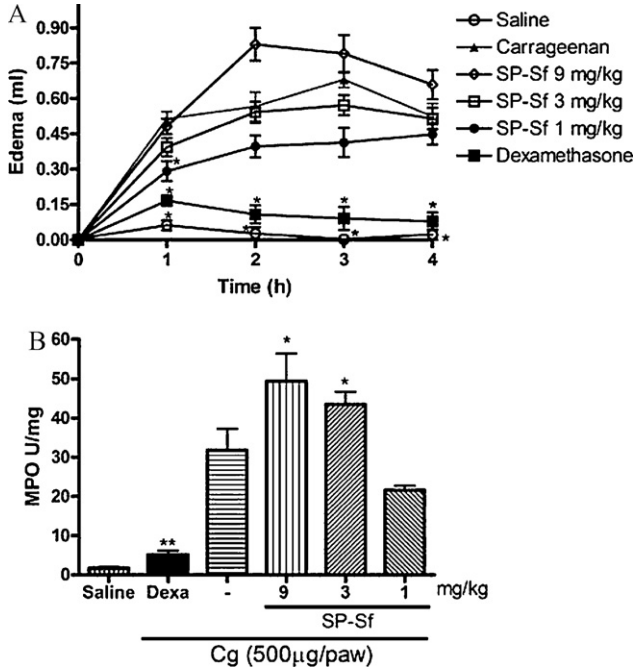

Fig. 4. (A) Effect of SP-Sf on paw edema induced by carrageenan in rats. Before receiving an injection of carrageenan (500 μg/paw; s.c.), animals received either SP-Sf (1, 3 and 9 mg/kg) or dexamethasone (1 mg/kg) via s.c. injections. Another group received only sterile saline without carrageenan. Data are expressed as means ± s.e.m. of six rats for each group (ANOVA; Bonferroni's test). *Significant difference from the carrageenan group ($P < 0.05$). (B) Activity of myeloperoxidase (MPO) in the supernatant of homogenates of paw sections injected with carrageenan, SP-Sf (1, 3 and 9 mg/kg), dexamethasone or sterile saline (s.c.), expressed as units of MPO activity per mg of tissue. Data are expressed as means ± s.e.m. of six rats for each group. *Significant difference from the carrageenan group ($P < 0.05$).

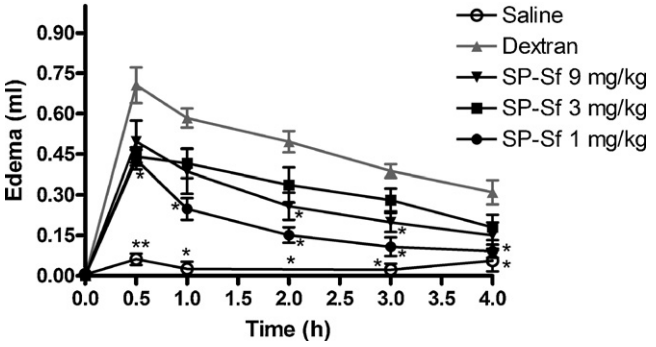

Fig. 5. Effect of SP-Sf on paw edema induced by dextran in rats. Before receiving an injection of dextran (400 μg/paw; s.c.), groups of animals received SP-Sf (1, 3 and 9 mg/kg). Another group received only sterile saline without dextran. Data are expressed as means ± s.e.m. of six rats for each group (ANOVA; Bonferroni's test). Pre-treatments with higher doses of SP-Sf (3 or 9 mg/kg, s.c.) did not alter dextran-induced edema. *Significant difference from the dextran group and **significant difference from the SP-Sf (1 mg/kg) group ($P < 0.05$).

ber and position of sulfate ester groups in the chemical structures. Overall, the different techniques used to obtain these polymers may also be responsible for the observed changes (Assreuy et al., 2008; Chotigeat, Tongsupa, Supamataya, & Phongdara, 2004).

In recent years, the medical potential of carrageenans has attracted the attention of researchers. In this study, we demonstrated that SP-Sf produces antinociceptive effects in mice. The writhing reaction, a stretching response in mice, has been used to evaluate the analgesic activity of non-steroidal anti-inflammatory drugs (Matsumoto et al., 1998). The inflammatory pain accompanying the writhing that occurs in response to i.p. injection of acetic acid is associated with the release of inflammatory mediators, such as bradykinin, substance P, prostaglandins and several cytokines, including IL-1β, TNF-α and IL-8 (Ribeiro et al., 2000). In this study, SP-Sf exhibited an antinociceptive effect in this model, suggest-

Table 1
Induction of paw edema by local injection of SP-Sf (9 mg/kg; 100 μl; s.c.). Prior to SP-Sf treatment, mice were injected with indomethacin (5 mg/kg; s.c.; 1 h); dexamethasone (1 mg/kg; s.c.; 1 h); L-NAME (30 mg/kg; i.p.; 30 min); pentoxifylline (90 mg/kg; s.c.; 1 h); or meclizine (40 mg/kg; s.c.; 1 h). Control animals received the same volume of SP-Sf or sterile saline. Data are expressed as means ± s.e.m. of six rats for each group (ANOVA; Bonferroni's test).

Experimental groups	Paw edema (ml)					
	30 min	1 h	2 h	3 h	4 h	5 h
Saline	0.06 ± 0.02*	0.06 ± 0.02	0.00 ± 0.00	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
SP-Sf (9 mg/kg)	0.49 ± 0.03	0.56 ± 0.07	0.83 ± 0.04	0.59 ± 0.07	0.64 ± 0.08	0.54 ± 0.06
Indomethacin + SP-Sf	0.59 ± 0.03	0.48 ± 0.02	0.49 ± 0.04*	0.60 ± 0.07	0.62 ± 0.08	0.52 ± 0.06
Dexamethasone + SP-Sf	0.46 ± 0.03	0.37 ± 0.04	0.44 ± 0.04*	0.42 ± 0.05	0.54 ± 0.07	0.36 ± 0.04
L-NAME + SP-Sf	0.55 ± 0.03	0.36 ± 0.07	0.56 ± 0.03*	0.63 ± 0.06	0.72 ± 0.06	0.75 ± 0.13
Pentoxifylline + SP-Sf	0.34 ± 0.03	0.20 ± 0.06*	0.44 ± 0.04*	0.50 ± 0.04	0.55 ± 0.05	0.49 ± 0.05
Meclizine + SP-Sf	0.38 ± 0.04	0.45 ± 0.07	0.80 ± 0.05	0.66 ± 0.05	0.67 ± 0.05	0.58 ± 0.08

* Significant difference from the SP-Sf (9 mg/kg) group ($P < 0.05$).

Table 2
Systemic effects of SP-Sf (9 mg/kg) in mice. Animals were weighed and injected once daily with SP-Sf over fourteen consecutive days. After fourteen days of treatment, animals were weighed, the blood samples were collected for biochemical dosage (AST, ALT and urea), mice were sacrificed, and the wet weight of organs determined. Values are reported as mean ± s.e.m. Student *t*-test for unpaired values.

Parameters	Treatment (9 mg/kg; i.p.)			
	Female		Male	
	Saline	SP-Sf	Saline	SP-Sf
Body mass (g) before	22.83 ± 0.47	22.83 ± 0.30	24.00 ± 0.85	23.50 ± 0.42
Body mass (g) after	25.67 ± 0.61	26.67 ± 0.33	31.83 ± 1.01	31.00 ± 0.54
Liver (g)/body mass	5.26 ± 0.18	5.17 ± 0.17	5.46 ± 0.14	5.45 ± 0.26
Kidney (g)/body mass	0.63 ± 0.02	0.66 ± 0.00	0.76 ± 0.03	0.82 ± 0.03
Heart (g)/body mass	0.67 ± 0.01	0.53 ± 0.05	0.57 ± 0.04	0.49 ± 0.03
Urea (mg/dl)	45.81 ± 0.05	32.83 ± 2.20	58.13 ± 0.12	36.90 ± 2.64
AST (U/l)	43.25 ± 13.90	70.61 ± 8.15	48.41 ± 14.44	68.06 ± 4.75
ALT (U/l)	19.31 ± 1.23	22.67 ± 1.96	15.06 ± 1.06	18.21 ± 1.99

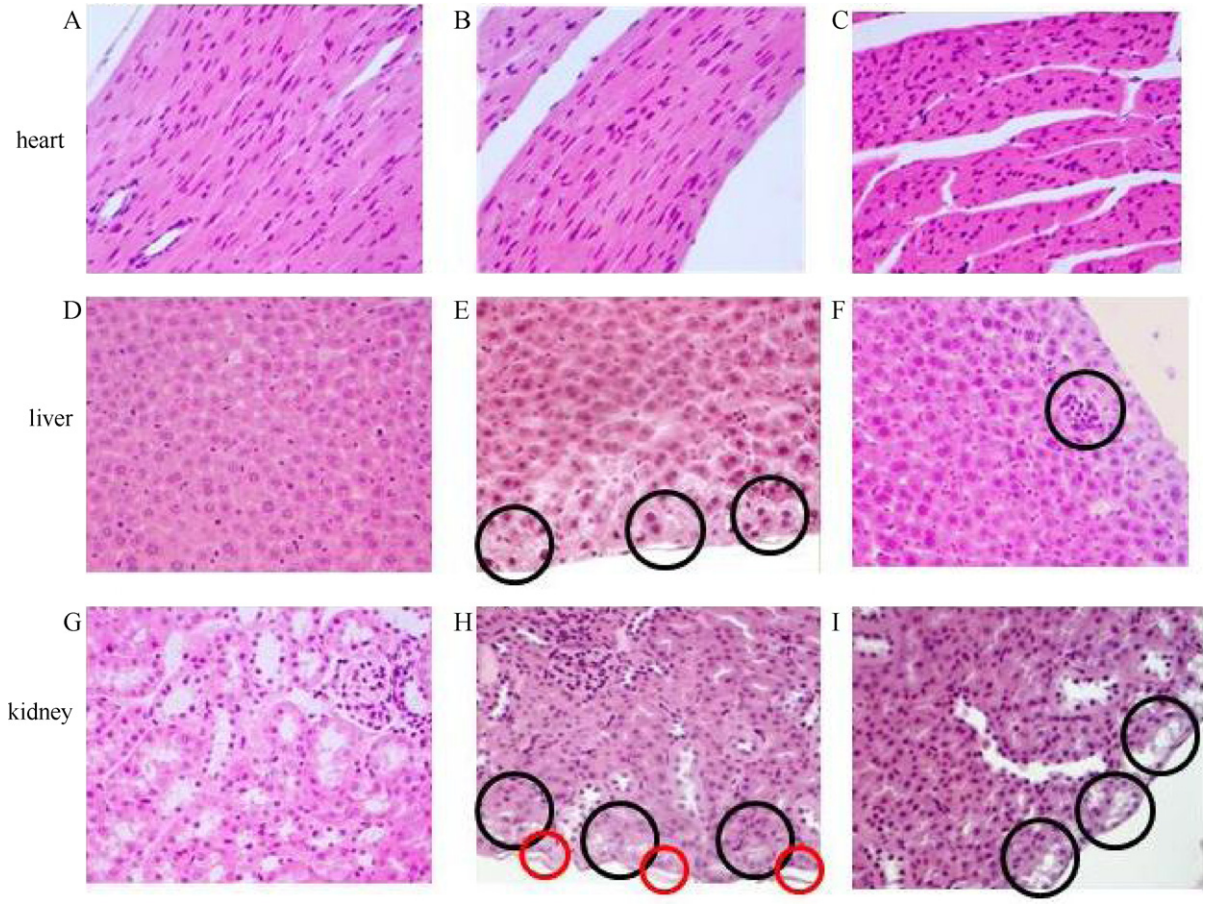

Fig. 6. Histopathological evaluation of mice organs after subchronic treatment with SP-Sf (9 mg/kg) for 14 days. Saline group: heart (A), liver (D), kidney (G). SP-Sf group: heart (B and C), liver (E and F), kidney (H and I). Organs were recovered and fixed with paraformaldehyde and stained with hematoxylin and eosin. Black circles indicate cellular tumefaction (E) and a slight subcapsular infiltration of mononuclear cells in the liver (F) as well as slight degeneration in tubular and subcapsular areas in the kidney (H and I). The red circle in (H) indicates the renal capsule without changes. The tissue sections were observed under a microscope at 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ing that action of SP-Sf on the ligands of these mediators and/or direct action on the nerve terminals are also possible. A similar effect has been observed in studies of sulfated galactans isolated from the red seaweeds *Champia feldmannii* (Assreuy et al., 2008) and *Bryothamnion seaforthii* (Viana et al., 2002).

The formalin test is a widely used model of persistent pain and is a mainstay for the development of novel agents for the treatment of postoperative pain (Shields, Cavanaugh, Lee, Anderson, & Basbaum, 2010). The test occurs in two phases. The first phase is characterized by neurogenic pain caused by the direct chemical stimulation of nociceptors. The second phase is characterized by inflammatory pain triggered by a combination of stimuli, including inflammation of the peripheral tissues and mechanisms of central sensitization (Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). This second phase results from the action of inflammatory mediators in peripheral tissues, such as prostaglandins, serotonin, histamine and bradykinin. Moreover, it is characterized by functional changes in neurons of the spinal dorsal horn that promote long-term facilitation of synaptic transmission at the spinal level (França et al., 2001; Oliveira, Sousa, & Almeida, 2008). In the formalin test, SP-Sf showed a greater inhibition in the second phase than in the first phase, suggesting that its antinociceptive effect is related to inflammatory pain. To distinguish between central and peripheral antinociceptive action, we performed the hot plate test. In this test, opioid agents exert their analgesic effects via supra-spinal and spinal receptors (Nemirovsky, Chen, Zelma, & Jurna, 2001; Yalcin, Charlet, Freund-Mercier, Barrot, & Poisbeau, 2009). In this study, morphine caused a significant increase in reaction time, but SP-Sf produced no significant antinociceptive effects. These results suggest that the antinociceptive action of SP-Sf occurs via a peripheral mechanism.

Because of the well-established link between the development of pain and inflammatory processes, we investigated the anti-inflammatory activity of SP-Sf in the paw edema model. In this test, injected SP-Sf (3 and 9 mg/kg; s.c.) failed to inhibit the edema evoked by carrageenan. These data agree with prior literature attributing anti-inflammatory effects to sulfated fucans but not to sulfated galactans (Assreuy et al., 2008, 2010; Campo et al., 2009; Silva et al., 2010). However, after 1 h, the lowest dose of SP-Sf (1 mg/kg) inhibited the edema induced by carrageenan. Increased doses of SP-Sf probably failed to inhibit edema because the maximum inhibition of neutrophil migration by SP-Sf was reached with the lowest dose (1 mg/kg). This effect corresponded with neutrophil influx and confirmed using MPO activity assays. Edema and inflammation induced by carrageenan can be characterized in three distinct phases. The first phase involves the release of histamine and serotonin. The second phase consists of the release of cytokines and prostaglandins are involved in the final phase (Lo et al., 1982). We demonstrated that SP-Sf (3 or 9 mg/kg; s.c.) did not inhibit the edema evoked by dextran, similar to its effects in the carrageenan-induced rat paw edema model. It has been demonstrated that the sulfated polysaccharides from red seaweed *C. feldmannii* do not inhibit the edema caused by dextran (Assreuy et al., 2008). However, SP-Sf (1 mg/kg) at 30 min inhibited edema following dextran challenge. These results suggest that the anti-

edematogenic response of SP-Sf in low doses is due to inflammatory events involving osmotic edema and may involve histamine, serotonin and bradykinin (Lo et al., 1982).

To better understand the differing edematogenic effects of various doses of SP-Sf, we examined its role as an inflammatory stimulator using a local route of administration. Subcutaneous injections of SP-Sf, especially at the highest dose, evoked intense paw edema that was significantly maintained until the fourth hour of development. To determine the mechanism of action of SP-Sf-induced edema, we injected SP-Sf (9 mg/kg) in combination with a series of anti-inflammatory drugs.

The paw edema caused by the highest dose of SP-Sf (9 mg/kg) involved cyclooxygenase enzymes, prostaglandins, NO and the primary cytokines IL-1 and TNF-α. Previous studies have revealed that the paw edema elicited by *C. feldmannii* evoked a pro-inflammatory response. This response was shown not to result from possible endotoxin contamination and was instead suggested to depend upon the release of primary cytokines, prostaglandins and histamines (Assreuy et al., 2008). Edema formation is a response to stimuli involving various inflammatory mediators and results in increased vascular permeability and/or blood flow (Williams & Peck, 1977).

The inflammatory reaction induced by sulfated polysaccharides from the red seaweed *S. filiformis* could be a defense mechanism of the immune system (Assreuy et al., 2010). This hypothesis is consistent with the observation that polysaccharides from sulfated galactans exhibit an immunostimulant activity (Bondu, Deslanches, Fabre, Berthou, & Guangli, 2010; Lins et al., 2009).

To evaluate the safety of SP-Sf administration, this study also evaluated the integrity of the heart, liver and kidneys in mice injected with SP-Sf. Biochemical analyses revealed no changes in the enzymatic activity of transaminases in the serum of treated mice. Histopathological analyses of liver revealed only slight changes, suggesting normal liver function in these animals. Serum dosages of urea and histopathological analysis of kidneys, which were used as parameters of renal function, were also slightly changed. Despite the fact that the serum dosage of urea did not show toxicity, the histopathological analysis of kidneys revealed edema and infiltration of lymphocytes, with preservation of the interstitial tissues, suggesting a possible reversibility of these morphological changes. The preservation of interstitial tissues in experimental animals was previously demonstrated in sulfated polysaccharides isolated from the red seaweed *C. feldmannii* (Lins et al., 2009). Previous toxicological studies have also reported that sulfated polysaccharides are well tolerated in experimental animals (Assreuy et al., 2008; Siqueira et al., 2010).

5. Conclusion

In this study, we demonstrate the efficacy of sulfated polysaccharides from the red seaweed *S. filiformis* in experimental models of nociception. Although the exact molecular mechanisms of SP-Sf activity remain unknown, our data demonstrate that the antinociceptive effects of SP-Sf occur via a peripheral mechanism. However, the edematogenic effects of SP-Sf suggest the involvement of prostaglandins, NO and primary cytokines (IL-1 and TNF-α). Furthermore, SP-Sf at effective doses did not show visible signs of toxicity. Taken together, these data suggest that this sulfated polysaccharide may be a key tool by which to study the inflammatory processes associated with nociception.

Conflict of interest statement

No conflict of interest to declare.

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