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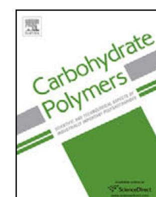

Polysaccharides isolated from *Digenea simplex* inhibit inflammatory and nociceptive responses

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ABSTRACT

Polysaccharides (PLS) have notably diverse pharmacological properties. In the present study, we investigated the previously unexplored anti-inflammatory and antinociceptive activities of the PLS fraction isolated from the marine red alga *Digenea simplex*. We found that the PLS fraction reduced carrageenan-induced edema in a dose-dependent manner, and inhibited inflammation induced by dextran, histamine, serotonin, and bradykinin. The fraction also inhibited neutrophil migration into both mouse paw and peritoneal cavity. This effect was accompanied by decreases in IL1-β and TNF-α levels in the peritoneal fluid. Pre-treatment of mice with PLS (60 mg/kg) significantly reduced acetic acid-induced abdominal writhing. This same dose of PLS also reduced total licking time in both phases of a formalin test, and increased latency in a hot plate test. Therefore, we conclude that PLS extracted from *D. simplex* possess anti-inflammatory and antinociceptive activities and can be useful as therapeutic agents against inflammatory diseases.

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1. Introduction

Presently, about 25–30% of all active compounds that are used as therapeutic treatments are derived from natural products (Silva, Moura, Oliveira, Diniz, & Barbosa-Filho, 2003), and natural marine products have been the focus for the efforts to discover new molecules of pharmacological and biomedical interest (Cabrita, Vale, & Rauter, 2010; Iannitti & Palmieri, 2010) Marine algae have received special attention since they have been shown to be valuable sources of structurally diverse bioactive compounds, such as polyphenols, carotenoids, pigments, enzymes, and polysaccharides (PLS) (Kusaykin et al., 2008; Wijesekara, Pangestuti, & Kim, 2010).

Many species of seaweed (marine macroalgae) are used as food and in traditional medicine because of their perceived health benefits. Red Seaweeds are sources of PLS, including some that have become valuable additives in the food industry because of their rheological properties (Kusaykin et al., 2008; Wijesekara et al., 2010). In addition, these PLS have a number of biological activities, including anticoagulant, antiviral, gastroprotective, antinociceptive, and anti-inflammatory properties (Brito et al., 2013; Chaves et al., 2013; Cumashi et al., 2007; Silva et al., 2011).

The Red Seaweed *Digenea simplex* (Wulfen) C. Agardh, a member of the Rhodomelaceae family, is used extensively in Japan as a parasiticide, and considered a good source of agar (El-Sayed, 1983; Tomoda, Nakatsuka, & Minami, 1972). In a previous study, the galactan content in the PLS of *D. simplex* was investigated by ion exchange chromatography, mass spectrometry, and infrared analysis and was found to be rich in common repeating galactan sulfate backbones (Takano, Shiomoto, Kamei, Hara, & Hirase, 2003). However, no study demonstrating the chemical characteristics of

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the polysaccharide fraction of this alga with habitat in Brazil was performed previously.

The inflammatory process is a temporally controlled phenomenon involving the participation of diverse mediators including histamine, serotonin, bradykinin, TNF-α, IL-1β, and prostaglandins, and is associated with intense migration of neutrophils from the blood into inflamed tissues (Carvalho et al., 1996; Hajare et al., 2001; Srinivasan et al., 2001). The biochemical mediators together stimulate a sequence of molecular events, as well as inflammation and nociception (Déciga-Campos, Palacios-Espinosa, Reyes-Ramírez, & Mata, 2007; Moncada & Higgs, 1993). It is clear that there is a strong association between the inflammatory process and the development of pain. Inflammatory pain, produced by the action of inflammatory mediators, is accompanied by the increased excitability of peripheral nociceptive sensory fibers (Linley, Rose, Ooi, & Gamper, 2010). Interestingly, there are no marine-derived anti-inflammatory natural products in clinical development currently (Mayer et al., 2010).

Thus, the aim of the present study was to investigate the antinociceptive and anti-inflammatory activities of a previously characterized PLS fraction was isolated from the marine red alga *D. simplex* by using experimental models of inflammation and nociception.

2. Experimental

2.1. Extraction of polysaccharide (PLS)

The extraction of the polysaccharide of *Gracilaria birdiae* was accomplished at the Laboratory of Biochemistry of Sea Algae at the Department of Biochemistry and Molecular Biology of the Federal University of Ceará. The Red Seaweed was harvested at Flexeiras Beach, Trairí, Ceará, Brazil, in December 1991, geographical localization: 03°13′25″ S and 39°16′65″ W. A voucher specimen (No. 4693) was deposited in the Herbarium Prisco Bezerra, Federal University of Ceará, Brazil. Samples cleaned of epiphytes were washed with distilled water and then submitted to extraction and fractionation in order to obtain of PLS using experimental protocol previously described (Takano et al., 2003). The dried tissue (5 g) was milled and suspended in 250 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 30 mg of papain (E. Merck), 5 mM EDTA, 5 mM cysteine and incubated at 60 °C for 6 h. The residue was removed by filtration and centrifuged at 2725 × *g* for 30 min at 4 °C. The PLS were precipitated by the addition of 48 mL of 10% cetylpyridinium chloride (CPC, Sigma Chemical). The mixture was centrifuged at 2725 × *g* for 30 min at 4 °C. The polysaccharides in the pellet were washed with 200 mL of 0.05% cetylpyridinium chloride solution, and then precipitated with 200 mL of ethanol (v/v), for 24 h at 4 °C. After further centrifugation (2725 × *g* for 30 min at 4 °C), the precipitate was washed twice with 200 mL of 80% ethanol and dried with acetone under hot air flow (60 °C).

2.2. Infrared spectroscopy

Fourier transform infrared (FT-IR) spectra of KBr pellets of the polysaccharides were recorded in a Shimadzu IR spectrophotomer (model 8300) scanning between 400 and 4000 cm^{-1}.

2.3. Nuclear magnetic resonance spectroscopy

^{13}C NMR spectra of 2.5% w/v solutions in D_2O were recorded at 353 K on a Fourier transform Bruker Avance DRX 500 spectrometer with an inverse multinuclear gradient probe-head equipped with z-shielded gradient coils, and with Silicon Graphics. Acetone was used as the internal standard (31.07 ppm for ^{13}C).

2.4. Animals

Male Swiss mice weighing 20–25 g were used. The animals were housed in temperature-controlled rooms and received food and water ad libitum. All experiments were conducted in accordance with the currently established principles for the care and use of COBEA (Colégio Brasileiro de Experimentação Animal), Brazil. The Animal Studies Committee of Universidade Federal do Ceará approved the experimental protocol.

2.5. Carrageenan-induced paw edema

The animals were randomly divided into 6 groups ($n = 5$), and edema was induced by the injection of 50 μL of a suspension of carrageenan (500 μg/paw) in 0.9% sterile saline into the right hind paw (group I). The mice were pretreated intraperitoneally (i.p.) with either 0.9% NaCl (group II, untreated control), 10 mg/kg indomethacin (group III, reference control), or 10, 30, or 60 mg/kg of PLS (groups IV, V, and VI, respectively) 1 h before carrageenan injection. Paw volume was measured with a plethysmometer (Panlab, Barcelona, Spain) immediately before (Vo), and at 1, 2, 3, and 4 h after carrageenan treatment (Vt) as previously described (Winter, Risley, & Nuss, 1962). The effect of pre-treatment was calculated as the percentage of inhibition of edema relative to the paw volume of the saline-treated controls as previously described (Lanhers, Fleurentin, Dorfman, Mortier, & Pelt, 1991) according to the following formula: % inibition of edema = (Vt − Vo)"Control" − (Vt − Vo)"Treated")/(Vt − Vo) "Control") × 100.

2.6. Paw edema induced by different inflammatory agents

To induce paw edema with different inflammatory agents, the animals were administered 50-μL injections of dextran (DXT, 500 μg/paw), serotonin (Ser, 1%, w/v), histamine (Hist, 1%, w/v), or bradykinin (Bk, 6 nmol) into the right hind paw. One group received 50 μL of 0.9% sterile saline and served as an untreated control group. PLS (60 mg/kg) or indomethacin (10 mg/kg, reference control) were injected i.p. 30 min before intraplantar injections of phlogistic agents. Paw volume was measured immediately before, and at selected interval of time.

2.7. Determination of myeloperoxidase activity

The extent of neutrophil accumulation in the mouse paw was measured using a myeloperoxidase (MPO) assay. To evaluate MPO activity, carrageenan was injected into the right plantar surface of the mice pre-treated with saline, carrageenan, indomethacin, or PLS (60 mg/kg). Four hours after carrageenan injection, we measured the MPO concentration in the right hind paw. Briefly, 50–100 mg of hind paw tissue was homogenized in 1 mL of potassium buffer with 0.5% hexadecyltrimethylammonium bromide (HTAB) for each 50 mg of tissue. The homogenate was centrifuged at 40,000 × *g* for 7 min at 4 °C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm by using *o*-dianisidine dihydrochloride and 1% hydrogen peroxide. The results are reported as MPO units/mg tissue. A unit of MPO (UMPO) activity was defined as that converting 1 mmol hydrogen peroxide to water over 1 min at 22 °C.

2.8. Peritonitis model

Mice were injected orally with 250 μL of sterile saline, indomethacin 10 mg/kg, or PLS 60 mg/kg. One hour later, they were injected with 250 μL of carrageenan (500 μg/cavity) into the peritoneal cavity. The mice were euthanized 4 h later and the peritoneal

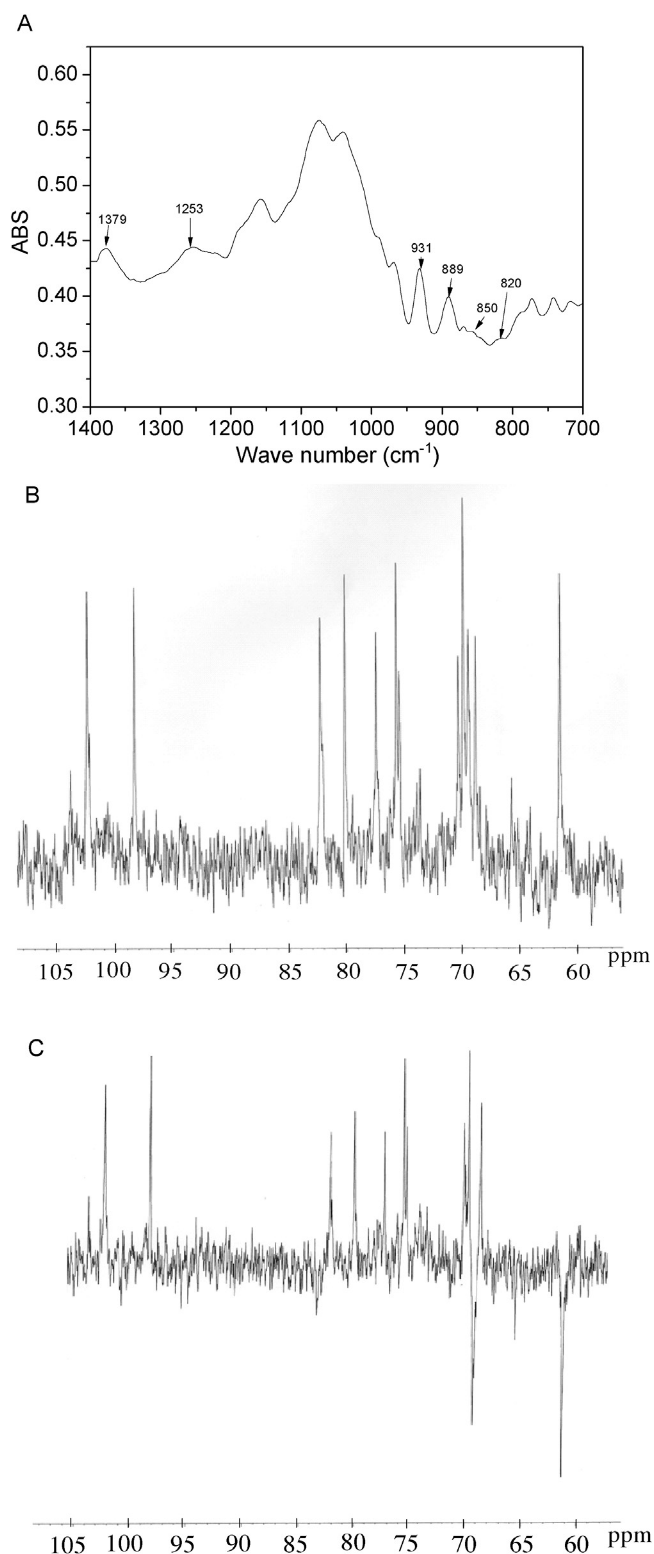

Fig. 1. FT-IR spectra (A) in KBr pellets; NMR spectra in D_2O, (B) ^{13}C-NMR spectrum s, (C) DEPT spectrum of PLS from *D. simplex*.

cavity was washed with 1.5 mL of heparinized phosphate-buffered saline (PBS) to harvest the cells contained in the peritoneal fluid. Total cell counts were performed using a Neubauer chamber, and differential cell counts (100 cells total) were carried out using cytocentrifuge slides stained with hematoxylin and eosin. The results are presented as the number of total leucocytes or neutrophils per milliliter of peritoneal exudates.

2.9. Measurement of IL-1β and TNF-α

After the peritonitis assay, samples of peritoneal fluid were collected and the levels of IL-1β and TNF-α were evaluated using a sandwich enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-1β were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). The kits consistently showed IL-1β and TNF-α levels to be over 4000 pg/mL and that they did not cross-react with other cytokines. The results are expressed as pg/mL of each cytokine per peritoneal cavity washed.

2.10. Acetic acid-induced writhing test

The acetic-acid writhing test was used to evaluate the analgesic activity (Collier, Dinneen, Johnson, & Schneider, 1968) of PLS. The mice (n = 6 per group) were injected (i.p.) with 0.6% acetic acid (10 mL/kg body weight), and the intensity of nociception was quantified by counting the total number of writhes over a period of 20 min, and included abdominal muscle contractions and hind paw extensions (Koster, Anderson, & De-Beer, 1959). The animals received PLS (60 mg/kg, i.p.) or sterile saline (control group, 0.9%, w/v) 30 min before acetic acid injection. Morphine (5 mg/kg, s.c.) was administered 30 min before acetic acid as a reference control.

2.11. Formalin test

This test, which results in a local tissue injury to the paw, has been used as a model for tonic pain and localized inflammatory pain (Hunskaar, Fasmer, & Hole, 1985). Twenty microliters of 2.5% formalin was administered (s.c.) into the right hind paw. The amount of licking time was recorded from 0 to 5 min (phase 1, neurogenic) and from 20 to 25 min (phase 2, inflammatory) after formalin injection (Hunskaar & Hole, 1987). The mice (n = 6 per group) were then treated with PLS (60 mg/kg, i.p.) or sterile saline (0.9%) 30 min before formalin injection. Morphine (5 mg/kg, s.c.) was also administered 30 min before formalin injection and used as a reference compound.

2.12. Hot-plate test

The hot plate test is another commonly used method to measure analgesic activity (Eddy & Leinback, 1953). Each mouse was placed twice onto a heated plate (51 ± 1 °C), separated by a 30-min interval. The first trial familiarized the animal with the test procedure, and the second trial served as the control for the reaction time (licking the paw or jumping). Animals showing a reaction time greater than 20 s were excluded. After the second trial (control reaction time), groups of animals (n = 6) received sterile saline (0.9%, i.p.), PLS (60 mg/kg, i.p.), or morphine (5 mg/kg, s.c.; reference drug). The reaction times were measured at time zero (0 time) and at 30, 60, 90, and 120 min after the compounds were administered, with a cut-off time of 45 s to avoid paw lesions.

2.13. Statistical analysis

The results are shown as the means ± S.E.M. For the peritonitis model experiments and cytokine measurements, ANOVA was performed followed by Bonferroni's test. For all other experiments, ANOVA was performed followed by Newman–Keuls tests; $p < 0.05$ was defined as statistically significant.

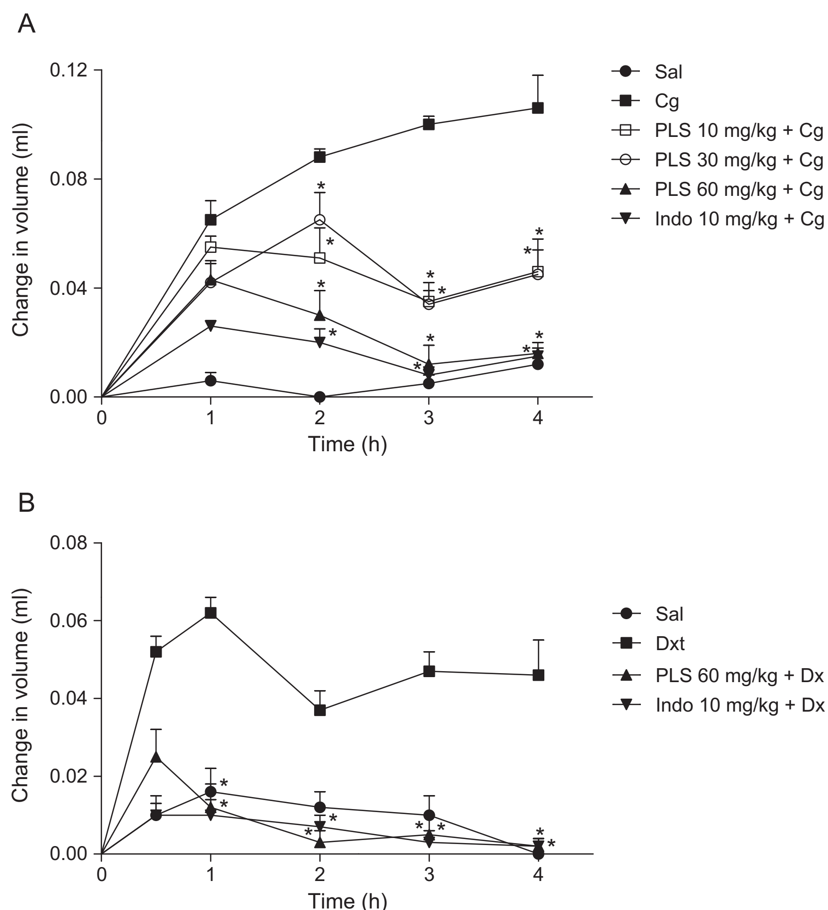

Fig. 2. Effect of PLS from *D. simplex* on paw edema induced by carragenan (A) and dextran (B). Paw edema was induced by carragenan (Cg: 500 μg/paw; Panel A) or dextran (Dx: 500 μg/paw; Panel B) injections into the plantar surface of the right paw. The change in paw volume was measured at the indicated time intervals. Mice were treated with polysaccharide (PLS: 10, 30 and 60 mg/kg; i.p.) and indomethacin (Indo: 10 mg/kg, i.p.; positive control). The values given are means ± S.E.M. ($n = 6$). *Statistical difference ($p < 0.05$) compared to carragenan (Panel A) or dextran (Panel B) (one-way ANOVA followed by Newman–Keuls post-test).

3. Results and discussion

3.1. Structural analysis of the PLS from D. simplex

The FT-IR spectrum of soluble polysaccharide from *D. simplex* is depicted in Fig. 1A. The bands in the region of 1400–700 cm^{-1} are characteristic of agarocolloids (Chopin & Whalen, 1993; Lahaye & Yaphe, 1988; Maciel et al., 2008; Melo, Feitosa, Freitas, & de Paula, 2002; Mollet, Rahaoui, & Lemoine, 1998; Prado-Fernandez, Rodriguez-Vazquez, Tojo, & Andrade, 2003; Rochas, Lahaye, & Yaphe, 1986). The band at 1253 and 931 cm^{-1} can be attributed to the S=O vibration of the sulphate groups C—O—C of 3,6-anhydrogalactose respectively. The region at 800–850 cm^{-1} is used for algal polysaccharides to characterize the sulfate pattern of agarocolloids. The presence of the low intensity bands at 850 and 820 cm^{-1} may suggest a small degree of sulfate substitution at C-4 and C-6 of galactose.

The anomeric region of ^{13}C NMR (Fig. 1B) shows two main signals, which were assigned based on literature data (Lahaye, Yaphe, Viet & Rochas, 1989; Miller & Furneaux, 1997; Usov, Yarotsky & Shashkov, 1980; Valiente, Fernandez, Perez, Marquina, & Velez, 1992) as C-1 of β-D-galactose linked to 3,6-α-L-anhydrogalactose at δ 102.6 and C-1 of 3,6-anhydro-α-L-galactopyranose at δ 98.4. A DEPT 135° experiment was used to investigate the presence of CH_2 groups, considering that the pulse sequence signals of the carbons bearing two protons have opposite amplitude to the CH and CH_3 carbons. The DEPT 135° spectrum of *D. simplex* (Fig. 1C) shows two intense CH_2 signals at δ61.5 and δ69.5 attributed to C-6 of β-D-galactose and 3,6-α-L-anhydrogalactose, respectively. The lower intense CH_2 signal observed at δ65.7 may be attributed as C-6 of α-L-galactose-6 sulfate.

The twelve main signals observed in the ^{13}C NMR spectrum can be attributed based in the literature data (Lahaye et al., 1989; Maciel et al., 2008; Melo et al., 2002; Miller & Furneaux, 1997; Usov et al., 1980; Valiente et al., 1992) to C-1–C-6 of β-D-galactose (102.5, 70.3, 82.3, 68.9, 75.5 and 61.5) and of 3,6-α-L-anhydrogalactose (98.4, 69.9, 80.2, 77.4, 75.7 and 69.5).

The ^{13}C-NMR and FT-IR spectra indicate that the main polysaccharide fraction extracted from *D. simplex* is a agarocolloid, corroborating with previous studies (El-Sayed, 1983; Tomoda et al., 1972).

3.2. The anti-inflammatory activity of PLS on paw edema induced by carrageenan and dextran

Since PLS derived from algae represent an important candidate therapeutic for the treatment of inflammation (Chen, Wu, & Wen, 2008; Groth, Grunewald, & Alban, 2009), the anti-inflammatory activity of PLS extracted from *D. simplex* was assessed using classical experimental models of inflammation. Fig. 2 summarizes the effects of PLS on paw edema in experimental animals. Treatment of mice with carrageenan (500 μg/paw; Panel A) or dextran (500 μg/paw; Panel B) induced a significant increase in paw volume over the specified time intervals ($p < 0.05$). A significant reduction in edema was observed in all the groups treated with PLS prior to carrageenan treatment, beginning within 1 h and lasting until

the fourth hour after inflammagen administration ($p < 0.05$). The maximum effect induced by PLS administration at doses of 10, 30 and 60 mg/kg was observed at the third hour, with inhibition rates of 65.0%, 66.0% and 87.5%, respectively, compared to animals that received carrageenan alone. The inhibitory effect of PLS on paw edema induced by carrageenan was dose-dependent. The most effective dose was 60 mg/kg and this dose was thus chosen for further evaluation, including that of its effects on dextran-induced paw edema. We subsequently observed that PLS also inhibited the edema induced by dextran. An inhibition rate of 80.6% was observed after the first hour, compared to the finding observed in the control group treated with dextran alone. The anti-oedematogenic effect of PLS against dextran-induced inflammation was maintained until the fourth hour of analysis in which the edema was almost completely absent (95.6% inhibition). It is well-documented that inflammatory events triggered by dextran administration lead to development of edema via resident cell degranulation, while those induced by carrageenan involve cell migration (Lo, Almeida, & Beaven, 1982). Our data are in agreement with those of other studies in which anti-inflammatory effects of PLS extracted from seaweed against carrageenan- or dextran-induced inflammation were reported (Chaves et al., 2013; Damasceno et al., 2013). Furthermore, PLS (60 mg/kg) was as efficacious as indomethacin ($p > 0.05$), a commercial drug used as an anti-inflammatory agent, reinforcing the pharmacologic potential of PLS extracted from this algal species.

3.3. Effect of PLS on paw edema induced by various inflammagens

Compared to injection of the saline control, the injection of various inflammagens into the subplantar surface of mouse hind paws produced a marked increase in paw volume ($p < 0.05$) (Fig. 3). The injection of PLS (60 mg/kg, i.p.) significantly reduced the edema induced by phlogistic agents during all tested time courses. At 30 min, the time point of the peak effect of the tested agents, the edema volume in the PLS group was 0.022 ± 0.004 mL compared to 0.117 ± 0.017 mL in the histamine group, corresponding to 80.7% inhibition (Fig. 3A). PLS was more effective than indomethacin, which produced only a 58.9% inhibition. PLS also significantly inhibited the increase in paw volume of animals treated with serotonin or bradykinin by 38.0% and 42.8%, respectively (Fig. 3B and C). It is well known that chemical mediators including histamine, serotonin, and bradykinin are involved in carrageenan-induced paw edema (Chen, Tsai, & Wu, 1995; Vinegar et al., 1987). In this model, the edema is believed to be biphasic, with the first phase being mediated by the release of histamine and serotonin, followed by the subsequent release of bradykinin and the late edema phase being dependent on cytokine production by resident cells and neutrophil infiltration (Barbosa et al., 2009; Kulkarni, Mehta, & Kunchandy, 1986; Vinegar, Schreiber, & Hugo, 1969). In contrast, dextran-induced paw edema is mediated by increased vascular permeability induced by mast cell degranulation of histamine and serotonin (Metcalfe, 2008). The oedematous fluid formed because of dextran injection contains little protein and few neutrophils (Gupta et al., 2003). Therefore, we can infer that the anti-oedematogenic action of PLS may be because of the differential inhibition of specific inflammatory mediators and modulation of neutrophil infiltration in the inflamed plantar tissue.

3.4. Effect of PLS on carrageenan-induced MPO activity in mouse paw tissue

As shown in Fig. 4A, at 4 h after treatment with inflammatory stimuli, a significant increase in MPO activity was found in the carrageenan-treated animals (15.23 ± 3.45 UMPO/mg plantar tissue) compared to those treated with saline (0.68 ± 0.11 UMPO/mg plantar tissue). Pre-treatment with PLS (60 mg/kg) consistently reduced MPO activity in paw tissue (4.17 ± 0.71 UMPO/mg of tissue); the effect was similar to that of indomethacin (5.03 ± 1.48 UMPO/mg plantar tissue). The carrageenan-induced inflammatory response in paw tissue is known to be accompanied by severe neutrophil infiltration into the inflammatory site (De Smet, 1997; Souza, Cunha, Mello, & Ferreira, 1988). MPO activity has been found in neutrophil azurophilic granules, indicating infiltration of this cell into tissues (Bradley, Priebat, Christensen, & Rothstein, 1982). Since MPO activity levels in PLS-treated animals were lower than those in the animals treated with carrageenan alone, it is plausible that this response involves the inhibition of neutrophil migration.

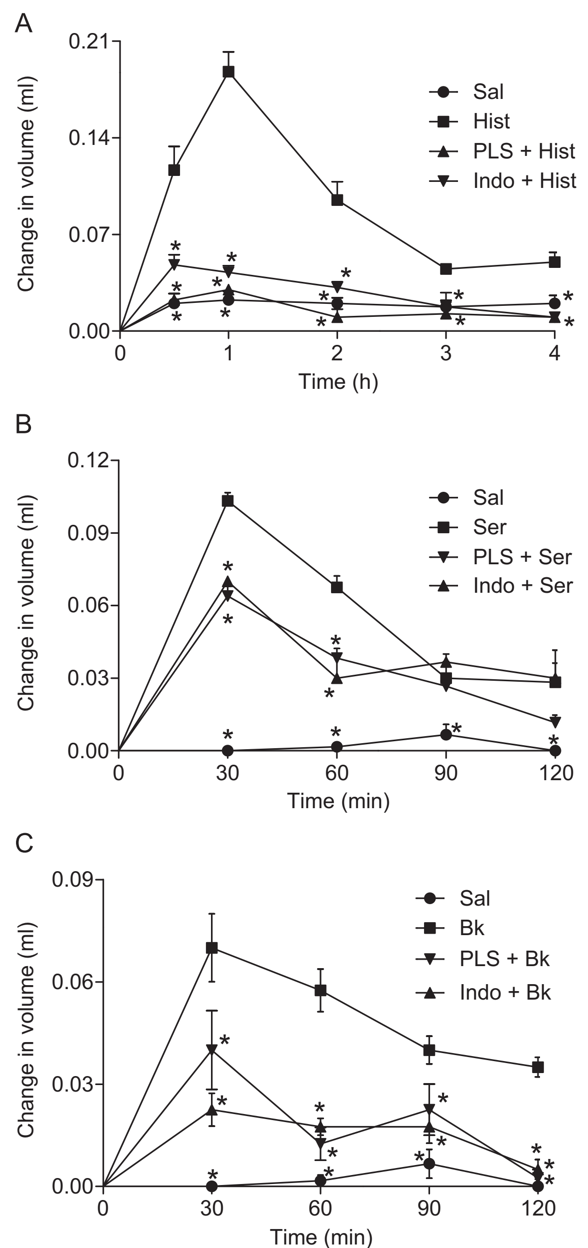

Fig. 3. Effect of PLS from *D. simplex* on paw edema induced by various inflammagens. Paw edema was induced by (A) histamine (Hist: 500 μg per paw), (B) serotonin (Ser: 500 μg per paw), or (C) bradykinin (Bk: 6 nmol, w/v, per paw) injections into the plantar surface of the right paw. The change in paw volume was measured at the indicated time intervals. Animals were pretreated with polysaccharide (PLS: 60 mg/kg; i.p.) and indomethacin (Indo: 10 mg/kg, i.p.) was used as a positive control. The values given are means ± S.E.M. ($n = 5$). *Statistical difference ($p < 0.05$) compared to inflammatory stimuli treatment (one-way ANOVA followed by Newman–Keuls post-test).

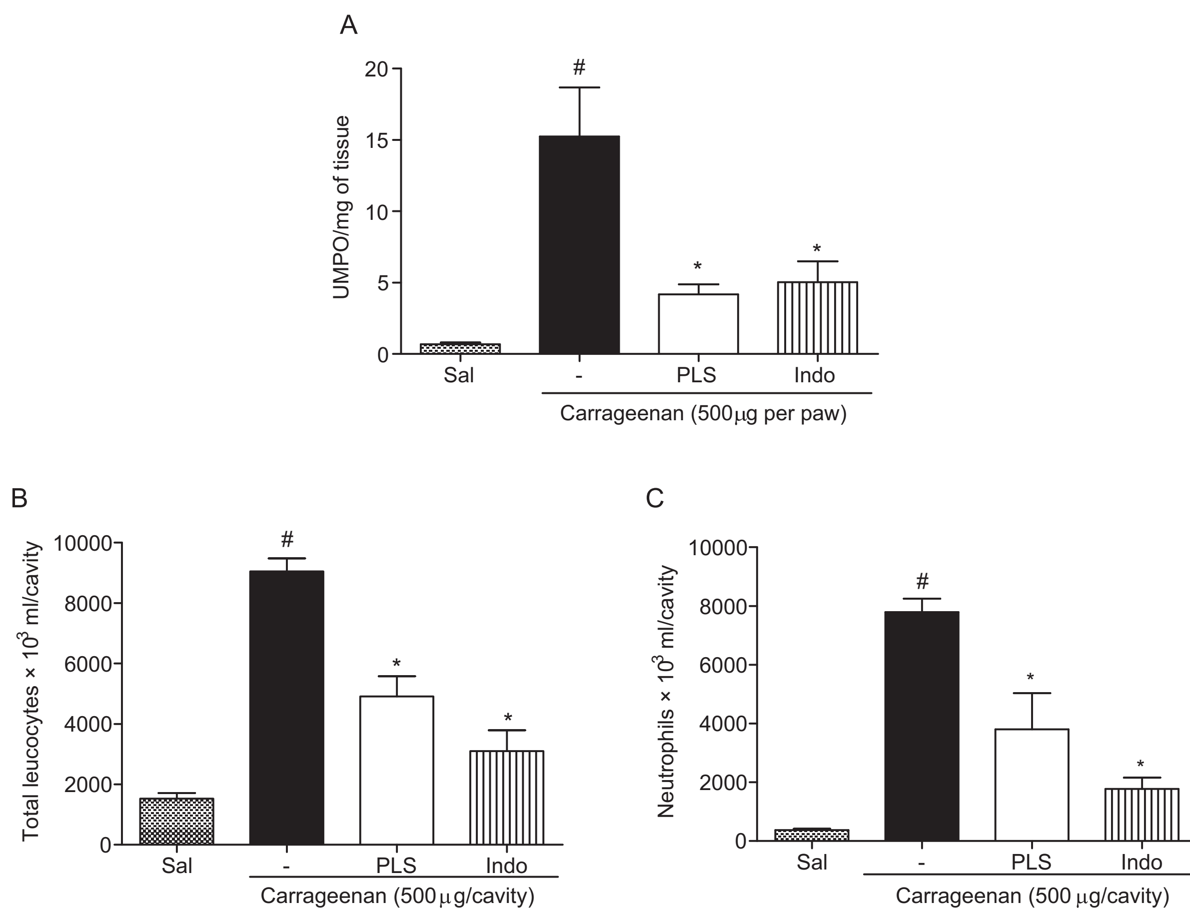

Fig. 4. The effects of PLS from *D. simplex* on myeloperoxidase (MPO) activity in paw tissue and cell migration in the peritoneal cavity. Animals received polysaccharide (PLS: 60 mg/kg; i.p.) 30 min before carrageenan administration, and myeloperoxidase activity or neutrophil migration were evaluated 4 h later. The values given are means ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg, i.p.) was used as a positive control. $^{\#}p < 0.05$ vs. saline group; $^{*}p < 0.05$ vs. carrageenan group (one-way ANOVA followed by Newman–Keuls post-test).

3.5. Anti-inflammatory effect of PLS on carrageenan-induced peritonitis in mice

The hypothesis that the anti-oedematogenic effect of PLS against carrageenan-induced inflammation was related to a reduction in neutrophil infiltration to the site of inflammation was further investigated in an experimental mouse model of peritonitis. This model allows quantification of cells and levels of several inflammatory mediators and is a well-characterized pharmacological tool for the examination of neutrophil migration (Montanher, Zucolotto, Schenkel, & Fröde, 2007). Compared to the animals treated with saline alone, those treated with

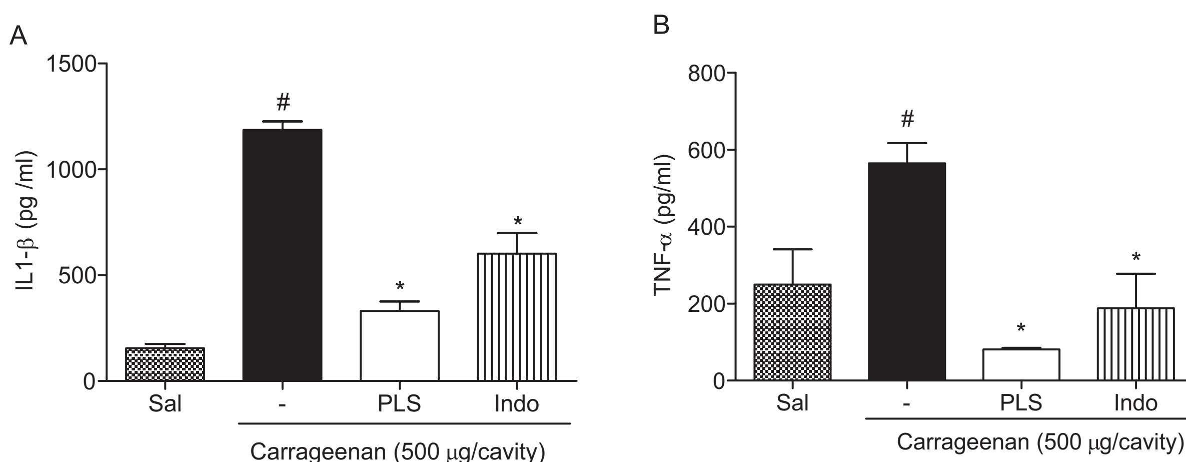

Fig. 5. Effect of PLS from *D. simplex* on carrageenan-induced cytokine production in peritonitis. Animals were injected with polysaccharide (PLS: 60 mg/kg) 30 min before carrageenan administration, and 4 h later the levels of IL-1β (A) and TNF-α (B) were measured in the peritoneal fluid. The values given are means ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg) was used as a positive control for anti-inflammatory activity. $^{\#,*}$Statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively (one-way ANOVA followed by Bonferroni's post-test).

Table 1
Antinociceptive effect of PLS from *D. simplex* on acetic acid induced writhes.

Treatment	Dose (mg/kg)	Number of abdominal constrictions (20 min)	Inhibition (%)
Control (acetic acid)	–	47.0 ± 5.6	–
Morphine	5	0.0 ± 0.0[a]	100
PLS	60	10.8 ± 3.6[a]	77.0

Values are given as the means ± S.E.M. of five animals, as analyzed by one-way ANOVA followed by Newman–Keuls test ($p < 0.05$). PLS: sulphated polysaccharides fraction.

[a] Compared with acetic acid control group.

intraperitoneal administration of carrageenan showed a significant increase in total leukocyte and neutrophil counts in the peritoneal fluid ($1525 \pm 185.4 \times 10^3$ total leukocytes/mL vs. $9050 \pm 429.1 \times 10^3$ total leukocytes/mL; $370.6 \pm 42.9 \times 10^3$ neutrophils/mL vs. $7789 \pm 459.1 \times 10^3$ neutrophils/mL; $p < 0.05$) (Fig. 4B and C). Pre-treating the animals with 60 mg/kg of PLS reduced the total leukocyte ($4913 \pm 668.4 \times 10^3$ cells/mL) and neutrophil migration ($3804 \pm 1228 \times 10^3$ cells/mL) to levels comparable to the corresponding levels observed in the saline and indomethacin groups ($p > 0.05$).

3.6. Effect of the PLS fraction on carrageenan-induced cytokine production in peritoneal exudates

The inflammatory response induced by carrageenan in the peritonitis model was associated with an increase in the levels of IL1-β and TNF-α (Fig. 5A and B). The levels of IL1-β and TNF-α in the peritoneal fluid of the saline control group were 155.4 ± 19.7 pg/mL and 249.5 ± 91.5 pg/mL, respectively; the corresponding levels were higher in the carrageenan-treated animals (IL1-β level: 1187 ± 39.8 pg/mL and TNF-α level: 564.9 ± 52.3 pg/mL). Carrageenan induces neutrophil migration into the peritoneal cavity through an indirect mechanism that involves the activation of macrophages and release of pro-inflammatory cytokines, such as IL-1β and TNF-α (Lo et al., 1982). Such increases in cytokine levels might result in plasma protein extravasation and cellular infiltration into the site of inflammation (Rosenbaum & Boney, 1991; Thorlacius, Lindbom, & Raud 1997). In the present study, compared to the carrageenan-treated group, the PLS-treated animals exhibited a significant reduction of both IL1-β and TNF-α levels in peritoneal exudates (330.9 ± 45.1 pg/mL and 81.1 ± 4.3 pg/mL, respectively) ($p < 0.05$). TNF-α and IL-1β are potent pro-inflammatory cytokines that possess multiple effects, including the activation of inflammatory cells, induction of several inflammatory proteins, edema formation, and neutrophil migration (Haddad, 2002; Hopkins, 2003). On the basis of this finding, we propose that PLS treatment decreased neutrophil migration by decreasing the production and release of pro-inflammatory cytokines.

3.7. Antinociceptive effect of PLS on acetic acid-induced writhing

There is a strong association between the development of pain and the inflammatory process (Bitencourt et al., 2008). It was previously demonstrated that inhibition of neutrophil migration reduces hypernociception induced by different inflammatory stimuli (Hopkins, 2003; Tjølsen & Hole, 1997). Several reports have demonstrated that PLS extracted from algae show promise as antinociceptive agents (Brito et al., 2013; Chaves et al., 2013; Damasceno et al., 2013). Thus, in the present study the

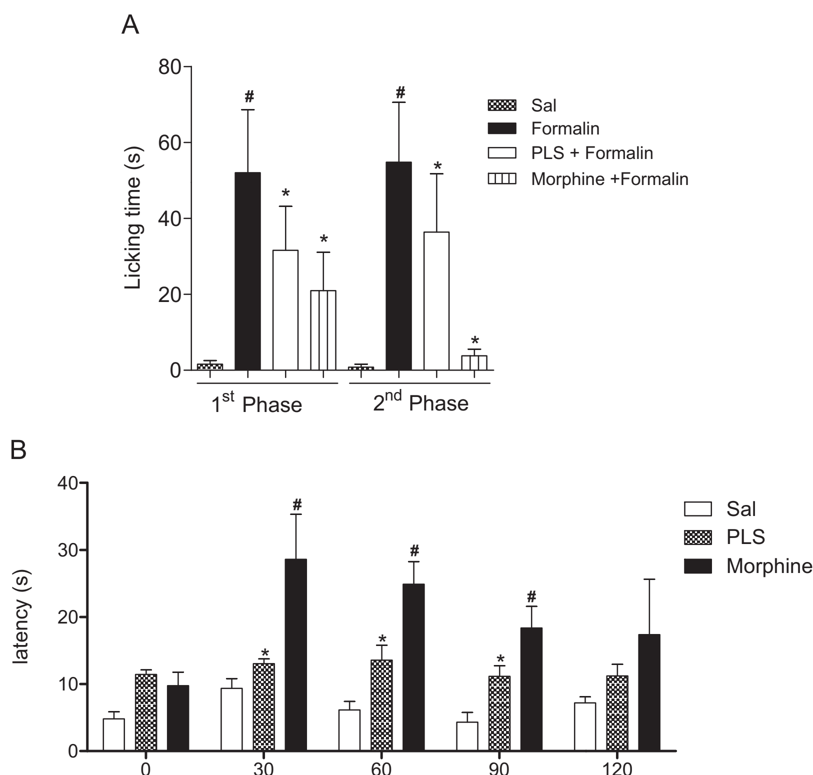

Fig. 6. Antinociceptive effect of PLS from *D. simplex* in formalin-induced paw licking (A) and hot plate test (B). Animals received PLS (60 mg/kg, i.p.) or morphine (5 mg/kg, sc) 30 min before 2.5% formalin administration by the intraplantar route. Licking time was recorded in the first 5 min (1st phase) and after 20 min (2nd phase) during 5 min. In the hot plate test the animals received the same doses of PLS or morphine to evaluated reaction times to thermal stimuli. The values given are means ± S.E.M. ($n = 6$). Panel (A) [#,*]Statistical difference ($p < 0.05$) compared to saline and formalin, respectively. Panel (B) [*,#]Statistical difference ($p < 0.05$) compared to saline (one-way ANOVA followed by Newman–Keuls post-test).

antinociceptive potential of PLS from *D. simplex* was also evaluated using three well-accepted murine pain models. Table 1 shows the antinociceptive effect of PLS on abdominal contractions induced by acetic acid. As expected, morphine showed a potent analgesic response compared to that observed in control animals ($p < 0.05$). The administration of PLS (60 mg/kg) 30 min prior to painful stimuli significantly reduced acetic acid-induced abdominal writhing (77.0% inhibition) compared to control ($p < 0.05$). The writhing test is commonly used for screening peripherally active analgesic compounds. Algogenic agents, such as acetic acid, provoke a stereotypical behavior in mice characterized by abdominal contractions, movements of the body as a whole, and twisting of dorsal abdominal muscles (Bars, Gozariu, & Cadden, 2001). This model involves different nociceptive mechanisms, such as release of biogenic amines (histamine and serotonin), bradykinin, and PGE2 (Collier et al., 1968; Duarte, Nakamura, & Ferreira, 1988). Furthermore, it is well established that the nociceptive response caused by acetic acid is also dependent on the release of some cytokines, such as TNF-α and IL-1β via modulation of macrophages and mast cells localized to the peritoneal cavity (Ribeiro et al., 2000). Thus, PLS may reduce writhing by inhibition of the release of nociceptive mediators in response to acetic acid.

3.8. Effect of PLS in the formalin test

Fig. 6A shows that intraplantar injection of formalin (2.5%/paw) significantly increased the total licking time in the first and second phases compared to saline treatment ($p > 0.05$). This event was strongly inhibited in both phases by pre-treatment with the PLS fraction (60 mg/kg). We observed that the licking time of PLS-treated animals was inhibited by 60.5% and 61.7% in the first and second phases, respectively, compared to the findings observed in the formalin group. The inhibitory effect of PLS was similar to that seen in the morphine group (First and second phases). Formalin-induced persistent pain in mouse paws involves two distinct phases. The first phase (neurogenic) is characterized by direct chemical stimulation of nociceptors, and the second phase is accompanied by the release of inflammatory mediators, such neuropeptides, prostaglandins, serotonin, histamine, and bradykinin (Hunskaar et al., 1985; Hunskaar & Hole, 1987; Murray, Porreca, & Cowan, 1988). Since PLS exhibited activity in both phases, it is suspected that it acts centrally, and a possible interaction with opioid receptors is also indicated (Shibata, Ohkubo, Takahashi, & Inoki, 1989).

3.9. Effect of PLS in the hot plate test

The effect of intraperitoneal administration of PLS (60 mg/kg) on animals evaluated in the hot plate test varied according to the observation time used (Fig. 6B). At time zero and 120 min, no significant antinociceptive effect was observed compared to controls ($p > 0.05$). At 30, 60, and 90 min, PLS increased the reaction times of mice by 34.4%, 122.9%, and 160.5%, respectively. The reference drug morphine, an opioid receptor agonist, induced a significant increase in latency, as expected. The hot-plate test is a well-known model for acute thermal nociception. Increases in reaction time indicate analgesic effects via supraspinal and spinal receptors (Nemirovsky, Chen, Zelman, & Jurna, 2001). Furthermore, the test specifically helps evaluate central nociception (Vilela, Padilha, Dos Santos, Silva, & Paiva, 2009) and measure complex responses to inflammation and nociception promoted by opioid agents (Bhandare, Kshirsagar, Vyawahare, Hadambar, & Thorve, 2010). On the basis of our results, we concluded that PLS can inhibit nociception by either peripheral or central mechanisms.

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