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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.

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