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Lectin obtained from the red seaweed *Bryothamnion triquetrum*: Secondary structure and anti-inflammatory activity in mice

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ABSTRACT

Seaweeds are sources of biomolecules with biological activities and pharmacological potential – for example, lectins, a group of proteins that can bind reversibly to carbohydrates or compounds containing them. The aim of this study was to elucidate the structural properties of a lectin extracted from the red seaweed *Bryothamnion triquetrum* (BtL) and to investigate its anti-inflammatory activity in mice. The lectin was purified by precipitation with ammonium sulfate and ion-exchange chromatography. Its secondary structure and tryptophan (Trp) microenvironment were analyzed by circular dichroism spectroscopy and steady-state fluorescence spectroscopy, respectively. The anti-inflammatory effect was evaluated by means of paw edema induced by carrageenan or dextran, myeloperoxidase activity in paw tissue, and by measurement of leukocyte and neutrophil migration and cytokine quantification in a peritonitis model. The secondary structure of BtL is mostly composed of β-strands and unordered conformation, and it is quite resistant to extremes of pH and temperature, preserving the exposure of Trp residues under these conditions. In an assessment of biological activities, groups of mice were subjected to pretreatment with BtL before the inflammatory stimulus. BtL had anti-inflammatory effects in the models tested, and hence may be considered a molecule with potential to be used in the pharmaceutical industry.

1. Introduction

Marine macroalgae (seaweeds) are a part of the traditional oriental diet. In Western countries, their major uses are associated with their rheological properties: they serve as gelling agents, thickeners, and stabilizers [1,2]. Nonetheless, in the last few decades, there was an increase in the pharmaceutical industry's interest in natural products of marine origin with biological activities, especially seaweeds, owing to the peculiarity of their chemical structures caused by the special features of the marine environment [3,5]. Many compounds with pharmacological properties are found in seaweeds, such as polyphenols, peptides, sulfated polysaccharides, and lectins [6,7]. Among these compounds, lectins obtained from seaweeds have shown a substantial therapeutic potential, exhibiting antinociceptive [8,9], antiviral [10], and antitumor effects [11].

Lectins are proteins, or glycoproteins, of non-immune origin which bind reversibly to sugar residues found ubiquitously in nature [12]. The wide structural and functional diversity of lectins in all major taxa of living organisms has been reported, e.g., in invertebrates, vertebrates, seaweeds, and microorganisms. These proteins are important glycobiology tools with the ability to recognize and decipher the codes found in oligosaccharides and glycosyl residues (i.e., glycol-codes) because of their highly specific binding to carbohydrates [13,14].

Inflammation is a complex reaction involving recognition of an infectious agent (or injurious stimulus) for its subsequent destruction and reconstruction of the damaged tissue. This recognition initiates an

Abbreviations: BSA, bovine serum albumin; BtL, *Bryothamnion triquetrum* lectin; CD, circular dichroism; DEAE-Sephacel, diethylaminoethanol covalently linked to Sephacel (a polysaccharide polymer material derived from agarose); ELISA, enzyme-linked immunosorbent assay; H_2O_2, hydrogen peroxide; HCl, hydrochloric acid; HU/mL, hemagglutination units; IL-1β, interleukin 1 beta; MPO, myeloperoxidase; MRW, mean residual weight; NaCl, sodium chloride; NaOH, sodium hydroxide; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor alpha; UFC, Federal University of Ceará.

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immune response activating and amplifying the release of inflammatory mediators, such as cytokines, chemokines, biogenic amines, and eicosanoids by immune-defense cells [15,16]. Nevertheless, when eradication of the pathogen and tissue repair do not proceed in an efficient and synchronized manner, the inflammatory response may lead to persistent tissue injury induced by leukocytes and collagenous accumulation, in addition to other substances that may be harmful to the organism [17]. Therefore, aberrations in the inflammatory process underlie several health problems, such as sepsis, asthma, arthritis, psoriasis, and autoimmune disorders [18,19] and contribute significantly to the development of cancer, diabetes mellitus, and cardiovascular disorders [20].

Several lectins from plants have been successfully evaluated in inflammatory models [21,22]; however, there are only a few studies on anti-inflammatory activities of lectins from seaweeds, in part because of the small amounts of lectins isolated. Nevertheless, this situation is changing, and the interest in seaweed lectins has been increasing notably due to their molecular structure and glycosidic bonding different from those found in plants or other life forms. Indeed, these lectins have unique features, such as low molecular weight, monomeric structure, thermostability (due to the presence of disulfide bridges), and high affinity for glycoproteins instead of monosaccharides [23,24].

A lectin obtained from the green seaweed *Caulerpa cupressoides* possesses an anti-inflammatory activity, inhibiting the paw edema induced by carrageenan and the recruitment of polymorphonuclear cells [8]. Among lectins from red seaweeds, those extracted from *Pterocladiella capillacea* [25] and *Hypnea cervicornis* [26] also exert anti-inflammatory effects in the paw edema model and in a neutrophil migration model, both based on carrageenan injection. A lectin obtained from the red seaweed *Solieria filiformis* yields similar results and can reduce the inflammation caused by dextran [27].

Lectins from the red seaweed *Bryothamnion triquetrum* were isolated and initially characterized by Ainouz et al. [28] and Calvete et al. [29]. Thenceforth, several researchers have demonstrated the pharmacological activities of lectins from *B. triquetrum*, for example, antinociceptive [30] and vasorelaxant effects [32]. However, there are no reports regarding a possible anti-inflammatory effect. Therefore, this study aims to elucidate the secondary structure properties of a lectin extracted from the red seaweed *Bryothamnion triquetrum* (BtL) and investigate its anti-inflammatory activity in mice using several classical models of inflammation.

2. Materials and methods

2.1. Animals

Female Swiss mice (20–25 g) were obtained from the Animal Care Unit of the Federal University of Ceará in Fortaleza, Brazil. They were housed in a temperature-controlled room and maintained under a 12-h light/dark cycle with free access to water and food. All the procedures and animal treatments were conducted in accordance with the guidelines set forth by the U.S. Department of Health and Human Services, and were approved by the Ethics Committee on Research at the Federal University of Ceará (protocol No. 12/2012).

2.2. Drugs and reagents

The following drugs and reagents were used: bovine serum albumin (BSA), papain, sulfuric acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), phosphate-buffered saline (PBS), carrageenan, dialysis sacks, indomethacin, dextran 70, o-dianisidine, hexadecyltrimethylammonium bromide, an avidin-peroxidase conjugate, and orthophenylenediamine dihydrochloride from Sigma (St. Louis, MO, USA). ELISA kits for TNF-α and IL-1β quantification were purchased from R&D Systems (Minneapolis, MN, USA), and DEAE-Sephacel from Merck (Darmstadt, Germany). Ammonium sulfate, sodium chloride (NaCl), violet gentian, and hydrogen peroxide (H_2O_2) were acquired from Synth (Diadema, SP, Brazil). All the reagents and lectin (BtL) were dissolved in distilled water or 0.9% sterile NaCl (saline).

2.3. The marine algae

Specimens of the red seaweed *B. triquetrum* were collected from the Atlantic coast in northeast Brazil (Fleixeiras Beach, Trairí – Ceará). A voucher sample of *B. triquetrum* was deposited at Prisco Bezerra Herbarium at the Institute of Marine Sciences (Federal University of Ceará, Fortaleza, Brazil) under the following identification number (EAC): 31,138.

2.4. BtL extraction and purification

The extraction of BtL was performed, with some modifications, as described by Ainouz et al. [28]. Seaweed samples were washed, dried, and grounded in liquid nitrogen. The powdered seaweed was subjected to protein extraction with 0.02 M phosphate-buffered saline (PBS) containing 0.15 M NaCl (pH 7.0) in the ratio 1:6 (w/v). The samples were incubated with the buffer for 4 h, and then, the mixture was filtered through a nylon mesh. The filtrate was centrifuged (8000 ×*g* at 4 °C for 30 min), and the pellet was discarded; the supernatant was acidified to pH 1.0 with 2 N HCl and incubated at 10 °C for 16 h. The liquid was centrifuged as mentioned above, and the pellet was discarded. The pH of the supernatant was adjusted to 7.0 with 2 N NaOH. Next, the supernatant was incubated with 60% ammonium sulfate for 16 h at 10 °C followed by centrifugation. The pellet was resuspended in 0.02 M PBS (pH 7.0) and dialyzed using dialysis sacks with an 8 kDa cut off against distilled water and then against the same buffer. The resulting solution was designated as Fraction F (0/60).

Fraction F was subjected to ion-exchange chromatography on a DEAE-Sephacel (19 × 27.5 cm) column equilibrated with 0.02 M PBS (pH 7.0) and was eluted by gravity flow. Solutions with different concentrations of NaCl were used to release adsorbed proteins from the column. The flow rate was maintained at 60 mL/h. Chromatographic fractions (3 mL/tube) were monitored for protein content at 280 nm and were analyzed for hemagglutinating activity.

2.5. Erythrocytes

Blood samples for the hemagglutination assays were collected from healthy adult New Zealand albino rabbits. The animals were maintained in the Animal Care Unit of the Federal University of Ceará, Fortaleza, Brazil.

2.6. Hemagglutinating activity

The hemagglutinating activity assay was performed on serial dilutions of the chromatographic fractions. Into each tube 100 μL of 0.15 M NaCl were pipetted. Into the first tube, 100 μL of a sample was added and serial twofold dilutions were prepared (1:2, 1:4, 1:8, … 1:64). We added 100 μL of 2% rabbit erythrocytes (trypsin-treated cells, at the concentration of 0.1 mg per 10 mL of blood). The reaction mixture was kept at 25 °C for 1 h. The tubes were then centrifuged at 2000 ×*g* for 30 s, and the results were expressed as a titer–the reciprocal of the highest twofold dilution that produced visible agglutination–in hemagglutination units (HU/mL). The active fractions were pooled, dialyzed, freeze-dried, and used for biological assays.

2.7. Circular dichroism (CD) spectroscopy

2.7.1. Structural composition of BtL under native conditions

CD spectroscopy of BtL (0.24 mg/mL) in 0.02 M PBS (pH 7.0) was performed on a Jasco J-815 (Tokyo, Japan) spectropolarimeter with constant N_2 flushing, in a rectangular quartz cuvette (0.1 cm path length), in the range from 190 to 250 nm, at 1-nm intervals, as an average of 8

scans at 25 °C. Final CD spectra were subtracted from the respective baseline and expressed in delta epsilon (Δε) units, using mean residual weight (MRW) of 110. Estimations of BtL secondary structure was done by CD spectrum deconvolution, using CD-Pro Package and ContinLL software.

2.7.2. The effect of pH on the native structure of BtL

BtL (0.24 mg/mL) was incubated for 30 min at extreme pH values by means of 0.02 M sodium acetate and sodium borate buffers at pH 4.0 and 11, respectively. The CD spectrum in each condition was recorded as described above and compared to the spectra at pH 7.0 to evaluate possible structural changes.

2.8. Steady-state fluorescence spectroscopy

2.8.1. The emission spectrum of BtL under native conditions

The fluorescence emission spectra of the Trp residues in BtL were acquired in a quartz cuvette (1-cm path length) at 25 °C in a circulating water bath from Fisher Scientific (Hampton, NH, USA). Analyses were carried out on an ISS K2 multifrequency phase fluorimeter (Champaign, IL, USA) with BtL at 0.1 mg/mL and excitation at 295 nm. The emission spectrum was monitored in the range from 300 to 450 nm. The reference spectra were subtracted in each analysis.

2.8.2. The effect of temperature on the BtL emission spectrum

Thermal stability of BtL was studied by heating the protein at 25 to 75 °C and by registering the successive emission spectra in 10 °C steps, with excitation performed at 295 nm. The final spectra were compared with those obtained under native conditions, and possible conformational changes were evaluated.

2.9. Experimental protocols

2.9.1. The paw edema model

The model proposed by Winter et al. [33] was used for the carrageenan-induced paw edema test. The animals were subdivided into the following groups: saline (negative control), carrageenan, BtL + carrageenan, and indomethacin + carrageenan. Dextran was also used, as a phlogistic agent, and all assays were conducted in accordance with the same protocol design in terms of carrageenan injection. Carrageenan (500 μg/paw, 100 μL) and dextran (500 μg/paw, 100 μL) were administered into the right hind paw by intraplantar injection (*i.pl.*). BtL was administered intraperitoneally (*i.p.*) 30 min before the inflammatory stimulus, at a dose of 1, 5, or 10 mg/kg. Indomethacin (10 mg/kg) was also injected via the *i.p.* route 30 min before the inflammatory stimulus. Right hind paw edema was measured by hydroplethysmometry (Ugo Basile, Gemonio, Italy) immediately before (time zero) the stimulus and at selected time points: at 1, 2, 3, and 4 h for carrageenan-induced paw edema and at 30 min and 1–4 h for dextran-induced paw edema. The results are expressed as a change in paw volume (mL) calculated as the difference from the basal volume (time zero).

2.9.2. Determination of myeloperoxidase (MPO) activity

The mice were subdivided into four groups: saline (negative control), carrageenan, BtL + carrageenan, and indomethacin + carrageenan. Carrageenan (500 μg/paw, 100 μL) was administered *i.pl.*, and BtL or indomethacin was injected *i.p.* at a dose of 10 mg/kg 1 h before the carrageenan injection. Four hours later, the animals were killed, and 50 mg of skin and subcutaneous cellular tissue were collected from each animal. The samples were resuspended in hexadecyltrimethylammonium buffer (pH 6.0, 50 mg of tissue per milliliter of buffer) and triturated with a tissue homogenizer. After that, the samples were centrifuged at 3000 ×*g* for 12 min at 4 °C, and the supernatant was collected. The MPO activity was determined according to the method described by Bradley et al. [34], with 0.0005% hydrogen peroxide (H_2O_2) as a substrate for MPO. The MPO unit was defined as the amount of the enzyme able to convert 1 μmol of H_2O_2 to water in 1 min at 22 °C. In the assay, the superoxide anion was produced while H_2O_2 was degraded. This anion reacted with o-dianisidine, resulting in a brown compound. The MPO activity was assayed by measuring changes in optical density at 450 nm and was expressed in units per milligram of tissue.

2.9.3. The peritonitis model

This experiment was conducted to test whether BtL decreases leukocyte and neutrophil migration (into the peritoneal cavity) induced by carrageenan. The animals were distributed into four groups: sterile saline (negative control), carrageenan, BtL + carrageenan, and indomethacin + carrageenan. Carrageenan (500 μg) was dissolved in 1 mL of sterile saline and administered *i.p.*; the animals received sterile saline alone (*i.p.*) or BtL (10 mg/kg) and indomethacin (10 mg/kg) (*i.p.*) 1 h before the leukocyte migration stimulus. Four hours later, the animals were killed, and the peritoneal cavity was washed with 15 mL of PBS containing 5 IU/mL heparin. Total leukocyte counts were determined in a Neubauer chamber, and differential leukocyte counting was carried out on cytocentrifuge slides stained with hematoxylin and eosin (H&E). The results are expressed in cells per microliter of the peritoneal wash.

2.9.4. Cytokine quantification

The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β were measured in the carrageenan-induced peritonitis model, as described above, by a sandwich ELISA. ELISA kits were purchased from the National Institute for Biological Standards and Control (Potters Bar, UK). Briefly, an anti-mouse TNF-α polyclonal antibody and an anti-mouse IL-1β antibody (2 μg/mL) were diluted in 50 μL of PBS and used to coat microtiter plates. The blocking of nonspecific binding sites was accomplished by incubation of the plates with PBS containing 2% BSA for 90 min at 37 °C. After incubation and washing of the plates in assay buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2), 50 μL of a standard (TNF-α or IL-1β) or a sample was added into each well and incubated overnight at 4 °C. After washing of the plates, 50 μL of the biotinylated rabbit polyclonal anti-mouse TNF-α antibody (1:1000 dilution with assay buffer plus 1% normal sheep serum) or the anti-mouse IL-1β antibody (1:1000) were added into the plate wells and incubated for 1 h at 25 °C. After incubation, the plates were washed again, and 50 μL of an avidin-peroxidase conjugate (1:5000 dilution) was added into all the wells. Next, the wells were incubated for 30 min with 50 μL of a substrate (40 μg/well, orthophenylenediamine dihydrochloride). After color development, the reaction was stopped by the addition of sulfuric acid (1 M). Absorbance was measured at 490 nm. These ELISA methods consistently detected TNF-α and IL-1β levels over 4000 pg/mL and did not cross-react with other cytokines. The results are expressed in picograms (pg) of each cytokine per milliliter.

2.10. Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM; $n = 6$). The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test (Prism 5.0 GraphPad software), when appropriate. Statistical significance was set to $p < 0.05$.

3. Results and discussion

3.1. BtL purification and hemagglutinating activity

Fraction F (0/60) was subjected to ion-exchange chromatography on a DEAE-Sephacel column and yielded two protein peaks. The first peak, i.e., the nonretained fraction (PI-DEAE), was eluted with 0.02 M PBS (pH 7.0) and was completely free of pigments. This peak also had hemagglutinating activity. The second peak (PII-DEAE) was eluted

with 0.02 M PBS (pH 7.0) containing 1 M NaCl and showed only weak hemagglutinating activity when compared to that of the first peak.

The apparent molecular weight was 9 kDa. The lectin isolation procedure was similar to that described by Ainouz et al. [28] for purifying two lectins from the red seaweeds *B. triquetrum* and *Bryothamnion seaforthii*, with minor modifications in the experimental design; however, they found a lectin in *B. triquetrum* with a molecular weight of 3.5 kDa. Therefore, a different molecule was obtained in our study.

The *B. triquetrum* lectin used in this study has major (9 kDa) specificity for glycoprotein mucin, as demonstrated by Pinto et al. [35] and Teixeira et al. [36], showing a hemagglutinating activity of 256 HU/mL.

Calvete et al. [29], using SDS-PAGE, discovered a lectin in *B. triquetrum* with the same molecular weight as in our study, strongly suggesting that they are the same lectin. Gel electrophoresis showed that when treated with a reducing agent, such as β-mercaptoethanol or dithiothreitol, BtL yields only a single band with the molecular weight of lectin, suggesting that BtL consists of a monomeric unit.

3.2. Secondary structure of BtL and structural analysis under extreme pH conditions

The CD spectra of BtL showed a small-intensity minimum at approximately 197 nm, which was assigned to the substantial proportion of a disordered conformation in the structure [37]. Moreover, this band is a feature shared with proteins belonging to the β_{II}-class, which is characterized by a high proportion of β-strands and disordered elements [38], but no α-helices (Fig. 1). An additional low-intensity minimum was observed in the region of 215 nm and may be assigned to the nπ* transitions in the β-sheet part of the protein structure [39]. Under native conditions (pH 7.0 at 25 °C), the CD spectra of BtL showed 3% of α-helices, 38% of β-sheets, 21% of turns, and 37% of the disordered conformation.

These data are corroborated by a study by Oliveira et al. [40] on the same *B. triquetrum* lectin (9 kDa) as BtL used in our work. They observed a similar CD spectrum, with the minimum at approximately 198 nm and low proportion of α-helices.

No significant conformational changes were observed in the CD spectra of BtL at pH 4.0 and 11.0. All the three CD spectra (Fig. 1) were nearly identical, albeit a small increase in the intensity at the ~197-nm minimum could be seen at both acidic and basic pH, indicating a small increase in the disordered state of the protein and confirming the structural stability of BtL under these extreme pH conditions. Furthermore, a hemagglutination assay was performed on BtL incubated at the three pH levels. The hemagglutination activity of BtL did not change much after incubation in the acidic or alkaline medium, thus supporting the results of CD spectroscopy. This high stability may be explained by the presence of intramolecular disulfide bridges [29].

Disulfide bonds are common in lectins from red seaweeds; Nagano et al. [41] found disulfide bridges in lectins extracted from the seaweeds *H. cervicornis* and *Hypnea musciformis*. Hori et al. [42] also found disulfide bonds in two lectins obtained from another species of the genus *Hypnea*, *Hypnea japonica*. Besides these seaweeds, in the red seaweed *B. seaforthii*, Nascimento-Neto et al. [43] detected a lectin with two intrachain disulfide bonds.

3.3. Analysis of intrinsic fluorescence of BtL

The fluorescence maximum emission of Trp residues in BtL was observed at 341 nm (Fig. 2). Maximum emission in the range between 336 and 344 nm is characteristic of partial exposure of Trp residues to an aqueous environment.

The BtL emission spectrum was also analyzed to determine the thermal stability of the protein by increasing the temperature from 25 to 75 °C. The exposure of the Trp residues in BtL to the aqueous environment did not change during the thermal treatment (Fig. 2); this finding suggests that the global structure of the protein was stable without drastic changes within this temperature range. The small decrease in fluorescence intensity observed during the heating process might be due to the preference for decay in the nonradiative process as the temperature was increased. The thermal stability of BtL in the whole range under study may also be explained by the presence of the disulfide bridges in the protein [29].

The physiological function of lectins occurs by deciphering the glycosylation patterns encoded in the structure of sugars attached to cell membrane glycoconjugates [44,45]. Thus, several biological processes, such as host-pathogen interactions, cell-cell communication and many others involving recognizing and binding carbohydrates are attributed to lectins [46]. These properties are common to lectins found in other living organisms; specifically in seaweeds the lectins play an essential role in recognition and adherence of gametes during sexual reproduction [47,48]. Thus, the stability of these molecules is a key-factor to face the hostile marine environment, with high variances of temperature, luminosity and salinity during low tides in coastal areas.

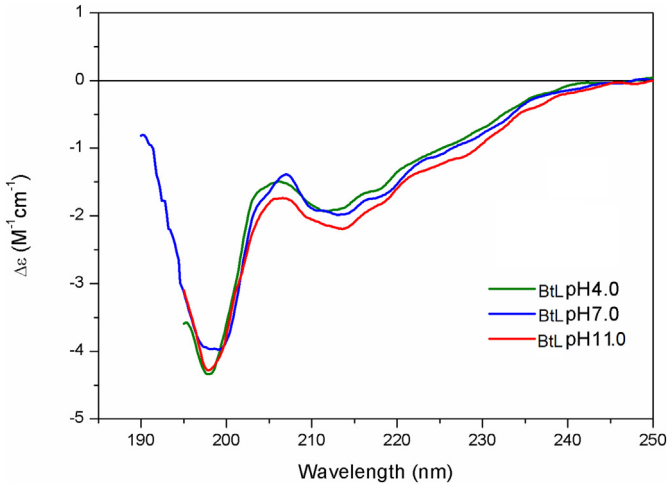

Fig. 1. The effect of pH on the secondary structure of BtL. Circular dichroism measurements of each sample was taken after 30 min incubation in a specific buffer to ensure a respective pH value (4.0, 7.0, and 11.0).

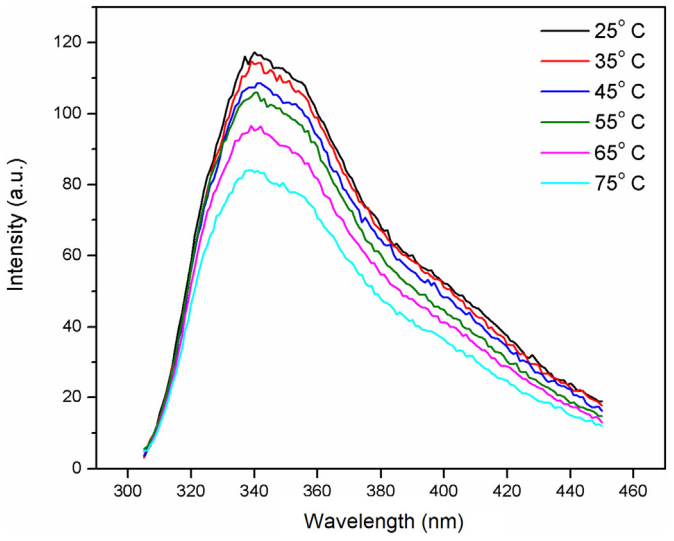

Fig. 2. The effect of temperature on the Trp emission spectrum of BtL. Excitation was performed at 295 nm, and BtL was incubated at each temperature (from 25 to 75 °C) for 30 min.

3.4. BtL inhibits paw edema induced by carrageenan and dextran

Paw edema induced by carrageenan is an experimental model widely used for anti-inflammatory studies on compounds with promising anti-inflammatory therapeutic properties [49]. In this model, carrageenan causes the release of several inflammatory mediators (bioactive amines, prostaglandins, and nitric oxide, among others) and induces edema characterized by a substantial neutrophilic infiltrate [50]. This infiltration may be identified by means of MPO enzymatic activity, a well-standardized marker of neutrophilic infiltrates [51].

BtL was administered *i.p.* at one of three doses: 1, 5, or 10 mg/kg. Indomethacin (10 mg/kg) served as a positive control. As shown in Table 1, the three doses of BtL significantly inhibited the paw edema induced by carrageenan. Pretreatment with BtL at a dose of 10 mg/kg was found to be the most effective dose in this experiment ($p < 0.05$) because it reduced the carrageenan-induced paw edema by 73.52%, 96.51%, 82.41%, and 91.39% at 1, 2, 3, and 4 h after induction, respectively, when compared to the carrageenan group. The anti-inflammatory effect of BtL (at the dose of 10 mg/kg) on carrageenan-induced paw edema is illustrated in Fig. 3A.

Similarly, indomethacin significantly inhibited ($p < 0.05$) the edematogenic response evoked by carrageenan at all the time points evaluated, mainly at 3 h (91.20%) and 4 h (91.39%) after carrageenan induction.

The lectin obtained from the green seaweed *C. cupressoides* administered intravenously (*i.v.*) reduced the inflammation induced by carrageenan (at the dose of 9 mg/kg), especially in the third hour after induction [8]. The lectin isolated from the red seaweed *P. capillacea* yielded a similar result, by significantly reducing the paw edema induced by carrageenan; however, it was less effective when compared to the positive control group treated with dexamethasone [25]. Our results obtained with BtL revealed a better activity when compared to *C. cupressoides* and *P. capillacea* lectins.

The edema caused by carrageenan proceeds in three phases. In the first hour after carrageenan injection, there is an increase in vascular permeability mediated by histamine and serotonin. In the second hour, this phenomenon is mediated by bradykinin. The third and most severe phase (between 3 and 4 h after induction) of edema induced by carrageenan is characterized by the presence of prostaglandins and leukotrienes promoting vascular permeability [52].

The reduction in paw edema at all the doses and time points tested suggests that BtL may interfere with the release of a cascade of inflammatory mediators. Therefore, the antiedematogenic property of BtL is probably related to the inflammatory events that involve neutrophil migration.

Dextran is a proinflammatory agent that causes a release of vasoactive amines, such as histamine and serotonin, thereby increasing vascular permeability, followed by osmotic edema. This edema is associated with low amounts of proteins and low neutrophil counts. Thus, this model was used here to determine whether BtL can reduce osmotic edema.

As presented in Fig. 3B, dextran produced its maximal effect 30 min after administration. BtL (10 mg/kg) reduced the dextran-induced paw edema significantly ($p < 0.05$) during all periods of the experiment. During the first 30 min, BtL reduced edema by 90.20%, thus showing a better result than that in the positive control group, which showed an inhibition rate of 46.34%. One hour after induction, BtL yielded 89.28% inhibition. BtL produced an inhibition rate near 100% in subsequent assays (2, 3, and 4 h after induction with dextran).

Indomethacin (10 mg/kg) also significantly reduced ($p < 0.05$) the paw edema in all periods of the experiment. By contrast, the edema was reduced by nearly 100% only in the last assay (4 h after induction with dextran).

There are a few studies about the effects of lectins from seaweeds on dextran-induced paw edema. The lectin extracted from the red seaweed *S. filiformis* significantly reduces the paw edema induced by dextran at all tested doses (1, 3, and 9 mg/kg), but without a statistically significant difference among the doses applied [27]. Previously, the lectin from *H. cervicornis* also was evaluated but was unable to reduce dextran-induced paw edema at the doses tested [26].

The model of dextran-induced paw edema allows for evaluation of new anti-inflammatory agents in processes mediated predominantly by histamine. There are three receptors mediating histamine action [53]. H1 receptors are present (among other tissues) in blood vessels, H2 receptors are located predominantly in the gastric mucosa, whereas H3 receptors are expressed predominantly in the central nervous system. H4 receptors are found in peripheral hematopoietic cells, eosinophils, neutrophils, and $CD4^+$ T cells [54,55].

Our findings indicate that BtL affects vascular events of inflammation. Thus, it is likely that BtL reduces edema by inhibiting the process of histamine release; however, its effects may also be caused by blockage of histaminergic and serotonergic receptors, through a lectin-receptor interaction.

3.5. BtL inhibits MPO activity in paw tissue

MPO is an enzyme present predominantly in azurophilic granules of neutrophils and in other cells of myeloid origin. It is widely known as a quantitative marker of neutrophil infiltration in inflammatory processes [51]. Therefore, the increase in MPO activity is a key indicator of the progression of inflammation. Thus, to confirm the anti-inflammatory effect of BtL, MPO activity was measured in mice with paw edema induced by carrageenan.

As depicted in Fig. 4, BtL at 10 mg/kg reduced the MPO activity by 92.93% when compared to the carrageenan group ($p < 0.05$). Pretreatment with indomethacin produced a similar result, reducing MPO activity by 92.65%. Accordingly, this result supports the findings in the carrageenan-induced paw edema assay at the dose of 10 mg/kg.

Table 1
The effect of BtL on paw edema induced by carrageenan.

Experimental groups	Paw edema (mL)			
	1 h	2 h	3 h	4 h
Saline	0.015 ± 0.005	0.018 ± 0.004	0.013 ± 0.008	0.008 ± 0.004
Carrageenan (Cg)	0.068 ± 0.004	0.086 ± 0.005	0.091 ± 0.004	0.093 ± 0.002
Cg + Indo (10 mg/kg)	0.018 ± 0.004 (73.04)*	0.020 ± 0.005 (76.74%)*	0.008 ± 0.004 (91.20%)*	0.008 ± 0.004 (91.39%)*
Cg + BtL (1 mg/kg)	0.036 ± 0.011 (47.05%)*	0.020 ± 0.007 (76.74%)*	0.017 ± 0.014 (81.31%)*	0.024 ± 0.008 (74.19%)*
Cg + BtL (5 mg/kg)	0.016 ± 0.007 (76.47%)*	0.015 ± 0.006 (82.55%)*	0.013 ± 0.007 (85.71%)*	0.018 ± 0.008 (80.64%)*
Cg + BtL (10 mg/kg)	0.018 ± 0.004 (73.52%)*	0.003 ± 0.002 (96.51%)*	0.016 ± 0.001 (82.41%)*	0.008 ± 0.004 (91.39%)*

Values are expressed as mean ± SEM (n = 6). The percentage of inhibition is shown in parentheses.
* $p < 0.05$ when compared to the carrageenan group (according to one-way ANOVA followed by the Newman-Keuls post hoc test).

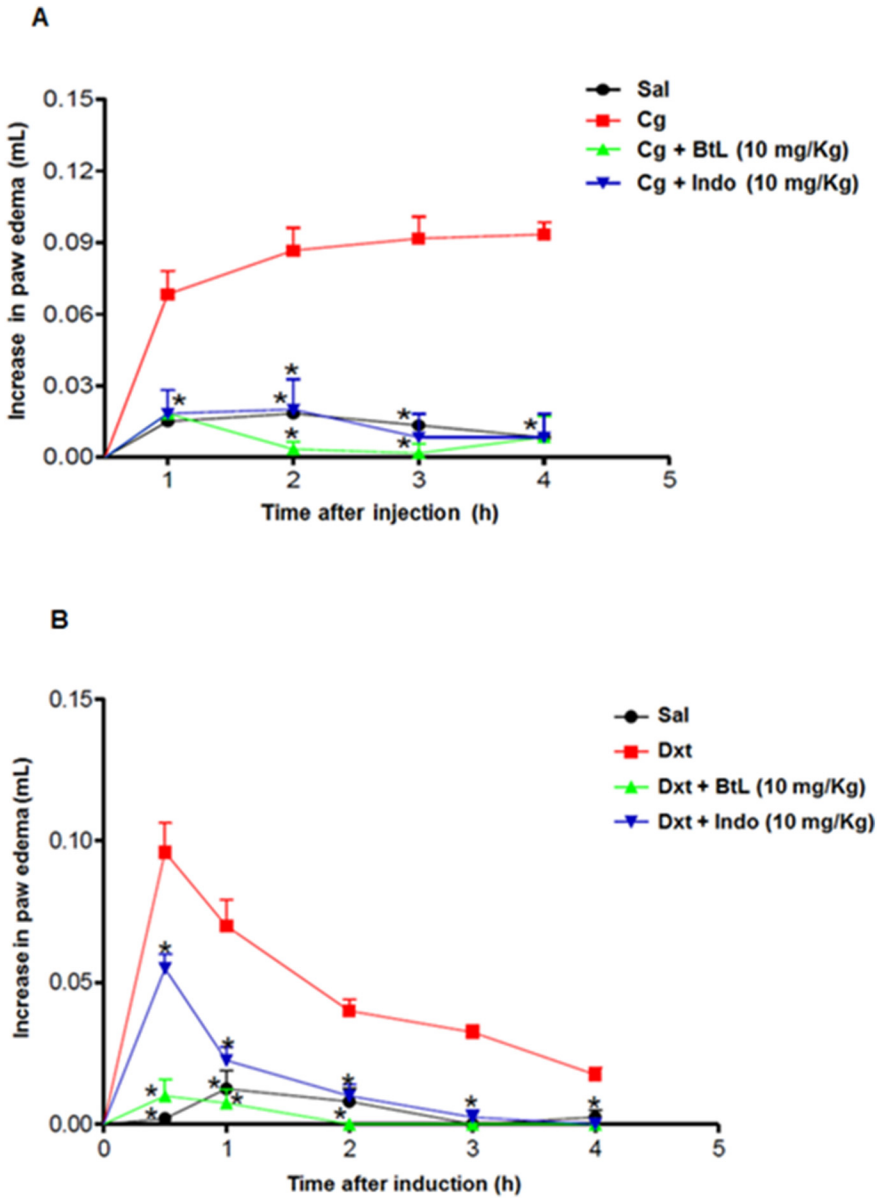

Fig. 3. Effects of BtL on paw edema induced by different phlogistic agents. (A) Paw edema induced by carrageenan. (B) Paw edema induced by dextran. Data are expressed as mean ± SEM ($n = 6$); $^{*}p < 0.05$ when compared with the carrageenan or dextran groups (according to one-way ANOVA followed by the Newman-Keuls post hoc test).

Similar results were obtained for other lectins isolated from seaweeds, such as the lectins extracted from *H. cervicornis* [24] and from *S. filiformis*, where the MPO activity was reduced by 60.00%, 73.00%, and 88.00% at doses of 1, 3, and 9 mg/kg, respectively [27]. These data confirm the anti-inflammatory therapeutic potential of these lectins and demonstrate a possible interaction between lectins from red seaweeds and selectins found on the leukocyte and endothelial cell surface through recognition of specific glycoconjugates. Thus, this anti-inflammatory effect may be explained by the interruption of leukocyte–endothelium interaction through competitive blocking of selectin binding sites, resulting in inhibition of leukocyte recruitment [56–58].

3.6. BtL decreases cell migration in peritonitis induced by carrageenan

Carrageenan induces neutrophil migration toward the peritoneal cavity through an indirect mechanism, i.e., via macrophage chemotaxis. The inhibition of leukocyte migration is the central mechanism of action of some anti-inflammatory drugs [59].

When administered to mice, BtL (10 mg/kg) significantly decreased the total leukocyte count at an inhibition rate of 91.84% ($p < 0.05$). Indomethacin decreased this migration by 86.82% (Fig. 5A). The differential leukocyte counts revealed that BtL (10 mg/kg) significantly decreased the neutrophil migration by 92.02% ($p < 0.05$), whereas indomethacin decreased neutrophil migration by 82.98% (Fig. 5B).

In another study using the lectin obtained from *B. triquetrum*, Neves et al. [60] obtained the opposite result. This lectin provoked neutrophil migration and was thus classified as a proinflammatory protein. Nonetheless, this phenomenon may be explained by the fact that this lectin is the same as the one found by Ainouz et al. [28], with molecular weight 3.5 kDa and no relation to the lectin used in our study.

In a study on the lectin extracted from *H. cervicornis*, Bitencourt et al. [26] observed a 90.00% reduction in neutrophil migration, at the dose of 10 mg/kg (*i.p.*). This effect was associated with leukocyte–endothelium interactions and an increase in nitric oxide production. The lectin obtained from *P. capillacea*, at the dose of 10 mg/kg, can also significantly reduce the neutrophil migration into the peritoneal cavity of rats [25]. Another lectin extracted from a red seaweed (*S. filiformis*) yielded a

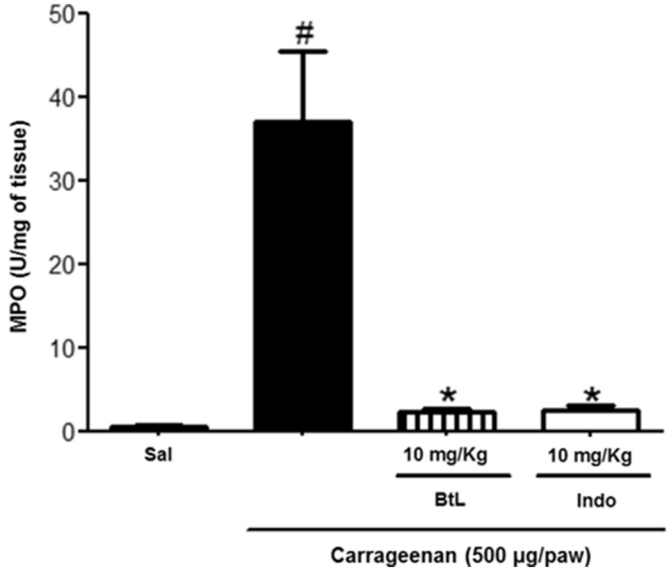

Fig. 4. Effects of BtL on MPO activity during carrageenan-induced paw edema. Data are expressed as mean ± SEM (n = 6); *p < 0.05 when compared with the carrageenan group; #p < 0.05 when compared with the saline group (according to one-way ANOVA followed by the Newman-Keuls post hoc test).

similar result when compared with BtL, by significantly decreasing the neutrophil migration (by 76.20%), when administered at a dose of 9 mg/kg [27].

The mechanisms of action of BtL involved in the reduction of neutrophil migration are still not completely elucidated; however, it is possible that BtL inhibits rolling and adhesion of neutrophils to endothelial cells and/or affects the release of chemoattractants by resident cells. All these activities are modulated by cytokines; hence, to assess the effects of BtL on the release of proinflammatory cytokines in carrageenan-induced peritonitis, the production of TNF-α and IL-1β was measured in the peritoneal cavity.

3.7. BtL decreased TNF-α and IL-1β production in carrageenan-induced peritonitis

The administration of BtL to mice (at 10 mg/kg) reduced the levels of TNF-α in carrageenan-induced peritonitis by 62.83% ($p < 0.001$), thus yielding a result similar to that obtained after indomethacin pretreatment, in which the TNF-α concentration decreased by 59.33% when compared to the carrageenan group ($p < 0.001$). The results on IL-1β are almost identical, the pretreatment of mice with BtL (10 mg/kg) significantly decreased ($p < 0.001$) the levels of this cytokine in the peritoneal cavity (by 54.52%). The pretreatment with indomethacin reduced this concentration by 53.17% ($p < 0.001$). All the results are summarized in Fig. 6 (A and B).

The peritonitis provoked by carrageenan, in addition to increasing the neutrophil migration into the peritoneal cavity (as demonstrated above), is also associated with an increase in cytokine production [61–63]. TNF-α is the first interleukin released by immune cells, mainly monocytes and macrophages, exerting its proinflammatory effects by stimulating transcription of various genes (such as those encoding other cytokines, chemokines, and cell adhesion molecules) via activation of transcription factors, such as nuclear factor (NF)-κB [64,65].

IL-1β is released most commonly by monocytes, macrophages, and mast cells; however, nonimmune cells, such as neuronal and glial cells, can synthesize and release IL-1β during cell injury or inflammation. Thus, IL-1β is associated with inflammation, pain, and autoimmune conditions, in addition to upregulating chemokines [66–68].

Lectins extracted from plant seeds possess known effects on cytokine levels, decreasing them to the basal level during peritonitis induced by carrageenan [22,69]. Nevertheless, researches into lectins from red seaweeds that evaluate cytokine production in peritonitis induced by carrageenan are still scarce.

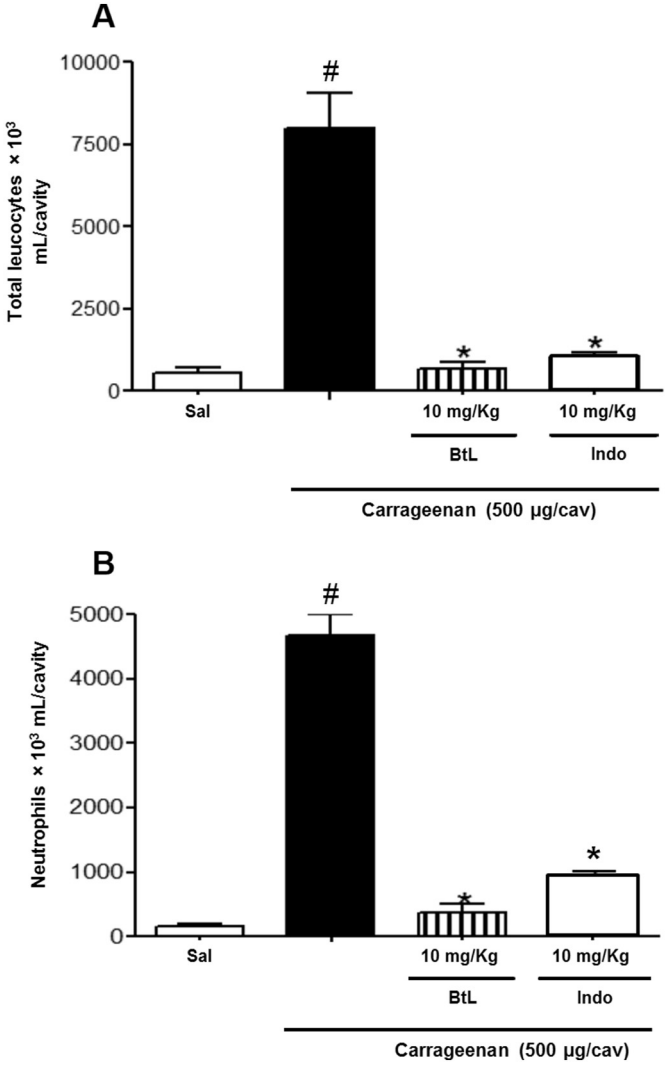

Fig. 5. Effects of BtL on cell migration in the carrageenan-induced peritonitis. (A) Leukocyte migration. (B) Neutrophil migration. Data are expressed as mean ± SEM (n = 6); *p < 0.05 when compared with the carrageenan group; #p < 0.05 when compared with the saline group (according to one-way ANOVA followed by the Newman-Keuls post hoc test).

In the present study, BtL inhibited vascular and cellular events of an acute inflammatory response. Therefore, the inhibition of neutrophil migration to inflammation sites via suppression of TNF-α and IL-1β production, as shown in carrageenan-induced peritonitis, suggests that the modulation of the process of leukocyte recruitment is a major mechanism behind the anti-inflammatory action of BtL.

4. Conclusions

According our results, we concluded that the lectin extracted from the red seaweed *B. triquetrum* is a stable protein, mostly composed of disordered secondary structure elements and β-strands, and possessing Trp residues partially exposed to an aqueous environment. This lectin is capable to preserve its native secondary structure unchanged even under extreme conditions, such as extreme pH, and high temperatures. The interesting anti-inflammatory activity of BtL in classical models of inflammation holds promise for evaluation in clinical trials.

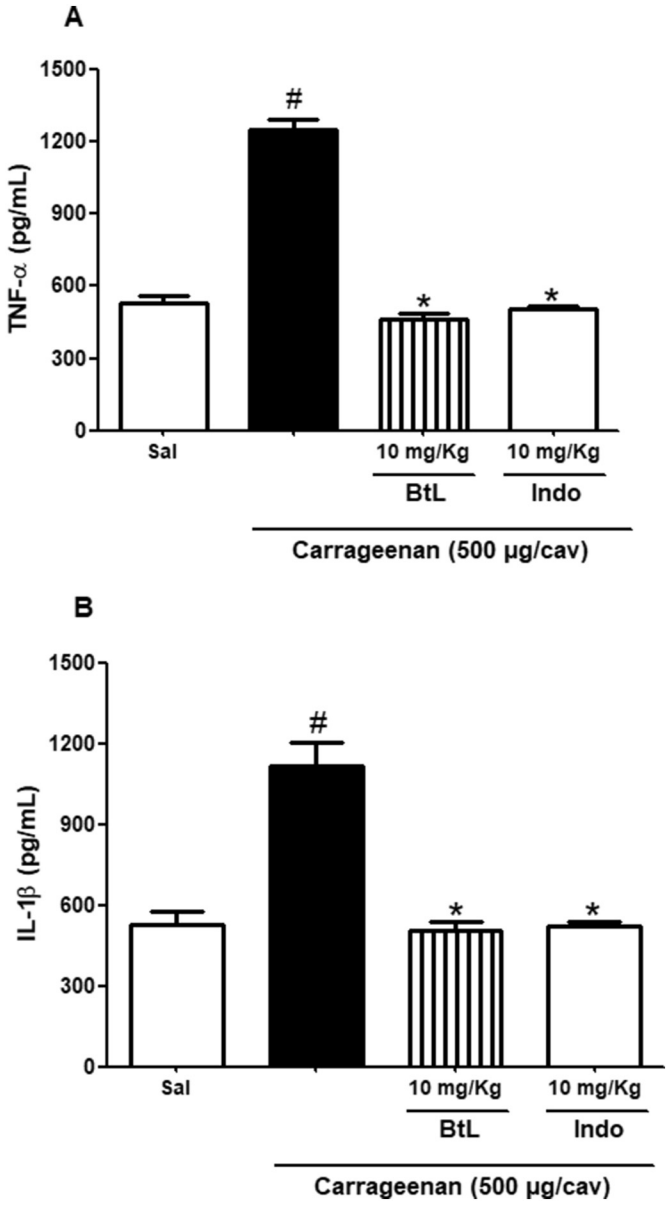

Fig. 6. Effects of BtL on TNF-α and IL-1β production during the carrageenan-induced peritonitis. (A) TNF-α levels. (B) IL-1β levels. Data are expressed as mean ± SEM (n = 6); $^{*}p < 0.001$ when compared with the carrageenan group; $^{\#}p < 0.001$ when compared with the saline group (according to one-way ANOVA followed by the Newman-Keuls post hoc test).

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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