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Chemical structure and anti-inflammatory effect of polysaccharide extracted from *Morinda citrifolia* Linn (Noni)

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ABSTRACT

Polysaccharides extracted from plants are very promising molecules in the field of pharmacotherapy. Knowing this, the aim of this study was to extract, characterize and evaluate the action of the polysaccharide of *Morinda citrifolia Linn* (Noni-PLS) in biological models of inflammatory processes. The characterization tests shown that sample refers to a heteropolysaccharide composed mainly of homogalacturonan and rhamnogalacturonan. This polysaccharide at dose of 10 mg/kg, when tested in our models of inflammation, showed significant activity in reducing carrageenan-induced paw oedema as well as all mediators edemas. This polysaccharide was able to inhibit the migration of leukocytes to the site of inflammation, and still reduced inflammatory nociception tests. This results, allows us to conclude that the polysaccharide extracted from *Morinda citrifolia linn* has anti-inflammatory potential since it reversed inflammatory parameters such as edema, leukocyte migration and nociception.

1. Introduction

Inflammation is a protective strategy to primarily combat injuries and infections (Lomax & Calder, 2009). The immune system rapidly responds to any unwanted changes in the tissues, leading to the recruitment of immune cells and several other inflammatory mediators. In other words, inflammation is a cleaning process of invading elements and noxious changes leading to the maintenance of homeostasis (Li, Manwani, & Leng, 2011). However, inflammatory responses that fail to regulate themselves can become chronic and contribute to the perpetuation and progression of diseases (Calder et al., 2009). Increasingly, studies have suggested that the onset and progression of cancers are closely associated with inflammation (Lin, Zhang, & Luo, 2016).

Plants have always been a common source of new medicines for a broad range of diseases (Yilmazer et al., 2016). Several reports have demonstrated that plant metabolites exert diverse therapeutic effects (Phosrithong & Nuchtavorn, 2016; Remila et al., 2015), such as anti-inflammatory action. The potential anti-inflammatory effects of several natural compounds are evidenced by the downregulation of key players in the development of inflammation with the modulation of inflammatory mediators (De Sousa et al., 2017).

Plant metabolites such as natural polysaccharides have been demonstrated to be promising candidates with anti-inflammatory activity, which affect multiple targets during inflammatory progression

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(Tzianabos, 2000). Hence, studies on polysaccharides isolated from natural sources have been of interest to characterize their biological activities (Jiao, Yu, Zhang, & Ewart, 2011). A polysaccharide fraction that has aroused interest is a fraction of Noni fruit (*Morinda citrifolia Linn*). Noni is an evergreen tree found in coastal regions and forest areas that is identifiable by its straight trunk, large, bright green and elliptical leaves, white tubular flowers, and its distinctive ovoid "grenade-like" yellow fruit (Pandy, Narasingam, & Mohamed, 2012). Due to its innumerable pharmacological effects, the consumption of Noni fruit juice outside the tropics has increased dramatically after the 2000's (West, Jensen, Palu, & Deng, 2011). A number of pharmacological activities have been reported for the fruit, leaf, and root extracts of Noni such as analgesic (Younos et al., 1990), anti-inflammatory (Basar, Uhlenhut, Hogger, Schone, & Westendorf, 2010), antioxidant (Chanda, Dave, & Kaneria, 2011; Su et al., 2005), and immunomodulatory (Palu et al., 2008).

Scientific evidence have demonstrated anti-inflammatory activity for parts of Noni and the different types of extracts (Singh, 2012). Based on the anti-inflammatory and immunomodulatory effects of the Noni components and knowing the potentiality attributed to natural polysaccharides, the present work aimed to isolate and characterize a fraction of the Noni polysaccharide (Noni-PLS), as well as to test the effect of the polysaccharide extracted on several models of inflammation induced in mice.

2. Experimental

2.1. Extraction of Noni-PLS

The fruits were harvested manually from a property located in Fortaleza - CE from August 2014 to February 2016. Afterwards, a preselection was performed, discarding the damaged fruits and those in advanced senescence phase. The fruits were washed in running water and then selected for the purpose of standardizing their maturation stage.

The extraction was performed according to an adaptation of the methodology described by Hirazumi & Furusawa (1999). The ripe fruits of *Morinda citrifolia Linn* had the peels removed manually and then were processed using the Mondial Juicer centrifuge to obtain the Noni purée, which was lyophilized to assess its dry mass. For every 5 g of the dried purée, 100 mL of distilled water was added and the suspension was kept under magnetic stirring at room temperature for 1 h. After this time, the suspension was centrifuged (6000 rpm, 20 min, 25 °C) and then the residue was discarded. The supernatant was filtered on a lyophilized nº 4 sintered plate funnel and then the dried residue obtained was dissolved in 50 mL of distilled water. The polysaccharide was precipitated slowly and with moderate agitation, in commercial 96% ethyl alcohol in the ratio of 1:4 (volume of Noni solution/volume of alcohol) (Bui, Bacic, & Pettolino, 2006). After precipitation, the suspension containing the polysaccharide was centrifuged (8000 rpm; 30 min; 10 °C) and the supernatant was discarded. Finally, the precipitate was dissolved in water and lyophilized, which was referred to as Noni-PLS.

2.2. Determination of monosaccharide composition

In order to gather information on the monosaccharide composition of the biopolymer, the Noni-PLS samples were subjected to acid hydrolysis, thus generating free monosaccharides after the breakdown of the glycosidic bonds.

Total acid hydrolysis of the polysaccharides was performed using approximately 10 mg of the sample dissolved in 2.0 mL of 4 mol.L^{-1} TFA at 100 °C for 5 h. After hydrolysis, the acid was removed from the samples by the addition of methanol and evaporation on a sand bath at 80 °C. The hydrolysis products and standard sugars were re-suspended in about 3.0 mL of distilled water and reduced with $NaBH_4$ at room temperature with alkaline medium (pH 9–10) for 24 h under magnetic stirring. After this period, cationic resins were added to the solutions and incubated under magnetic stirring for approximately 2 h in order to exchange the Na^+ of the solution with the H^+ of the resin, thus neutralizing the OH- in solution. The boric acid formed was co-evaporated as trimethyl borate by successive washes with methanol (Wolfrom & Thompson, 1963).

The alditols formed during the reduction process were then acetylated with a mixture of acetic anhydride and pyridine (1:1, v/v) for 24 h at room temperature. Acetylation was stopped by addition of ice, and the alditol acetates were extracted with chloroform. The residual pyridine present in the chloroform fraction was removed by successive washes with 5% $CuSO_4$ solution (Wolfrom & thompson, 1963).

Gas chromatography with mass spectrometer (GC–MS) analyses were carried out using a gas chromatograph GCMS QP-2010 Shimadzu equipped with DB-5 ms Agilent (30 m × 0.25 mm I.D.). The split injector temperature was 250 °C. Oven temperature gradient profile: 60 °C (2 min), 5 °C/min to 280 °C (20 min), 20 °C/min (3 min), and back to initial. Helium (purity 99.999%) was used as a carrier gas at an inlet pressure of 100 kPa and a flow rate 1.61 mL/min.

Protein was calculated by nitrogen percentage determined by elemental micro analysis using a Perkin Elmer 2400 CHN equipment. The conversion factor of %N to protein was 5.87 (Azero & Andrade, 2002).

2.3. Size exclusion chromatography

The peak molar mass was determined by size exclusion chromatography using a Shimadzu LC 20 equipment with refraction index and UV–vis detectors. The analysis was carried out using a PolySep Linear (7.8 mm × 300 mm), flow of 1.0 mL/min with 0.1 mol/L $NaNO_3$ as the eluent maintained at 30 °C. The injection sample volume was 50 µL and a calibration curve was constructed using Pullulan Shodex P-82 standards (equation 1).

$$\log M_{peak} = -1.007 V_{EL} + 13.94 \ (R^2 = 0.992) \quad (1)$$

where M_{peak} is the peak molar mass and V_{EL} is the elution volume

2.4. Infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy

The Fourier transform infrared spectra (FT-IR) of Noni-PLS were recorded with a Shimadzu IR spectrophotometer (model 8300) scanning between 400 and 4000 cm^{-1}. The samples were analyzed as KBr pellets. NMR analyses were performed in one dimension (13C NMR decoupled) using a Bruker model Avance DRX-300, belonging to the Northeastern Center for the Application and Use of Nuclear Magnetic Resonance at the Federal University of Ceará (CENAUREMN). The chemical shifts, expressed in δ (ppm), were determined using D2O as an internal standard.

2.5. 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, Brazil. The Animal Studies Committee of Federal University of Piauí approved the experimental protocol (123/2015).

2.6. Carrageenan-induced paw edema

The animals were randomly divided into 6 groups (n = 6), and an 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 (intraplantar injection; i.pl.). One hour before induction of the paw edema, the mice were pre-treated with an intraperitoneal (i.p.) injection of indomethacin at 10 mg/kg or 30 min before with Noni-PLS at

doses of 0.1, 1.0, or 10 mg/kg. The paw volume was measured by a plethysmometer (Panlab LE75000) with readings performed before the experiment (basal volume, Vo) and at 1, 2, 3, and 4 h after treatment with carrageenan (Vt) (Winter, Risley, & Nuss, 1962). The effect of the 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: % inhibition of edema = $(Vt - Vo)_{Control} - (Vt - Vo)_{Treated})/(Vt - Vo)_{Control}) \times 100$.

2.7. Histological evaluation of the microscopic scores of inflammatory lesions

Segments from the sub-plantar tissue of the study groups were removed after carrageenan injection for histological evaluation. The samples were fixed in 10% formalin solution for 24 h. Then, samples were transferred to a solution of 70% alcohol. The material was then embedded in paraffin and sectioned; 4-μm-thick sections were stained with hematoxylin/eosin and evaluated by an experienced pathologist. The scores applied in this test verified the total scores of lesions (sum of the microscopes scores of lesions) in the paw tissue and was presented as the stages of severity of the neutrophil infiltration, edema, and tissue bleeding (variation 1 to 4).

2.8. Paw edema induced by different inflammatory agents

In order to induce paw edema with different inflammatory agents, the animals received 50-μL injections of dextran (500 μg/paw), serotonin (5-HT, 1%, w/v), histamine (1%, w/v) or bradykinin (6 nmol/paw) into the right hind paw (i.pl.). One group received 50 μL of 0.9% sterile saline and served as an untreated control group. Noni-PLS (10 mg/kg) or indomethacin (10 mg/kg, reference control) received i.p. 30 min before i.pl. injections of phlogistic agents. Paw volume was measured immediately before and at the selected intervals of time.

2.9. Determination of myeloperoxidase (MPO) activity

For the MPO assay, hind paw tissue was homogenized in hexadecil trimethylammonium (HTAB) buffer, 50–100 mg of hind paw tissue was homogenized in 1 mL of the HTAB buffer. The homogenated tissue was centrifuged at 40,000 ×g for 7 min at 4 °C. The MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. A unit of MPO (UMPO) activity was defined as that converting 1 mmol hydrogen peroxide into water in 1 min at 22 °C. The results were reported as MPO units/mg tissue (De Brito et al., 2013).

2.10. Analysis of glutathione levels from peritoneal exudate

The peritoneal lavage was centrifuged at 1500 rpm for 15 min at 4 °C. Aliquots of 400 μL of the liquid were mixed with 320 μL of distilled water and 80 μL of 50% trichloroacetic acid (TCA) for precipitation of the proteins. This material was again centrifuged at 3000 rpm for 15 min at 4 °C. Next, 800 μL of 0.4 M Tris buffer (pH 8.9) and 20 μL of 0.01 M Ellman Reagent were added to 400 μL of the supernatant. The absorbance was read at 412 nm in a spectrophotometer. The concentrations of non-protein sulfhydryl groups were expressed in mg of NP-SH/ml (Sedlak & Lindsay, 1968).

2.11. Induction of peritonitis

Either 250 μL of 0.9% saline solution (untreated group I) or 250 μL of the solution with 500 μg Carrageenan was administered i.p. in the peritoneal cavity. Before the injection of Carrageenan, the animals of the treated groups received an i.p. injection of Indomethacin at 10 mg/kg 1 h or 30 min before the Noni-PLS injection at 10 mg/kg. The mice were sacrificed after 4 h and the peritoneal cavity was washed with 1.5 mL of phosphate buffer saline heparinized to collect the fluid and peritoneal cells.

2.12. Writhes induced by acetic acid

The mice received a 250 μL i.p. injection of 0.9% saline solution (untreated group I) or 0.6% acetic acid solution (groups II, positive control). Group III and IV (control reference) were subcutaneously (s.c.) treated with Noni-PLS (10 mg/kg) and Morphine (5 mg/kg), respectively. After 30 min of treatment, the animals of groups III and IV received an i.p. injection of 250 μL of a 0.6% solution of acetic acid. After 10 min of administration of the acid solution, the number of abdominal writhes was determined for each mouse for a period of 20 min A contortion was identified as an extension of the hind paws, accompanied by constriction of the abdomen (Koster, Anderson, & De-Beer, 1959).

2.13. Formalin test

The mice received 20 μL of 0.9% saline solution by i.pl. injection (group I - untreated) or 20 μL of 2.5% formalin solution (groups II and III). They were treated with Noni-PLS at 10 mg/kg (group II) or morphine at 5 mg/kg by s.c. injection (group III). After 30 min of treatment, formalin was administered by i.pl. injection on the right hind paw of each animal. The paw licking time was recorded, in seconds, from 0 to 5 min (phase 1, direct stimulation of nociceptors) and 20–25 min (phase 2, release of inflammatory mediators) (Hunskaar & Hole, 1987; Fasmer, Berge, & Hole, 1986).

2.14. Statistical analysis

Data were described as either the mean ± SEM or median. Analysis of variance (ANOVA), followed by Student-Newman-Keuls test, was used to compare the means. The histopathological parameters were analyzed using the Kruskal − Wallis non-parametric test, followed by multiple-comparison Dunn's test. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Noni-PLS characterization

The Noni-PLS isolation method yielded 13% from the total solids of the fruit juice. Subsequently, the chemical characterization and biochemical tests were performed with this sample.

The monosaccharide composition obtained by GC–MS analysis of hydrolysated polysaccharide from Noni juice, fruit collected in Brazil, included galacturonic acid (GalA) (29.1 mol%), galactose (Gal) (30.9 mol%), arabinose (Ara) (31.0 mol%), rhamnose (Rham) (5.4 mol %), and mannose (3.6 mol%). The Noni fruit grown in Vietnam showed a different monosaccharide composition (Table 1), which included more GalA and Rham, and less Gal and Ara. Other difference included the presence of xylose (Xyl), glucose (Glu), and GluA in the Noni fruit grown in Vietnam that were not detected in Brazilian fruit. Fucose also present in Asiatic fruit was not analyzed in American fruit. The protein

Table 1
Comparison between monosaccharide composition (mol%) of Noni juice precipitated from fruit of different origin.

Sugar Origin	GalA	Gal	Ara	Rham	Man	Xyl	Glu	GluA	Fuc
Brazil	29.1	30.9	31.0	5.4	3.6	NI	NI	NI	NA
Viet Nam	53.6	17.9	13.6	9.5	0.7	1.2	2.2	1.1	0.3

NI – not identified, NA – not analyzed.

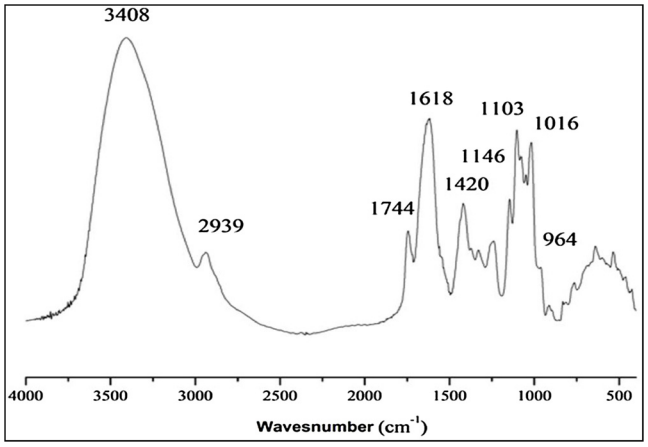

Fig. 1. FT-IR spectrum for KBr pellets of Noni-PLS.

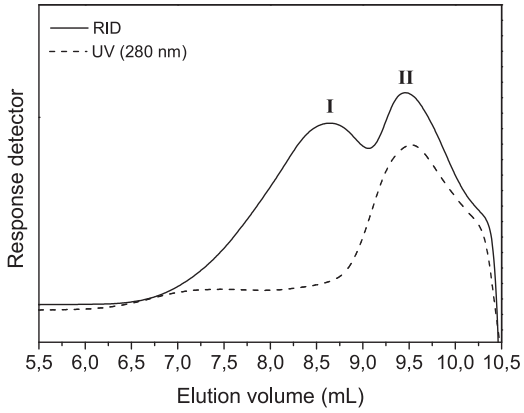

Fig. 3. Size exclusion chromatogram of Noni-PLS in aqueous solution.

content in the Brazilian fruit was 13.6 wt% higher than the content in the Vietnam juice (2–6 wt%). According to Assi et al. (2017), the chemical composition of *M. citrifolia* juice depends upon the method of juice extraction. The Noni juice analyzed by Bui et al. (2006) was seeped out for 1–3 days, which was different from the analysis in the present work (processed soon after peeled). Furthermore, it has been demonstrated that the polysaccharide composition depends on its geographic origin (de Paula & Rodrigues, 1995), which explains the different monosaccaride composition.

The FT-IR spectrum of Noni-PLS as shown in Fig. 1 shows a band at 3408 cm^{-1}, which refers to the axial stretch of the O—H bond. The absorption at 2939 cm^{-1} was characteristic of the axial stretching of the C–H bond of methyl clusters, the band at 1146 cm^{-1} was relative to the C—O—C deformation of the glycosidic linkage, and the band at 1016 cm^{-1} was related to C—O stretching of primary alcohol. In the polysaccharides, the carbonyl stretching vibration of the carboxylic acid occurs between 1710–1740 cm^{-1}, while the asymmetric and symmetric vibration of the carboxylate group appears in the regions of 1600–1651 cm^{-1} and 1408–1450 cm^{-1}, respectively (Oliveira, 1998). The observed values of νC=O from acid, and νCOO- were in agreement with the wavenumbers previously reported in the literature (Oliveira, 1998). The results also indicated that a portion of the COO- was in the salt form and a portion was protonated (acid form).

The ^{13}C NMR spectrum of Noni-PLS (Fig. 2) shows two high-field signals at 173.4 and 177.8 ppm, which are characteristic of carbonyl and can be attributed to C-6 (COOH and $COOCH_3$) of the galacturonic acid units (Petersen, Meier, Duus, & Clausen, 2008). The analysis of the anomeric region of the spectrum shows more intense signals in 109.8, 103.1, 101.8, 98.90, and 94.90 ppm, which can be attributed to C-1 of the Ara, Gal (substituted and unsubstituted), Gala, and Rham units, respectively (Agrawal, 1992; Vriesmann, 2012). Signals at 17.70 and 55.60 ppm correspond respectively to C-6 of Rham (Agrawal, 1992) and the carbon of methyl ($COOCH_3$) groups of the esterified units of GalρA present in the heteropolysaccharide. The main content of Gala was in the form of methoxyl groups, consistent with previous observations by Bui et al. (2006). The presence of acetyl groups was evidenced by the signal at 23.00 ppm (Jungles, 2013; Vriesmann, 2012).

The chromatogram depicted in Fig. 3 shows a bimodal curve. The fraction I eluted in 8.62 mL is of high molar mass ($M_{peak} = 1.71 \times 10^5$ g/mol). The fraction II eluted in 9.46 mL has $M_{peak} = 2.57 \times 10^4$ g/mol and contains protein as showed by the absorption at 280 nm detected by UV–vis. The fractions were present in similar proportion at 50 wt%. The percentage of protein was 13.6 wt%. Taking in account that the average molar mass of amino acids is 110 g/mol, and the average molar mass of monosaccharides from Noni juice (Table 1) is 174 g/mol, the content of protein is 21.6 mol%.

The type of polysaccharide identified by Bui et al. (2006) in Noni juice were as follow: homogalacturonan, rhamnogalacturonan I, arabinan and arabinogalactan type I, and arabino-galactan-protein type II. The molar content of Ara and Gal were similar, and were present as an arabinogalactan (31.0 mol%). The protein content was 21.6 mol%, and

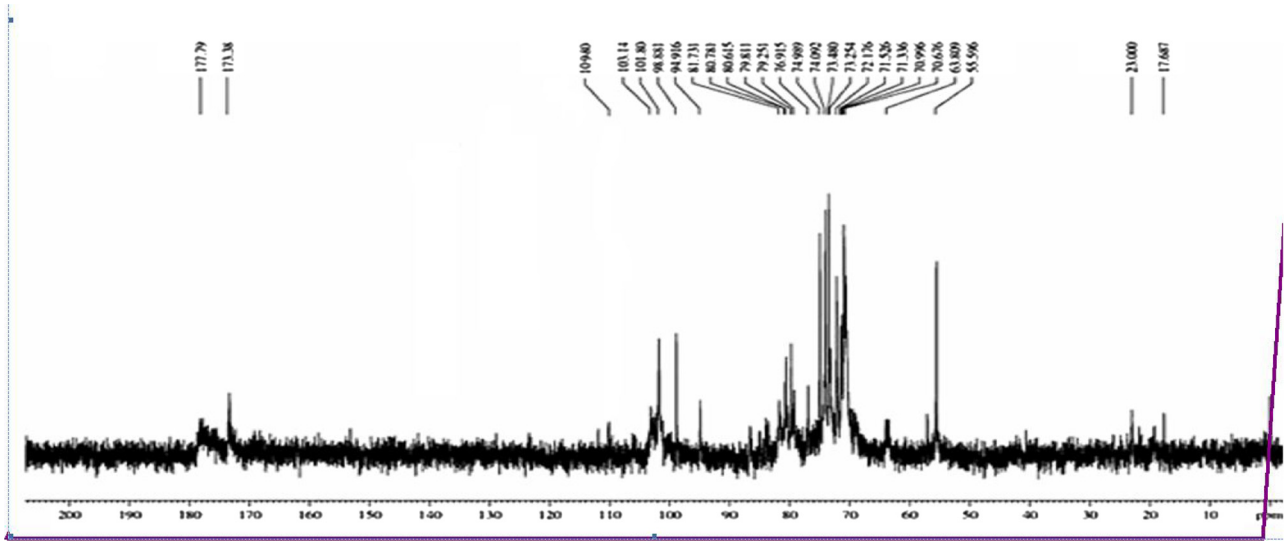

Fig. 2. ^{13}C NMR spectrum of the Noni-PLS fraction. Chemical shifts (δ) expressed in ppm. Internal calibration with D_2O.

the content of arabinogalactan-protein (type II) was estimated to be 21.6 mol%. The residual (11.4 mol%) consisted of arabinogalactan (type I). All of the Rham were in a rhamnogalacturonan form at 5.4 mol %. The residual GalA was 23.7 mol% and was probably present as a homogalacturonan.

GalA had the highest molar mass (194.1 g/mol) of the identified monosaccharides in Noni-PLS. The polysaccharide that contains this monosaccharide probably had a higher M_{peak}. We deduced that fraction I contains homogalacturonan and rhamnogalacturonan, and fraction II contains arabinogalactan type I and arabinogalactan-protein type II. These residues are important to reverse some inflammatory conditions (de Oliveira, do Nascimento, Iacomini, Cordeiro, & Cipriani, 2017). Based on this information, we decided to study this compound, since it was rich in these residues, in classical models of inflammation and inflammatory pain.

3.2. Noni-PLS reduced paw edema induced by λ-carrageenan

The *Morinda citrifolia Linn* polysaccharide at doses of 0.1, 1.0, and 10 mg/kg was tested in the paw edema induced by λ-carrageenan to establish the best dose with a significant anti-inflammatory effect to be used in subsequent experiments.

Table 2 shows that the dose of Noni-PLS at 10 mg/kg showed a significant reduction in edema throughout the duration of the effect tested (1 h, 0.024 ± 0.013; 2 h, 0.018 ± 0.009; 3 h, 0.017 ± 0.006; 4 h, 0.022 ± 0.007) when compared with animals that received only the inflammatory agent (1 h, 0.038 ± 0.005; 2 h, 0.09 ± 0.017; 3 h, 0.100 ± 0.013; 4 h, 0.082 ± 0.008). For having presented a better result, the dose of the Noni-PLS used for the other inflammatory and antinociceptive tests was 10 mg/kg via i.p. injection.

Carrageenan edema causes a biphasic inflammatory response characterized in paw edema. This is dependent on an increase in the levels of inflammatory mediators in the first phase, as well as neutrophil infiltration and prostaglandin production in the second phase, operating subsequently to produce the inflammatory response (Vinegar, Schreiber, & Hugo, 1969; Damasceno et al., 2013; Florentino et al., 2017).

Histopathological data of the paw tissue show that the treatment with Noni-PLS at 10 mg/kg (Fig. 4D) also decreased the microscopic inflammatory lesion in mouse paw tissue caused by the i.pl. carrageenan administration (Fig. 4B). This fact corroborates the significant decrease in mean scores for the inflammatory tissue damage in the group receiving Noni-PLS, compared with that in the group that received only carrageenan into the paw. These results reinforce the findings of the Table 1, which show that the treatment with Noni-PLS was equally efficient in both phases of carrageenan-induced paw edema, in which they promoted a significant reduction in the paw volume. That led us to believe that the anti-inflammatory effects of this polysaccharide could be associated with the inhibition of pro-inflammatory mediators.

In order to test this hypothesis, we induced paw edemas with different inflammatory mediators. We also assessed the migration of immunological cells to the site of inflammation.

3.3. Noni-PLS reduced paw edema induced by different inflammatory mediators

The i.pl. administration of dextran at a dose of 500 µg/kg (0.076 ± 0.011 mL, Fig. 5A), histamine (0.083 ± 0.009 mL, Fig. 5B), serotonin (0.096 ± 0.004 mL, Fig. 5C) and bradykinin (0.065 ± 0.007 mL, Fig. 5D) caused intense edema in the paw of the mice with a maximum peak at 30 min when compared with the mice of the groups that received only 0.9% saline in the paw. Noni-PLS injected via i.p. at a dose of 10 mg/kg, 30 min before the administration of these agents, significantly reduced the edema maximum peak at 30 min, as shown in Fig. 5, caused by Dextran (0.022 ± 0.002 mL), histamine (0.036 ± 0.003 mL), serotonin (0.035 ± 0.002 mL), and also bradykinin (0.023 ± 0.004 mL).

Dextran paw edema occurs through the release of amines such as histamine and serotonins after degranulation of mast cells (Carneiro et al., 2014; Motoyama et al., 2016). Histamine and serotonin trigger a rapid action on the vasculature (Ashley, Weil, & Nelson, 2012) causing vasodilation and increased vascular permeability during the inflammation process (Gosslau, Li, Ho, Chen, & Rawson, 2011). This, in turn, causes redness and a consequent increase of blood in the site that suffered the injury (Sherwood & Toliver-Kinsky, 2004); dextran promotes extravasation of fluid with few proteins (Coura et al., 2015).

Noni-PLS decreases the dextran-induced paw edema dependent on the increase in vascular permeability (Fig. 5A). That also accounts for the inhibition of edema induced by rapid mediators such as histamine and serotonin.

Another important inflammatory mediator that also had its effect antagonized by Noni-PLS was bradykinin. This is a vasoactive peptide formed in the plasma involved in the pain process. Among the mediators of inflammation (Hariz et al., 2008), it increases the permeability of capillaries and venules with fluid leakage facilitating the arrival of soluble cells and factors, such as antibodies and acute phase proteins, to the site of tissue injury (Aller et al., 2007; Dias et al., 2014).

The Noni-PLS activity in reduced paw edema promoted by different mediators corroborates with the reduction of the first phase of edema induced by carrageenan (Florentino et al., 2017). In order to evaluate how the Noni-PLS acts to reduce the second phase of carrageenan-induced edema, we analyzed the neutrophil migration to the inflammatory site through direct and indirect observations.

3.4. Noni-PLS reduced MPO and GSH levels in the tissue of paw and peritoneal fluid, respectively

Table 3 shows that the group treated with Noni-PLS at 10 mg/kg (3.270 ± 0.807 UMPO/mg) presented a significant reduction in enzyme concentration when compared with that in the group receiving carrageenan i.pl. (26.84 ± 4.541 UMPO/mg). The reduction of MPO activity in the group treated with Noni-PLS even approached the baseline values of the 0.9% saline group (3.360 ± 0.636 UMPO/mg). There was also no significant difference in the results of the group that received Noni-PLS and the one that received indomethacin.

In order to measure GSH levels, there was a previous induction of

Table 2
Effect of Noni-PLS on carrageenan-induced paw edema. Values are expressed as mean ± S.E.M. of six animals per group. The % inhibition of paw edema is indicated in parentheses. Carrageenan (Cg); Noni-PLS (polysaccharide fraction of *Morinda citrifolia*); Indometacin (Indo).

Treatment	Dose mg/kg	1h	2h	3h	4h
Sal		0.011 ± 0.004	0.001 ± 0.001	00.00 ± 00.00	0.006 ± 0.003
Cg		0.038 ± 0.005	0.090 ± 0,017	0.100 ± 0.005	0.082 ± 0.008
Indo	10	0.028 ± 0.007 (26.40%)*	0.016 ± 0.004 (82.30)*	0.014 ± 0.008 (86.00%)*	0.014 ± 0.007 (83.00%)*
Noni-PLS	0.1	0.068 ± 0.006 (-78.90%)*	0.082 ± 0.010 (08.90%)	0.096 ± 0.009 (04.00%)	0.083 ± 0.012 (-01.20%)
Noni-PLS	1.0	0.044 ± 0.007 (-15.80%)	0.046 ± 0.006 (48.90%)*	0.070 ± 0.018 (30.00%)	0.058 ± 0.014 (29.30%)
Noni-PLS	10	0.024 ± 0.013 (36.90%)*	0.022 ± 0.011 (75.60%)*	0.017 ± 0.006 (83.00%)*	0.022 ± 0.007 (73.20%)*

Indicates *P < 0.05, as compared to Cg group. One-way ANOVA followed by the Newman Keuls test.

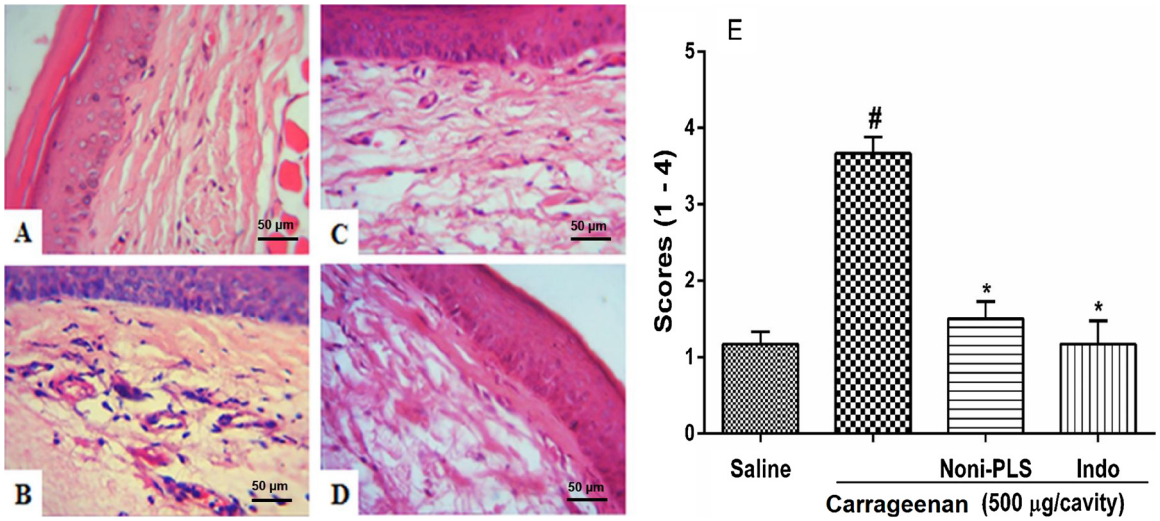

Fig. 4. Noni polysaccharide (Noni-PLS) fraction reduces histopathological damage induced by carrageenan in the paw tissue. Saline (A), Carrageenan (B), Carrageenan + Indomethacin (C) Carrageenan + Noni-PLS (D). Microscopic histological scores of inflammatory lesion (E). # $p < 0.05$, when compared with saline group. *$P < 0.05$, when compared Noni-PLS group.

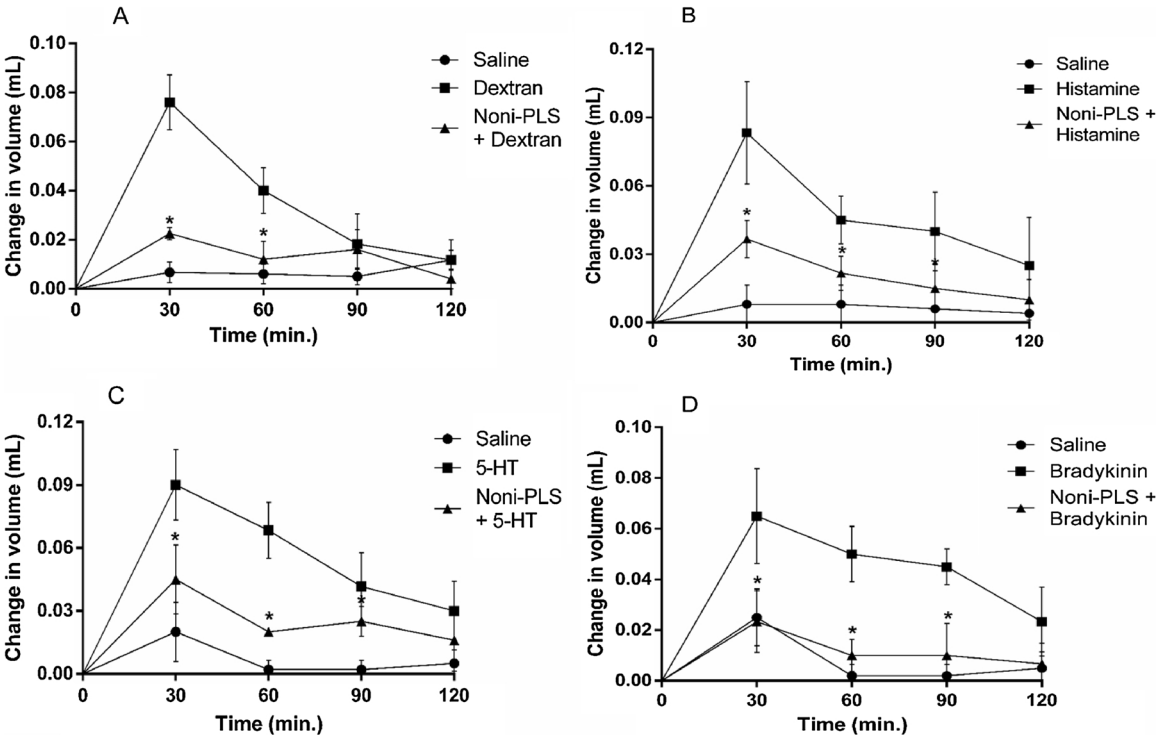

Fig. 5. Noni-PLS fraction reduces paw edema induced by dextran (A), histamine (B), Serotonin (C) and Bradykinin (D). *$P < 0.05$ indicates a significant difference from the dextran, histamine, serotonin and bradykinin groups.

Table 3
Effect of Noni-PLS on MPO levels in paw tissue and GSH levels in the peritoneal fluid on carrageenan-induced inflammation.

Experimental group	MPO (UMPO/mg tissue)	GSH (µg/g tissue)
Saline 0,9%	3.367 ± 0.636	140.6 ± 18.19
Carrageenan	26.84 ± 4.541#	67.75 ± 8.128#
Noni-PLS (10 mg/kg) + Carrageenan	3.270 ± 0.807*	145.4 ± 8.120*
Indometacin (10 mg/kg)	1.793 ± 0.579*	122.6 ± 7.722*

Results are expressed as the means ± S.E.M. at least 5 mice per group. # $P < 0.05$, when compared with saline group.*$P < 0.05$, when compared with Carrageenan group.

carrageenan peritonitis. According to Table 2, it was evidenced that the group that received the 500 µg injection of carrageenan (67.75 ± 8.128 µg/g) presented high glutathione consumption when compared with the saline group (140.6 ± 18.19 µg/g). It was also observed that the Noni-PLS + Cg group (145.4 ± 8.120 µg/g) maintained GSH levels significantly when compared with the carrageenan i.p. group, and did not present any statistical differences from the saline group and the group receiving Indomethacin 10 mg/kg (122.6 ± 7.72 µg/g).

The MPO enzyme is stored in the azurophil granules of neutrophils, which besides being a marker for the degree of inflammation of several tissues, is also involved in oxidative stress, contributing to the pathogenesis of inflammation (Bradley, Priebat, Christensen, & Roth- stein,

1982; Odobasic, Kitching, & Holdsworth, 2016). The recruitment and activation of neutrophils during acute inflammation contribute to the overproduction of reactive oxygen and nitrogen species that overwhelm the tissue antioxidant protective mechanisms, resulting in oxidative stress (Cuzzocrea, Riley, Caputi, & Salvemini, 2010). On the contrary, GSH (an endogenous antioxidant) protects the cells against oxidative damage, keeping the sulfhydryl groups (-SH) of proteins reduced and preventing them from reacting with free radicals (Amirshahrokhi, Bohloolia, & Chinifroush, 2011).

Glutathione in reduced form protects cells by removing reactive metabolites due to their conjugation to reactive forms. Their levels are a very sensitive indicator of cell functionality and viability (Rodrigues Neto, 2010). The GSH data reinforce the data obtained with the MPO enzyme, which indirectly showed a lower infiltration of neutrophils determined by the treatment with Noni-PLS, because a maintenance of glutathione levels also indicates fewer infiltration cells producing reactive oxygen species in the microenvironment of inflammation. Thus, it has been suggested that the anti-inflammatory effect of *M. citrifolia linn* polysaccharide should involve the inhibition of leukocyte migration and neutrophil infiltration during inflammation. In order to confirm this evidence, we performed an experiment of cell migration into the peritoneal cavity induced by carrageenan and treated with Noni-PLS.

3.5. Noni-PLS reduced leukocytes migration in carrageenan-induced peritonitis

We can see (Fig. 6A) that the total leukocyte infiltration was caused by i.p. of Cg ($11.85 \pm 2.31 \times 10^3$ leucocytes/mL) comparing the values with the group that received only saline at 0.9% via i.p. ($2.163 \pm 0.402 \times 10^3$ leucocyte/mL). However, according to Fig. 6A, Noni-PLS 10 mg/kg decreased the migration of leukocytes to the peritoneal cavity of the mice to 46.41% ($5.500 \pm 0.460 \times 10^3$ leucocytes/mL). The group that received Indomethacin at 10 mg/kg ($3.350 \pm 0.405 \times 10^3$ leucocytes/mL) showed no statistical difference in their results when compared with the Noni-PLS-treated group.

Similarly, the Cg injection (500 μg) caused increased migration of neutrophils ($9.456 \pm 1.759 \times 10^3$ neutrophils/mL) in the peritoneal cavity of the animals (Fig. 6B) compared with that in the group that received 0.9% saline only ($0.400 \pm 0.400 \times 10^3$ neutrophils/mL). Regarding the group that received treatment with Noni-PLS, the decrease in neutrophil count was very expressive ($4.800 \pm 0.512 \times 10^3$ neutrophils /mL).

Neutrophils are the most abundant leukocytes in the human circulation and play key roles in inflammatory responses; recently, their contribution to chronic inflammation has been appreciated (Soehnlein, Stffens, Hidalgo, & Weber, 2017). These cells comprise one of the first lines of defense against pathogens, and their infiltration into inflamed tissues is one of the hallmarks of the acute innate inflammatory response (Sostres, Gargallo, Arroyo, & Lanas, 2010). In this study, we demonstrated that inflammatory cell recruitment to the paw tissue 4 h after carrageenan administration was markedly reduced in mice treated with Noni-PLS as determined using histological analysis.

3.6. Noni-PLS attenuated the inhibition of nociception that was induced by acetic acid and formalin

Inflammatory pain results from the increased excitability of peripheral nociceptive sensory fibers produced by the action of inflammatory mediators such as histamine, serotonin, and bradykinin (Linley, Rose, Ooi, & Gamper, 2010). In the nociception model induced by formalin, we found that the polysaccharide *Morinda citrifolia Linn* 10 mg/kg had a significant antinociceptive effect in the inflammatory phase of the formalin test (Fig. 7A). The antinociceptive effect of Noni-PLS was accentuated in the second phase of the test and decreased the reactivity of the animals caused by the pain effect by 84.04% (92.75 ± 16.78 s) when compared with the group that received only 2.5% formalin (second phase, 69 ± 18.63 s), also acting like morphine (4.2 ± 1.98 s), the absence of statistical difference between Noni-PLS and morphine groups. When we tested this polysaccharide in the model of abdominal writhing induced by acetic acid, as we can see in Fig. 7B, Noni-PLS significantly reduced the number of abdominal writhing after 30 min of administration, with an observed reduction of 61.34% (27.6 ± 7724 writhing) in the number of writhes in the Noni-PLS group compared with the group that received only 0.6% acetic acid.

The formalin test is biphasic and evaluates two phases of pain sensitivity: the first phase of formalin-induced nociception is characterized as neurogenic pain. It results from direct chemical stimulation of the nociceptors and release of substance P. The second phase (inflammatory) is triggered by a combination of stimuli, including inflammation of the peripheral tissues and mechanisms of central sensitization (McNamara et al., 2007).

The acetic acid-induced writhing reaction in mice has been largely used for the assessment of analgesic or anti-inflammatory properties of drugs (Tjølsen & Hole, 1997). Thus, the writhing test with an acetic acid induction is a chemical method used to induce pain of peripheral origin by the injection of an irritant in mice. Analgesic activity of the Noni-PLS is inferred from the decrease in the frequency of writhing (Gawade, 2012). Thus, ours results show that in the nociceptive tests, the analgesic action of the studied fraction can be due to the ability to inhibit the release of inflammatory mediators and thereby diminishes pain by antagonizing the inflammatory response.

4. Conclusion

The results obtained in this work suggest that *Morinda citrifolia Linn* has two polysaccharide fractions with different molecular weights (fraction I, 1.71×10^5 g/mol; and fraction II, 2.57×104 g/mol) and composed mainly of homogalacturonan and arabinogalactan residues.

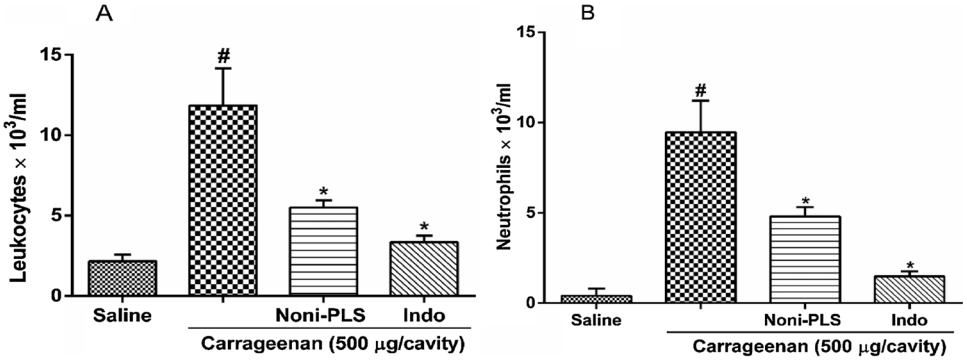

Fig. 6. Noni polysaccharide (Noni-PLS) fraction reduces immunological cell infiltration in carrageenan-induced peritonitis. Leukocytes (A) and neutrophils (B). *P < 0.05 indicates a significant difference from the carrageenan group; #P < 0.05 indicates a significant difference from the saline group.

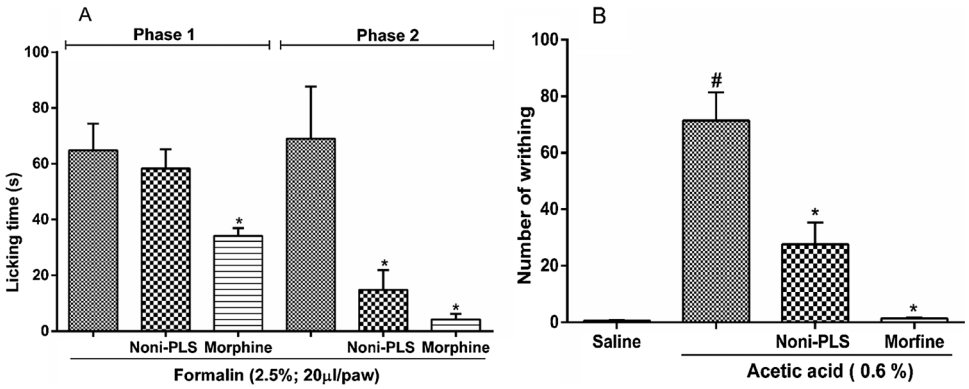

Fig. 7. Noni-PLS fraction reduces nociception induced by formalin (A) and acetic acid (B). $^{\#}p < 0.05$, when compared with saline group. *$P < 0.05$, when compared acetic acid or formalin.

In addition, we suggest with our pharmacological test that these polysaccharide structures possess anti-inflammatory and antinociceptive activities. Noni-PLS was able to significantly reduce the inflammatory parameters analyzed in paw edema induced by carrageenan, dextran, serotonin, histamine, and bradykinin; in biochemical evaluations of MPO and GSH, we assessed the migration of leukocytes into the peritoneal cavity as well as the histological evaluation of inflamed subplantar tissue. The polysaccharide fraction extracted from Noni also significantly decreased the pain parameters in mice tested using the abdominal writhing test induced by acetic acid and formalin test. Overall, all observations obtained in this study lead us to believe that the anti-inflammatory action of this polysaccharide was due to its ability to block the action of inflammatory mediators and signaling molecules derived from this condition, which were responsible to promote tissue dysfunction in several inflammatory diseases.

Conflicts of interest

The authors declare no conflict of interest.

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