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Anti-inflammatory polysaccharides of *Azadirachta indica* seed tegument

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Abstract: *Azadirachta indica* A. Juss., Meliaceae, or Indian neem is a plant used to treat inflammatory disorders. Total polysaccharide (TPL) and FI (fractioned by ion exchange chromatography) from the seed tegument of *A. indica* were evaluated in models of acute inflammation (paw edema/peritonitis) using Wistar rats. Paw edema (measured by hydroplethysmometry) was induced *s.c.* by λ-carrageenan (300 µg), histamine (100 µg), serotonin (20 µg), compound 48/80 (10 µg), prostaglandin (PGE_2 30 µg) or L-arginine (15 µg). Peritonitis (analyzed for leukocyte counts/protein dosage) was induced *i.p.* by carrageenan (500 mg) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP 50 ng). Animals were treated *i.v.* with TPL (1 mg/kg) or FI (0.01, 0.1, 1 mg/kg) 30 min before stimuli. FI toxicity (at 0.1 mg/kg, *i.v.* for seven days) was analyzed by the variation of body/organ mass and hematological/biochemical parameters. TPL extraction yielded 1.3%; FI, presenting high carbohydrate and low protein content, at 0.1 mg/kg inhibited paw edema induced by carrageenan (77%), serotonin (54%), PGE_2 (69%) and nitric oxide (73%), and the peritonitis elicited by carrageenan (48%) or fMLP (67%), being well tolerated by animals. FI exhibited potent anti-inflammatory activity, revealing to be important active component in traditionally prepared remedies to treat inflammatory states.

Article

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Introduction

Azadirachta indica A. Juss., Meliaceae, is a medicinal plant known as Indian neem or lilac, Persian lilac or margosa tree, originated from Asia, but worldwide cultivated. In Brazil, neem or nim is well adapted in the northeast Caatinga biome being largely recognized by its repellent and insecticide properties causing no environment pollution (Martinez, 2002). In folk medicine, oil seeds, bark and leaf extracts have been used to treat gastrointestinal, analgesic and inflammatory disorders. Experimental data demonstrated immunomodulator and anti-inflammatory activities of bark and leave extracts and also antipyretic and anti-inflammatory effects of oil seeds (Arora et al., 2011; Biswas et al., 2002).

Many compounds have been isolated from the oil seeds (mixture of tetranortripterpenes) and barks (polysaccharides) of *A. indica*. Although studies have described anti-inflammatory effect of polysaccharide extracts (Biswas et al., 2002), little has identified the bioactive components. Based on previous knowledge of *A. indica* constituents and their occurrence in traditional medicinal preparations, this study evaluated the effects of isolated polysaccharide fractions of *A. indica* seed tegument in models of inflammation in vivo.

Materials and Methods

Plant material

Azadirachta indica A. Juss., Meliaceae, was collected in Quixadá, Ceará-Brazil, identified by Ms. Vaneicia dos S. Gomes (State University of Ceara) and excicata deposited in the Prisco Bezerra herbarium of Federal University of Ceará (voucher nº 46084).

Extraction and isolation of polysaccharides from A. indica *seed tegument*

Seeds were washed with distilled water, dried at 40 °C before kernels exclusion. Dried tegument was grounded into fine particles for extraction. Absolute MetOH (250 mL) was added to 5 g of powder (1:50 w/v, 76 ºC) for removal of methanol-soluble materials

- procedure repeated twice. The insoluble portion was extracted (three times) with 0.1 M NaOH (1:50 w/v, 97 ºC) and centrifuged (2496 x *g*; r.t.). The alkaline polysaccharide extracts were pooled, neutralized in 1 M HCl, precipitated with 4 volumes of absolute EtOH (~800 mL) and re-centrifuged. Supernatant was dialyzed against running water for 72 h, centrifuged (2496 x *g*; r.t.) and the final supernatant (Total Polysaccharides-TPL) was lyophilized (Yoon et al., 2002).

Ten milligrams of TPL, dissolved in 20 mL of H_20 (1:2, w/v), was applied to ion exchange chromatography (DEAE-cellulose), equilibrated and washed with distilled water for removal of neutral polysaccharides, being the acidic polysaccharides eluted (60 mL/h) with NaCl (0.25-1 M). TPL and major polysaccharide fractions were dialyzed and further lyophilized. Content of carbohydrate (Dubois et al., 1956), uronic acid (Dische, 1947) and protein (Bradford et al., 1976) were assessed using D-galactose, D-galacturonic acid and BSA as respective standards.

Animals

Wistar rats (150-200 g) were maintained at 25 ºC, under a 12/12 h light/dark cycle, with free access of food and water. Protocols were approved by the Institutional Animal Care and Use Committee of the State University of Ceará-UECE (09204632-0) in accordance with the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication n° 85-23, revised 1985).

Inflammation models

Paw edema

Paw edema was induced by subcutaneous (*s.c.*) injection of λ-carrageenan or dextran (300 µg), histamine (100 µg), serotonin (20 µg), compound 48/80 (10 µg), prostaglandin (PGE_2 30 µg) or L-arginine (15 µg/paw). Control animals were treated 15 min prior to injection of the inhibitor of nitric oxide synthase L-NAME (30 mg/kg, *i.v.*) or 30 min prior to injection of the antagonist of serotonin receptor methisergide (5 mg/kg, *i.p.*). Edema was measured by plethysmometry (Panlab-LE-7500) before (zero time) and at ½, 1, 2-6 h thereafter and calculated by the difference in paw volume displacement (mL) or by the trapezoid method expressed as area under curves-AUC (arbitrary units) (Landucci et al., 1995). Animals were treated *i.v.* (0.1 mL/100 g body weight) with TPL (1 mg/kg), FI (0.01, 0.1, 1 mg/kg) or saline 30 min prior administration of flogistic agents.

Peritonitis

Peritonitis was induced *i.p.* with carrageenan (500 mg) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP 50 ng). Animals were euthanized 4 h later and peritoneal fluid harvested with 10 mL saline (5 IU heparin) for total and differential leukocyte counts (neutrophils, eosinophils, mast cells and mononuclear cells) (Souza & Ferreira, 1985) and protein leakage (Bradford, 1976). Animals received FI (0.1 mg/kg, *i.v.*) or saline 30 min before stimuli.

Toxicity assay

Rats were weighted before and after *i.v.* treatment with F1 (0.1 mg/kg) or saline during seven days. Peripheral blood was collected for hematological and biochemical analysis from anesthetized animals via intramuscular (*i.m.*) injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Animals were euthanized by cervical dislocation for remotion and weighting of heart, spleen, kidney, stomach and liver (expressed as g wet weight/100 g body mass).

Statistical analysis

Data are presented as mean±SEM of animals (n=6-7) and analyzed by ANOVA and Bonferroni's test. $p<0.05$ was considered significant.

Results and Discussion

Total Polysaccharides extraction yielded 1.3% of dry material, containing 54% carbohydrate, 4% protein and 51% uronic acid. TPL ion exchange chromatography (DEAE-cellulose) provided three major fractions (FI, FII, FIII) eluted at 0.25, 0.5 and 1.0 M NaCl, respectively (Figure 1). FI showed the best yield (40%) and highest carbohydrate (54%) and uronic acid (31%) contents, but low levels of protein (3%) (Table 1). Both polysaccharide extraction and fractioning were shown to be efficient, considering the low content of protein contaminants and high content of carbohydrate and uronic acid, an important feature of plant cell walls (Drozdova & Bubenchikov, 2005).

Systemic treatment of animals with TPL at 1 mg/kg inhibited in 17% the paw edema induced with dextran (87.5±10.82; TPL 72.5±4.14 AUC) and in 55% that induced with carrageenan (120.3±6.80; TPL 54.5±11.50 AUC) (Figure 2A). FI, fraction showing the highest content of carbohydrate, inhibited the entire edema formation elicited by carrageenan, at all doses (0.01, 0.1, 1 mg/kg), from 60 min (carrageenan 0.47±0.02 mL; 0.01 mg/kg 0.25±0.04 mL; 0.1 mg/kg 0.20±0.03 mL; 1 mg/kg 0.23±0.02 mL) to 300 min (carrageenan 0.23±0.02 mL; 0.01 mg/kg 0.08±0.03 mL; 0.1 mg/kg 0.02±0.02 mL; 1 mg/kg 0.17±0.01 mL) (Figure 2B). FI maximal effect

was obtained at 0.1 mg/kg (27.00±2.50 AUC) about 77% compared to the control carrageenan (118.02±6.50 AUC) (Figure 2A). Paw edema induced by carrageenan is characterized by protein leakage in the first two hours, synthesis of initial phase (histamine, serotonin, bradykinin) and late phase (nitric oxide, prostaglandins) inflammatory mediators and neutrophil infiltrate (Di Rosa et al., 1971). On the other hand, dextran induces an osmotic edema followed by protein extravasation without neutrophil infiltrate (Lo et al., 1982).

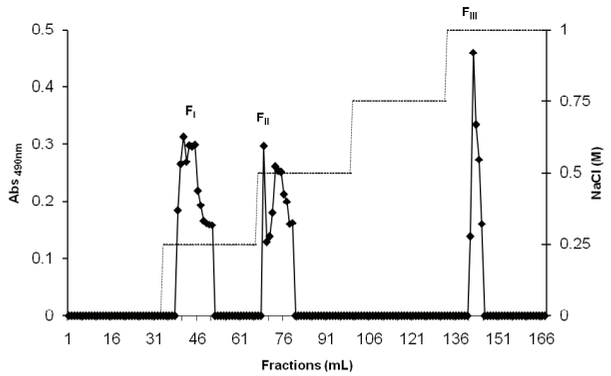

Figure 1. TPL ion exchange chromatography. TPL (10 mg) were dissolved in 20 mL distilled water and applied to DEAE-cellulose (9.8 x 2.0 cm). Resin was washed with water and the acidic polysaccharide fractions eluted (60 mL/h) with a linear NaCl (- - -) gradient. Carbohydrates (♦) were assayed by Dubois (1956).

Table 1. Yield and chemical analysis of *A. indica* polysaccharide fractions.

Polysaccharide fractions	[a]Yield (%)	[b]Carbohydrate (%)	[c]Protein (%)	[d]Uronic acid (%)
FI	40	54	3	31
FII	31	37	2	31
FIII	13	3	0.3	5

[a]FI, FII and FIII (10 mg); [b]Dubois (1956); [c]Bradford (1976); [d]Dische (1947)

FI (0.1 mg/kg) did not alter the paw edema elicited by histamine (169.3±17.89; FI 146.8±4.41 AUC), but efficiently reduced that of serotonin (165.3±17.89; FI 74.7±7.82 AUC) in 54% and that of compound 48/80 (109.4±7.36; FI 59.1±4.33 AUC) in 46% (Figure 2C). Besides, pre-treatment of animals with methysergide inhibited the edema elicited by serotonin in 84% (25.5±6.9 AUC) (Figure 2C). Our data indicates that FI did not antagonize histamine receptors, but could stabilize mast cell membranes, preventing the release of its content (histamine and serotonin) stimulated by compound 48/80 (Brito et al., 2007). This data is supported in literature, showing that polysaccharide fractions of aerial parts of *Viola odorata* and *Malva pusilla* plants presented anti-inflammatory activity (Drozdova & Bubenchikov, 2005). Also, polysaccharide fractions of *Orbihnya phalerata* fruit and *Cyrtopodium cardiochilum* pseudobulbs reduced plasma leakage in rats (Pereira da Silva & Paz Parente, 2001; Barreto & Parente, 2006).

FI (0.1 mg/kg) reduced in 69% the paw-edema elicited by PGE_2 (66.5±4.56; FI 20.25±2.94 AUC) and in 73% that of L-arginine (55.2±3.49; FI 14.5±3.90 AUC), a substrate of nitric oxide (NO) generation. Additionally, pre-treatment of animals with L-NAME inhibited in 84% (7.0±4.1 AUC) the edema elicited with L-arginine (Figure 2D). Based on the biphasic nature of carrageenan-induced edema and in the late inhibitory effect of FI, the polysaccharide possibly causes better interference with late-phase mediators. In this line, plant extracts of *Trichilia dregeana* (Jäger et al., 1996), *Cryptocarya latifolia, Euclea natalensis, Felicia muricata, Mohria caffrorum* (McGaw et al., 1997) and *Culcasia scandens* (Okoli & Akah, 2004) show anti-inflammatory activity, inhibiting PGE_2 release. Moreover, nimbidin, the major active principle of *A. indica* oil seed inhibits NO and PGE_2 from rat peritoneal macrophages stimulated with lipopolysccharide (Kaur et al., 2004).

As showed in the paw edema model, FI effectively inhibited the effect of late-phase inflammatory mediators involved in cell events. In fact, FI (0.1 mg/kg) reduced the number of total leukocyte migration in 67% when stimulated with fMLP (2.74±0.15; FI 0.89±0.13 x 10^3) and in 48% when stimulated with carrageenan (7.79±0.03; FI 4.07±0.04 x 10^3) (Figure 2E). Neutrophils were the most reduced cells, about 75%, when stimulated with fMLP (1.50±0.20; FI 0.37±0.02 x 10^3) and about 55% when stimulated with carrageenan (5.01±0.03; FI 4.07±0.04 x 10^3) (Figure 2E). It is well known that in acute inflammatory responses the neutrophil rolling/adhesion along endothelium is a result of direct (fMLP) or indirect activation (carrageenan), via resident cells, that culminate in chemotactic release of mediators (Ribeiro et al., 1997). Thus, FI may be interfering in both pathways. Accordingly, pectic polysaccharide fractions from aerial parts of *Comarum palustre* (Popov et al., 2005) and the acidic polysaccharide from flower-heads of *Cyrtopodium cardiochilum* (Barreto & Parente, 2006) were shown to inhibit cell migration. Further, FI (±0.03 x 103) reduced vascular leakage elicited by carrageenan (0.9±0.06 x 103 mg/mL) in 81% (Figure 2F), corroborating the initial phase action of FI in the model of paw edema. This finding stretches the role of FI in inflammatory vascular events, as described for polysaccharide fractions of *Orbignya phalerata* mesocarp (Pereira da Silva & Paz Parente, 2001).

Moreover, FI was well tolerated by the animals, since the observed lack of lethality or other external symptoms. However, minor alterations were seen in the wet weigh (g) of stomach (1.53±0.05; control 1.76±0.16)

and kidney (0.93±0.03; control 0.80±0.03). Alanine and aspartate transaminases (ALT; AST), creatinine, albumin and globulin levels were not affected, but urea levels increased. The number of circulating lymphocytes were also raised (Table 2), which could be accredited in part to the immunomodulatory action of plant polysaccharides (Arora et al., 2011).

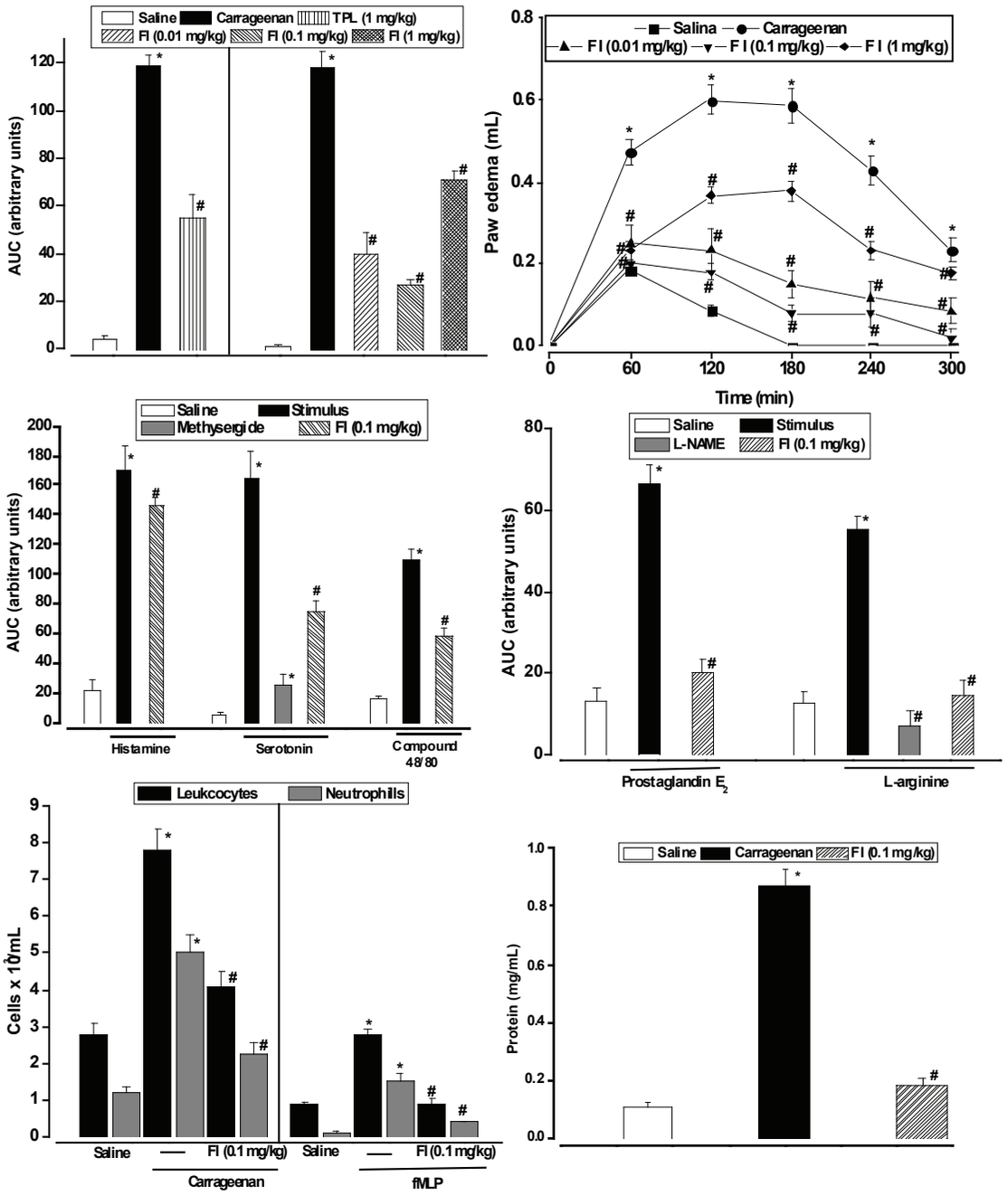

Figure 2. Anti-inflammatory effect. TPL (1 mg/kg) or FI (0.01, 0.1 and 1 mg/kg) was injected *i.v.* 30 min before *s.c*. edema induction with A. carrageenan (300 µg); B. histamine (100 µg), serotonin (20 µg) or compound 48/80 (10 µg); C. PGE_2 (30 µg) or L-arginine (15 µg; *s.c.*) in animals pre-treated or not 15 min prior L-NAME (30 mg/kg, *i.v.*) or before *i.p.* peritonitis induction with D. carrageenan (500 µg) or F. fMLP (50 ng); E. protein leakage induced with carrageenan (500 µg). Controls received saline (0.9%). Edema was measured at ½, 1, 2-6 h and peritonitis at 4 h after stimuli. Mean±SEM. (n=6). ANOVA and Bonferroni tests. *p<0.05 compared to saline, #p<0.05 compared to stimuli.

Table 2. Biochemical and hematological parameters of animals treated with *Azadirachta indica* polyssacharide.

[b]Parameters	[a]Treatment (100 µL/100 g)	
	Saline	FI (0.1 mg/kg)
ALT (U/l)	[c]33.85±2.14	[b]33.85±1.01
AST (U/l)	79.0±3.7	72.85±4.58
Urea (mg/dl)	46.42±3.14	60.42±2.01*
Creatinine (mg/dl)	0.46±0.01	0.48 ± 0.02
Total protein (mg/dl)	5.91 ± 0.10	5.78±0.14
Hematocrit (%)	45.35±0.87	43.4±0.45
Hemoglobin (g/dl)	14.65±0.37	14.65±0.14
Erythrocytes (10^6/µl)	7.56±0.19	7.33 ±0.08
Platelets (10^3/µl)	846.0±62.04	975.71±38.38
Lymphocytes (%)	22.14±2.19	78±2.21*
Monocytes (%)	2.57±0.48	2.28 ±0.18
Eosinophils (%)	1±0	1.42±0.29

[a]Rats were treated daily in single doses with FI (0.1 mg/kg) or saline (0.9%) during 7 days; [b]Analysis of biochemical and hematological parameters after animals sacrifice; [c]Mean±SEM (n=7); ANOVA and Student's t test; ALT: alanine transaminase; AST: aspartate transaminase.

In conclusion, TPL and FI fractioned from *A. indica* seed tegument exhibited potent anti-inflammatory activity, interfering not only in vascular, but especially in cellular inflammatory events, involving serotonin, PGE_2 and NO. These polysaccharides could be revealed as important active components in traditionally prepared remedies to treat inflammatory diseases.

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