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Polysaccharide extract of *Mimosa tenuiflora* stem barks stimulates acute inflammatory response via nitric oxide

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ABSTRACT. *Mimosa tenuiflora* (Mimosaceae) or "jurema-preta" is well distributed in the northeast Brazil, being popularly used to treat skin lesions, burns and inflammation. The healing effect of the alcoholic extract prepared with its barks corroborates the popular use. This study aimed to evaluate the inflammatory response of polysaccharides extracted from *M. tenuiflora* barks (EP-Mt) by methanol/NaOH and ethanol precipitation. Inflammatory activity was assessed in rat models of acute inflammation (paw edema and peritonitis), by the following parameters: edema, vascular permeability, leukocyte migration, myeloperoxidase activity and pharmacological modulation of nitric oxide and prostaglandins. EP-Mt presented 3.8% yield, 41% carbohydrate and 0.34% protein. EP-Mt (0.01, 0.1, 1.0 mg kg^{-1}) injected by subcutaneous route elicited paw edema that lasted from 30-420 min, with maximal effect at 1 mg kg^{-1} (40x *vs.* saline), and was inhibited by L-NAME (52%) and dexamethasone (26%). EP-Mt (1 mg kg^{-1}, via intraperitoneal) stimulated leukocytes migration (2.2x), mainly neutrophils (6.5x) and MPO activity (96%). The leukocyte migration elicited by EP-Mt was inhibited by dexamethasone (39%) and L-NAME (38%). EP-Mt containing high carbohydrate content induces acute inflammation via nitric oxide, which open perspectives of application in pathological conditions of immunosuppression.

Keywords: Mimosaceae, medicinal plant, carbohydrate, inflammation.

Extrato polissacarídico oriundo das cascas do caule de *Mimosa tenuiflora* induz resposta inflamatória aguda via óxido nítrico

RESUMO. *Mimosa tenuiflora* (Mimosaceae) ou "jurema-preta", amplamente distribuída no nordeste brasileiro, é utilizada popularmente no tratamento de lesões de pele, queimaduras e inflamação. O efeito cicatrizante do extrato alcoólico de suas cascas corrobora o uso popular. Avaliou-se o efeito inflamatório dos polissacarídeos da casca de *M. tenuiflora* (EP-Mt), obtidos por extração com metanol/NaOH e precipitação com etanol. O efeito inflamatório foi avaliado em modelos agudos em ratos (edema de pata, peritonite) por meio dos seguintes parâmetros: edema, permeabilidade vascular, migração leucocitária, atividade da mieloperoxidase e modulação farmacológica de prostaglandinas e óxido nítrico. EP-Mt apresentou 3,8% de rendimento, 41% de carboidratos totais e 0,34% de proteína. EP-Mt (0,01, 0,1, 1,0 mg kg^{-1}), administrado por via subcutânea, induziu edema de pata com duração de 30 a 420 min e efeito máximo na dose de 1 mg kg^{-1} (40x *vs.* salina), o qual foi inibido por L-NAME (52%) e dexametasona (26%). EP-Mt (1 mg kg^{-1}, via intraperitoneal) estimulou a migração de leucócitos (2,2x *vs.* salina), principalmente de neutrófilos (6,5x), com aumento da atividade da mieloperoxidase (96%). A migração de leucócitos foi inibida por dexametasona (39%) e L-NAME (38%). EP-Mt contendo elevado teor de carboidratos induz inflamação aguda via óxido nítrico com perspectivas de aplicação em condições patológicas de imunossupressão.

Palavras-chave: Mimosaceae, plantas medicinais, carboidratos, inflamação.

Introduction

Mimosa tenuiflora (Wild.) Poiret (Mimosaceae) is a plant commonly found in the northeast Brazil, where is popularly known as "jurema-preta" (Oliveira, Chiavone-Filho, Rodrigues & Medeiros, 1999). The bark powder is used in folk medicine to treat skin burns, wounds and inflammation (Grether, 1988; Camargo-Ricalde, Grether, & Martínez-Bernal, 1994). *In vivo* experiments demonstrated the healing effect in human venous ulceration of the alcoholic extracts from *M. tenuiflora* cortex (Rivera-Arce et al., 2006) and the antinociceptive and anti-inflammatory activities of

its barks (Cruz et al., 2016). *In vitro*, arabinogalactans (5 to 140 kDa) isolated from *M. tenuiflora* stimulates fibroblast and keratinocyte proliferation (Zippel, Deters, & Hensel, 2009). Besides, polysaccharides derived from higher plants are recognized for their relative nontoxicity, being ideal candidates for immunomodulatory, anti-tumor and wound-healing therapies (Meng et al., 2014; Pereira et al., 2012a; Zhou, Shi, Jiang, Zhou, & Xu, 2014; Lazareva et al., 2002). The aim of this study was to evaluate the inflammatory response of the polysaccharides extracted from *M. tenuiflora* stem barks in models of acute inflammation in rats.

Material and methods

Plant material

Barks of *M. tenuiflora* were collected directly in nature, in the District of Daniel de Queiroz-Quixadá, Ceará, Brazil. The plant was identified by the biologist Regis C. Paulino and its exsiccate (n° 46085) was deposited at the Botanical Laboratory (*Universidade Estadual do Ceará*) and Herbarium Prisco Bezerra (*Universidade Federal do Ceará*).

Compounds

N-Nitro-L-arginine methyl ester hydrochloride (L-NAME), D-galactose, bovine serum albumin (BSA), Coomassie brilliant blue, G-250, formamide and Evans blue were obtained from Sigma Chemical-Aldrich (St. Louis, MO, USA); dexamethasone and indomethacin were from *Prodome Química e Farmacêutica*, Campinas, SP (Brazil). EP-Mt and drugs were solubilized in sterile saline (0.9% NaCl), except for indomethacin, which was dissolved in 10% dimethyl sulfoxide (DMSO).

Animals

Wistar rats (150-250 g) were maintained under 12/12 h light/dark cycle, at 25°C, with free access of food and water. Experimental protocols were in compliance with the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH n° 85-23, 1985) and approved (n° 10130208-8/40) by the Animal Care and Use Committee of the *Universidade Estadual do Ceará*, Brazil.

Polysaccharide extraction

Mimosa tenuiflora stem barks were washed with distilled water, dried at 40°C, grounded into powder (5 g), suspended in methanol (1:50, w/v, 60°C) and filtered to remove methanol-soluble material (repeated two times). The insoluble portion was extracted in 0.1 M NaOH at 90°C and centrifuged (2496 × g; r.t.) (repeated three times). The alkaline extracts were pooled, neutralized with 1 M HCl, precipitated with four volumes of ethanol and centrifuged. The pellet was dialyzed (membrane cut-off 14 kDa; 72 h) against running water, centrifuged (2496 × g; r.t.), the final supernatant lyophilized (Pereira et al., 2012a) and named extract polysaccharide of *M. tenuiflora* (EP-Mt).

Chemical analysis

The content of carbohydrate (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and protein (Bradford, 1976) of EP-Mt was assessed by spectrophotometry, using D-galactose (A_{490} nm) and BSA (A_{595} nm), respectively, as standards.

Inflammation models

Paw edema was induced by subcutaneous (s.c.) intraplantar injection of EP-Mt (0.01, 0.1 and 1 mg kg^{-1}) as inflammatory stimulus. Control animals received sterile saline (0.9% NaCl). Dexamethasone (5 mg kg^{-1}) or L-NAME (25 mg kg^{-1}) was injected by intravenous route (i.v.) 30 min prior to EP-Mt. The edema was measured by plethysmometry (Panlab LE-7500) before edema induction (zero time), at 30 min, and from 60 to 420 min thereafter, and expressed in µL or area under the curve-AUC (arbitrary units) (Landucci et al., 1995). For evaluation of plasma leakage, animals received Evans blue (25 mg kg^{-1}) 30 min before edema induction by EP-Mt. The paws were excised, incubated with 1 mL formamide (37°C, 72 hours) and the increase in vascular permeability assessed by spectrophotometry (A_{600} nm). Data was expressed in µg Evans blue/g paw wet weight using linear regression based on Evans blue standard curve (Wilhelm, 1982).

Peritonitis was induced by intraperitoneal (i.p.) injection of EP-Mt (1 mg kg^{-1}; 500 µL) and evaluated 180 min later. After sacrifice, peritoneal cells were harvested with 3 mL phosphate-buffered saline (PBS) containing heparin (5 IU) for total and differential leukocyte counts. Results were expressed as the number of peritoneal leukocytes (x 10^3 mL^{-1}). Animals received saline (i.v.), dexamethasone (5 mg kg^{-1}; i.v.), L-NAME (25 mg kg^{-1}; i.v.) or indomethacin (10 mg kg^{-1}; i.v.) 30 min before stimulation with EP-Mt. Vascular permeability was quantified by protein dosage of the peritoneal fluid (Bradford, 1976).

The activity of mieloperoxidase (MPO) was measured in peritoneal fluid collected 180 min after stimulation. The samples were homogenized, mixed

with *o*-dianisidine dihydrochloride (0.167 mg mL^{-1} in 50 mM phosphate buffer), containing H_2O_2 (0.005%) and measured at A_{460} nm. Results were expressed as units of MPO (UMPO mL^{-1}). One unit of MPO was defined as the conversion of 1 µmol of hydrogen peroxide to water in 1 min at 22°C (Bradley, Chritensen, & Rothstein, 1982).

Statistical analysis

Results were expressed as mean ± S. E. M. (n=6) and analyzed by One-way/Two-way ANOVA and Bonferroni test (Prism 5.0, GraphPad Software Inc., California, USA). Differences were considered for $p \leq 0.05$.

Results and Discussion

The brown colored polysaccharide extract of *M. tenuiflora* stem barks (EP-Mt) yielded 3.8%, higher than those obtained from *Caesalpinia ferrea* pods (2.8%) (Pereira et al., 2012a) and *Azadirachta indica* teguments (1.3%), extracted by similar procedures (Pereira, Silva, Silva, Assreuy, & Pereira, 2012b). Yield variations of polysaccharide extraction can be explained by the changes in their distribution in plant parts and also by the type of extraction employed (Gottlieb & Borin, 2004). EP-Mt revealed high content of carbohydrate (41%), which was superior in comparison with that obtained from *C. ferrea* (31%) (Pereira et al., 2012a) and *Erigeron canadensis* flowers (34.1%) (Pawlaczyk et al., 2011). Importantly, the protein content (0.34%) was much less compared to the polysaccharides of *Geoffroea spinosa* barks (9%) (Silva et al., 2015), *C. ferrea* (3%) (Pereira et al., 2012a) and *A. indica* (4%) (Pereira et al., 2012b), both extracted by the same protocol. The low content of protein and high content of carbohydrate are justified by the conditions of polysaccharide extraction: high temperature, depigmentation by methanol, alkaline extraction and precipitation with ethanol (Yoon et al., 2002, Pereira et al., 2012a).

Although in the present study the analysis of phenolic compounds had not been assessed, in other study performed with *M. tenuiflora* barks, the presence of these compounds (minor residual amounts) was observed in the total polysaccharide extract. It is important to note that the authors could correlate the presence of phenolic compounds with the toxicity observed in cultured skin fibroblasts. Such effect was abolished after removal of the phenolic compound, which was associated to the polysaccharide (Zippel et al., 2009). In addition, previous phytochemical studies demonstrated presence of phenolic compounds (Jiag et al., 1991; Rivera-Arce et al., 2007), saponin, alkaloids, glucose, xylose, rhamnose, arabinose, lupeol, phytosterols, lipids, calcium oxalate crystals and starch (Jiag et al., 1991) in *M. tenuiflora* barks.

EP-Mt evoked paw edema in rats at all doses tested (0.01, 0.1 and 1 mg kg^{-1}), showing a time course that was initiated at 30 min being maximal at 180 min. At 0.01 and 0.1 mg kg^{-1} the edema lasted up to 240 min and at 1 mg kg^{-1} up to 420 min (Figure 1A). The area under the edema time-course curve (AUC) showed that EP-Mt increased paw volume in 10 fold at 0.01 mg kg^{-1} (AUC: 26.9 ± 3.5) and 0.1 mg kg^{-1} (25.3 ± 0.7), while at 1 mg kg^{-1} the paw edema increase was about 40 fold (AUC: 102.5 ± 9.3) compared to saline (AUC: 2.5 ± 0.9 arbitrary units) (Figure 1B). The edematogenic effect of EP-Mt (1 mg kg^{-1}) was inhibited mainly by L-NAME (52%) and by dexamethasone (26%) (Figure 1C).

In the peritonitis model, EP-Mt (1.0 mg kg^{-1}, i.p.) increased the number of total leukocytes in 2.2 fold (6.980 ± 0.707 *vs*. saline: 3.140 ± 0.531 x 10^3 mL^{-1}), mainly neutrophils in 6.5 fold (4.648 ± 0.467 *vs*. saline: 0.705 ± 0.158 x 10^3 mL^{-1}) (Figure 2A). EP-Mt also increased MPO activity by 96% (14.3 ± 3.1 *vs*. saline: 4.9 ± 0.8 UMPO mL^{-1}) (Figure 2B), an enzyme highly expressed in neutrophils that has been associated with the nitric oxide (NO) bioavailability (Galijašević, 2013). Accordingly, the pre-treatment with L-NAME (nitric oxide synthase inhibitor) reduced EP-Mt leukocyte migration by 38% (5.683 ± 0.481 x 10^3 mL^{-1}). In addition, the treatment of animals with dexamethasone (a non-specific anti-inflammatory glucocorticoid) 180 min after EP-Mt administration, significantly reduced leukocyte migration by 39% (5.584 ± 0.193 x 10^3 mL^{-1}). On the other hand, indomethacin (cyclooxygenase inhibitor) did not alter the leukocyte migration elicited by EP-Mt (Figure 2C).

The vascular permeability evaluated by Evans blue leakage at 30 min in the paw edema model was not altered, indicating that EP-Mt edematogenic effect is not of osmotic nature, different from that elicited by the polysaccharide dextran (Lo, Almeida, & Beaven, 1982). This data was confirmed by the lack of alteration in vascular permeability, seen by the protein dosage by the Bradford method evaluated at 180 min after peritonitis induction (Table 1).

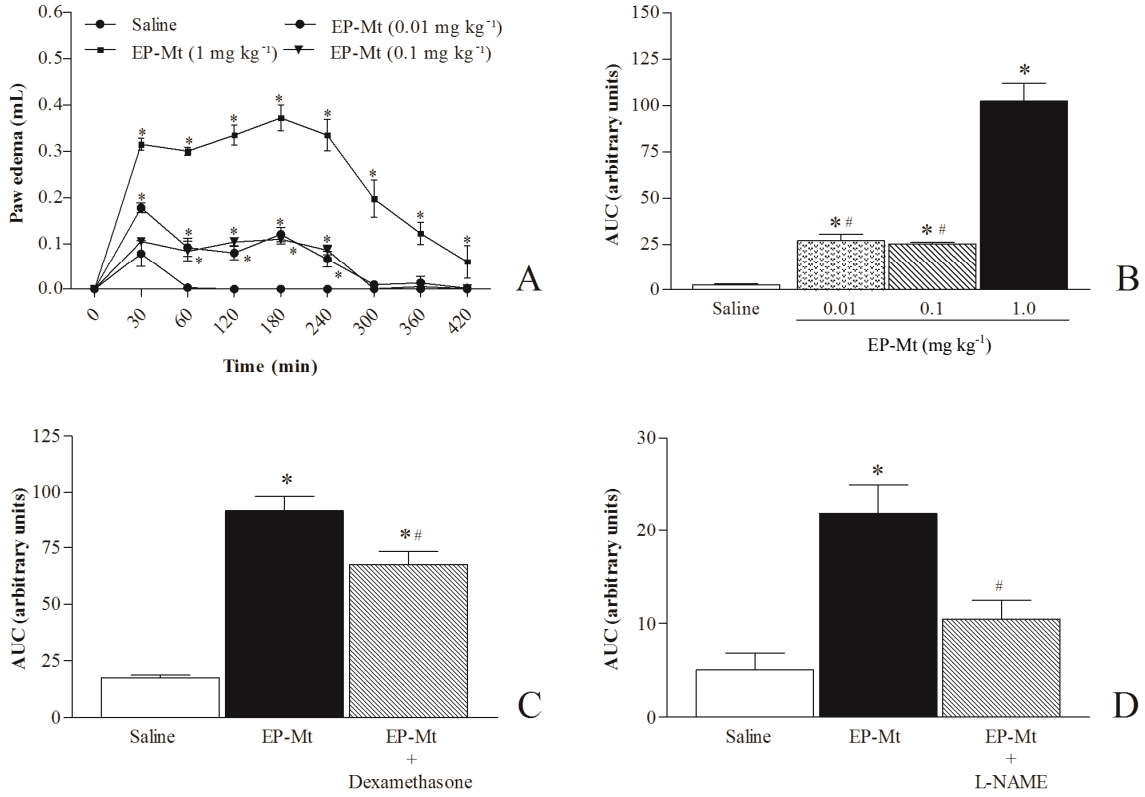

Figure 1. EP-Mt edematogenic effect is inhibited by L-NAME and dexamethasone. EP-Mt (0.01, 0.1, 1 mg kg^{-1}) was injected s.c. and the paw edema evaluated at zero time and from 30-420 min thereafter. (A) Edema time course (mL); (B) area under curve (AUC); (C) AUC of EP-Mt (1 mg kg^{-1}) or EP-Mt + dexamethasone (5 mg kg^{-1}; i.v.): evaluation from 30-240 min or; (D) EP-Mt + L-NAME (25 mg kg^{-1}; i.v.): evaluation from 30-120 min. Mean ± S.E.M. (n = 6). One-way ANOVA and Bonferroni test. *p<0.05 *vs.* saline; #p<0.05 *vs.* 1 mg kg^{-1} EP-Mt.

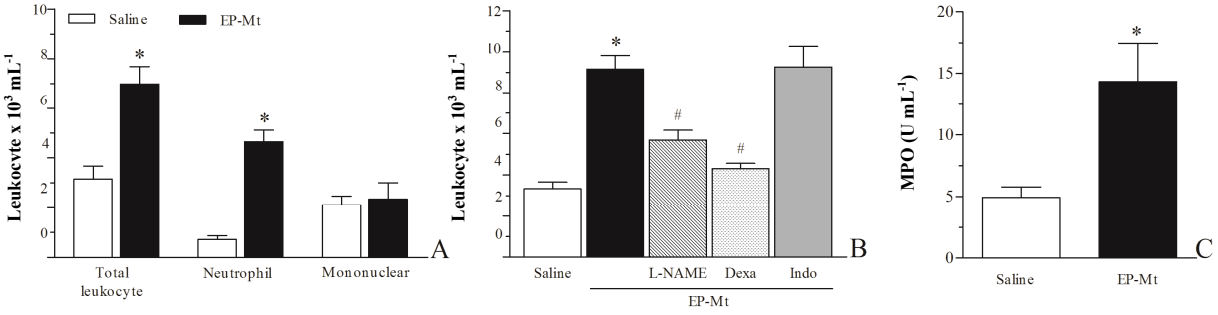

Figure 2. EP-Mt elicits leukocyte migration. Peritonitis was induced by EP-Mt (1 mg kg^{-1}; i.p.) in animals (A) naive or treated 30 min before with (B) dexamethasone (Dexa, 5 mg kg^{-1}; i.v.), L-NAME (25 mg kg^{-1}; i.v.) or indomethacin (Indo, 10 mg kg^{-1}; i.v). Total and differential leukocyte counts or (C) MPO activity was evaluated 180 min after peritonitis induction; Mean ± E.P.M. (n=6). Two-way ANOVA and Bonferroni. *p < 0.05 *vs.* saline; #p < 0.05 *vs.* EP-Mt.

Table 1. EP-Mt does not alter vascular permeability.

Models	Control	EP-Mt
[a]Paw edema	4.5 ± 0.2	3.8 ± 1.7
[b]Peritonitis	10.4 ± 0.4	10.0 ± 0.4

[a]Evans Blue leakage (µg Evans blue/g paw); [b]Bradford method (mg mL^{-1})

In acute inflammatory processes, macrophages, mast cells and lymphocytes have a modulator role in neutrophil migration releasing chemotactic factors, such as leukotrienes (LTB_4) and cytokines: tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1) (Ranki, Sylvester, Smith, Yoshimura, & Leonard, 1990). The inhibitory effect of dexamethasone on EP-Mt chemotactic effect could be explained by the blockage of leukotrienes and/or cytokines release. Besides, the anti-inflammatory mechanism of dexamethasone includes the

inhibition of the expression of various inflammatory genes, such as that of the inducible nitric oxide synthase (iNOS), leading to decreased production of NO (Korhonen, Lahti, Hämäläinen, Kankaanranta, & Moilanen, 2002; Secco et al., 2006; Moncada, Palmer, & Higgs, 1991). This data reinforces the inhibitory effect of L-NAME on the neutrophil migration stimulated by EP-Mt. Prostaglandins are mediators that also play a complex role in primary aspects of acute inflammatory response, such as pain, neutrophil migration, swelling and plasma exudation (Menezes et al., 2008). Our data demonstrated that the pre-treatment of animals with indomethacin did not alter the leukocyte migration, suggesting that prostaglandins are not crucial for the occurrence of leukocyte migration elicited by EP-Mt. In addition, it is possible to suggest that EP-Mt stimulates neutrophil chemotaxis, rather than vascular permeability. Furthermore, although not detected at the time-points evaluated in the peritonitis model, the possible EP-Mt effect in permeability could occur prior to its neutrophil chemotactic effect, which partially explains the inhibitory effect of L-NAME.

The effect of polysaccharides of higher plants in leukocytes, via macrophage activation, is extensively described in literature (Park et al., 2001; Sugawara, Lee, & Wong, 1984; Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005; Schepetkin & Quinn, 2006). It has been shown that these compounds might stimulate macrophage phagocytic and cytotoxic activities against tumoral cells and micro-organisms, production of reactive oxygen species (ROS), and secretion of cytokines and chemokines. The polysaccharide of *Juniperus scopolorum* showed potent immunostimulant effect, via increase of NO production and release of proinflammatory cytokines by macrophages (IL-1, IL-6, TNF-α) (Schepetkin et al, 2005). In addition, the polysaccharide obtained from *Dendobrium officinal* raised macrophage levels of TNF-α, IL-1β (He et al., 2016) and NO production (Xia et al., 2012). Based on these, we believe that EP-Mt might stimulate leukocytes to release chemotactic substances, being considered a promisor candidate to be used in immunostimulant therapies.

Conclusion

The results reported in the present study clearly demonstrate that the polysaccharides extracted from *M. tenuiflora* stem barks, possessing high content of carbohydrate, elicit acute inflammation with the involvement of nitric oxide. It is worth mentioning the necessity to investigate molecules from natural sources presenting immunomodulatory effect, aiming the development of novel therapeutic agents for being used in pathological conditions of the immune system.

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