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Original Article

Laticifer proteins from *Plumeria pudica* inhibit the inflammatory and nociceptive responses by decreasing the action of inflammatory mediators and pro-inflammatory cytokines

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A B S T R A C T

Some publications have described the pharmacological properties of latices proteins. Thus, in the present study proteins from *Plumeria pudica* Jacq., Apocynaceae, latex were evaluated for anti-inflammatory and antinociceptive activities. Obtained data showed that an intraperitoneal administration of different doses of latex was able to reduce the paw edema induced by carrageenan in a dose-dependent manner (better dose 40 mg/kg; 72.7% inhibition at 3rd and 78.7% at 4th hour) and the edema induced by dextran (40 mg/kg; 51.5% inhibition at 30 min and 93.0% at 1st hour). Inhibition of edema induced by carrageenan was accompanied by a reduction of myeloperoxidase activity. Pre-treating animals with latex (40 mg/kg) also inhibited the paw edema induced by histamine, serotonin, bradykinin, prostaglandin E_2, compound 48/80. Additionally, the latex (40 mg/kg) reduced the leukocyte peritoneal migration induced by carrageenan and this event was followed by reduction of IL-1β and TNF-α in peritoneal fluid. The latex-treatment (40 mg/kg) reduced the animal abdominal constrictions induced by acetic acid and the first phase on paw licking model induced by formalin. When latex was treated with heat (at 100 °C for 30 min), anti-edematogenic and myeloperoxidase activities were significantly reduced, indicating the involvement of heat-sensitive proteins on anti-inflammatory effect. Our results evidence that latex fluids are a source of proteins with pharmacological properties.

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Introduction

Latex is a milkweed fluid produced by specialized plant cells named laticifers that is often released after suffering mechanical injury (Kekwick, 2001; Pickard, 2007). It is mainly composed by lipids, hydrolytic enzymes, natural polymers, defense proteins and numerous secondary metabolites (Morcelle et al., 2004; Hagel et al., 2008a; Mazoir et al., 2008). The latex is commonly known as a rich source of active compounds bearing pharmacological properties (Dewan et al., 2000; Kumar et al., 2001; Frade et al., 2004; Chin et al., 2006). In traditional medicine, the latex of different plant species are usually diluted in water and used for the treatment of inflammation, inflammatory pain, tumors, gastritis and others (Devprakash et al., 2012; Hagel et al., 2008b).

The curative properties of latices have been confirmed by the scientific community. The fluid collected from different plants has undergone extraction procedures with organic or aqueous solvents and extracts were evaluated using *in vivo* and *in vitro* models involving animals (Sharma and Sharma, 1999; Choedon et al., 2006). In general, these studies described the presence of pharmacological activities and no information about putative molecules involved is proposed. Otherwise, several molecules with pharmacological importance, such as morphine, papaverine and codeine were found in latex (Lindner, 1985; Hagel et al., 2008b).

Recently, proteins from laticifer fluids have received special attention since they have exhibited the ability to inhibit

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inflammatory and pain process as well as tumor growth (Alencar et al., 2004; Soares et al., 2005; Oliveira et al., 2010). In these studies, the latices were collected in water and submitted to centrifugation and dialysis steps to obtain a protein fraction free of rubber and small molecules. In parallel, characterization of proteins in latex fluids was performed and important advances in the elucidation of mechanism of active proteins were achieved.

Plumeria pudica Jacq. is plant belonging to Apocynaceae known by its intense latex production. The plant is found abundantly in northeastern Brazil, where its latex is mentioned by the poor population to be used for the treatment of skin diseases and tooth pain. In the literature, several reports demonstrated the pharmacological properties of different parts of plants belonging to the genera *Plumeria*. These studies demonstrated properties such as anti-inflammatory, antinociceptive, anti-diarrhoeal, skin healing and others (Hamburger et al., 1991; Gupta et al., 2006), On the other hand, there are no scientific investigations confirming these properties in *P. pudica* latex.

The investigations on latex proteins have demonstrated that these proteins are capable to interfere in important events of inflammatory response, such as neutrophil migration, cytokine and inflammatory mediator production (Alencar et al., 2006; Ramos et al., 2009; Matos et al., 2013). Taking into consideration these previous observations, a protein fraction recovered from *P. pudica* latex was obtained and evaluated to exhibit anti-inflammatory and antinoceptive effects. In addition, the protein nature and the possible mechanisms of such actives were investigated.

Materials and methods

Latex and extraction of laticifer proteins (LP)

Plants of *Plumeria pudica* Jacq., Apocynaceae, growing around the neighbors of Parnaíba-PI, Brazil, were used as the source of fresh latex. The plant material was identified and the voucher N.52534 was deposited in the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil.

The latex was collected in distilled water to give a dilution rate of 1:2 (v/v) and the mixture was gently agitated during collection. Later, the samples were centrifuged at $3600 \times g$ for 15 min at 25 °C. The rubber-rich precipitate was removed and the supernatant was exhaustively dialyzed against distilled water using membranes of 8000 molecular weight cut-off. The dialysis water was renewed every four hours to flow out ions, salts and other small organic metabolites and again centrifuged using the conditions described above. The clean, rubber-free supernatant was lyophilized named laticifer proteins (LP) and used for further experiments. Concomitantly, the latex was collected in distilled water containing 3 mM iodoacetamide (IAA), and the LP_{IAA} fraction was obtained according to the procedures described above.

Treatment of LP through heat and dithiothreitol (DTT)

To provide evidence of the protein nature of the active molecules, LP fraction was dissolved in saline (0.9%) and heated at 100 °C for 30 min ($LP_{100\,°C}$) in the attempt to denature protein structure. We also dissolved LP in saline containing 3 mM DTT and incubated at 37 °C for 10 min (LP_{DTT}). LP_{DTT} was further used to investigate the possible involvement of latex cysteine proteinase-containing proteins.

Electrophoresis analysis of LP and LP treatments

LP and LP treatments (LP_{DTT}, LP_{IAA} and $LP_{100\,°C}$) were examined through 12.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) according to Laemmli (1970). Samples were treated with 0.0625 M tris buffer pH 6.8 containing 2% SDS and applied into the gel. Runs were performed at 40 mA at 25 °C. Gels were stained with 0.1% coomassie brilliant blue (R-350) solution in water:acetic acid:methanol (8:1:3.5). Unbound dye was removed with the same solution without the dye.

Proteolytic activity of LP and LP treatments on gelatin-containing gels

The samples were separated by means of electrophoresis through 12.5% polyacrylamide gels containing 0.1% gelatin at 25 °C (Macedo et al., 2004). After electrophoresis, the gels were immersed in water containing 2.5% of Triton X-100 and gently shaken for 30 min at 25 °C to wash out the SDS and re-nature proteins. The gels were then incubated in 50 mM PBS pH 6.0, for 2 h at 37 °C containing 3 mM DTT. The incubated gels were stained and revealed as before. Proteolytic activity was detected as a transparent band that was not stained by Coomassie Brilliant Blue.

Animals

Female Swiss mice (*Mus musculus*) weighing 20–30 g were obtained from the central animal house of the Federal University of Ceará, Brazil for the present study. Animals were housed in cages with free access to food and water and were maintained under a 12-h light–dark cycle (lights on at 6 am) at 25 °C. All experimental procedures were approved and performed in accordance with the guidelines of Institutional Animal Ethics Committee (Protocol N. 041/14).

Anti-inflammatory assays

Paw edema model

Paw edema was induced by carrageenan (500 μg/paw) or dextran (500 μg/paw) prepared in 0.9% sterile saline. A volume of 50 μl was injected into the right hind paw following each treatment. Paw volume was measured immediately before the irritant injection and at selected time intervals, with a plethysmometer (Panlab, Barcelona, Spain) (Winter et al., 1962). LP (1, 5, 10 and 40 mg/kg) or LP treatments ($LP_{100\,°C}$; LP_{DTT}; LP_{IAA}; 40 mg/kg) were injected intraperitoneally 1 h before injection of carrageenan or dextran. Control groups received only sterile saline or indomethacin (Indo: 10 mg/kg; *i.p.*). Results are expressed according to increase in paw volume (Δ ml) calculated by subtracting the basal volume.

Determination of myeloperoxidase activity

After the paw edema assay, myeloperoxidase (MPO) activity was measured in the paw of animals injected with carrageenan and submitted to LP and LP treatments at dose of 40 mg/kg. After 4 h of inflammatory stimulus administration, 50–100 mg of the tissue from the hind paws was collected and MPO activity was determined as previously described by (Bradley et al., 1982).

Paw edema induced by different inflammatory agents

Initially, mice were pre-treated with LP (40 mg/kg; *i.p.*). After 1 h, the animals received injections of 50 μl of histamine (50 ng/paw), serotonin (10 ng/paw), compound 48/80 (5 ng/paw), bradykinin (0.5 ng/paw) or prostaglandin E_2 (0.1 ng/paw) into the right hind paw. Paw volume was measured as described above. Control groups received only sterile saline or indomethacin (10 mg/kg; *i.p.*).

Peritonitis model

Carrageenan (500 μg) was injected intraperitoneally in 250 μl of sterile saline. Four hours later, mice were euthanized and the peritoneal cavity was washed with 1.5 ml of heparinized phosphate buffered saline (PBS) to harvest peritoneal fluid contained

Table 1
Recoveries of rubber and soluble proteins from latex of *Plumeria pudica*.

Dry mass of rubber		Dry mass of lyophilized LP		Soluble proteins in whole latex (mg/ml)[a]	Soluble proteins in LP (mg/ml)[a,b]
mg	%	mg	%		
561.10 ± 168.40	98.62 ± 0.32	7.80 ± 0.10	1.38 ± 0.36	0.417 ± 0.013	0.650 ± 0.040

Four independent samples of 20 ml of latex were prepared as described in Section 'Materials and Methods'. Values are given as mean ± S.E.M. Dry mass of lyophilized proteins (LP) was considered as the fraction free of rubber.
[a] Soluble proteins were estimated by Bradford.
[b] Measurement performed using 1 mg/ml solution of LP.

in cells. Total cell counts and differential cell counts were performed as described previously (Souza and Ferreira, 1985). LP (40 mg/kg) was injected via intraperitoneal route 1 h before injection of carrageenan. Control groups received only sterile saline or indomethacin (Indo: 10 mg/kg; *i.p.*). Results are presented as the number of neutrophils per ml of peritoneal exudates.

Measurements of IL-1β and TNF-α

After the peritonitis assay, samples of peritoneal fluid were collected and the levels of and IL-1β and TNF-α were evaluated using sandwich Enzyme-Linked Immunoabsorbent Assay (ELISA) (purchased from R&D Systems, Minneapolis, USA) according to the supplier's protocol. The results are expressed as picograms (pg/ml) of each cytokine per peritoneal cavity washed.

Antinociceptive assays

Abdominal contortions induced by acetic acid

Mice were injected *i.p.* with 0.6% acetic acid (10 ml/kg body weight). After 10 min, a recording of the number of constrictions was initiated, which lasted for 20 min, as it has been previously described (Koster et al., 1959). In the experimental group, the animals were pretreated (*i.p.*) with LP (40 mg/kg) 1 h before the administration of acetic acid. The control animals were injected with sterile saline (0.5 ml). Morphine (5 mg/kg, subcutaneously) was used as a reference drug.

Formalin test

Animals were pretreated with either LP (40 mg/kg, *i.p.*) or morphine (5 mg/kg, *s.c.*) 1 h before the administration of 20 μl of 2.5% formalin into the right hind paws of the mice. The licking time was determined in two time ranges, from 0 to 5 min (phase 1, neurogenic) and from 20 to 25 min (phase 2, inflammatory), after the intraplantar formalin injection, according to Hunskaar et al. (1985). Control animals were injected with sterile saline (0.5 ml).

Hot-plate test

For the hot-plate test, mice were submitted to a plate heated to 55–56 °C according to the methodology of Eddy and Leimbach (1953), with some modifications. Mice were treated with a LP dose of 40 mg/kg (*i.p.*), and the control group received sterile saline (0.5 ml). Measurements were performed at time zero (0 min) and at 30, 60, 90 and 120 min after the protein fraction administration. The hot plate cut-off time was 45 s to avoid animal paw lesions. Morphine (5 mg/kg, *s.c.*) was used as a reference drug.

Statistical analysis

The results are given as the means ± S.E.M. of $n = 5$. For the peritonitis model experiments and cytokine measurements, the statistical analysis was performed through ANOVA followed by Bonferroni's test. For all the other experiments, the statistical analysis was performed through ANOVA followed by Newman Keuls tests. $p < 0.05$ was defined as statistically significant. Data were analyzed using GraphPad Prism 5 software.

Results

After collected, the latex of *P. pudica* was submitted to centrifugation and dialysis steps to remove rubber and water-soluble low molecular compounds. These procedures rendered a rich protein fraction (LP) which comprises only 1.38% of the latex dry mass (Table 1). The whole content of the latex is remarkably composed by rubber, representing 98.62% of dry mass. Soluble protein content in LP measured through Bradford's method showed that the dialysis step was efficient in concentrate proteins from whole latex. Water-soluble low molecular substances were not recovered and were not included in the estimation of the latex dry mass.

In order to investigate the anti-inflammatory activity of LP, mice paw edema was induced by administration of carrageenan into the animal's right hind paw. Since peak inflammation induced by carrageenan occurs between 3 and 4 h after injection (Souza et al., 1988), paw edema formation was evaluated at these time intervals. The carrageenan administration promoted significant increase on mice paw edema when compared to control animals treated only with saline (Fig. 1). No statistical differences were observed among control carrageenan-group and LP-treated animals at doses of 1 or 5 mg/kg ($p > 0.05$). On the other hand, the administration of doses 10 or 40 mg/kg of LP prior to inflammatory stimuli reduced significantly the edematogenic effect promoted by carrageenan ($p > 0.05$). The highest inhibition rate was seen when animals received 40 mg/kg dose (72.7% and 78.7%; 3rd and 4th hour, respectively). As expected, indomethacin (10 mg/kg) inhibited significantly the paw edema. Because 40 mg/kg dose exhibited higher inhibition rate, it was chosen for further evaluations.

Latex proteins were submitted to chemical (DTT and IAA) or physical (100 °C) treatments and the effects of different treatments upon anti-edematogenic activity were evaluated. As observed in Fig. 1, LP_{DTT} and LP_{IAA} administered via intraperitoneal route still inhibited the increase in the animals' paw volume at evaluated time intervals ($p < 0.05$). No statistical differences were observed comparing LP_{DTT} and LP_{IAA} groups to LP-treated animals at dose of 40 mg/kg ($p > 0.05$). However, the heat treatment of LP for 30 min at 100 °C prior to injection in mice significantly reduced the anti-edematogenic effect of LP ($p > 0.05$).

After the paw edema measurements, the myeloperoxidase activity (MPO) was evaluated in mice's paw tissue. As observed in Fig. 2, carrageenan administration provoked an increase in MPO activity (9.904 ± 2.006 UMPO/mg of tissue) compared to saline group (1.475 ± 0.372 UMPO/mg of tissue). Significant reduction of MPO activity was observed in mice injected with LP (2.151 ± 0.639 UMPO/mg of tissue) 1 h prior to carrageenan and this effect was also observed in groups treated with LP_{DTT} (4.678 ± 1.281 UMPO/mg of tissue) or LP_{IAA} (2.016 ± 0.613 UMPO/mg of tissue). On the other hand, heat-treated LP did not reduce significantly myeloperoxidase activity (7.965 ± 0.876 UMPO/mg of tissue).

To verify the effect of chemical and physical treatments on protein content in LP, latex proteins and their treatments were evaluated through electrophoresis in gel of polyacrylamide and zymogram in gelatin-containing gels (Fig. 3a and b). Electrophoresis

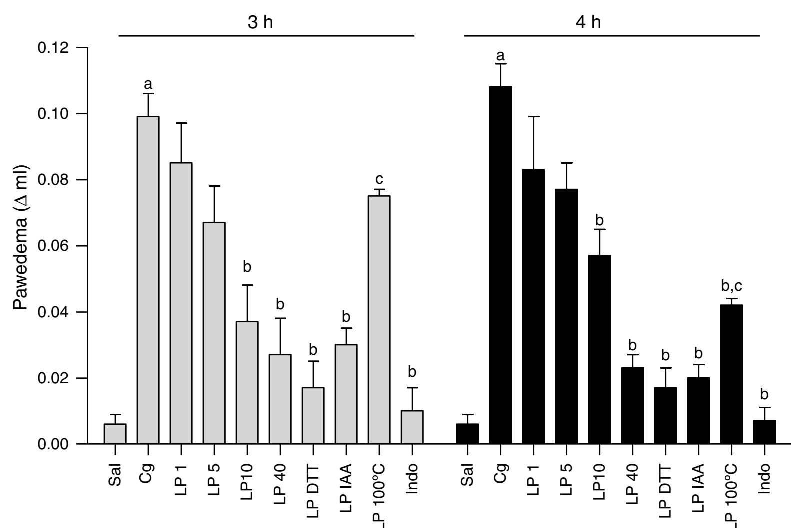

Fig. 1. The inhibitory effect of LP from *Plumeria pudica* and LP treatments on paw edema induced by carrageenan. Animals received LP (1, 5, 10 or 40 mg/kg; *i.p.*) or LP treatments (40 mg/kg; *i.p.*) one hour before the carrageenan (Cg) administration (500 µg/paw), and paw edema changes were measured at indicated time intervals. The values are given as the mean ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg, *i.p.*) was used as a positive control. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively. Letter "c" indicates difference ($p > 0.05$) compared to LP 40 mg/kg (ANOVA followed by Neuman Keuls post-test).

revealed that LP possesses proteins with molecular weight ranging from 14.0 to 45.0 kDa with a predominant band of around 22.0 kDa. Similar protein profile was observed in LP_{DTT}. However, LP submitted to IAA and heat treatments exhibited marked changes in protein content (Fig. 3a). Heat treatment drastically reduced the presence of proteins in LP. Zymogram analysis showed that both IAA and heat treatment abolished proteolytic activity found in LP (Fig. 3b).

The LP fraction was also evaluated to inhibit paw edema induced by dextran (Fig. 4). The administration of dextran into the paw of animals was accompanied by intense edema when compared to control treated with saline. The pre-treatment of animals with LP (40 mg/kg) inhibited the peaks of inflammatory effect of dextran by 51.5% and 93.0% at 30 min and 1 h, respectively. Inhibition rate of 84.5% (30 min) and 87.8% (1 h) were observed for animals injected with indomethacin.

The effect of LP upon paw edema induced by different flogistic agents was further investigated. As shown in Fig. 5, the injection of various inflammagens into the subplantar surface of the mouse's hind paw produced a noticeable increase in paw volume when compared to the saline group ($p < 0.05$). At 30 min, the time in which the peak of the agents tested is observed, the edema volume

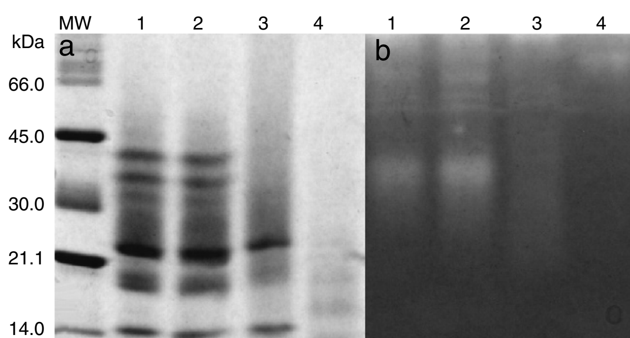

Fig. 3. Polyacrylamide gel (12.5%) electrophoresis of LP and LP treatments (a) and corresponding zymogram showing proteinase activity upon gelatin (0.1%) (b). 1: LP; 2: LP_{DTT}; 3: LP_{IAA} and 4: $LP_{100\,°C}$. Molecular weight markers (MW) were as follows: albumin (66.0 kDa); ovalbumin (45.0 kDa); carbonic anhydrase (30.0 kDa); trypsin inhibitor (20.1 kDa); and lactalbumin (14.4 kDa).

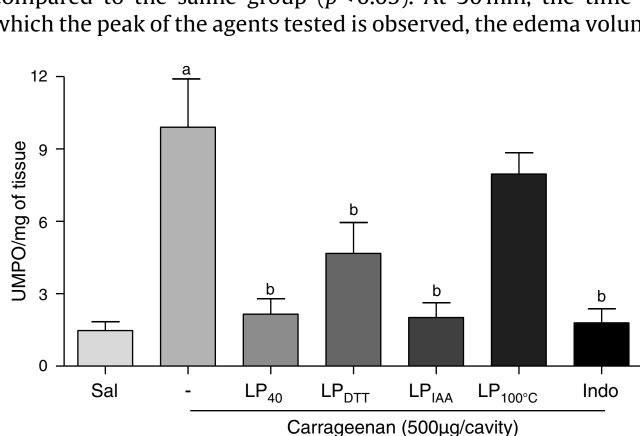

Fig. 2. The inhibitory effect of LP and LP treatments on carrageenan-induced myeloperoxidase activity in mice paws tissue. Animals received LP or LP treatments (40 mg/kg; *i.p.*) one hour before the carrageenan administration (500 µg/paw), and 4 h later, the myeloperoxidase activity was evaluated. The values are given as the mean ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg, *i.p.*) was used as a positive control. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively (ANOVA followed by Neuman Keuls post-test).

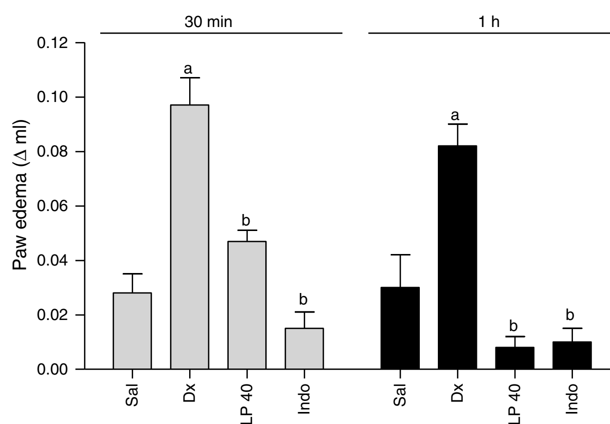

Fig. 4. Anti-inflammatory activity of LP from *Plumeria pudica* on paw edema induced by dextran. Animals received LP (40 mg/kg; *i.p.*) one hour before dextran (Dx) administration, and paw edema changes were evaluated at indicated time intervals. The values are given as the mean ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg, *i.p.*) was used as a positive control. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively (ANOVA followed by Neuman Keuls post-test).

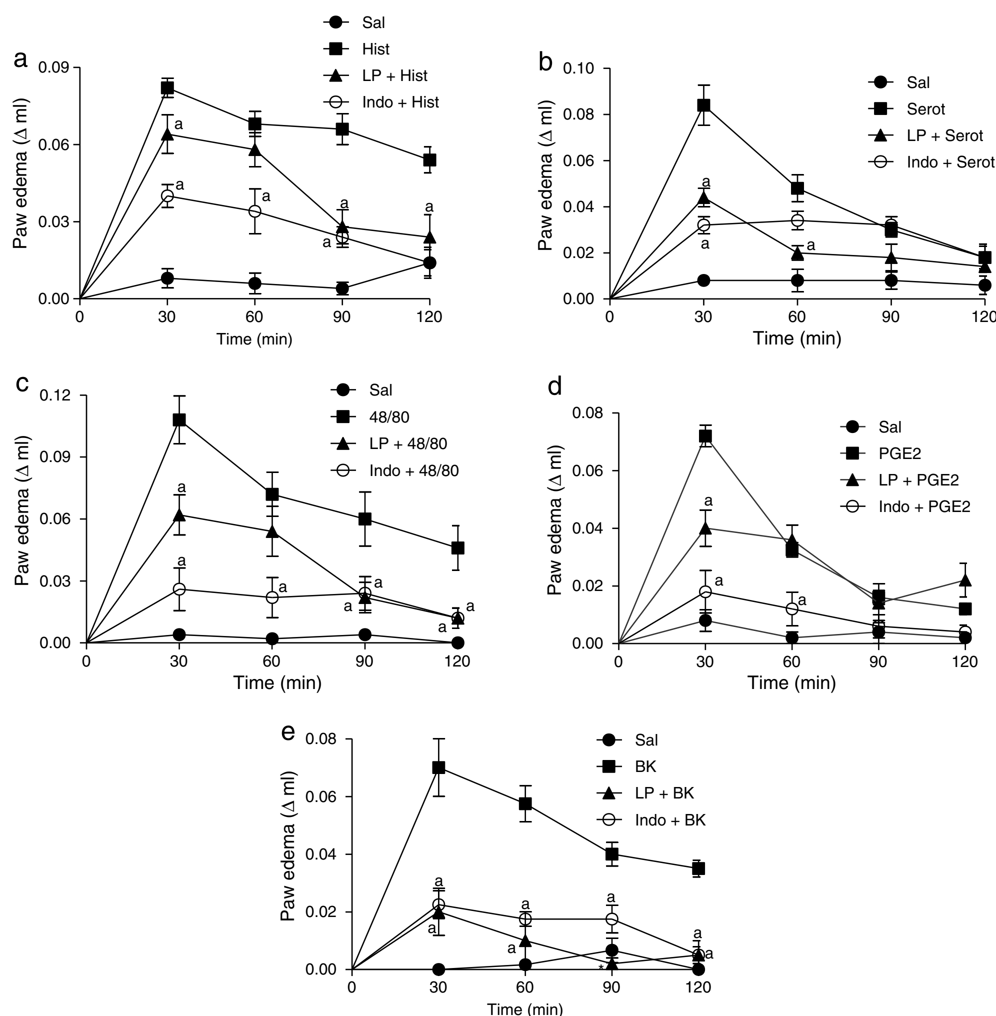

Fig. 5. Effect of LP from *P. pudica* on paw edema induced by various inflammagens. Paw edema was induced by (a) histamine (Hist), (b) serotonin (Serot), (c) compound 48/80 (48/80: 14 µg), (d) bradykinin (BK) or (e) prostaglandin E_2 (PGE_2) injections into the plantar right paw. The change in paw volume was measured at indicated intervals of time. Animals were pretreated with LP (40 mg/kg; *i.p.*) and indomethacin (Indo: 10 mg/kg, *i.p.*) was used as a positive control. The values are given as the mean ± S.E.M. ($n = 5$). Letter "a" indicates statistical difference ($p < 0.05$) compared to inflammatory stimuli treatment (ANOVA followed by Neuman Keuls post-test).

in LP group was 0.043 ± 0.010 Δ ml against 0.067 ± 0.006 Δ ml in the histamine group, corresponding to an inhibition rate of 35.5% (Fig. 5a). A marginal inhibition rate was observed against inflammation induced by prostaglandin E_2 (15.5%) (Fig. 5d). LP fraction, however, was more effective in inhibiting the paw volume induced by the other evaluated inflammagens. Inhibition rates of 53.3%, 47.5%, 81.73% were obtained for serotonin, compound 48/80, and bradykinin, respectively (Fig. 5b, c and e). Indomethacin also significantly inhibited all the paw edema ($p < 0.05$).

The anti-inflammatory activity of LP was also confirmed on peritonitis model and the data obtained corroborated to MPO measurements (Fig. 6). It was observed that animals treated with LP (40 mg/kg) 1 h before intraperitoneal injection of carrageenan exhibited a significant reduction in total leukocyte migration (1970 ± 0.354 cells × 10^3 ml) and neutrophil count (1244 ± 0.187 cells × 10^3 ml) when compared to carrageenan-treated animals (13,220 ± 2610 total leukocytes × 10^3 ml and 11,410 ± 2392 neutrophils × 10^3 ml). Indomethacin also inhibited cell migration to peritoneal cavity of animals ($p < 0.05$).

The peritoneal cell-migration inhibition displayed by LP was accompanied with the reduction of levels of pro-inflammatory cytokines in the mice's peritoneal fluid (Fig. 7). The peritoneal fluid amount of IL1-β and TNF-α in carrageenan-treated animals was 1995.0 ± 13.19 pg/ml and 202.2 ± 19.97 pg/ml, respectively and significantly decreased in LP-treated mice (IL1-β: 1306.0 ± 189.2 pg/ml and TNF-α: 94.9± 12.73 pg/ml). As expected, indomethacin significantly reduced cytokine levels in the animals' peritoneal fluid ($p < 0.05$).

Furthermore, the LP fraction was studied in three distinct experimental models of nociception. In the acetic acid-induced writhing model, the LP showed a strong antinociceptive effect (Fig. 8). The administration of LP (40 mg/kg) 30 min prior to stimuli significantly reduced the acetic acid-induced abdominal writhings (88.1% inhibition) compared to control group ($p < 0.05$). The reference drug morphine inhibited the abdominal constrictions by 100%.

The LP also exhibited analgesic effect in the formalin test (Fig. 9). The intraplantar injection of formalin increased significantly the licking time at first and second phases compared with saline group ($p > 0.05$). Treating the animals with a 40 mg/kg dose of LP prior formalin injection reduced the paw licking time in the first phase (64.2%; $p < 0.05$). However, no statistical significance was observed with the control in the second phase (24.5%; $p > 0.05$). The reference drug morphine significantly inhibited the formalin-induced paw licking in both phases.

Fig. 10 shows the antinociceptive effect of LP measured in mice during the hot-plate test. At this model, administration of LP via the *i.p.* route did not increase the reaction time at the different intervals tested, when compared to the control ($p > 0.05$).

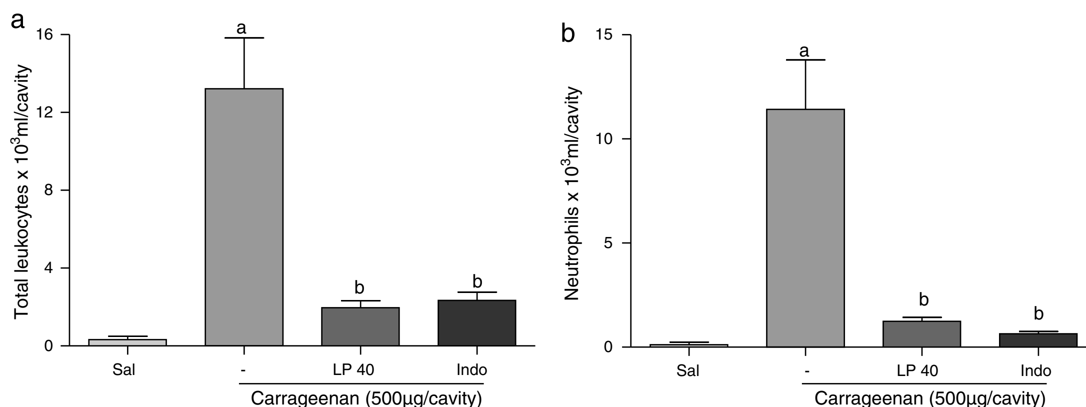

Fig. 6. The inhibitory effect of LP from *Plumeria pudica* on cell migration induced by carrageenan in peritonitis model. Mice received LP fraction (40 mg/kg; *i.p.*) 1 h before an *i.p.* injection of carrageenan, and the total leucocytes (a) and neutrophil migration (b) were counted in the peritoneal fluid four hours later. The values are given as the mean ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg) was used as a positive control for the anti-inflammatory activity. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively (ANOVA followed by Bonferroni's post-test).

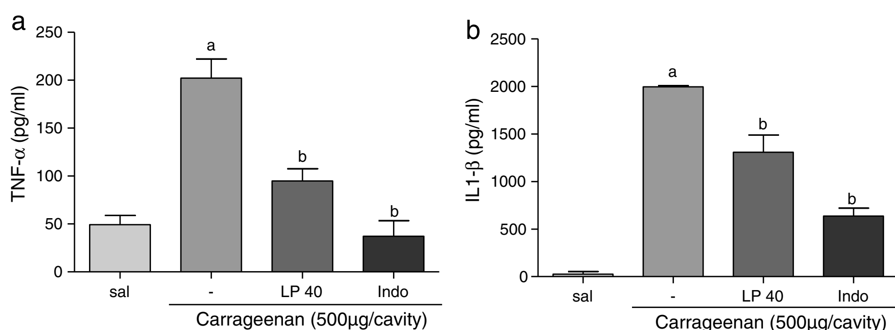

Fig. 7. The inhibitory effect of LP from *Plumeria pudica* on carrageenan induced cytokine production in peritonitis. Mice received LP fraction (40 mg/kg; *i.p.*) one hour before an *i.p.* injection of carrageenan, and 4 h later the levels of TNF-α (a) and IL-1β (b) were measured in the peritoneal fluid. The values are given as the mean ± S.E.M. ($n = 5$). Indomethacin (Indo: 10 mg/kg) was used as a positive control for the anti-inflammatory activity. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and carrageenan, respectively (ANOVA followed by Bonferroni's post-test).

Discussion

An increasing number of scientific investigations have been reported dealing with pharmacological properties found within plant latex such as anti-inflammatory, analgesic and antitumor activities. Some of these studies were developed with a water-soluble fraction and it was demonstrated that proteins are involved in such activities (Ramos et al., 2009; Oliveira et al., 2007; Mousinho et al., 2011). Besides investigating the presence of active proteins in pharmacological models, the authors obtained a

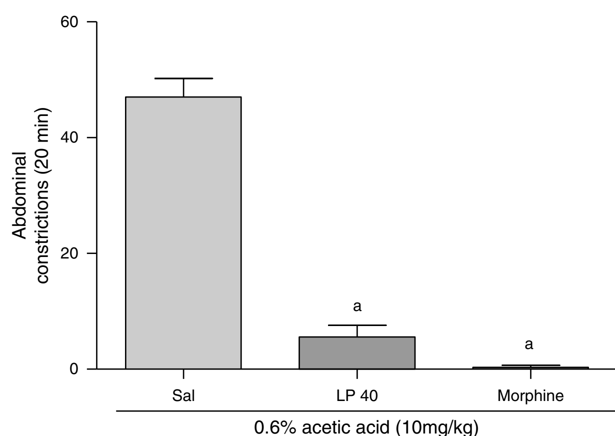

Fig. 8. Antinociceptive effect of LP from *Plumeria pudica* on acetic acid induced writhes. Mice received LP (40 mg/kg; *i.p.*) one hour before an *i.p.* injection of 0.6% acetic acid (10 ml/kg body weight) and abdominal constrictions were count for 20 min. The values are given as the mean ± S.E.M. ($n = 5$). Morphine (5 mg/kg, *s.c.*) was used as a positive control for the antinociceptive activity. Letter "a" indicates statistical difference ($p < 0.05$) compared to acetic acid group (ANOVA followed by Neuman Keuls post-test).

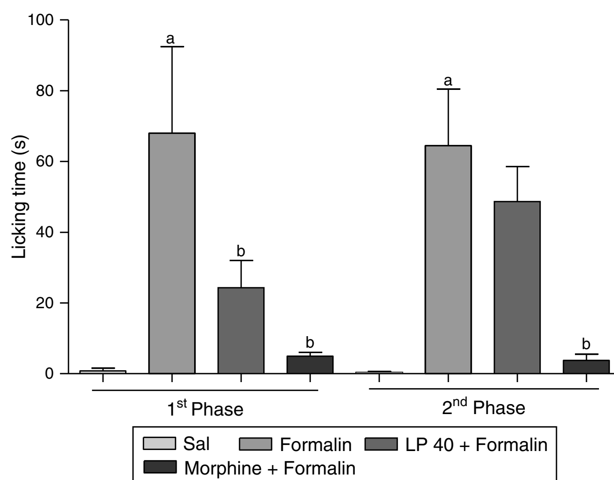

Fig. 9. Antinociceptive effect of LP from *Plumeria pudica* in the formalin induced paw licking. Animals received LP (40 mg/kg) 30 min before a 2.5% formalin administration by intraplantar rout. Licking time was recorded in the first 5 min (1st phase) and after 20 min (2nd phase) during 5 min. Each column represents the mean ± S.E.M. ($n = 5$). Morphine 5 mg/kg, *s.c.* was used as a positive control for the antinociceptive activity. Letters "a" and "b" indicate statistical difference ($p < 0.05$) compared to saline and formalin, respectively (ANOVA followed by Neuman Keuls post-test).

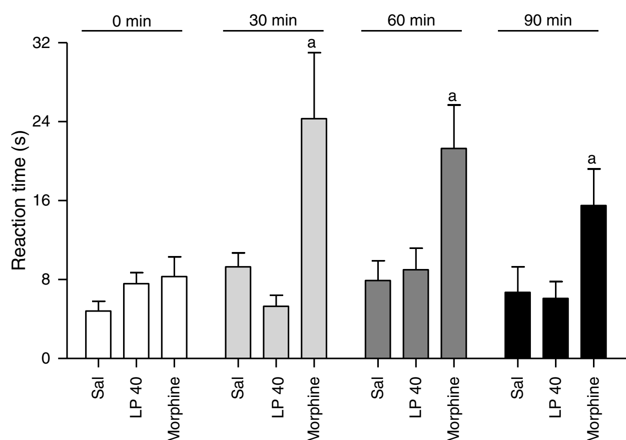

Fig. 10. Antinociceptive effect of LP from *Plumeria pudica* in the hot-plate test. Mice received LP (40 mg/kg; *i.p.*) and one hour later submitted to a plate heated to 55–56 °C. The reaction time was recorded at indicated time intervals. The values are given as the mean ± S.E.M. ($n = 5$). Morphine (5 mg/kg, *s.c.*) was used as a positive control for the antinociceptive activity. Letter "a" indicates statistical difference ($p < 0.05$) compared to acetic acid group (ANOVA followed by Neuman Keuls post-test).

significant advance concerning the identification of active molecules and the mechanism of action. This was the main idea behind the present study. The latex of *P. pudica*, which is used for therapeutic treatment in the folk medicine, was evaluated for its anti-inflammatory and analgesic effects and to detect active proteins.

The paw edema is an experimental animal model for acute inflammation, which is widely used for screening new anti-inflammatory drugs (Winter et al., 1962). Herein, it was demonstrated that LP was able to inhibit the edematogenic effect promoted by carrageenan or dextran. It is well established that the inflammatory process triggered by carrageenan involves two distinct phases. The earlier is sustained by a release of histamine, serotonin from mast cells, while the late phase involves prostaglandin release, cytokine production and intense neutrophil migration (Vinegar et al., 1969; Kulinsky, 2007). On the other hand, the inflammation promoted by the administration of dextran leads to the development of edema as a consequence of the release of histamine and serotonin by resident mast cells on an edema characterized by few neutrophil migrations (Lo et al., 1982; Metcalfe, 2008). Taking into account the results obtained, it is suggested that anti-inflammatory effect of LP on paw edema model seems to be related to the inhibition of neutrophil migration into the inflammatory site, as well as the blockage of the release of inflammatory mediators.

During the early phase of the inflammatory process, several mediators are released and they will act on the vascular endothelium promoting fluid and protein extravasations into the inflammatory site (Di Rosa et al., 1971). This process involves the action of chemical inflammatory mediators such as histamine, serotonin, bradykinin and PGE_2 (Vinegar et al., 1969, 1982). The protein fraction recovered from *P. pudica* was able to block the edema induced by the administration of histamine, serotonin, bradykinin and PGE_2. Our data indicate that LP neutralizes the vascular component of edema, which involves a reduction in the action of inflammatory mediators. This idea was reinforced since LP also inhibited edema induced by compound 48/80, which is known to release endogenous histamine and serotonin stored in the mast cells (Irman-Florjanc and Erjavec, 1983).

The anti-inflammatory effect of LP on paw edema model induced by carrageenan was also accompanied by the inhibition of myeloperoxidase activity in paw tissue. MPO is an enzyme found in neutrophil azurophilic granules (Bradley et al., 1982) and the reduction of its activity in LP-treated animals indicates inhibition of infiltration of this cell into the tissues. These data were further confirmed by a significant reduction of cell migration into the peritoneal cavity of animals submitted to LP treatment prior to carrageenan administration. The carrageenan induces neutrophil migration into the peritoneal cavity through an indirect mechanism that involves the activation of macrophages and the release of pro-inflammatory cytokines, such as IL-1β and TNF-α (Lo et al., 1982). Such increases in cytokine levels might result in plasma protein extravasations and cellular infiltration into the site of inflammation (Rosenbaum and Boney, 1991; Thorlacius et al., 1997). In agreement with that, it was demonstrated that the levels of IL-1β and TNF-α were drastically reduced in LP-treated animal proposing that LP decreased neutrophil migration by decreasing the production and release of pro-inflammatory cytokines.

Experimental studies have demonstrated that the inhibition of neutrophil migration reduces hypernociception induced by different inflammatory stimuli (Levine et al., 1984; Hopkins, 2003; Verri et al., 2004). The LP fraction reduced cell migration due to the inhibition of inflammatory mediator release and cytokine production. These combined events have also been described with other latex materials (Prabha et al., 2008). This fact is very important because pain is a classical sign of inflammation and one of the main reasons that lead people to seek treatment. In our experiments, the LP fraction was able to reduce significantly writhing induced by acetic acid. This test is commonly used for screening peripherally active analgesic compounds and involves different nociceptive mechanisms, such as release of biogenic amines, bradykinin, and PGE_2 (Collier et al., 1968; Duarte et al., 1988). Furthermore, the nociceptive response caused by acetic acid is also dependent on the release of some cytokines, such as TNF-α and IL-1β via modulation of macrophages and mast cells located in the peritoneal cavity (Ribeiro et al., 2000). According to this information, the LP effect may be related to the inhibition of the release of nociceptive mediators in response to acetic acid, such as those described above.

To verify the effectiveness of the analgesic effect of LP, we used the formalin test. The formalin test involves two phases. The first one is characterized by releasing of intense noxious mediators, such as substance P and bradykinin, promoted a direct chemical stimulation of the nociceptors involved with painful state (Shibata et al., 1989). The second phase is accompanied by the release of inflammatory mediators (neuropeptides, prostaglandins, serotonin, histamine and bradykinin) and these mediators stimulated the periferic nociceptive nervous by an indirect mechanism (Hunskaar et al., 1985; Murray et al., 1988). The administration of LP during the formalin test promoted pronounced antinociception only in the first phase of the model. Thus, based on our results, we can infer that LP promotes peripheral analgesia by blocking directly the action of bradykinin and other mediators on nociceptive neurons.

Although antinociception in the hot-plate test has already been described with the latex collected from other plants (Soares et al., 2005; Prabha et al., 2008), the LP fraction from *P. pudica* was not capable to promote an increase in the animals' reaction time. This model is performed to evaluate central nociception (Vilela et al., 2009) indicating analgesic effects via supraspinal and spinal receptors (Nemirovsky et al., 2001). Since no positive effect was observed at this model, we concluded that latex proteins cannot inhibit nociception by central mechanisms.

In the present work we have demonstrated the presence of proteins in a water-soluble fraction recovered from *P. pudica* latex with pharmacological properties. Our results strongly suggest the involvement of proteins in the effects observed. This idea was supported by the observation that after treating LP at 100 °C for 30 min, the LP anti-edematogenic effect was significantly reduced and this

effect was accompanied by an increment in MPO activity in animals' paw tissue. In parallel, a drastic reduction of soluble proteins in $LP_{100\,°C}$ was seen through electrophoresis, indicating that the heat treatment promoted a reduction in protein solubility, which can interfere in their activities. Similar results were observed in protein fractions recovered from latex of *Calotropis procera* and *Himatantus drasticus*. After treating proteins with heat, their anti-inflammatory activities were abolished (Alencar et al., 2004; Matos et al., 2013).

Some publications have reported the anti-inflammatory activity promoted by plant cysteine proteinases on experimental animals (Hale et al., 2005; Eric et al., 2007; David et al., 2008). To investigate the involvement of this class of proteins in LP, this fraction was submitted to DTT treatment (LP_{DTT}), an activator of cysteine proteinase, or recovered after collecting the latex in the presence of IAA (LP_{IAA}), a non-natural cysteine proteinase inhibitor. The presence of white bands on zymography evidenced that DTT treatment activated proteolytic activity in LP while IAA provoked its inhibition (Fig. 3). No changes were seen on anti-inflammatory efficacy of LP treated with DTT or IAA. These data evidence that cysteine proteinases activity is not related to the investigated activity promoted by LP.

In summary, our results showed that LP exhibits anti-inflammatory and antinociceptive effects by inhibiting the action of various inflammatory mediators, neutrophil infiltration and pro-inflammatory cytokines. Proteins present in LP are involved in the activities evaluated. Our results support latex as a rich source of molecules with interesting properties in inflammatory pharmacological models. LP is now a target of fractionation by chromatography steps in order to purify and identify the molecule responsible for evaluated activities. This was the first work developed investigating anti-inflammatory and nociceptive properties in protein latex content recovered from *P. pudica*.

Author contributions

HBF, DLM, ACTCP, GPF collected the latex to obtain LP and carried out some experiments of inflammation and pain. JMD, TVB, JAB, ROS performed paw edema, MPO measurements and peritonitis assays. ALRB and JVRM were responsible for nociceptive evaluations. MVR performed enzymatic activity and was the supplier of animals. KSA and RAR evaluated the level of cytokines on peritoneal fluids of animals. JSO was the leader of the project and wrote the paper. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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