



Original Article

Anti-inflammatory action of *Justicia acuminatissima* leaves

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ABSTRACT

In Amazonas State (Brazil), *Justicia acuminatissima* (Miq.) Bremek., Acanthaceae, leaf teas are used in folk medicine to treat several inflammatory illnesses. In order to validate this medicinal application, we analyzed the acute toxicity and antioxidant, antiedematogenic and antinociceptive potentials of an aqueous extract of this species, using culture cells and animal models. The aqueous extract did not cause toxic effects on human lymphocytes in high concentration (400 µg/ml), neither on mice treated with high doses (5000 mg/kg) in an acute toxicity analysis by oral route, and also did not cause lesions in the gastric mucosa of animals treated with 300 mg/kg, which was the maximal dose used in the anti-inflammatory screening. The aqueous extract caused inhibition of inflammatory pain in formalin-induced paw licking test with all tested doses, 30, 100 and 300 mg/kg, and antiedematogenic activity at 100 and 300 mg/kg. Additionally, the aqueous extract presented statistically significant action on the release of nitric oxide by lipopolysaccharide-activated macrophages. These results and other preliminary studies support the folk use of this species, and further investigation of its action mechanism by inhibition of COX-2 or related metabolite would be interesting.

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Introduction

Inflammation plays a crucial role in the pathogenic mechanisms of various diseases, since it is triggered by various stimuli of physical, chemical and biological origin (Ganesh, 2014). Two of the most prominent molecular mechanisms implied in the inflammatory response, among many others, are the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the release of prostaglandins by cyclooxygenases (COX). While the use of iNOS inhibitors as anti-inflammatory medicines still requires more studies (Rochette et al., 2013), non-steroidal anti-inflammatory drugs (NSAIDs) are an important group of drugs largely utilized in treatment of inflammatory diseases, acting by inhibition of COX enzymes (Blackler et al., 2014). However, the use of these medicines is frequently related with disorders on gastric mucosa, as well as the renal and cardiovascular systems (Harirforoosh et al., 2013).

Plant extracts are valuable leads for multi-target drugs due the interplay of their multiple components. It becomes more evident that, for complex diseases like inflammation, an interference with

multiple targets is superior to targeting a single key factor regarding drug efficiency, side effects and adverse compensatory mechanisms (Koeberle and Werz, 2014). Indeed, various research groups have studied different plants used in traditional medicine for the discovery of new anti-inflammatory drugs, which are more safe and efficient.

In traditional medicine around the world, the largest genus of Acanthaceae family, *Justicia*, is used for treating various health complications, including toothache, heart diseases, gastrointestinal disturbances and even AIDS. In Brazil, different species are used for treating pain, fever, respiratory problems and headaches, and many pharmacological properties were scientifically proven, including anti-inflammatory activity (Côrrea and Alcântara, 2012). On the other hand, leaf tea of *Justicia acuminatissima* (Miq.) Bremek., a native subshrub of the Brazilian Amazon rainforest, known as “sara-tudo”, is usually used in folk medicine to treat inflammatory illness in the Amazonas State, although its pharmacological potential was not known until recently.

A study investigated phytochemically and pharmacologically a few organic extracts and substances obtained from this species (Côrrea, 2013). However, the anti-inflammatory potential and the possible toxic effects of an aqueous extract after oral consumption, mimicking traditional use, remained unknown. Therefore,

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this study aims to investigate the anti-inflammatory potential of *J. acuminatissima* aqueous extract by oral route in order to validate its popular use. We analyzed cytotoxic effects on human lymphocytes, acute toxicity on experimental animals and a possible ulcerogenic effect after oral administration to evaluate the safe of its use. We analyzed its inhibition capacity of the release of NO by human macrophages stimulated by lipopolysaccharides, and also the analgesic and antiedematogenic activities in experimental animals in order to formulate the preliminary considerations about its alleged anti-inflammatory potential.

Materials and methods

Plant material

The collection of samples was authorized by the Managing Council of the Genetic Patrimony (CGEN), chaired by the Ministry of Environment (Brazil), under the registration 034/2008. Leaves of *Justicia acuminatissima* (Miq.) Bremek., Acanthaceae, were collected at Careiro Castanho, 100 km south of Manaus, capital of Amazonas state, Brazil. A voucher specimen (224414) was deposited at the Herbarium of Instituto Nacional de Pesquisas da Amazônia, after identification and taxonomic authentication by José Lima.

Preparation of the extract

The leaves were dried at 40 °C in a stove for 72 h (WHO, 2003), and then scraped and pulverized using a mechanical grinder. The powder was macerated with distilled water under agitation for 72 h (10% aqueous extract). The extract, named PMFA in this work, was then vacuum-filtered, lyophilized and conserved 2–8 °C for further use.

Cell culture

Human lymphocytes were isolated using a gradient of Ficoll – Histopaque (1 blood: 1 ficoll) from peripheral venous blood (5 ml) collected in heparinized vials from one normal, healthy donor, with no history of smoking/drinking or chronic use of medication. J774 murine macrophage cells were obtained from Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil, and cultured at 37 °C in a humidified incubator with 5% CO₂ in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma).

Animals

Male and female Swiss mice (30 ± 5 g) and Wistar rats (160–220 g) were maintained in a temperature-controlled room (23 ± 2 °C) with a 12/12 h light–dark cycle and acclimatized in the experimental environment for at least 24 h before the tests. Water and a balanced diet were continually provided *ad libitum*, but food was withdrawn 12 h before the tests. The tests were performed in accordance with the Conselho Nacional de Controle da Experimentação Animal and were authorized by the Ethical Committee on the Use of Animals of Universidade Federal do Amazonas, under registration 002/2015.

Cytotoxicity on human lymphocytes

Peripheral blood lymphocytes (PBL) were seeded in 96-well plates (10⁵ cells per well), and were treated with PMFA, and the Alamar Blue™ assay was performed on them (Ahmed et al., 1994). PMFA (400–6.25 µg/ml) was dissolved in dimethyl sulfoxide (DMSO), added to each well, and was incubated for 72 h. Doxorubicin was used as positive control. Control groups received

the same amount of DMSO (0.1%). Four hours before the end of the incubations, 10 µl of Alamar Blue™ was added to each well. The fluorescent signal was monitored using a multiplate reader using 530–560 nm excitation wavelength and 590 nm emission wavelength. The fluorescent signal generated from the assay was proportional to the number of living cells in the sample.

Acute toxicity

Six mice (three male and three female) received orally the maximal dose of 5000 mg/kg of PMFA dissolved in fresh water. Two animals (1 male and 1 female) were used as negative control and received 10 ml/kg of fresh water. Abnormal morphological and behavior signs of toxic/pharmacological effects (Hippocratic screening) were observed during the first 4 h after administration of extract and at every 24 h for 14 days (Malone and Robichaud, 1962).

Ulcerogenic effect

Three different groups of six rats (three male and three female) received orally 300 mg/kg of PMFA, 10 mg/kg of indomethacin (positive control) and 10 ml/kg of fresh water (vehicle, negative control). The animals were then anesthetized and sacrificed by cervical dislocation. Their stomachs were removed and opened along the great curvature, and their inner surface was examined in order to look for gastric lesions (Lapa et al., 2008).

NO production assay

After the pre-incubation of J774 murine macrophages (1 × 10⁵ cells/ml) with different concentrations of PMFA for 2 h, the cells were washed and incubated for 24 h either with or without bacterial lipopolysaccharide (LPS, 1 µg/ml) at 37 °C in a 5% CO₂ incubator. NO was measured as nitrite (NO₂[−]), in the culture supernatant by reaction with Griess reagent, followed by measuring at 532 nm using a microplate reader (DTX 800, Beckman Coulter®, California, EUA). Sodium nitrite was used as a standard to calculate nitrite concentrations (Verma et al., 2012).

Formalin-induced paw licking test

Groups of six mice (three male and three female) received 30, 100 and 300 mg/kg of PMFA, 10 mg/kg of indomethacin (positive control 1), 50 µl/kg of fentanyl (positive control 2) and 10 ml/kg of fresh water (vehicle, negative control), all orally administered. One hour after, formalin at 2.0% was injected in the right hind paw of all the animals, and the duration of paw licking was counted in periods of 0–5 min (early phase) and 15–30 min (late phase). The results were compared with the negative control (Lapa et al., 2008).

Carrageenan-induced paw edema

Doses of 30, 100 and 300 mg/kg of PMFA in fresh water were administered orally in different groups of six rats (three male and three female). Two other groups received indomethacin at 10 mg/kg (positive control) and water (negative control). After 1 h, 0.3 ml of λ-carrageenan 2% in normal saline was injected in the right hind paw of all animals, and the paw edema progression was measured using a digital plethysmometer, in hourly intervals, for 5 h (Winter et al., 1962).

Statistical analysis

The results were expressed as the mean ± standard deviation. The results were compared by one or two-way analysis of variance

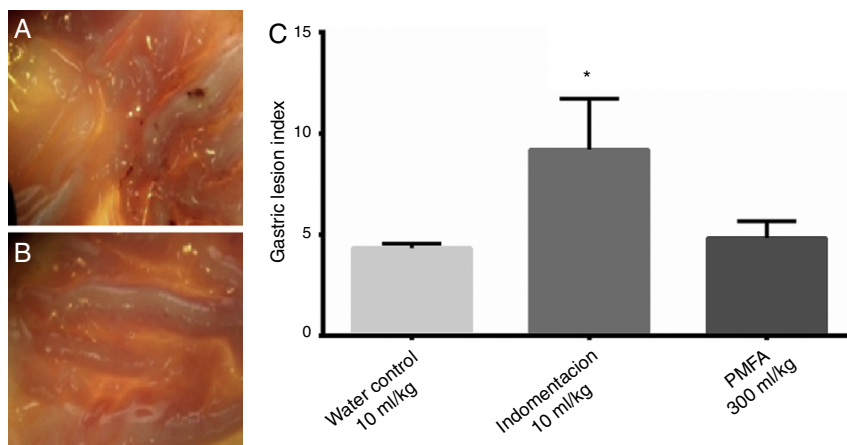


Fig. 1. Effect of aqueous extract of *Justicia acuminatissima* on the gastric mucosa of rats. (A) Gastric mucosa damage after indomethacin administration at 10 mg/kg. (B) Gastric mucosa after administration of the extract PMFA at 300 mg/kg. (C) Comparison of the highest concentration of PMFA analyzed and positive and negative controls analyzed by one-way analysis of variance (ANOVA)/Dunnett's test, *p*-values versus negative control: **p* < 0.05.

(ANOVA) using GraphPad Prism6® software (San Diego, California, USA), considering values of *p* < 0.05 statistically significant.

Results and discussion

We did not observe any cytotoxic effects in human lymphocytes exposed to maximal dose of 400 µg/ml of PMFA during 72 h. In addition, the animals exposed to very high dose of PMFA (5000 mg/kg *p.o.*) did not present toxicity signs and no death occurred during Hippocratic screening. These results suggested that the use of *J. acuminatissima* leaf tea by oral route is safe, although toxicity after long time exposition should be further investigated. Thus, three spaced doses, 30, 100 and 300 mg/kg, were selected for analysis of their anti-inflammatory potential.

Considering that ulceration is one of the more deleterious collateral effects of traditional anti-inflammatory medicines, the ulcerogenic potential of PMFA was evaluated after the administration of the higher dose tested in the anti-inflammatory activity study. In this assay we observed that PMFA at 300 mg/kg did not induce stomach ulcer, whereas indomethacin 10 mg/kg induced a significant ulceration, with *p* < 0.05 (one-way ANOVA/Dunnett's test) in comparison to negative control (Fig. 1).

Traditional NSAIDs inhibit both isoforms of COX and their adverse gastrointestinal toxicities are related to the inhibition of gastroprotective prostaglandins, produced by COX-1 pathway. The results of evaluation of gastric lesions after exposition to high dose of PMFA are in agreement with clinical studies where non-selective NSAID was compared with COX-2 inhibitors and the ulcer index was observed, suggesting that PMFA might act by a mechanism independent of COX-1 inhibition (Kangwan et al., 2014). Emphasizing this, in a previous study we demonstrated that PMFA has no effect on platelet aggregation *in vitro* (Verdam et al., 2009), which is dependent on action of this enzyme (Dovizio et al., 2014).

NO released by activated macrophages has a critical role in the inflammatory response, including activation of the COX enzymes, resulting in exacerbated production of pro-inflammatory prostaglandins. Furthermore, NO is a potent vasodilator and had the ability to increase vascular permeability and edema through changes in local blood flow (Gamper and Ooi, 2014). PMFA significantly inhibit the formation of NO₂⁻, an oxidized form of NO released by LPS-activated macrophages. Since this NO production also reflects the activity of iNOS (Verma et al., 2012), this effect can be due to radical scavenging activity or inhibitory effect on enzyme (Fig. 2).

In biological medium, the oxidation of NO to cytotoxic radical anion peroxynitrite (ONOO⁻), by combination with anion superoxide radical (O₂⁻), is an important event in the aggravation of inflammatory processes, including those induced by carrageenan (Salvemini et al., 1996a), since it has already been demonstrated that inhibition of excessive production of NO reduces edema (Salvemini et al., 1996b). On the other hand, evidences showed that NO and ONOO⁻ facilitate PGE₂-induced hyperalgesia, but has lower hyperalgesic action when acting alone (Ndengele et al., 2008).

The injection of dilute formalin produces a persistent biphasic nociceptive stimulation noted by the behavioral pain responses of licking, flexing and jerking of the injected paw (Lariviere et al., 2011). The early phase of response (within 0–5 min after injection) comprises the neurogenic pain, while the late phase (within 15–60 min after injection) comprises the inflammatory pain (hyperalgesia) (Lapa et al., 2008). In our study, PMFA did not inhibit the early phase of the pain, indicating that the extract is not effective to control neurogenic pain. However, PMFA presented anti-hyperalgesic activity statistically significant with all tested doses (Fig. 3), without difference between them, which indicates, in advance, the significant anti-inflammatory potential of *J. acuminatissima* and reinforces the hypothesis of a possible action of PMFA on COX-2 or a derivate.

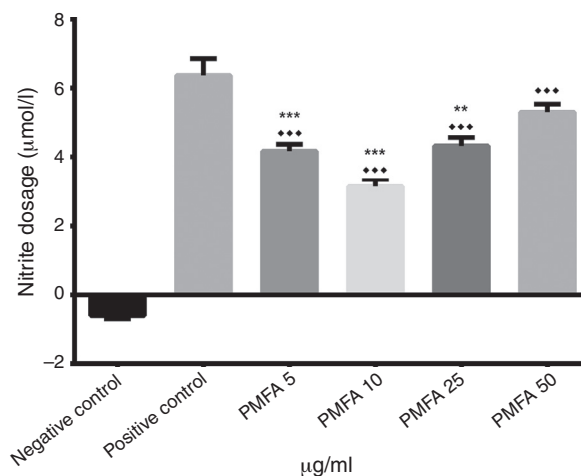


Fig. 2. Effect of different concentrations of PMFA extracts on nitrite production by murine macrophages J774 stimulated with lipopolysaccharide (LPS 1 µg/ml) for 24 h. Comparison of the concentrations analyzed by the Holm–Sidak test, *p*-values versus positive control: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *p*-value versus negative control: ♦*p* < 0.05, ♦♦*p* < 0.01, ♦♦♦*p* < 0.001.

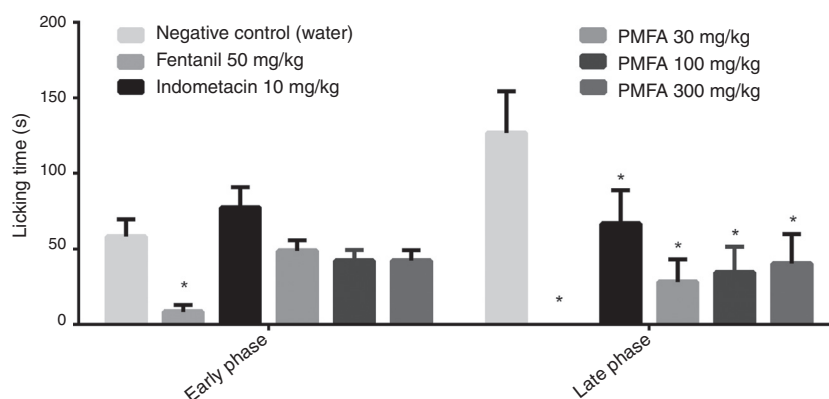


Fig. 3. Effect of aqueous extract of *Justicia acuminatissima* p.o. (30, 100 and 300 mg/kg), on the control of the neurogenic (early phase) and inflammatory pain (late phase) of the formalin test. Two-way ANOVA/Bonferroni, *p*-values versus negative control: **p* < 0.05.

Edema is another important sign of inflammation. In the carrageenan-induced paw edema model, rats paw volume is recorded at different time points using a plethysmometer, and this parameter may be used for inference on anti-inflammatory activity of various samples (Winter et al., 1962). Two phases of inflammatory response caused by carrageenan are well established. The first phase occurs one hour after polysaccharide injection, and is mainly related to the release of histamine, serotonin, bradykinin, platelet activating factor and leukotrienes. The second phase occurring within the first and third hours (peak of the inflammatory response) is due to the products release from COX-1 and COX-2 metabolism (Vinegar et al., 1969). PGs such as PGE₂ and PGI₂ produced via the COX-2 pathway magnify the degree of inflammation initiated by other mediators of inflammation such as histamine and bradykinin leading to increased vascular permeability and edema (Kawahara et al., 2014).

PMFA presented statistically significant antiedematogenic activity in both phases after carrageenan injection (Fig. 4), presenting no difference in relation to positive control (indomethacin at 10 mg/kg weight). This result, however, reaffirms the anti-inflammatory potential of the studied species. Comparing the results of the evaluation of the anti-hyperalgesic and antiedematogenic activities of PMFA, it can be inferred that the popular reports of healing of inflammatory processes by users of the *J. acuminatissima* leaf teas are more directly related to pain relief, which is the most troublesome symptom of inflammation, since all tested doses were active.

We previously showed that condensed tannins, coumarins and saponins form the majority of the secondary metabolites in *J. acuminatissima* leaves (Verdam et al., 2009). The saponins inhibit

the glucocorticoid receptors, which are related to inflammatory response (Augustin et al., 2011). The pharmacological interest by coumarins is due, particularly, to their bacteriostatic and anti-tumor activities and their capacity of undoing high protein edemas (Jain and Joshi, 2011), which may also contribute for the antiedematogenic effect of PMFA. In turn, the presence of condensed tannins in *J. acuminatissima*, that are easily extracted by water, suggests good antioxidant potential for PMFA, as shown in the NO production assay.

Côrrea and colleagues (2014b) investigated the anti-inflammatory properties of other organic extracts and compounds isolated from *J. acuminatissima*, getting good results. They also isolated four luteolin heterosides of an aqueous extract of *J. acuminatissima* leaves, and confirmed its anti-inflammatory activity (Côrrea et al., 2014a). Interestingly, these researchers used a model where the inflammatory response was inflicted by physical damage (contusion on hind right paw) and the substances were administered topically. Here, we evaluate the capacity of *J. acuminatissima* to inhibit the inflammatory response caused by chemical stimuli (formalin and carrageenan) after oral administration, and the results have been promising. Thus, the anti-inflammatory potential of *J. acuminatissima* was demonstrated by different routes and stimuli, preliminarily by basing it on its ethnopharmacological employment.

In order to add more information to the knowledge of the chemical composition of the aqueous extract of *J. acuminatissima*, we investigated the presence of twelve substances (friedelin, gallic acid, 1,8-hydroxyquinone, lupeol, rutin, β -stigmaterol, quercetin, *p*-cumaric acid, kaempferol, naringenin, scopoletin and umbelliferone) in our extract by HPLC–DAD. None of the substances screened were detected by this method, however, the substances previously identified in the aqueous extract support the observed results (Côrrea et al., 2014a).

With the results of studies with *J. acuminatissima* until now, we can conclude that the species is rich in biologically active compounds and has good anti-inflammatory capacity, being able to significantly inhibit inflammation caused by different types of stimuli, especially chemical and physical, without critical effects after acute exposure to high doses. Thus, these results can contribute to the validation of the main popular use of the species. As noted in our work, it is interesting to investigate in an upcoming study whether PMFA interferes with the action of PGE₂ or other related mediators involved in the physiology of inflammatory pain.

Authors' contributions

MCSV contributed in collecting plant sample and identification, confederation of herbarium, running the laboratory work and analysis

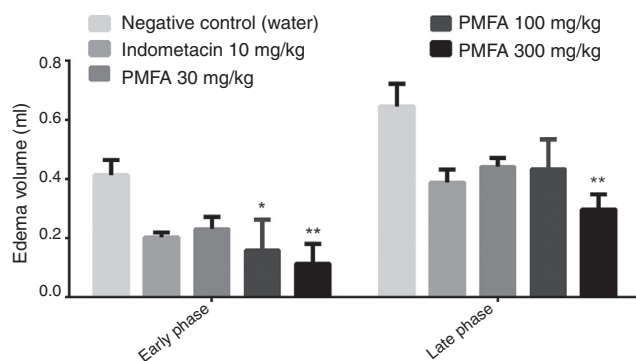


Fig. 4. Effect of aqueous extract of *Justicia acuminatissima* p.o. (30, 100 and 300 mg/kg) in the early and late phases of the carrageenan-induced paw edema test. Two-way ANOVA/Bonferroni, *p*-values versus negative control: **p* < 0.05, ***p* < 0.01.

of the data. FGS contributed in analysis of the data and drafted the paper. GSB, ALM, CIFBO, PDOA and MCV contributed to biological studies. TMM contributed to chromatographic analysis and analysis of the data. ESM contributed to critical reading of the manuscript. DTO and MMP designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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