

Evaluation of the antiinflammatory efficacy of *Lotus pedunculatus*

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The aim of this study was to investigate the antiinflammatory effect of the crude extract of *Lotus pedunculatus*. Aerial parts of *Lotus pedunculatus* were extracted by maceration with ethanol 96%. The solvent was evaporated under low pressure at 55°C to dryness, yielding crude extract. The antiinflammatory effects of *Lotus pedunculatus* were analyzed using carrageenan-induced mouse pleurisy. Significance was determined by analysis of variance, Dunnett's or Student's *t*-tests. Values of $P < 0.05$ were considered significant. The crude extract of *Lotus pedunculatus* inhibited leukocyte, neutrophil, myeloperoxidase, and adenosine-deaminase activities, as well as exudation, nitric oxide, interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) levels ($P < 0.05$). These results indicate that *Lotus pedunculatus* has an important antiinflammatory action and its inhibitory activity may be due to not only to the inhibition of proinflammatory mediators, but also to the inhibition of the activated leukocytes.

Key words: Air pouch, antiinflammatory activity, cytokines, enzymes, exudation, Leukocytes, *Lotus pedunculatus*

INTRODUCTION

The genus *Lotus* (Fabaceae) has a widespread and traditional usage as a forage plant on many continents, including Eurasia and the Mediterranean. It has about 173 species,^[1] some of which have been used in folk medicine as a contraceptive and to treat sexually transmitted diseases.^[2]

In Brazil, the most abundant species is the *Lotus pedunculatus*, also widely used as forrage, to increase the absorption of methionine and cysteine that are considered as important amino acids involved in improving the metabolism of sheep.^[3] Further, this species is also used as an antihelmintic substance in ruminants,^[4,5] as well as providing a mean of reducing voluntary plant intake and promoting fiber digestibility in sheep.^[6,7]

Motivated by the scarce pharmacological studies of *Lotus pedunculatus*, the aim of this study was to evaluate the antiinflammatory effect of the crude extract of *Lotus pedunculatus* upon leukocyte migration, myeloperoxidase (MPO) and adenosine-deaminase activities (ADA), as well as the exudation, nitric oxide (NO_x), interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) levels in the inflammation caused by carrageenan in the mouse model of the air pouch.

MATERIALS AND METHODS

Plant Material

Lotus pedunculatus was collected in November 2006 in Lages, Santa Catarina State, Brazil, at the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A. (EPAGRI). The voucher has a registration at the Ministério da Agricultura, Pecuária e Abastecimento (MAPA), in Lages (SCS313 SERRANA).

Preparation of the Crude Extract

The aerial parts of *Lotus pedunculatus* were dried at room temperature with air circulation. The dried and ground material (1000 g) was extracted by maceration with ethanol 96% for one month. The solvent was evaporated under low pressure at 55°C to dryness, yielding 155 g of crude extract (CE).

Animals

Swiss mice, weighing 18-25 g, were housed under standardized conditions (room at constant temperature (22 \pm 2°C) with alternating 12 h periods of light and darkness), humidity 50-60%, and were allowed free access to a standard mouse diet and water before use. This study was approved by the Committee for Ethics in Animal Research of our university (Protocol number-PP00180), and experiments were performed in accordance with the norms of the Brazilian College of animal experimentation. Efforts were made to minimize animal suffering and to reduce the number of animals

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used. No side effects were observed in any of the studied animal groups.

Experimental Protocol

Firstly, at the beginning of the experiments, different groups of mice received air injection (1.5 mL) on three alternate days to induce the air pouch. On the sixth day, the mice received carrageenan (1%) administered by subcutaneous route (s.c), and 24 h later they were killed by an overdose of ether.^[8,9] The mice were fixed on a surgical table and an incision in the dorsal skin was made to perforate the air pouch. The cavity was washed with 1.0 mL of sterile phosphate-buffered saline (PBS; pH 7.6; composition in mmol: NaCl 137, KCl 2.7 and phosphate buffer salts 10) containing heparin (20 IU/mL). Leukocyte migration, myeloperoxidase (MPO) and adenosine-deaminase activities (ADA), as well as exudation, nitrate/nitrite (NO_x), interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) levels were evaluated 24 h after phlogogen administration.

Indometacin (5.0 mg/kg) and dexamethasone (0.5 mg/kg) were used as reference antiinflammatory drugs.

For analysis of the dose-response, different groups of animals were pretreated (0.5 h) with different doses of the crude extract (CE) (10, 25 and 50 mg/kg) administered by intraperitoneal route (i.p.). The inflammatory parameters (leukocytes and exudation) were analyzed after 24 h. In parallel, different groups of animals were treated with innocuous substances only (sterile saline or the diluent used for the crude extract) acting as vehicle controls. The same inflammatory parameters were analyzed after 24 h.

In another set of experiments employed to establish the time-course profile, different groups of mice were pretreated with a single dose of the crude extract described above at different time points (0.5-2 h), and the same inflammatory parameters were evaluated after 24 h.

According to this protocol, the crude extract (25 mg/kg), administered 0.5 h before carrageenan induction, was elected as the dose to be used in the following experiments in other groups of animals. The reference drugs indometacin and dexamethasone were also administered by intraperitoneal route 0.5 h before inflammation induction.

Quantification of Leukocytes and Exudation

After killing the mice, samples of exudate from the air pouch cavity were collected to determine the total and differential leukocyte contents, and exudation. The leukocytes were determined in a Neubauer chamber, and cytopsin preparations of exudates were stained with May Grünwald-Giemsa for the differential count.^[8,9]

The degree of exudation was determined by measuring the amount of Evans blue dye extravasation in the exudate as previously described.^[8,9] Thus, in different groups of animals, except those used to analyze the MPO and ADA activities, as well as NO_x , TNF- α and IL-1 β levels, mice were challenged 5 minutes after the inflammation induction with a solution of Evans blue dye (25 mg/kg) administered by intravenous route (i.v.) to evaluate the exudation in the air pouch cavity. On the day of the experiment, a batch of stored samples was thawed at room temperature and the amount of dye was estimated by colorimetry methods using an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) at 620 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01-50 $\mu\text{g/mL}$.

Quantification of Myeloperoxidase Activity

Standard samples with different concentrations of myeloperoxidase (from human neutrophils, Sigma: M6908; Sigma, St Louis, MO, USA) were prepared in order to obtain a standard curve in the range of 0.07- 140 mU/mL. The exudate (40 μL) from the air pouch cavity and standard samples were transferred to cuvettes and the reaction was initiated with the addition of 360 μL of assay buffer (0.167 mg/mL of *o*-dianisidine 2HCl and 0.0005% H_2O_2). The reaction was stopped with sodium azide (1%). Afterwards, the samples were centrifuged at 50 $\times g$ for 5 min, the supernatants were separated, and the rates of changes in absorbancy at 520 nm were determined. MPO activity was estimated by interpolation from the standard curve by means of colorimetric measurement on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA).^[10] Results were expressed as mU/mL.

Quantification of Adenosine-deaminase Activity

Initially, standard samples with different volume concentrations of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (35 mM), $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ (15 mM) and NH_3SO_4 (15mM) were prepared in order to obtain a standard curve in the range of 10-50 U/L. The samples of fluid exudate (20 μL) from the air pouch cavity were transferred to cuvettes and the reaction was initiated by the addition of adenosine phosphate buffered solution (pH 6.5, 500 μL , composition in mM: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 35, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 15 and adenosine 0.5). After incubation for 1 h at 37°C, the reaction was halted with the addition of a solution (1000 μL) of phenol (1 mM) and nitroprussiate (0.17 mM), plus alkaline buffer (1000 μL ; NaCl: 11 mM). This solution was also added to the cuvettes with the different standard samples. Afterwards, the rate of change in absorbance at 620 nm was determined. Adenosine-deaminase activity was estimated by interpolation from the standard curve by means of colorimetric measurements on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA).^[11] Results were expressed as U/L.

Quantification of Nitrate/Nitrite Level

Nitric oxide and its breakdown products nitrite (NO_2^-) and nitrate (NO_3^-) were measured using the Griess method.^[12] Samples of exudate from the air pouch cavity were collected, separated and stored at -20°C . Concentrations of nitrate/nitrite were determined as previously described by Saleh *et al.*^[13] and levels were estimated by means of colorimetric measurement at 450 nm on an ELISA plate reader (Organon, Teknika, Roseland, NY, USA) estimated by interpolation from a standard curve (0-150 μM). Results were expressed as μM .

Quantification of IL-1 β and TNF- α Levels

Samples of exudate from the air pouch cavity were collected and immediately prepared for the analysis of cytokine levels. In this protocol, commercially available kits were used with monoclonal specific antibodies for each cytokine. The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA), using the kits according to the manufacturer's instructions. The ranges of values detected by these assays were: IL-1 β = 100 - 6400 pg/mL and TNF- α = 5 - 2000 pg/mL. The intra- and inter-assay coefficients of variation (CV) for IL-1 β and TNF- α were: Intra CV: IL-1 β = $6.2 \pm 0.4\%$ and TNF- α = $7.8 \pm 0.9\%$; inter CV: IL-1 β = $5.1 \pm 0.6\%$ and TNF- α = $9.6 \pm 2.1\%$, with sensitivity values of IL-1 β = 1.7 pg/mL and TNF- α = 5.0 pg/mL. All cytokine levels were estimated by means of colorimetric measurements at 450 nm on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve.

Acute Toxicity

Different groups of animals ($n = 10$ animals per group) were treated with different doses of crude extract of *Lotus pedunculatus* (100, 200 and 500 mg/kg) administered by intraperitoneal route. Animals were maintained for 10 days in an appropriate environment in order to evaluate the morbidity and/or mortality.

Chemicals

The following drugs and reagents were used: Carrageenan (degree IV), human neutrophil myeloperoxidase, indometacin, (Sigma Chemical Co., St Louis, MO, USA), dexamethasone, (Ache pharmaceutical laboratories S.A., São Paulo, SP, Brazil), Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of rat IL-1 β (IBLImmuno Biological Laboratories Co. Ltd, Fujioka-city, Gunma, Japan), mouse TNF- α (BD-Biosciences Pharmingen, San Diego, CA). Other reagents used were of analytical grade and were obtained from different commercial sources.

Data Analysis

The data are reported as the mean \pm s.e.m. Significant

differences between groups were determined by analysis of variance complemented with Dunnett's or Student's *t*-tests (or both). $P < 0.05$ was considered significant.

RESULTS

Effect of *Lotus Pedunculatus* on Leukocyte Migration and Exudation

The crude extract of *Lotus pedunculatus* (10 to 50 mg/kg) significantly produced a dose-dependent inhibition of leukocyte migration from $22.37 \pm 6.52\%$ ($P = 0.0395$) to $93.96 \pm 0.61\%$ ($P = 0.0001$) [Figure 1a]. This extract, at the doses 25 and 50 mg/kg, also significantly inhibited neutrophils by $84.32 \pm 0.27\%$ ($P = 0.0001$) and $95.91 \pm 0.45\%$ ($P = 0.0001$) [Figure 1b], mononuclears by $42.80 \pm 9.80\%$ ($P = 0.0408$) and $61.36 \pm 9.09\%$ ($P = 0.0309$) [Figure 1c] and exudation by $53.62 \pm 2.91\%$ ($P = 0.0019$) and $65.30 \pm 6.34\%$ ($P = 0.0006$), respectively [Figure 1d].

The time-course profile for this extract showed that only the pretreatment period of 0.5 h significantly inhibited leukocyte migration ($P = 0.0001$), neutrophils ($P = 0.0001$), mononuclears ($P = 0.0408$) and exudation ($P = 0.0019$) in the studied inflammation model [Figure 2a-d].

As expected, indometacin (5.0 mg/kg) and dexamethasone (0.5 mg/kg) significantly inhibited leukocyte migration by $66.82 \pm 6.81\%$ ($P = 0.0004$) and $51.83 \pm 7.62\%$ ($P = 0.0029$), neutrophils by $75.17 \pm 2.35\%$ ($P = 0.0005$) and $64.31 \pm 5.59\%$ ($P = 0.0012$) and exudation by $53.98 \pm 3.33\%$ ($P = 0.0028$) and $36.36 \pm 5.19\%$ ($P = 0.0233$), respectively. In this protocol the reference drugs also significantly increased the mononuclears by $93.18 \pm 1.23\%$ ($P = 0.0463$) and $229.54 \pm 15.36\%$ ($P = 0.0075$), respectively [Figures 1 and 2].

The suppressive effects of the CE (25 mg/kg) upon leukocytes and exudation were effective only when the animals were pretreated at 0.5 h before carrageenan induction [Figure 2].

Effect of *Lotus Pedunculatus* on Myeloperoxidase, Adenosine-deaminase Activity, Nitrate/Nitrite and Cytokines Levels

The pretreatment of animals with the crude extract (25 mg/kg) caused a significant decrease in myeloperoxidase by $58.12 \pm 7.95\%$ ($P = 0.0001$) [Figure 3a] and adenosine-deaminase activities by $29.81 \pm 6.80\%$ ($P = 0.0108$) [Figure 3b].

This herb also significantly inhibited nitrate/nitrite levels by $37.37 \pm 12.94\%$ ($P = 0.0020$) [Figure 3c]. Indometacin and dexamethasone pretreatment significantly inhibited myeloperoxidase by $62.72 \pm 1.08\%$ ($P = 0.0029$) and $83.31 \pm 8.56\%$ ($P = 0.0010$), and adenosine-deaminase activities by $44.09 \pm 3.29\%$ ($P = 0.0349$) and $81.12 \pm 7.43\%$ ($P = 0.0067$) and nitrate/nitrite levels by $85.25 \pm 10.39\%$ ($P = 0.0172$) and

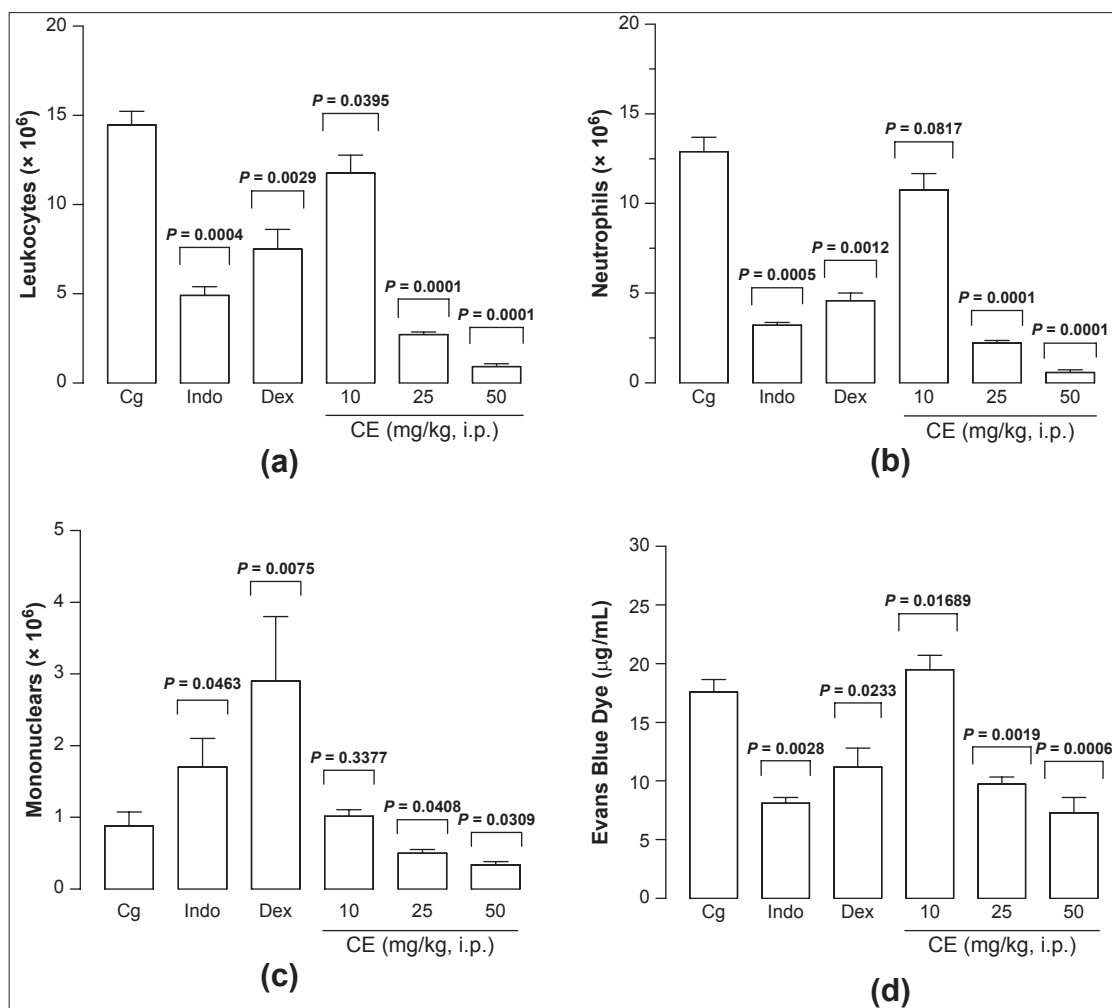


Figure 1: Effect of crude extract (CE: 10 - 50 mg/kg, i.p.) of *Lotus pedunculatus* administered 0.5 h before carrageenan (Cg 1%/cav.) upon leukocytes (a) neutrophils (b) mononuclears (c) and exudation (d) in the mouse model of air pouch. Cg = responses in animals treated only with carrageenan, Indo = responses in animals pretreated with indomethacin (5.0 mg/kg, i.p.), Dex = responses in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Each group represents the mean of 6 animals and the vertical bars the SEM

90.65 \pm 6.97% ($P = 0.0145$), respectively [Figure 3a-c].

Further, this extract at the same dose also significantly decreased IL-1 β by 57.30 \pm 4.22% ($P = 0.0008$) [Figure 4a] and TNF- α levels by 48.62 \pm 9.14% ($P = 0.0362$) [Figure 4b]. Indometacin and dexamethasone also significantly inhibited IL-1 β levels by 68.74 \pm 15.21% ($P = 0.0089$) and 98.82 \pm 5.80% ($P = 0.0001$), respectively [Figure 4a]. On the other hand, dexamethasone, but not indometacin, inhibited TNF- α levels by 47.02 \pm 3.02% ($P = 0.0143$) [Figure 4b].

Acute Toxicity

No deaths were observed in the group of animals pretreated with different doses (100, 200 and 500 mg/kg) of the crude extract of *Lotus pedunculatus* [Figure 5].

DISCUSSION

Our results demonstrated that *Lotus pedunculatus* has an important antiinflammatory property by inhibiting

leukocyte migration, exudation, proinflammatory mediators and enzymes that are recognized to participate in the inflammation caused by carrageenan in a murine model of the air pouch.^[8,9]

The inhibitory effect of *Lotus pedunculatus* upon leukocytes was associated with a marked decrease of myeloperoxidase, as well as adenosine-deaminase activities.

Myeloperoxidase, a heme protein abundantly expressed in neutrophils, is considered an important marker of activated neutrophils and also contributes to prolongation of the inflammatory response.^[14,15]

In relation to adenosine-deaminase, studies have demonstrated that this enzyme is a potent modulator of inflammation, and it is considered to be an important marker of mononuclears.^[14,16]

Since *Lotus pedunculatus* caused a significant inhibition of

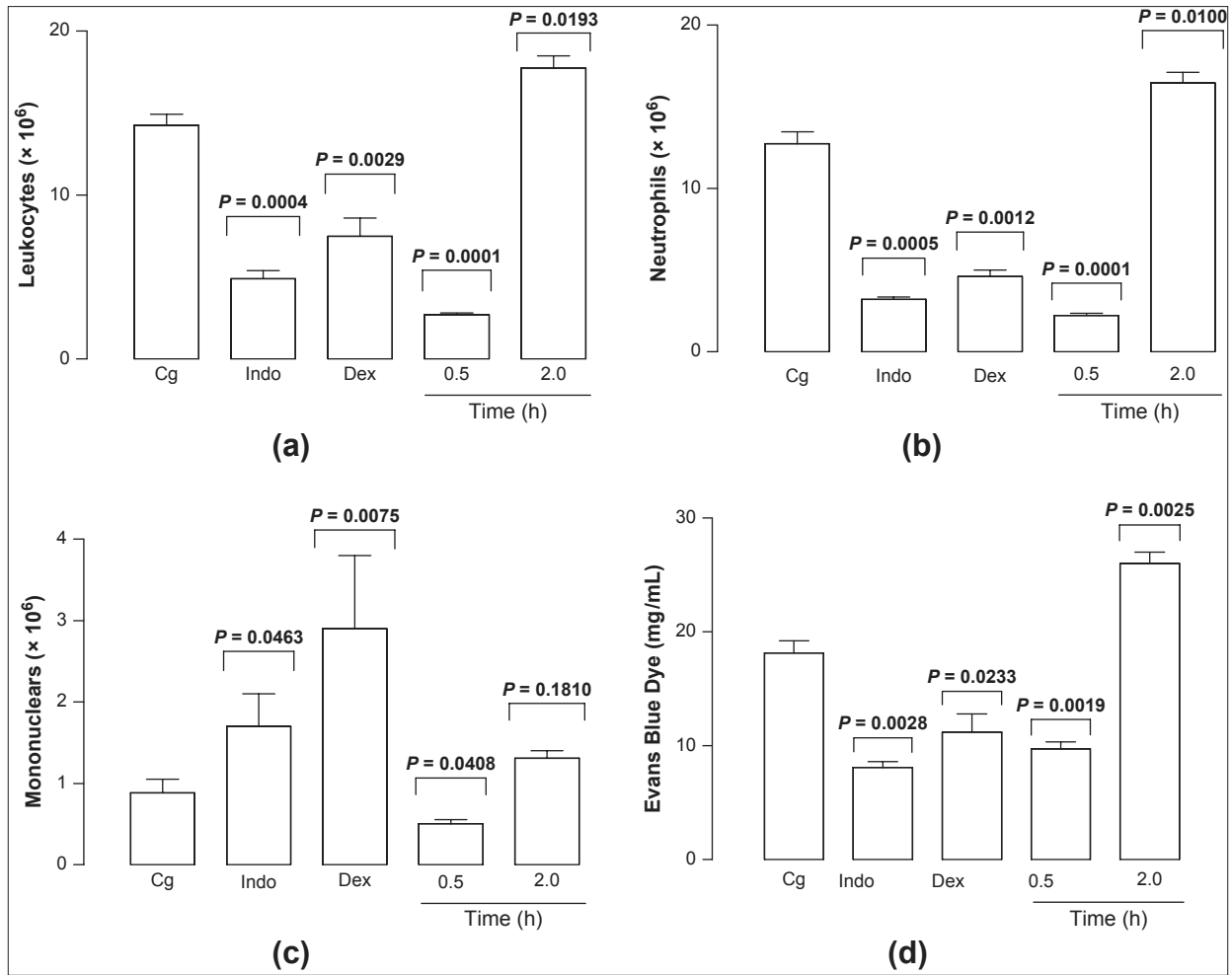


Figure 2: Effect of crude extract (CE: 25 mg/kg, i.p.) of *Lotus pedunculatus* administered 0.5 and 2 h before carrageenan (Cg 1%/cav.) upon leukocytes (a) neutrophils (b) mononuclears (c) and exudation (d) in the mouse model of air pouch. Cg = responses in animals treated only with carrageenan, Indo = responses in animals pretreated with indomethacin (5.0 mg/kg, i.p.), Dex = responses in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Each group represents the mean of 6 animals and the vertical bars the SEM

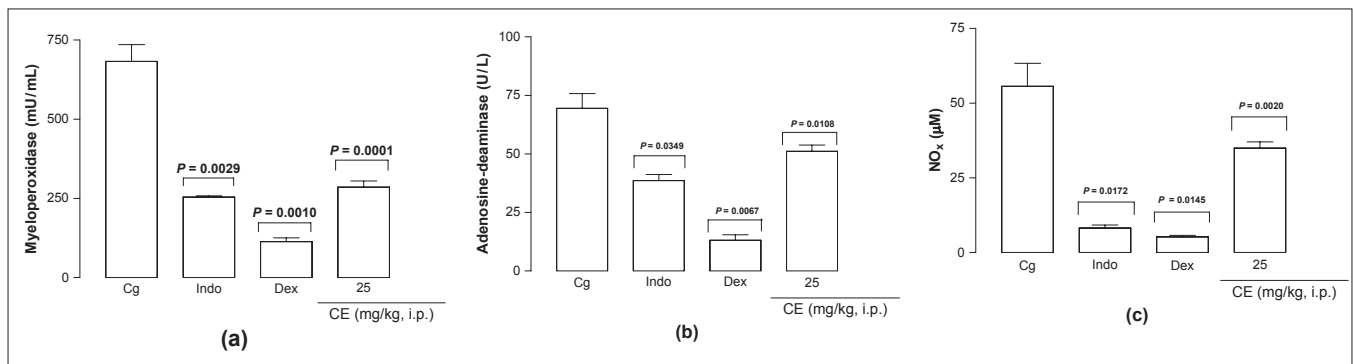


Figure 3: Effect of crude extract (CE: 25 mg/kg, i.p.) of *Lotus pedunculatus* administered 0.5 h before carrageenan (Cg 1%/cav.) upon: Myeloperoxidase (a) and adenosine-deaminase (b) activities and nitrite/nitrate levels (c) in the mouse model of air pouch. Cg = responses in animals treated only with carrageenan, Indo = responses in animals pre-treated with indomethacin (5.0 mg/kg, i.p.), Dex = responses in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Each group represents the mean of 6 animals and the vertical bars the SEM

both myeloperoxidase and adenosine-deaminase activities, these results show that this herb not only inhibited activated leukocytes, but also leukocyte migration to the air pouch cavity in the inflammation induced by carrageenan.

Cytokines are soluble protein mediators that are important for the orchestration of inflammatory responses. In inflammation, a balanced ratio of proinflammatory cytokines is crucial to determine magnitude of the antiinflammatory effect of *Lotus pedunculatus*. In this study

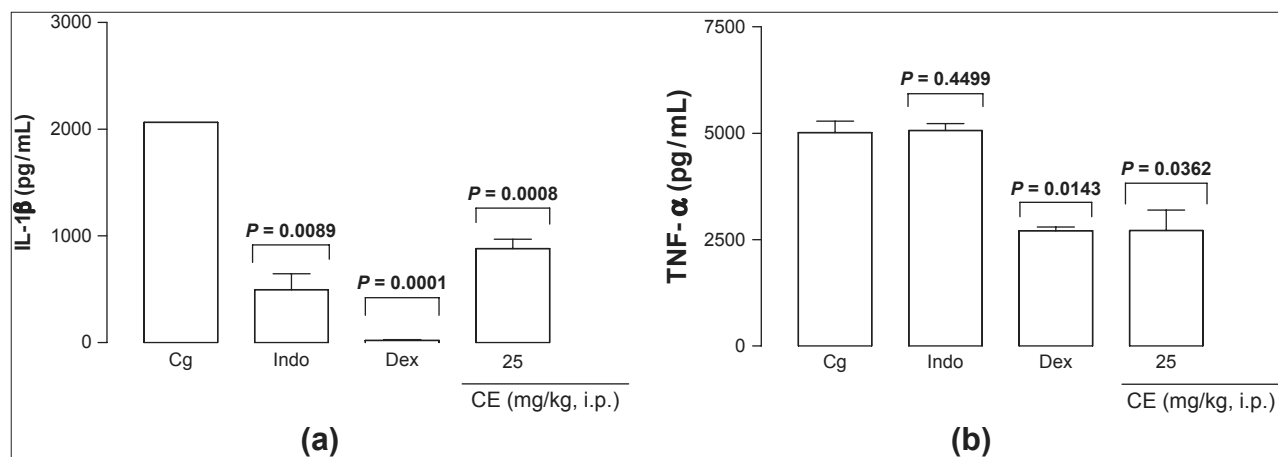


Figure 4: Effect of crude extract (CE: 25 mg/kg, i.p.) of *Lotus pedunculatus* administered 0.5 h before carrageenan (Cg 1%/cav.) upon IL-1 β (a) and TNF- α (b) levels, in the mouse model of air pouch. Cg = responses in animals treated only with carrageenan, Indo = responses in animals pretreated with indomethacin (5.0 mg/kg, i.p.), Dex = responses in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Each group represents the mean of 6 animals and the vertical bars the SEM

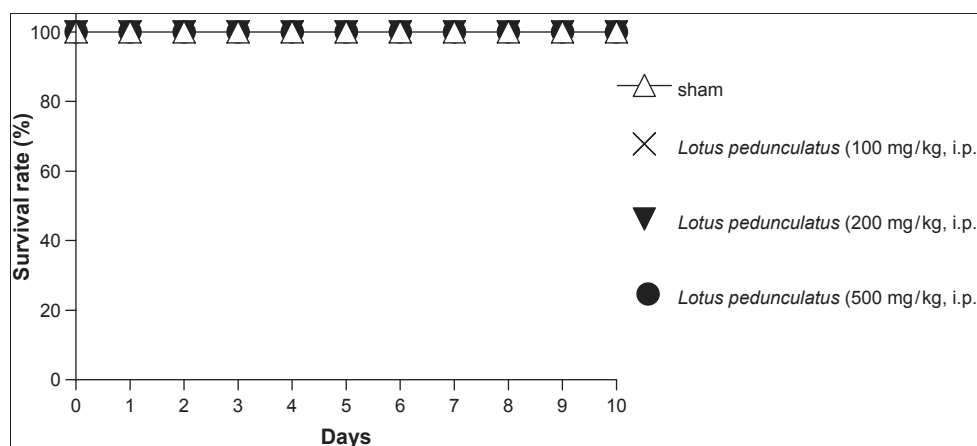


Figure 5: Survival rate of mice after treatment with different doses of crude extract of *Lotus pedunculatus* (CE: 100 - 500 mg/kg, i.p.).

we also measured the levels of TNF- α and IL-1 β in the animals pretreated with crude extract of *Lotus pedunculatus*. The role of TNF- α and IL-1 β in the inflammatory response is well known. Mazzoni *et al.*^[17] showed that these cytokines promote the adhesion molecule and inducible nitric oxide expression, as well as neutrophil chemotaxis, in the carrageenan-induced inflammation in the paw edema model, in mice. A similar result was obtained with studies from Bombini *et al.*^[18] who demonstrated the participation of these cytokines in the neutrophil recruitment during immune inflammation in the arthritis model, in rats.

Nitric oxide is also known to be involved in multiple biologically-important inflammatory reactions.^[19] A study by Razavi *et al.*^[20] has also demonstrated that knock-out mice to iNOS (iNOS $^{-/-}$) show less neutrophil infiltration into the pulmonary microvascular in the sepsis model induced by cecal ligation/perforation than in the wild type (iNOS $^{+/+}$). Another study has demonstrated that this mediator is also involved in the exudation formation.

Yet another study by Durland *et al.*^[21] has shown that the blockade of NO synthesis with a N-nitro-l-arginine methyl ester promotes a significant inhibition of both albumin exudation and inflammatory cell influx into the nasal cavity in the animal model of allergy induced by histamine. To better understand the modulation of NO by *Lotus pedunculatus*, the nitrite and nitrate levels were measured in the exudate air pouch cavity of the inflamed animals pretreated with *Lotus pedunculatus*. In this protocol the studied herb also significantly suppressed the nitrite/nitrate levels.

In conclusion, further studies are required to better clarify the antiinflammatory mechanism of action of *Lotus pedunculatus*. The potent *in vivo* inhibition of NO $_x$, TNF- α , IL-1 β , and also leukocytes and exudate, as well as the proinflammatory enzymes (MPO and ADA) may account for the antiinflammatory activity of *Lotus pedunculatus*, and our results indicate that this herb has some potential for the future development of a new therapeutic drug to treat

inflammatory disease.

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