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# *Lotus corniculatus* Regulates the Inflammation Induced by Bradykinin in a Murine Model of Pleurisy

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**ABSTRACT:** This study evaluated the anti-inflammatory efficacy of the crude extract (CE), the fractions derived from hexane (HEX), ethyl acetate (AcOEt), *n*-butanol (BuOH), and aqueous (Aq) and isolated compounds (oleanolic acid or kaempferitrin) obtained from the aerial parts of *Lotus corniculatus* var. São Gabriel in mice with bradykinin-induced pleurisy. Swiss mice were used for the In Vivo experiments. Inflammatory parameters [leukocytes; exudate concentrations; myeloperoxidase and adenosine-deaminase activities, and nitric oxide and interleukin-17 levels] were evaluated 4 h after pleurisy induction. The crude extract of *Lotus corniculatus*, its derived fractions, and isolated compounds inhibited leukocytes and the exudate. This inhibitory effect was associated with decreased of myeloperoxidase and adenosine-deaminase activities, nitric oxide products, and IL-17A levels. *Lotus corniculatus* presented important anti-inflammatory action by inhibiting leukocyte influx and exudate concentrations. This effect was directly related to the inhibition of nitric oxide and interleukinin17 levels. Oleanolic acid and kaempferitrin can account for these anti-inflammatory effects.

**KEYWORDS:** *Lotus corniculatus*, bradykinin, pleurisy, anti-inflammatory effect, kaempferitrin, oleanolic acid, myeloperoxidase, adenosine-deaminase, nitric oxide, interleukin-17

## INTRODUCTION

The genus *Lotus* (Fabaceae) has about 173 species and is used as a forage plant in many parts of the world.[1] *Lotus corniculatus* plays an important role in ruminant nutrition by promoting the enhancement of energy, protein, and minerals.[2] In Brazil, *Lotus corniculatus* var. São Gabriel, also known as cornichão, is used as ruminant feed because it promotes weight gain, wool growth,[3] essential amino acid absorption,[4] milk production, and composition and ovulation rate.[5] This herb is also used as an antihelmintic in sheep and cattle.[6]

Phytochemical studies of *Lotus corniculatus* showed that the plant contains substances well known to have important anti-inflammatory properties, such as flavonoids (quercetin and kaempferol),[7] triterpene (oleanolic acid), and saponins.[8] The anti-inflammatory properties of these isolated compounds have been demonstrated in several In Vivo and/or in vitro studies. Quercetin acts as an antioxidant[9] and an anti-inflammatory agent.[10] Kaempferol seems to exert its antinociceptive and anti-inflammatory actions by inhibiting pro-inflammatory mediators and transcription factors such as nuclear factor-$\kappa$B (NF-$\kappa$B).[10−12] Also, oleolic acid and saponins exhibited anti-inflammatory properties via the inhibition of leukotriene synthesis,[13] nitric oxide release,[14] and levels of tumor necrosis factor-alpha (TNF-$\alpha$), prostaglandin $E_2$ ($PGE_2$), and cyclooxygenase-2 (COX-2).[15]

The aim of this study was to confirm and extend the investigation of the anti-inflammatory effects of the crude extract, derived fractions and isolated compounds from the aerial parts of *Lotus corniculatus* var. São Gabriel bradykinin (BK)-induced pleurisy in mice. We evaluated their effects on cell migration, exudate concentrations, and nitric oxide, myeloperoxidase, and adenosine-deaminase activities as well as on interleukin-17A levels.

## MATERIALS AND METHODS

**Chemicals.** The following drugs and reagents were used: bradykinin, human neutrophil myeloperoxidase, indomethacin, Evans blue dye, sodium hydroxide, vanadium chloride (Sigma Chemical Co., St. Louis, MO, USA), dexamethasone (Ache Pharmaceutical Laboratories S.A., São Paulo, SP, Brazil), *N*-(1-naphthyl) ethylenediamine dihydrochloride (Merck, São Paulo, SP, Brazil), heparin (Liquemine, Roche, São Paulo, SP, Brazil), phosphoric acid ($H_3PO_4$ 85%) (Biotech, São Paulo, SP, Brazil), and an enzyme-linked immunosorbent assay (ELISA) for quantitative determination of mouse IL-17A (eBioscience, Inc. San Diego, CA, USA). Other reagents used were of analytical grade and were obtained from different commercial sources.

**Plant Material.** *Lotus corniculatus* var. São Gabriel was collected in November 2006 in Lages, Santa Catarina State, Brazil. The material was identified by the botanist Professor Dr. Daniel de Barcelos Falkenberg.

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A voucher specimen was deposited in the Herbarium at the Federal University of Santa Catarina, Florianópolis, SC, Brazil (FLOR 18.770).

**Preparation of Crude Extract and Fractions.** The aerial parts of *Lotus corniculatus* var. São Gabriel were air-dried at room temperature (25 °C) for one week. Subsequently, the dried aerial parts (620 g) were ground into particles (1.5 mm) using a knife mill (Mill TE-651, Tecnal, Piracicaba, SP, Brazil). The ground material was extracted using ethanol, as previously described in detail by Koelzer et al.,[16] yielding 78 g of crude extract (CE). The CE was fractionated by successive liquid−liquid extraction using solvents of increasing polarity, resulting in a hexane fraction (HEX, 7.82 g), an ethyl acetate fraction (AcOEt, 11.4 g), an *n*-butanol fraction (BuOH, 5.24 g), and an aqueous fraction (Aq, 30.8 g).

**High-Performance Liquid Chromatography Profile (HPLC) of the Hexane and Ethyl Acetate Fractions of *Lotus corniculatus* var. São Gabriel.** The high-performance liquid chromatography (HPLC) profile of the HEX and AcOEt fractions were obtained using Varian ProStar 310 equipment with a UV/vis detector monitoring at 210 and 280 nm, respectively (Walnut Creek, CA, USA), a manual injector, and StarFinder 70 version 5.5 software. The analysis time of these fractions was performed from zero to 40 min. The HPLC apparatus was equipped with a ChromSpher 5 C18 column (4.6 mm × 250 mm i.d.) (Walnut Creek, CA, USA).

**Isolation of the Compounds.** The HEX fraction was subjected to silica gel column chromatography and eluted with a gradient of HEX/EtOAc, resulting in the isolation of a white powder with a melting point of 279−282 °C (Compound **1**). A chromatographic fractionation on silica gel of the EtOAc fraction afforded a yellow crystal from EtOAc/EtOH with a melting point of 198−201 °C (compound **2**).

**Nuclear Magnetic Resonance of Isolated Compounds (NMR).** *Compound **1** (Oleanolic Acid).* NMR $^1$H ($CDCl_3$, 400 MHz) $\delta$: (0.74 (3H, s), 0.76 (3H, s), 0.89 (3H, s), 0.90 (3H, s), 0.91 (3H, s), 0.97 (3H, s), 1.12 (3H, s), 1.24 (3H, s), 1.00−2.00 (m), 2.81 (1H, dd, $J$ = 14 Hz), 3.20 (1H, dd, $J$ = 6.8 Hz), 5.27 (1H, m, H-12). NMR $^{13}$C ($CDCl_3$, 100 MHz) $\delta$: 15.29; 15.52; 17.11; 18.25; 22.87; 23.36; 23.55; 25.91; 27.13; 27.65; 28.07; 30.65; 32.40; 32.56; 33.05; 33.75; 37.05; 38.34; 38.72; 39.22; 40.92; 41.54; 45.83; 46.48; 47.58; 55.1; 79.0; 122.59; 143.56; 183.33.

*Compound **2** (Kaempferitrin).* NMR $^1$H ($CDCl_3$, 400 MHz) $\delta$: 0.96 (3H, d, $J$ = 6.2 Hz), 1.25 (3H, d, $J$ = 6.2 Hz), 3.30 (1H, m), 3.30 (1H, m), 3.33 (1H, m), 3.47 (1H, t, $J$ = 9.2 Hz), 3.59 (1H, m), 3.71 (1H, dd, $J$ = 3.1 e 9.2 Hz), 3.82 (1H, dd), 4.01 (1H, dd), 5.38 (1H, d, $J$ = 1.8 Hz), 5.55 (1H, s), 6.45 (1H, d, $J$ = 2.2), 6.71 (1H, d, $J$ = 2.2 Hz), 6.93 (2H, d, $J$ = 8.8 Hz), 7.78 (2H, d, $J$ = 8.8 Hz). NMR $^{13}$C ($CDCl_3$, 100 MHz) $\delta$: 17.67; 18.08; 71.29; 71.68; 71.79; 71.90; 72.05; 72.78; 73.14; 73.58; 95.58; 99.83; 100.5; 103.52; 107.53; 116.57; 122.37; 132.00; 136.47; 158.07; 159.79; 161.77; 163.00; 163.52; 179.77. The results obtained were compared with published spectral data.[17,18]

**Animals.** Swiss mice weighing 18 to 25 g were housed under standardized conditions (room maintained at 22 ± 2 °C, with alternating 12-h periods of light and dark and with 50 to 60% humidity) 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 PP00180), and the experiments were performed in accordance with the norms of the Brazilian College of Animal Experimentation.

**Induction and Analysis of Pleurisy.** As previously described,[19] pleurisy was induced by a single intrapleural (ipl.) injection of 0.1 mL of sterile saline (NaCl 0.95%) plus bradykinin (BK, 20 nmol). In this study, the animals treated with BK were also treated with captopril (5 mg/kg) administered by the intraperitoneal route (i.p.) 0.5 h before the induction of inflammation to prevent the action of kininases.[20] The inflammatory parameters were evaluated 4 h after pleurisy induction.

After sacrificing the animals with an overdose of ether, the thorax was opened, and the pleural cavity was washed with 1.0 mL of sterile phosphate buffered saline (PBS) (pH 7.6) containing NaCl (130 mM), $Na_2HPO_4$ (5 mM), and $KH_2PO_4$ (1 mM) in distilled water containing heparin (20 IU/mL). Several samples of pleural fluid were collected for further determination of the total and differential leukocytes, exudate concentrations, and myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitric oxide products ($NO_x$) and interleukin-17A (IL-17A) levels.

**Experimental Protocol.** To analyze the dose−response curve, different groups of animals were treated with different doses of the crude extract (CE) (25, 50, 100, and 200 mg/kg, i.p.), hexane fraction (HEX), ethyl acetate fraction (AcOEt), butanolic fraction (BuOH), and aqueous fraction (Aq) at doses of 5, 10, and 25 mg/kg i.p, oleanolic acid (1, 2.5, and 5 mg/kg, i.p.) or kaempferitrin (1, 5, and 10 mg/kg i.p.) 0.5 h before pleurisy.

According to the results obtained in the experiments above, we chose the lower doses of the CE and its derived fractions and the isolated compounds that inhibited total and differential leukocytes, as well as exudate concentrations. The doses of CE (50 mg/kg), HEX fraction (10 mg/kg), AcOEt fraction (10 mg/kg), BuOH fraction (10 mg/kg), Aq fraction (25 mg/kg), oleanolic acid (1 mg/kg), or kaempferitrin (5 mg/kg) were chosen to determine the other inflammatory parameters, such as myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities and nitrate/nitrite ($NO_x$) and interleukin-17A (IL-17A) levels.

Further, a previous study in our laboratory revealed that the CE and its derived fractions, as well as the isolated compounds, were effective in inhibiting leukocyte migration and exudate concentrations only when they were administered 0.5 h before pleurisy induction by carrageenan.[16] This time point of pretreatment was also chosen in our experiments to analyze the inflammatory parameters in the pleurisy induced by bradykinin.

In parallel, different groups of animals were treated with (1) only 0.1 mL of BK (20 nmol, ipl.), considered the positive control group, (2) only 0.1 mL of sterile saline or vehicle (NaCL 0.95%, ipl.), considered the negative control group, (3) indomethacin (5.0 mg/kg, i.p.,0.5 h before) plus BK (20 nmol, ipl.), or (4) dexamethasone (0.5 mg/kg, i.p., 0.5 h before) plus BK (20 nmol, ipl.).

**Quantification of Leukocytes and Exudate Concentrations.** Total leukocyte counts were performed in a Neubauer chamber, and cytospin preparations of samples of the fluid leakage of the pleural cavity were stained by the May-Grunwald-Giemsa technique for the differential leukocyte counts, which were performed under an oil immersion objective. All animals, except in the experiments that analyzed the enzyme activities, nitric oxide products, and cytokine level, were previously challenged (1 h) with a solution of Evans blue dye (25 mg/kg, i.v.) to evaluate the extent of exudation into the pleural space. A sample (500 $\mu$L) of the fluid was collected from the pleural cavity, and the amount of dye was estimated by colorimetry using an enzyme-linked immunosorbent assay (ELISA) plate reader (Organon Tecknica, Roseland, NJ, USA) at 620 nm by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 $\mu$g/mL.

**Quantification of Myeloperoxidase and Adenosine-Deaminase Activities.** In-house assays of both myeloperoxidase and adenosine-deaminase were employed according to the methods developed by Rao et al.[21] and Giusti and Galanti.[22] By using conventional reagents, the concentration of each enzyme was estimated in the fluid leakage of the pleural cavity by means of colorimetric measurements (absorbances of 450 and 630 nm, respectively) in an ELISA plate reader (Organon Tecknica, Roseland, NJ, USA). The results were expressed as mU/mL (MPO) and U/L (ADA). Detailed descriptions of these assays have been published previously.[23]

**Quantification of Nitrate/Nitrite Levels.** Nitric oxide was measured as its breakdown products of nitrite ($NO_2^-$) and nitrate

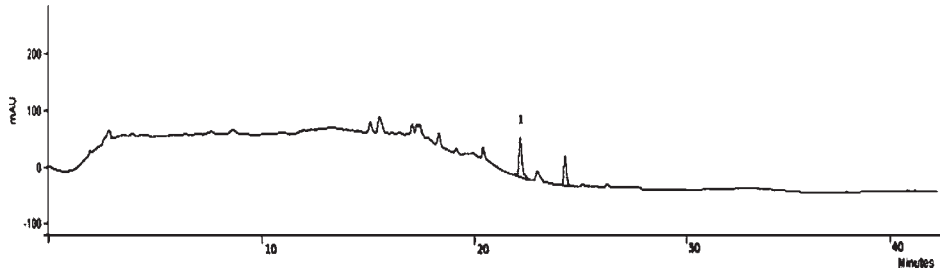


**Figure 1.** Chromatographic profile (HPLC) (210 nm) of the hexane fraction isolated from *Lotus corniculatus* var. São Gabriel. Peak 1 represents compound **1** (oleanolic acid).

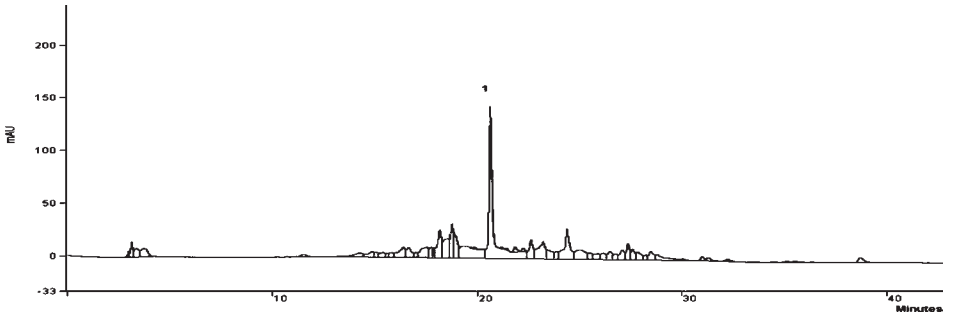


**Figure 2.** Chromatographic profile (HPLC) (280 nm) of the ethyl acetate fraction isolated from *Lotus corniculatus* var. São Gabriel. Peak 1 represents compound **2** (kaempferitrin).

($NO_3^-$) using the Griess method.[24] Samples of the fluid leakage of the pleural cavity obtained from controls and treated animals were collected, separated, and stored at −70 °C, and the levels of nitrate/nitrite were determined as previously described.[16] The results were expressed in $\mu$M.

**Quantification of IL-17A Levels.** To analyze IL-17A levels, samples of fluid leakage of the pleural cavity were collected and immediately prepared for the analysis of cytokine levels. This protocol used a commercially available kit with monoclonal specific antibodies against IL-17A. The cytokine levels were measured with an enzyme-linked immunosorbent assay Kit according to the manufacturers' instructions. The results were expressed in pg/mL.

**Data Analysis.** The data were reported as the mean ± SEM. Comparisons of parameters between groups were performed by two-way analysis of variance (ANOVA) followed by Dunnett's and/or Student's *t*-tests for post hoc analysis, as necessary. *P* values of less than 0.05 were considered statistically significant, and all tests were two-tailed.

## RESULTS

**Isolation and Identification of Compounds.** The hexane fraction was subjected to silica gel column chromatography and eluted with a gradient of HEX/AcOEt (70:30, v/v), resulting in the isolation of triterpenoid oleanolic acid (Compound 1) (25 mg). A chromatographic fractionation on silica gel of the AcOEt fraction afforded a crude flavonoid, kaempferitrin (compound **2**) (45 mg), from the AcOEt/EtOH (50:50, v/v) eluate, which was further purified by flash chromatography using ethyl acetate/water/formic acid/acetic acid (70:20:3:2, v/v/v/v).

**High-Performance Liquid Chromatography Profile (HPLC) of Hexane and Ethyl Acetate Fractions of *Lotus corniculatus* var. São Gabriel.** The first chromatogram profile (HEX fraction), obtained at 210 nm, contained several distinct peaks. The isolated compound of this fraction (oleanolic acid) had a retention time of 22.22 min and represented 9.5% of the hexane fraction (9.5 mg/100 mg) (Figure 1). The second compound (kaempferitrin), isolated from the AcOEt fraction, showed a major peak (at 280 nm) with a retention time of 20.46 min that represented 31.6% of the ethyl acetate fraction (31.6 mg/100 mg) (Figure 2).

**Effects of *L. corniculatus* var. São Gabriel on Leukocytes and Exudates.** In the mouse model of pleurisy induced by bradykinin, we observed a significant increase of either leukocyte influx or pleural exudate 4 h after phlogogen administration compared with that in saline-treated animals (negative-control group).[19]

In our study, the crude extract of *L. corniculatus* (CE 50 to 200 mg/kg) significantly decreased leukocytes in the pleural fluid leakage from 48.7 ± 7.0% to 50.0 ± 2.3% ($p < 0.01$). This inhibitory effect was associated with a significant decrease in neutrophils from 66.4 ± 4.8% to 73.1 ± 2.0% ($p < 0.01$) and exudate from 28.7 ± 4.3% to 40.2 ± 1.7% ($p < 0.05$). At 25 mg/kg, CE also inhibited exudate concentrations by 29.0 ± 12.9% ($p < 0.05$) (Table 1).

With respect to the hexane fraction, we observed that all tested doses of this fraction inhibited BK-induced inflammation. This fraction (5 to 25 mg/kg) significantly reduced leukocytes from 27.8 ± 5.9% to 41.6 ± 9.3% ($p < 0.05$), neutrophils from 55.0 ± 5.7% to 65.1 ± 8.5% ($p < 0.01$), and exudate concentrations from 19.8 ± 4.2% to 29.9 ± 7.0% ($p < 0.05$) (Table 1).

The ethyl acetate fraction showed an anti-inflammatory pattern similar to that of the HEX fraction. Both fractions inhibited the measured inflammatory parameters with a similar dose dependence. The AcOEt fraction (5 to 25 mg/kg) inhibited leukocyte migration from 27.7 ± 8.8% to 63.2 ± 7.0% ($p < 0.05$) and neutrophils from 54.8 ± 10.0% to 67.3 ± 2.6% ($p < 0.01$), and exudate concentrations from 52.5 ± 4.3% to 59.0 ± 10.4% ($p < 0.01$). At 25 mg/kg, this fraction also inhibited mononuclear cells by 62.0 ± 9.2% ($p < 0.01$) (Table 1).

Similar to the HEX and AcOEt fractions, the butanol fraction (5 to 25 mg/kg) produced a significant decrease in leukocytes from 27.3 ± 4.1% to 46.5 ± 3.8% ($p < 0.05$) and neutrophils

**Table 1. Effects of the Crude Extract Isolated from *Lotus corniculatus* var. São Gabriel and Its Derived Fractions and Isolated Compounds on Leukocyte Migration and Exudate Concentrations in Mice with Bradykinin-Induced Pleurisy[a]**

| groups/doses (mg/kg) | leucocytes ($\times 10^6$) | neutrophils ($\times 10^6$) | mononuclear cells ($\times 10^6$) | exudate ($\mu$g/mL) |
|---|---|---|---|---|
| S[b] | 0.67 ± 0.06 | 0.30 ± 0.02 | 0.37 ± 0.03 | 1.14 ± 0.09 |
| BK-treated[b] | 1.84 ± 0.18 | 1.13 ± 0.19 | 0.71 ± 0.07 | 1.59 ± 0.13 |
| CE 25[c] | 1.64 ± 0.24 | 0.96 ± 0.13 | 0.68 ± 0.12 | 1.16 ± 0.17* |
| CE 50[c] | 0.93 ± 0.04** | 0.37 ± 0.05** | 0.56 ± 0.05 | 0.95 ± 0.03** |
| CE 100[c] | 1.17 ± 0.08** | 0.47 ± 0.03** | 0.70 ± 0.04 | 0.88 ± 0.13** |
| CE 200[c] | 0.96 ± 0.02** | 0.28 ± 0.02** | 0.68 ± 0.03 | 1.12 ± 0.07** |
| HEX 5[c] | 1.11 ± 0.18** | 0.45 ± 0.11** | 0.66 ± 0.08 | 1.11 ± 0.11* |
| HEX 10[c] | 1.37 ± 0.11* | 0.43 ± 0.06** | 0.94 ± 0.09 | 1.14 ± 0.13* |
| HEX 25[c] | 1.31 ± 0.10* | 0.42 ± 0.04** | 1.05 ± 0.10** | 1.23 ± 0.09* |
| AcOEt 5[c] | 1.33 ± 0.16* | 0.36 ± 0.06** | 0.97 ± 0.11 | 0.76 ± 0.07** |
| AcOEt 10[c] | 1.25 ± 0.09* | 0.37 ± 0.03** | 0.88 ± 0.06 | 0.73 ± 0.03** |
| AcOEt 25[c] | 0.70 ± 0.13** | 0.43 ± 0.07** | 0.27 ± 0.06** | 0.64 ± 0.16** |
| BuOH 5[c] | 1.34 ± 0.07* | 0.54 ± 0.10** | 0.79 ± 0.04 | 1.22 ± 0.15 |
| BuOH 10[c] | 1.25 ± 0.12* | 0.45 ± 0.05** | 0.80 ± 0.08 | 1.20 ± 0.19* |
| BuOH 25[c] | 1.02 ± 0.07** | 0.28 ± 0.03** | 0.74 ± 0.10 | 1.11 ± 0.10* |
| Aq 5[c] | 1.68 ± 0.03 | 0.71 ± 0.06* | 0.97 ± 0.04 | 1.35 ± 0.15 |
| Aq 10[c] | 1.46 ± 0.19 | 0.54 ± 0.10** | 0.89 ± 0.11 | 1.13 ± 0.15* |
| Aq 25[c] | 0.75 ± 0.12** | 0.21 ± 0.01** | 0.53 ± 0.11 | 0.67 ± 0.14** |
| oleanolic acid 1[c] | 1.11 ± 0.11** | 0.30 ± 0.04** | 0.81 ± 0.08 | 1.37 ± 0.21 |
| oleanolic acid 2,5[c] | 0.77 ± 0.14** | 0.20 ± 0.05** | 0,57 ± 0.09 | 1.17 ± 0.19 |
| oleanolic acid 5[c] | 0.51 ± 0.14** | 0.11 ± 0.04** | 0.40 ± 0.10* | 1.02 ± 0.14* |
| kaempferitrin 1[c] | 1.45 ± 0.16 | 0.30 ± 0.04** | 1.13 ± 0.12 | 1.60 ± 0.21 |
| kaempferitrin 5[c] | 1.30 ± 0.16* | 0.40 ± 0.14** | 0.90 ± 0.18 | 1.20 ± 0.11 |
| kaempferitrin 10[c] | 1.29 ± 0.18* | 0.60 ± 0.08** | 0,68 ± 0.09 | 0.94 ± 0.07** |
| Dex 0.5[c] | 0.97 ± 0.07** | 0.19 ± 0.02** | 0.79 ± 0.07 | 0.45 ± 0.08** |
| Indo 5[c] | 0.82 ± 0.10** | 0.20 ± 0.03** | 0.64 ± 0.08 | 0.64 ± 0.03** |

[a] Crude extract (CE, 25 to 200 mg/kg, i.p.) isolated from *Lotus corniculatus* and its derived fractions: hexane fraction (HEX, 5 to 25 mg/kg, i.p.), ethyl acetate fraction (AcOEt, 5 to 25 mg/kg, i.p.), butanol fraction (BuOH, 5 to 25 mg/kg, i.p.), aqueous fraction (Aq, 5 to 25 mg/kg, i.p.), and isolated compound, compound **1**, oleanolic acid (1 to 5 mg/kg, i.p.), and compound **2**, kaempferitrin 1 to 10 mg/kg, i.p.), administered 0.5 h before the induction of pleurisy by bradykinin (20 nmol/i.pl.). S = response in animals treated only with sterile saline (NaCl 0.95%). Bk-treated = response in animals treated only with bradykinin. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg, i.p.). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg, i.p.). *$p < 0.05$ and **$p < 0.01$. The data are reported as the mean ± SEM. $N = 6$ animals. [b] Administered by intrapleural route [c] administered by intraperitoneal route.

from 52.0 ± 9.1% to 73.4 ± 2.8% ($p < 0.01$). At 10 and 25 mg/kg, the BuOH fraction also decreased exudate concentrations from 23.4 ± 12.0% to 29.5 ± 6.2% ($p < 0.05$) (Table 1).

The aqueous (Aq) fraction was not as potent an anti-inflammatory agent as the other fractions. The Aq fraction significantly inhibited the measured inflammatory parameters only at the highest dose tested (25 mg/kg). The extent of inhibition (%) by the Aq fraction was as follows: leukocytes, 60.3 ± 6.5%; neutrophils, 78.8 ± 1.7%; and exudate concentrations, 57.0 ± 8.8% ($p < 0.01$). Additionally, the Aq fraction (5 and 10 mg/kg) inhibited neutrophils by 47.6 ± 9.4% and 51.0 ± 10.6%, respectively ($p < 0.05$), and at a dose of 10 mg/kg, it also inhibited exudate concentrations by 27.9 ± 9.6% ($p < 0.05$) (Table 1).

Because the HEX and AcOEt fractions presented better anti-inflammatory effects than the Aq fraction, the next step was to isolate compounds from these *Lotus corniculatus* fractions to determine which of them was responsible for inhibiting bradykinin-induced inflammation.

Compound **1** (oleanolic acid) revealed important anti-inflammatory effects at 1 to 5 mg/kg, inhibiting leukocytes from 39.5 ± 6.0% to 72.4 ± 7.4% ($p < 0.01$) and neutrophils from 73.0 ± 3.7% to 90.6 ± 3.4% ($p < 0.01$). At 5 mg/kg, compound **1** also inhibited both mononuclears by 43.4 ± 14.6% ($p < 0.05$) and exudate concentrations by 36.1 ± 8.8% ($p < 0.05$) (Table 1).

Compound **2** (kaempferitrin) at 5 and 10 mg/kg also significantly decreased leukocytes from 29.4 ± 8.8% to 30.0 ± 9.8% ($p < 0.05$) and neutrophils from 46.7 ± 7.5% to 66.8 ± 13.8% ($p < 0.01$). At 10 mg/kg, compound **2** inhibited exudate concentrations by 41.0 ± 4.6% ($p < 0.01$), and at 1 mg/kg, it also inhibited neutrophils by 73.9 ± 3.5% ($p < 0.01$) (Table 1).

It is important to note that in this study, compound **1** more potently decreased BK-induced inflammation than compound **2**: lower doses (1 to 5 mg/kg) of compound **1** inhibited the same inflammatory parameters (leukocytes and exudate concentrations) as kaempferitrin did at higher doses (5 to 10 mg/kg).

As expected, dexamethasone and indomethacin significantly inhibited leukocytes, neutrophils, and exudate concentrations ($p < 0.01$) (Tables 1).

**Effects of *Lotus corniculatus* var. São Gabriel on Myeloperoxidase and Adenosine-Deaminase Activities and Nitrate/Nitrite Levels.** Our results clearly demonstrated that *Lotus corniculatus* inhibited leukocyte influx and exudate concentrations. Because myeloperoxidase and adenosine-deaminase

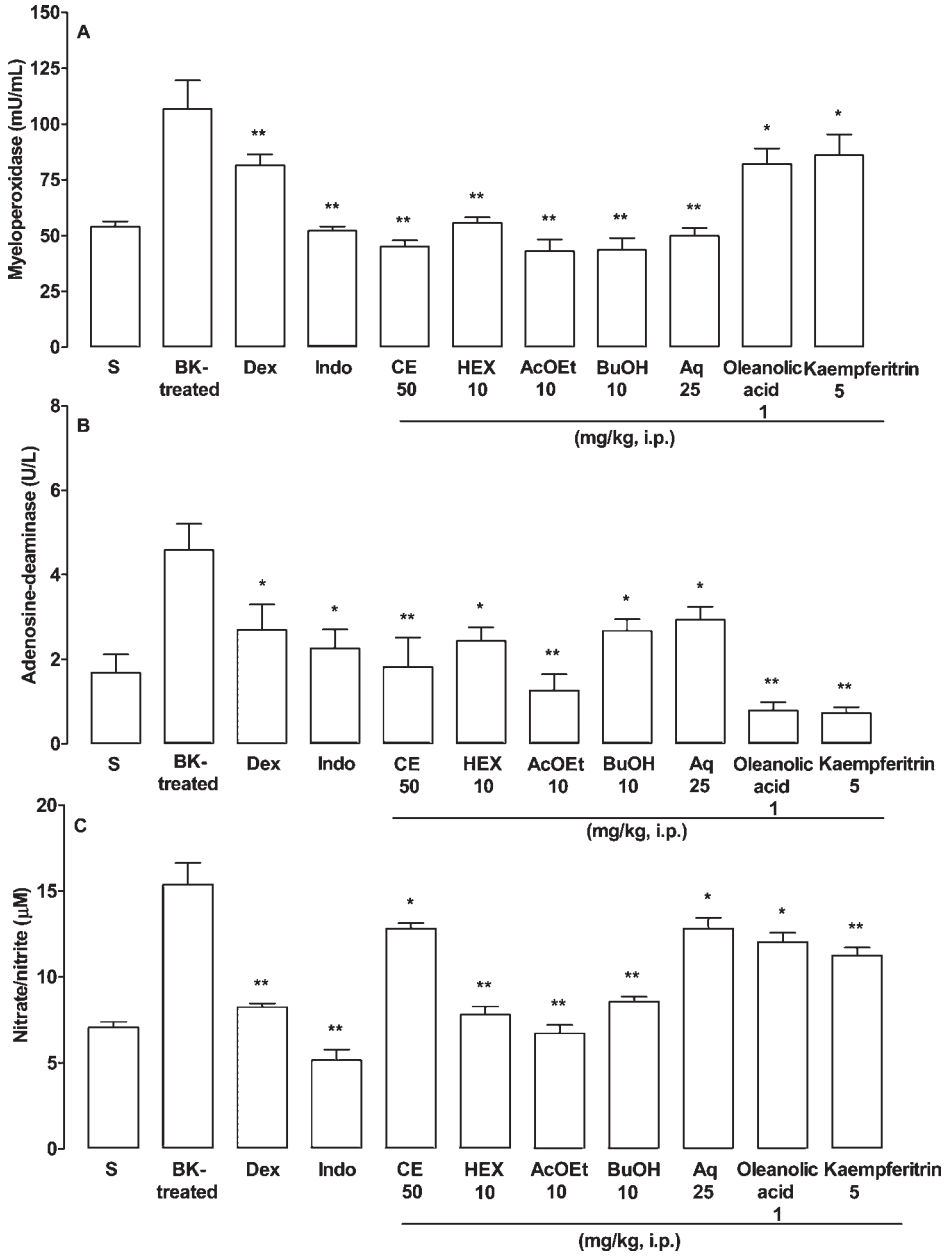


**Figure 3.** Effect of crude extract (CE: 50 mg/kg, i.p.) isolated from *Lotus corniculatus* and its hexane fraction (HEX, 10 mg/kg, i.p.), ethyl acetate fraction (AcOEt, 10 mg/kg, i.p.), butanol fraction (BuOH, 10 mg/kg, i.p.), aqueous fraction (Aq, 25 mg/kg, i.p.), compound **1** (oleanolic acid, 1 mg/kg, i.p.), and compound **2** (Kaempferitrin, 5 mg/kg, i.p.), administered 0.5 h before the induction of pleurisy by bradykinin (20 nmol/i.pl.), on myeloperoxidase (A) and adenosine-deaminase (B) activities and nitrite/nitrate concentrations (C). S = response in animals treated only with sterile saline (NaCl 0.95%). BK-treated = response in animals treated only with bradykinin. Dex = response in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Indo = response in animals pretreated with indomethacin (5.0 mg/kg, i.p.). $^{*}p < 0.05$ and $^{**}p < 0.01$. The data are reported as the mean ± SEM. $N = 6$ animals.

are considered important markers of leukocyte activation[23] and because nitric oxide is related to exudate and leukocyte chemotaxis,[25] in the next experiments, we assessed whether *Lotus corniculatus* extracts affected these pro-inflammatory markers in BK-treated mice. The pretreatment of mice with the CE of *L. corniculatus* and its derived fractions, as well as with the individual components of the extracts, caused a significant decrease in myeloperoxidase activity. The percent inhibition of myeloperoxidase by the various treatments (at the indicated doses) was as follows: CE (50 mg/kg), 39.9 ± 11.6; HEX fraction (10 mg/kg), 58.0 ± 5.8; AcOEt fraction (10 mg/kg), 49.2 ± 2.7; BuOH fraction (10 mg/kg), 59.2 ± 4.9; Aq fraction (25 mg/kg), 53.3 ± 3.3; oleanolic acid (1 mg/kg), 33.2 ± 16.3; and kaempferitrin (5 mg/kg), 19.5 ± 8.8 ($p < 0.05$) (Figure 3A). *Lotus corniculatus* extracts and derivatives also significantly inhibited adenosine-deaminase activity. The percent inhibition of adenosine-deaminase by the different treatments (at the indicated doses) was as follows: CE (50 mg/kg), 60.3 ± 15.1; HEX fraction (10 mg/kg), 47.0 ± 7.4; AcOEt fraction (10 mg/kg), 72.5 ± 8.5; BuOH fraction (10 mg/kg), 41.1 ± 6.4; Aq fraction (25 mg/kg), 36.0 ± 6.8; oleanolic acid (1 mg/kg), 82.8 ± 4.3; and kaempferitrin (5 mg/kg), 84.2 ± 3.1 ($p < 0.05$) (Figure 3B).

Under the same conditions, fractions of *Lotus corniculatus* and components thereof also decreased nitrate/nitrite

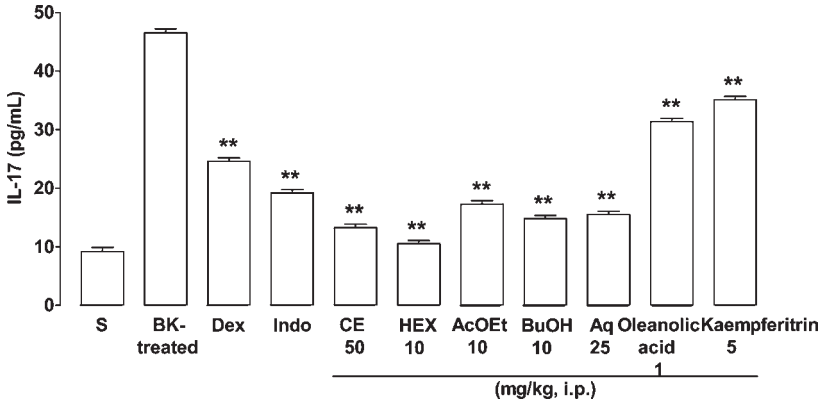


**Figure 4.** Effect of crude extract (CE, 50 mg/kg, i.p.) isolated from *Lotus corniculatus* and its hexane fraction (HEX, 10 mg/kg, i.p.), ethyl acetate fraction (AcOEt, 10 mg/kg, i.p.), butanol fraction (BuOH, 10 mg/kg, i.p.), aqueous fraction (Aq, 25 mg/kg, i.p.), compound **1** (oleanolic acid, 1 mg/kg, i.p.), and compound **2** (Kaempferitrin, 5 mg/kg, i.p.), administered 0.5 h before the induction of pleurisy by bradykinin (20 nmol/i.pl.), on IL-17 levels. S = response in animals treated only with sterile saline (NaCl 0.95%). BK-treated = response in animals treated only with bradykinin. Dex = response in animals pretreated with dexamethasone (0.5 mg/kg, i.p.). Indo = response in animals pretreated with indomethacin (5.0 mg/kg, i.p.). $^{**}p < 0.01$. The data are reported as the mean ± SEM. $N = 6$ animals.

concentrations. The percent inhibition of nitrate/nitrite levels by the individual treatments (at the indicated doses) was as follows: CE (50 mg/kg), 16.7 ± 2.1; HEX fraction (10 mg/kg), 49.2 ± 3.0; AcOEt fraction (10 mg/kg), 56.4 ± 3.2; BuOH fraction (10 mg/kg), 44.4 ± 1.9; Aq fraction (25 mg/kg), 16.8 ± 4.1; oleanolic acid (1 mg/kg), 21.8 ± 3.4; and kaempferitrin (5 mg/kg), 26.9 ± 3.1 ($p < 0.05$) (Figure 3C).

Dexamethasone and indomethacin were effective at inhibiting these pro-inflammatory parameters ($p < 0.05$) (Figure 3A, B, and C).

**Effects of *L. corniculatus* var. São Gabriel on IL-17A Levels.** One of the most important pro-inflammatory cytokines studied in the past few years is IL-17. This critical cytokine is responsible for amplifying the inflammatory process.[26]

In our experiments, the CE of *L. corniculatus* and its derived fractions, as well as individual components of the extracts, caused a significant decrease in IL-17A. The percent inhibition of IL-17A in each treatment group (with the indicated doses) was as follows: CE (50 mg/kg), 71.4 ± 1.2; HEX fraction (10 mg/kg), 77.4 ± 1.2; AcOEt fraction (10 mg/kg), 62.9 ± 1.3; BuOH fraction (10 mg/kg), 68.2 ± 1.3; Aq fraction (25 mg/kg), 66.6 ± 1.2; oleanolic acid (1 mg/kg), 24.6 ± 1.2; and kaempferitrin (5 mg/kg), 32.2 ± 1.3 ($p < 0.01$) (Figure 4).

Dexamethasone and indomethacin were effective at inhibiting IL-17A levels ($p < 0.01$) (Figure 4).

## DISCUSSION

Inflammation can be defined as a condition or state into which tissues enter as a response to injury or insult. This event is caused, among other factors, by the release of pro-inflammatory mediators, such as nitric oxide, and cytokines, including IL-17, and/or the activation of different cell types.[26,27]

Furthermore, neutrophils are the most abundant leukocyte to be delivered to the site of the inflammatory response, providing the first line of defense of the innate immune system.[28]

Another point to be considered is that the evidence suggests that rat/mouse pleurisy is a useful model for characterizing and/or screening plants with anti-inflammatory properties because it is possible to evaluate simultaneously both total and differential cell count, as well as the exudate concentrations and inflammatory mediators in a closed cavity.[16,19,23]

Our results showed that *Lotus corniculatus* and its derived fractions significantly inhibited pro-inflammatory enzymes (myeloperoxidase and adenosine-deaminase) and mediators (nitric oxide and IL-17), as well as activated leukocytes and exudate concentrations at the site of the inflammatory process. Our results are in accordance with published studies demonstrating the anti-inflammatory effects of *Lotus corniculatus* var. São Gabriel in a mouse model of pleurisy induced by carrageenan.[16]

In this study, *Lotus corniculatus* inhibited leukocytes, especially neutrophils, in the mouse model of pleurisy induced by bradykinin. The inhibitory effect upon leukocytes was associated with a decrease in myeloperoxidase activity. Myeloperoxidase, a heme protein abundantly expressed in neutrophils, has long been viewed to function primarily as a bactericidal enzyme centrally linked to innate host defense. Studies suggest that MPO is involved in the regulation of cellular homeostasis and may play a central role in the initiation and propagation of acute and chronic inflammatory disease.[29]

A significant inhibition of adenosine-deaminase activity was also detected. Adenosine-deaminase is an enzyme that regulates tissue function by activating different adenosine receptors, A1, A2A, A2B, and A3, on different types of cells in an inflammatory environment. Animal models of inflammation have also helped to elucidate the regulatory roles of adenosine in dictating the development and progression of disease.[30]

It is interesting to note that for leukocytes, exudate, nitric oxide, and MPO and ADA, the AcOEt, HEX, and BuOH fractions were more potent in inhibiting these pro-inflammatory parameters than the Aq fraction because lower doses of these fractions (10 mg/kg) promoted the same inhibitory effect as the higher dose (25 mg/kg) of the Aq fraction.

Another substance that is a potent modulator of inflammation is IL-17. This cytokine induces the release of IL-6, TNF-α, IL-1$\beta$, hematopoietic growth factors (granulocyte colony stimulating factor and granulocyte and macrophage colony stimulating factor), and chemokines (IL-8, CXC ligand 1, and monocyte chemoattractant protein 1) by activated lymphocytes. Additionally, IL-17 promotes the recruitment of neutrophils and monocytes to the site of inflammation.[26] Once again, *Lotus corniculatus* decreased IL-17A levels in BK-induced inflammation. For this parameter, the HEX, AcOEt, and BuOH fractions showed the same pattern of anti-inflammatory action.

Furthermore, in the case of *Lotus corniculatus*, the compounds oleanolic acid and kaempferitrin were isolated from the HEX and AcOEt fractions. These compounds presented an anti-inflammatory effect by inhibiting leukocyte migration, myeloperoxidase and adenosine-deaminase activities, as well as nitric oxide and IL-17A levels in the mouse pleural cavity. Furthermore, oleanolic acid was more potent in inhibiting the inflammation caused by bradykinin than kaempferitrin because a lower dose of oleanolic acid (1 mg/kg) caused the same effect as a higher dose (5 mg/kg) of kaempferitrin. These results are also corroborated by Giner-Larza et al.,[13] who reported that oleanolic acid displayed anti-inflammatory properties in mice by inhibiting 12-deoxyphorbol-13-phenyl acetate-induced edema in the ear. In this study, the authors also showed that this compound caused a significant decrease of paw edema induced by bradykinin or phospholipase $A_2$.

Kaempferitrin is also well known to have anti-inflammatory actions. For example, the compound decreased edema in croton-oil-induced inflammation in the ear of mice. Kaempferitrin also inhibited carrageenan-induced inflammation in the peritoneal cavity of mice.[11] Kaempferol exhibited an anti-inflammatory effect by inhibiting cyclooxygenase-2 levels, inducible nitric oxide synthase (iNOS) activity, monocyte chemoattractant protein-1 levels, and T-cell activation via the suppression of nuclear factor-kappaB (NF-kappaB) in the kidney of aged rats.[12] It is notable that in several aspects, *Lotus corniculatus* presents similar anti-inflammatory effects as the reference drugs, suggesting a common mechanism of anti-inflammatory action.

In our studies, we demonstrated that *Lotus corniculatus* displayed considerable anti-inflammatory action by inhibiting leukocyte influx and exudate concentrations induced by bradykinin. This effect was directly related to the inhibition of either $NO_x$ or IL-17A levels. Oleanolic acid and kaempferitrin can account for these anti-inflammatory effects and thus are important agents for future studies.

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## ABBREVIATIONS USED

AcOEt, ethyl acetate; ADA, adenosine deaminase; Aq, aqueous; BK, bradykinin; BuOH, *n*-butanol; CE, crude extract; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbant assay; eNOS, endothelial nitric oxide synthase; HEX, hexane; HPLC, high-performance liquid chromatography; IL-1, interleukin-1; IL-1$\beta$, interleukin-1$\beta$; IL-2, interleukin-2; IL-3, interleukin-3; IL-6, interleukin-6; IL-8, interleukin-8; IL-17, interleukin-17; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NF-$\kappa$B, nuclear factor-$\kappa$B; NO, nitric oxide; $PGE_2$, prostaglandin $E_2$; TNF-$\alpha$, tumor necrosis factor-alpha.

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