



Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: [www.elsevier.com/locate/jphs](http://www.elsevier.com/locate/jphs)

## Full Paper

# *Dermatophagoides pteronyssinus*-induced pro-inflammatory responses mediated via STAT3 and NF-kappaB signaling pathways in human bronchial epithelial cells – Inhibitory effects of *Lafoensia pacari* and ellagic acid

Ana Letícia Júlio de Souza <sup>a</sup>, Aline Beatriz Mahler Pereira <sup>a</sup>, Jhony Robison de Oliveira <sup>a</sup>, Luciana Santos Ramalho <sup>a</sup>, Henrique Ismarsi de Souza <sup>a</sup>, Altina Lacerda Nascimento <sup>b</sup>, Mohib Uddin <sup>c</sup>, Paulo Sérgio Pereira <sup>b</sup>, David Nascimento Silva Teixeira <sup>a</sup>, Paulo Roberto da Silva <sup>a</sup>, Deijanira Alves de Albuquerque <sup>d</sup>, Lucia Helena Faccioli <sup>e</sup>, Alexandre de Paula Rogério <sup>a,\*</sup>

<sup>a</sup> Institute of Health Sciences, Department of Clinical Medicine, Laboratory of Experimental Immunopharmacology, Federal University of Triângulo Mineiro, Uberaba, MG 38025-350, Brazil

<sup>b</sup> Programa de Pós-Graduação em Agroquímica, Instituto Federal Goiano, Rio Verde, GO, Brazil

<sup>c</sup> Respiratory Global Medicines Development, AstraZeneca, Gothenburg, Sweden

<sup>d</sup> Departamento de Ciências Básicas em Saúde, Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil

<sup>e</sup> Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

## ARTICLE INFO

## Article history:

Received 29 April 2019

Received in revised form

19 December 2019

Accepted 7 January 2020

Available online xxx

## Keywords:

Allergic asthma

Human bronchial epithelial cells

*Lafoensia pacari*

Ellagic acid

*Dermatophagoides pteronyssinus*

## ABSTRACT

The house dust mite allergen *Dermatophagoides pteronyssinus* (Der p) is a major driver of allergic asthma. Studies from our group demonstrated anti-eosinophilic effects of ethanolic extract of *Lafoensia pacari* stem bark (and ellagic acid, isolated from *L. pacari* extract), used as traditional medicine in Brazil to naturally treat inflammatory conditions. Here, we extended these results through performing phytochemical analysis of the constituents of *L. pacari* using liquid chromatography-mass spectrometry and evaluating the anti-inflammatory effects of both *L. pacari* and ellagic acid in the human BEAS-2B bronchial epithelial cell line stimulated with Der p. Ellagic acid (major constituent), gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid, but not flavonoids (rutin, kaempferol, luteolin and quercetin), were found in the *L. pacari*. Pro-inflammatory mediators, IL-6, IL-8 and CCL-2 production were increased in BEAS-2B stimulated with Der p (10 µg/mL, 24 h) compared to control. *L. pacari* (250 µg/mL) and ellagic acid (100 µM) significantly reduced the concentration of these mediators. *L. pacari* increased the production of the anti-inflammatory cytokine, IL-10. These results were associated with the downregulation

\* Corresponding author. Department of Clinical Medicine, Laboratory of Experimental Immunopharmacology, Institute of Health Sciences, Federal University of Triângulo Mineiro, Street Manoel Carlos 162, 38025-350 Uberaba, MG, Brazil.

E-mail addresses: [analejsouza@gmail.com](mailto:analejsouza@gmail.com) (A.L. Júlio de Souza), [alinebmp@gmail.com](mailto:alinebmp@gmail.com) (A. Beatriz Mahler Pereira), [jhony\\_sacra@hotmail.com](mailto:jhony_sacra@hotmail.com) (J. Robison de Oliveira), [lsantosramalho@yahoo.com.br](mailto:lsantosramalho@yahoo.com.br) (L. Santos Ramalho), [henrique\\_ismarsi@hotmail.com](mailto:henrique_ismarsi@hotmail.com) (H. Ismarsi de Souza), [altinalacerda@yahoo.com.br](mailto:altinalacerda@yahoo.com.br) (A. Lacerda Nascimento), [mohib.uddin@astrazeneca.com](mailto:mohib.uddin@astrazeneca.com) (M. Uddin), [paulo.pereira@ifgoiano.edu.br](mailto:paulo.pereira@ifgoiano.edu.br) (P. Sérgio Pereira), [david.teixeira@uftm.edu.br](mailto:david.teixeira@uftm.edu.br) (D. Nascimento Silva Teixeira), [pauloroberto@cefes.ufmg.edu.br](mailto:pauloroberto@cefes.ufmg.edu.br) (P. Roberto da Silva), [albuquerque@yahoo.com](mailto:albuquerque@yahoo.com) (D. Alves de Albuquerque), [faccioli@fcrp.usp.br](mailto:faccioli@fcrp.usp.br) (L. Helena Faccioli), [alexandre.rogerio@uftm.edu.br](mailto:alexandre.rogerio@uftm.edu.br) (A. de Paula Rogério).

Peer review under responsibility of Japanese Pharmacological Society.

<https://doi.org/10.1016/j.jphs.2020.01.004>

1347-8613/© 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Please cite this article as: Júlio de Souza AL et al., *Dermatophagoides pteronyssinus*-induced pro-inflammatory responses mediated via STAT3 and NF-kappaB signaling pathways in human bronchial epithelial cells – Inhibitory effects of *Lafoensia pacari* and ellagic acid, Journal of Pharmacological Sciences, <https://doi.org/10.1016/j.jphs.2020.01.004>

of NF- $\kappa$ B and STAT3 pathways. These findings indicate a novel anti-inflammatory action for *L. pacari* and ellagic acid in the airways allergic inflammatory response.

© 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

The house dust mite *Dermatophagoides pteronyssinus* (Der p) is commonly implicated as allergen causing allergic disorders such as asthma, atopic dermatitis, rhinoconjunctivitis and rhinitis.<sup>1</sup> The complex interaction between immune and structural cells including the epithelium is associated to pathological mechanisms of chronic inflammation of allergic asthma.<sup>2</sup> The airway epithelium is the first line of defense against inhaled aeroallergens which plays a significant role in innate and adaptive immune responses as well as mucosal airway inflammation.<sup>3</sup> Der p activates airway epithelial cells causing the release of pro-inflammatory chemokines and cytokines that attract and/or activate immune cells to airway inflammatory milieu.<sup>4</sup> In addition, Der p skews the immune system toward a Th2-mediated immune response.<sup>5</sup>

Using experimental models of allergic asthma in vivo which recapitulate key molecular and pathological features of allergic responses, our group have demonstrated the pharmacologic activities of plant extracts and their isolated compounds in reducing some of the pathological features of asthma.<sup>6</sup> *Lafoensia pacari* Jaumes St. Hilaire (Lythraceae) extract, used in traditional medicine by Brazilian population to treat gastric ulcers and inflammation, and its isolated compound, ellagic acid has previously been demonstrated to exhibit significant anti-inflammatory effects in dampening eosinophilic and neutrophilic-dominant airway inflammation.<sup>7,8</sup>

Here in this study, we extended these previous results and evaluated the anti-inflammatory effects of both *L. pacari* extract and ellagic acid in human BEAS-2B bronchial epithelial cell line stimulated with *D. pteronyssinus*. In addition, we also carried a phytochemical analysis of ethanolic extract of *L. pacari* stem bark.

## 2. Material and methods

### 2.1. Ethanolic extract

*L. pacari* stem bark was collected through permission in the city of Cuiabá (state of Mato Grosso, Brazil). A voucher specimen was deposited at the Central Herbarium of the Universidade Federal do Mato Grosso. The crude ethanolic extract was prepared as described previously.<sup>7</sup>

### 2.2. Analysis of *L. pacari* extract by liquid chromatography mass spectrometry (LC-MS)

*L. pacari* extract were analyzed in an LC-MS for the identification and quantification of gallic acid, ferulic acid, ellagic acid, rosmarinic acid, apigenin, kaempferol + luteolin, rutin and quercetin. The limits of quantification (LQ) considered as the first point of curve generating a signal/noise higher than 5 are gallic acid (25 ppb), ferulic acid (50 ppb), apigenin (25 ppb), kaempferol + luteolin (25 ppb), quercetin (250 ppb), rosmarinic acid (25 ppb), and rutin (250 ppb). Compound solubilization was performed in triplicate by adding methanol. The extract solution was filtered on a 0.45  $\mu$ m nylon filter prior to analysis. To prepare the curve, standards with concentrations ranging from 25 to 3000 ppb were used. Compound

separation was carried out in a C18 reverse column (250 mm  $\times$  4.6 mm  $\times$  5  $\mu$ m) and, for quantification, an SQ 300 MS mass spectrometer (PerkinElmer®) was used. Solvents used in the elution were (A) water + acetic acid (0.1%) and (B) methanol. The total running time was 32 min, with 2 min of equilibrium with 60% of solvent A and 40% of solvent B. Running was started with 60% of solvent A and 40% of solvent B, evolving to 100% of solvent B within 25 min. One hundred percent of solvent B remained for 5 min, returning to the initial condition (60% of solution A and 40% of solution B) for equilibrium for 2 min before the next injection. The ions monitored were gallic acid (160.1), ferulic acid (191.1), ellagic acid (301.1), chlorogenic acid (353.1) e rosmarinic acid (359.4), apigenin (269.1), kaempferol + luteolin (285.1), quercetin (301.1) and rutin (609.2).

### 2.3. Ellagic acid

Ellagic acid was isolated from ethanolic extract of *L. pacari* as described previously<sup>9</sup>

### 2.4. Bronchial epithelial cells

The BEAS-2B human bronchial epithelial cell line (purchased from ATCC; CRL-9609) was cultured in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 10% foetal bovine serum and 1% penicillin + streptomycin and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% ambient air.

### 2.5. Stimulus and treatment

BEAS-2B cells ( $1 \times 10^5$  cells/mL) were cultured in 96-well plates. *L. pacari* extract (10, 50 or 250  $\mu$ g/mL)<sup>7</sup> or ellagic acid (1, 10 or 100  $\mu$ M)<sup>10</sup> or vehicle (DMSO 0.05%) was administrated 1 h before the crude extract of *D. pteronyssinus* stimulation (10  $\mu$ g/mL; purchased from FDA Allergenic, Brazil).<sup>11</sup>

### 2.6. Cytotoxicity assay

Cell viability to the *L. pacari* extract (250  $\mu$ g/mL) and ellagic acid (100  $\mu$ M) were evaluated 24 h after treatments using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide reagent (MTT; Sigma–Aldrich, USA) as described previously with some modifications.<sup>12</sup> Briefly, after 24 h, 5 mg/mL MTT solution was added to each culture well and incubated in darkness for 4 h at 37 °C. At the end of incubation, the purple formazan crystals were dissolved adding 100  $\mu$ L/well of dimethyl sulfoxide for 30 min. Absorbance was measured with an automatic enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. Negative controls were obtained by measuring untreated cell medium. Data from control and treated cells were calculated and expressed as percentage of viable cells.

### 2.7. IL-8, CCL2, IL-6 and IL-10 concentrations

The supernatant was collected at 24 h after Der p stimulation, and IL-8, IL-6, CCL2 and IL-10 concentrations were determined by

enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (BD Pharmingen).

## 2.8. NF- B, I B  and STAT3 expression

The effect of *L. pacari* extract and ellagic acid on NF- B, I B  and STAT3 pathways were assessed by cytometry according to Oliveira et al., 2017.<sup>11</sup> Briefly, 24 h after Der p stimulation, cells were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37  C. After centrifugation, the cells were permeabilized in ice-cold methanol for 30 min and then stained with mouse monoclonal antibodies to Alexa Fluor 488 mouse anti-serine 529 phosphorylated NF- B p65 (p-p65<sup>S529</sup>) (BD Biosciences), PE mouse anti-I B  or Alexa Fluor 488 mouse anti-Stat3 (pS727) (BD Biosciences) their corresponding mouse IgG2b and IgG1 isotypes, respectively, for 60 min at 10  C in dark. The cells were then washed, resuspended, and subjected to analysis. The expression of intracellular phosphorylated signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACS Calibur; BD). We have chose flow cytometry once it most commonly used for the analysis of multiple kinases and provide quantitative data similar to the Western blot without using significant amount of cells or necessity of cell lysis and solubilization of the samples. In addition, flow cytometry has greater sensitivity and precision than Western blot in many proteins involved in signal transduction pathways whose expression is at low levels.<sup>13,14</sup>

## 2.9. Statistical analysis

The data are reported as mean   SEM. The means from different treatments in each individual experiment were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made with the Tukey's test. Values of  $P < 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Chemical analysis of *L. pacari* extract by liquid chromatography-mass spectrometry (LC-MS)

The following constituents were present in LC-MS analysis: ellagic acid, gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid. The monitored molecular ion of ellagic acid in 301.1 is also the characteristic ion for quercetin and glycosylated derivatives, but in both LC-MS and liquid chromatography with UV/diode array

detection (LC/DAD), flavonoids (rutin, kaempferol, luteolin and quercetin) were not detected in this extract. In this way, ellagic acid is the major constituent from *L. pacari* extract (Fig. 1 and Table 1).

### 3.2. Effects of *L. pacari* and ellagic acid exposure on cells viability in BEAS-2B stimulated with Der p

MTT assay showed that *L. pacari* and ellagic acid exposure, at higher dose (250  g/mL), did not induce any significant difference in cell viability cells compared to control (considered as 100%) (Fig. 2).

### 3.3. Ethanolic extract of *L. pacari* and ellagic acid reduced the IL-6 concentration in BEAS-2B stimulated with Der p

We next evaluated the dose-response effect of *L. pacari* extract (10–250  g/mL) and ellagic acid (1–100  M) on IL-6 production in bronchial epithelial cells stimulated with Der p in vitro. Compared to control group, Der p increased significantly the IL-6 concentration (Fig. 3). *L. pacari* decreased IL-6 productions in a dose dependent manner when compared to cells stimulated with Der p (Fig. 3A). The IL-6 production was reduced from  $280 \pm 26.97$  in Der p group to  $72.11 \pm 29.17$  (*L. pacari* at 10  g/mL), to  $28.61 \pm 24.18$  (*L. pacari* at 50  g/mL) and to  $16.86 \pm 6.32$  (*L. pacari* at 250  g/mL) (mean   SEM for  $n = 4-8$ ;  $p < 0.05$ ). Ellagic acid significantly decreased the IL-6 production when compared to Der p group (Fig. 3B) reducing from  $304.40 \pm 29.67$  in Der p group to  $145 \pm 23.03$  (ellagic acid at 10  M) and to  $66.32 \pm 20.96$  (ellagic acid at 100  M) (mean   SEM for  $n = 5-7$ ;  $p < 0.05$ ). No significant alteration in the IL-6 production was observed in the cells treated with ellagic acid at 1  M when compared to cells stimulated by Der p. BEAS-2B cells stimulated with Der p and treated with *L. pacari* (50 and 250  g/mL) and ellagic acid (100  M) decreased IL-6 productions when compared to control group.

No significant differences were observed in the IL-6 concentration in cells stimulated with Der p and treated with the vehicle when compared to cells only stimulated with Der p (supplementary Figure 1). In addition, no significant alteration of IL-6 production was observed in the cells only treated with *L. pacari* (250  g/mL) or ellagic acid (100  M) when compared to control group (supplementary Figure 2).

*L. pacari* at 250  g/mL and ellagic acid at 100  M were chosen as the maximally effective concentrations for the subsequent experiments.

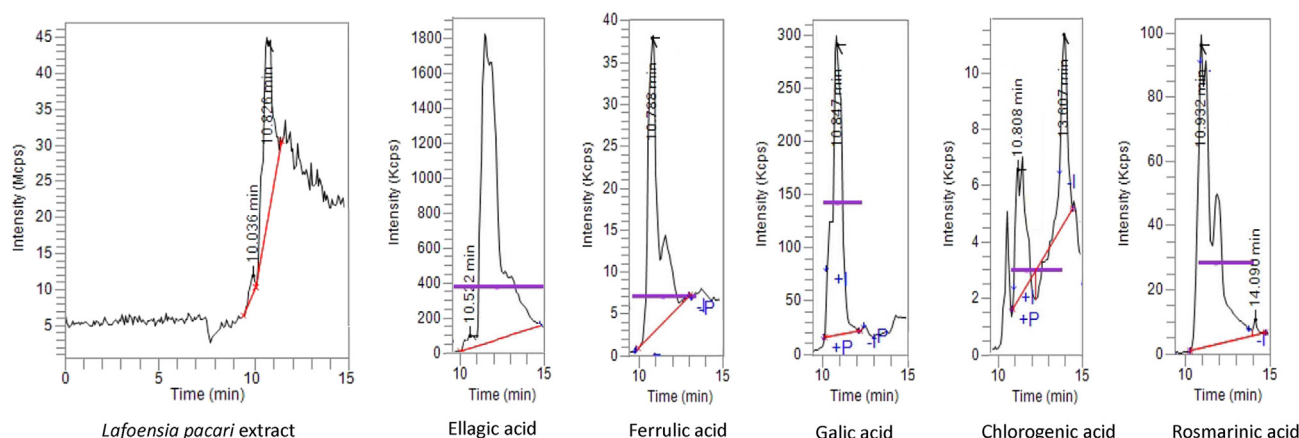
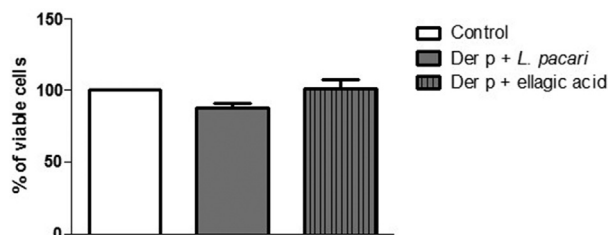


Fig. 1. Results of chromatographic profile of *Lafoensia pacari* extract.

**Table 1**HPLC-MS analysis of phenolic acids from ethanolic extract of *Lafoensia pacari* stem bark.

| Identified compound | Average $R_t$ (min) | RSD (%) | $[M-H]^+$ , $m/z$ | Concentration in stem bark, mg/100 g of dry weight |
|---------------------|---------------------|---------|-------------------|----------------------------------------------------|
| Ellagic acid        | 10.603              | 0.686   | 301               | 543.2 $\pm$ 6.6                                    |
| chlorogenic acid    | 10.831              | 4.520   | 353               | 21.4 $\pm$ 18.9                                    |
| Gallic acid         | 10.889              | 4.160   | 169               | 76.5 $\pm$ 1.6                                     |
| rosmarinic acid     | 11.004              | 3.842   | 359               | 9.5 $\pm$ 0.6                                      |
| Ferulic acid        | 11.447              | 3.306   | 193               | 25.6 $\pm$ 0.7                                     |

Compounds listed in order of retention time;  $R_t$  = retention time (as min).**Fig. 2.** Effects of *L. pacari* and ellagic acid exposure on cells viability. BEAS-2B cells were treated with *L. pacari* extract (250  $\mu$ g/mL) or ellagic acid (100  $\mu$ M) for 24 h. Cells viability was measured using MTT method. The data are reported as the means  $\pm$  SEM (n = 5/group) from three experiments.

### 3.4. *L. pacari* and ellagic acid reduced IL-8 and CCL2 concentrations while only *L. pacari* increased the IL-10 concentration in BEAS-2B stimulated with Der p

Compared to control group, Der p increased significantly IL-8 and CCL-2 concentrations (Fig. 4A and B, respectively), but no significant alteration was observed in IL-10 concentration (Fig. 4C). *L. pacari* extract (250  $\mu$ g/mL) and ellagic acid (100  $\mu$ M) decreased IL-8 and CCL-2 productions when compared to cells stimulated with Der p (Fig. 4A and B, respectively). The IL-8 production was reduced from 853.5  $\pm$  39.56 in Der p group to 131  $\pm$  10.04 (*L. pacari*) and to 388.40  $\pm$  71.23 (ellagic acid) (mean  $\pm$  SEM for n = 7–12;  $p < 0.05$ ). The CCL2 production was reduced from 1847  $\pm$  25.74 in Der p group to 21.85  $\pm$  9.32 (*L. pacari*) and to 107.10  $\pm$  25.22 (ellagic acid) (mean  $\pm$  SEM for n = 6–9;  $p < 0.05$ ). *L. pacari* significantly increased the production of IL-10 when compared to cells stimulated with Der p (Fig. 4C). The IL-10 production was increased from 10.59  $\pm$  5.64 in Der p group to 40.53  $\pm$  5.54 (*L. pacari*) (~4 folds; mean  $\pm$  SEM for n = 9;  $p < 0.05$ ). No significant alteration in the IL-10 production was observed in the cells treated with ellagic acid when compared to cells stimulated by Der p. BEAS-2B cells stimulated with Der p and treated with *L. pacari* and ellagic acid

(100  $\mu$ M) decreased IL-8 and CCL2 productions when compared to control group. On the other hand, *L. pacari* increased the IL-10 compared to cells non-stimulated.

No significant differences were observed in the IL-8, IL-10 and CCL2 productions in cells stimulated with Der p and treated with the vehicle when compared to cells stimulated with Der p (supplementary Figure 1). In addition, no significant alteration of IL-8, IL-10 and CCL2 production was observed in the cells only treated only with *L. pacari* or ellagic acid when compared to control group (supplementary Figure 2).

### 3.5. *L. pacari* extract and ellagic acid downregulated the phosphorylation of NF- $\kappa$ B in BEAS-2B stimulated with Der p

Der p induced the phosphorylation of NF- $\kappa$ B p65 Serine 529 (p-p65<sup>S529</sup>) when compared to the control group (Fig. 5A). *L. pacari* and ellagic acid significantly decreased the phosphorylation of NF- $\kappa$ B when compared to cells stimulated by Der p. p-p65<sup>S529</sup> was reduced from 0.30  $\pm$  0.02 in Der p group to 0.21  $\pm$  0.02 (*L. pacari*) and to 0.20  $\pm$  0.02 (ellagic acid) (mean  $\pm$  SEM for n = 4–6;  $p < 0.05$ ).

We also evaluated the effect of both *L. pacari* and ellagic acid in I $\kappa$ B $\alpha$  degradation. Der p induced I $\kappa$ B $\alpha$  degradation when compared to control group. Of note, *L. pacari* and ellagic acid significantly abolished the Der p-induced I $\kappa$ B $\alpha$  degradation (Fig. 5B).

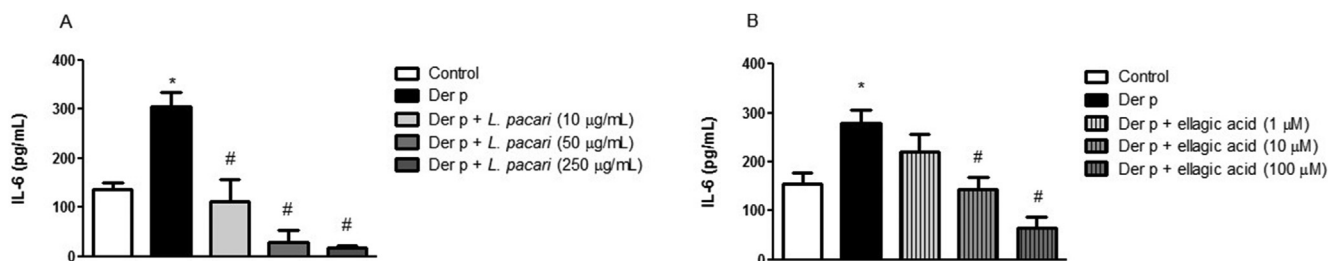
### 3.6. *L. pacari* extract and ellagic acid decreased the activation of phosphorylated STAT3 in BEAS-2B stimulated with Der p

Der p induced the phosphorylation of STAT3 (p-STAT3) when compared to control group (Fig. 6). *L. pacari* and ellagic acid significantly decreased the phosphorylation of STAT3 when compared to cells stimulated by Der p. p-STAT3 was reduced from 0.15  $\pm$  0.04 in Der p group to 0.06  $\pm$  0.01 (*L. pacari*) and to 0.10  $\pm$  0.01 (ellagic acid) (mean  $\pm$  SEM for n = 3–8;  $p < 0.05$ ).

## 4. Discussion

In this study, the phytochemical analysis of *L. pacari* extract found ellagic acid as the major compound, in addition to other compounds (gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid). Pharmacologically, *L. pacari* extract and ellagic acid was able to suppress Der p-induced IL-6, IL-8 and CCL-2 release from human bronchial epithelial cell lines in vitro. We also show for the first time that such anti-inflammatory effects were associated with downregulation of STAT3 and NF- $\kappa$ B signaling pathways and/or increase of IL-10 production.

Phenolic acids (such as ellagic acid, gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid) are widely distributed in the fruits, seeds, vegetables and medicinal plants. They are known mainly for the antioxidant activity but demonstrate also anticancer

**Fig. 3.** *L. pacari* extract and ellagic reduced the production of IL-6 on bronchial epithelial cells stimulated with *D. pteronyssinus* (Der p). BEAS-2B cells were treated or not with *L. pacari* (10–250  $\mu$ g/mL) or ellagic acid (1–100  $\mu$ M) and after 1 h the cells were stimulated or not with Der p (10  $\mu$ g/mL). After 24 h, the culture supernatants were collected and the IL-6 concentrations were measured using an ELISA kit. The data are reported as the means  $\pm$  SEM (n = 4–8/group). \*P < 0.05 versus control group. #P < 0.05 versus Der p group.

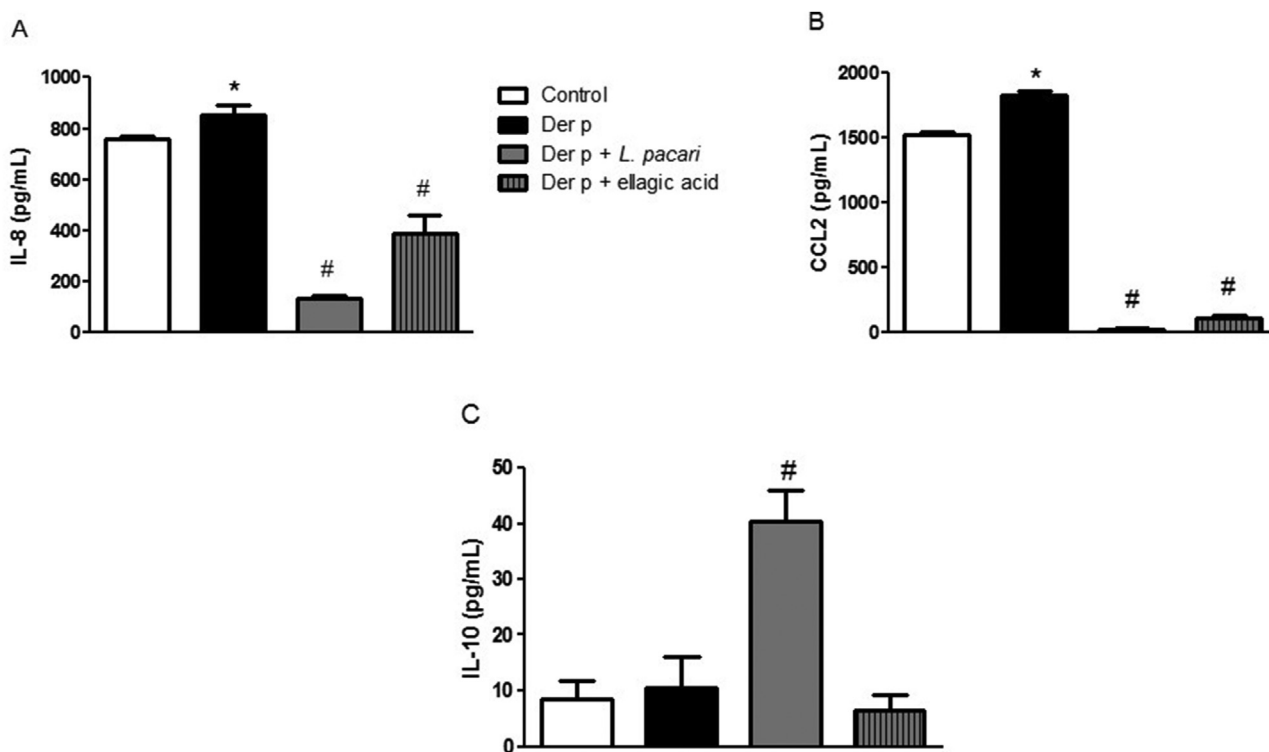


**Fig. 4.** *L. pacari* extract and ellagic acid reduced the production of IL-8 and CCL-2 but only *L. pacari* increased the IL-10 concentration in bronchial epithelial cells stimulated with *D. pteronyssinus* (Der p). BEAS-2B cells were treated or not with *L. pacari* (250 µg/mL) or ellagic acid (100 µM) and after 1 h the cells were stimulated or not with Dp (10 µg/mL). After 24 h, the culture supernatants were collected, and IL-8 (A), CCL-2 (B) and IL-6 (C) concentrations were measured using an ELISA kit. The data are reported as the means ± SEM (n = 6–12/group). \*P < 0.05 versus control group. #P < 0.05 versus Der p group.

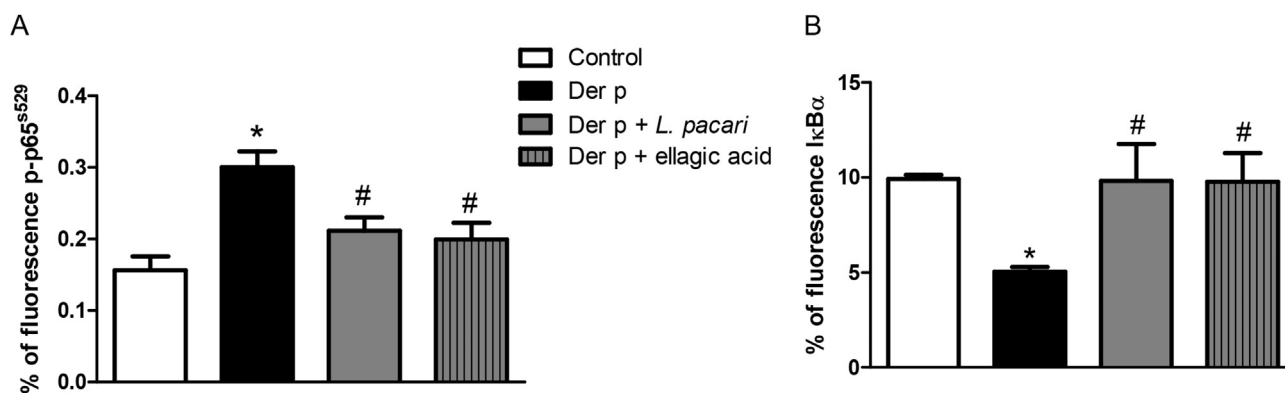


**Fig. 5.** *L. pacari* and ellagic acid downregulated the activation and translocation of NF-κB in bronchial epithelial cells stimulated with *D. pteronyssinus* (Der p). BEAS-2B cells were treated or not with *L. pacari* extract (250 µg/mL) or ellagic acid (100 µM) and after 1 h the cells were stimulated or not with Der p (10 µg/mL). After 24 h, the cells were recovered to evaluate the activation (phosphorylated NF-κB p65; p-p65<sup>S529</sup>) (A) and translocation (IκBα) (B) of NF-κB. The percentage of fluorescence in 50,000 cells was analyzed using cytometry. The data are reported as the means ± SEM (n = 4–6/group), from three independent experiments. \*P < 0.05 versus control group. #P < 0.05 versus Der p group.

and anti-inflammatory effects. Specifically in the inflammatory process, their target are multiple inflammatory components including immune cell regulation, proinflammatory cytokines synthesis and pathways such as NF-κB and arachidonic acids, and others.<sup>15–19</sup> Taken together, these effects make phenolic acids interesting compounds that can play a beneficial role in the prevention and the progress of inflammatory chronic diseases such as asthma, neurodegeneration and cardiovascular diseases, among other conditions.

Bioassay-guided fractionation of the ethanolic extract stem bark of *L. pacari* in a model of experimental acute eosinophilic (peritonitis induced by fraction of the *Histoplasma capsulatum* yeast wall in

mice) led to identify ellagic acid. In the chemical analysis of ethanolic extract stem bark of *L. pacari* by LC-MS, ellagic acid was found as the major active compound. This result is in agreement with other previous published studies.<sup>20,21</sup> In addition, the phytochemical analyses demonstrated the presence of gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid. Flavonoids (rutin, kaempferol, luteolin and quercetin) were undetectable. Reichert et al.<sup>21</sup> carried out phytochemical analysis of methanolic extract of *L. pacari* stem bark and found more than sixty substances among them ellagic acid, catechin, saponins and triterpenes. Other active compounds such as kaempferol, quercetin-3-O-xylopyranoside quercetin-3-O-rhamnopyranoside, flavan-3-ols have been detected

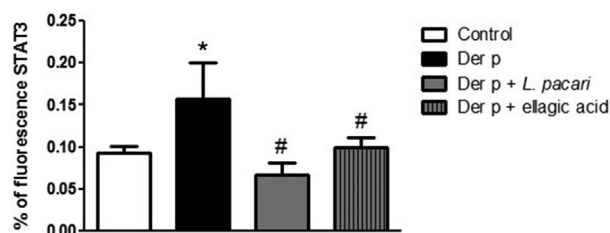


**Fig. 6.** *L. pacari* and ellagic acid downregulated STAT-3 pathways in bronchial epithelial cells stimulated with *D. pteronyssinus* (Der p). BEAS-2B cells were treated or not with *L. pacari* extract (250  $\mu$ g/mL) or ellagic acid (100  $\mu$ M) and after 1 h the cells were stimulated or not with Dp (10  $\mu$ g/mL). After 24 h, the cells were recovered, and the percentage of fluorescence in 50,000 cells was analyzed using cytometry. The data are reported as the means  $\pm$  SEM. (n = 3–8/group), from three independent experiments. \*P < 0.05 versus control group. #P < 0.05 versus Der p group.

in the leaves extracts.<sup>22,23</sup> The differences from phytochemical profiles these studies from ours could be associated to solvent extract (ethanol to methanol) and sample of crude extract (leaves to stem bark). However, in all of the published studies including ours, ellagic acid appears to be the main active constituent of the plant and thus could represent as a phytochemical marker for the *Lafoensia* species. So, ellagic acid is a hallmark from *L. pacari* extracts. In addition, all histories of our group with medicinal plants were mainly focused in the ellagic acid.<sup>6,24,25</sup> In this way, we chosen only the ellagic acid to carried out the experiments. The ellagic acid content in extract *L. pacari* corresponded to 17.9  $\mu$ M. So, we used a concentration range from 1 to 100  $\mu$ M of ellagic acid to carry out the experiments. These doses are in agreement with the previous study from our group (phagocytosis assay).<sup>10</sup>

Bronchial epithelial cells are essential to airways homeostasis and play a significant role in airways inflammation in lung diseases.<sup>3,26</sup> Bronchial epithelial cells express pattern recognition receptors such as Toll-like receptors (TLR) and protease-activated receptors (PARs), which recognize microorganisms and allergens such as *D. pteronyssinus* (Der p), respectively.<sup>1</sup> Der p induces the production of chemokines, cytokines and the expression of adhesion molecules in bronchial epithelial cells<sup>27</sup> that can influence dendritic cell maturation and T cell differentiation and consequently modulate the airway inflammation in asthma patients.<sup>28</sup> We found that *L. pacari* extract and ellagic acid effectively reduced the production of IL-6, IL-8 and CCL-2 in bronchial epithelial cells stimulated with Der p. IL-6 and IL-8 are important epithelial-derived mediators generated by the asthmatic epithelium that contribute to mucosal airway inflammation.<sup>26</sup> CCL2 is chemotactic to monocytes/macrophages, basophils, eosinophils, and Th2 cells. In addition, CCL2 is involved in the polarization of Th2 cells.<sup>29</sup> Our data also confirms previous results from our laboratory, which demonstrated that ethanolic extract of *L. pacari* and ellagic acid markedly dampened airway inflammation driven by an eosinophilic<sup>3,10,30</sup> or neutrophilic<sup>8</sup> component in the experimental models employed in vivo. In this regard, *L. pacari* and ellagic acid might favor the control and/or prevention of the development of Der p-induced allergic airway inflammatory responses seen in asthma.

A significant therapeutic target for the control of inflammatory processes is the transcription factor, NF- $\kappa$ B which regulates the production of IL-6, IL-8, and CCL2 as well as other inflammatory mediators associated with asthma pathogenesis.<sup>31</sup> Interestingly, *L. pacari* extract and ellagic acid reduced the phosphorylation of NF- $\kappa$ B (p-p65<sup>S29</sup>) in BEAS-2B cells stimulated by Der p. The diminished productions of IL-6, CCL2 and IL-8 are consistent with the down-regulation of NF- $\kappa$ B by *L. pacari* and ellagic acid. In resting cells, NF- $\kappa$ B is localized to the cytoplasm because of binding to inhibitory

protein I $\kappa$ B $\alpha$ . Pro-inflammatory stimulus leads to ubiquitination of the I $\kappa$ B $\alpha$ , targeting it for rapid degradation which allowed NF- $\kappa$ B to translocate into the nucleus where it can up-regulate transcription NF- $\kappa$ B-dependent genes. *L. pacari* and ellagic acid inhibit the I $\kappa$ B $\alpha$  degradation and consequently inhibit the NF- $\kappa$ B translocation to nucleus.<sup>32</sup> Thus, *L. pacari* and ellagic acid might have the therapeutic anti-inflammatory potential to control airways inflammation associated with allergic asthma.

STAT3 is a transcription factor associated with proliferation, apoptosis and differentiation cells that can modulate both innate and adaptive responses through several cytokines and chemokines, including IL-6,<sup>33</sup> IL-8<sup>34</sup> and CCL2.<sup>35</sup> *L. pacari* and ellagic acid reduced the phosphorylation of STAT3 induced by Der p in bronchial epithelial cells. These effects, in synergism with the down-regulation of NF- $\kappa$ B, could be related to the reduction of IL-6, IL-8 and CCL2 observed and consequently to the potential anti-inflammatory effects of *L. pacari* and ellagic acid.

IL-10 is an anti-inflammatory cytokine that blocks the synthesis of many inflammatory proteins.<sup>36</sup> An inverse association between severity of asthma and IL-10 concentration has been observed.<sup>37</sup> In addition, IL-10 has a known suppressive effect on peripheral tolerance to allergens inducing anergy of immune system.<sup>38</sup> We found that Der p did not induce IL-10 production. In addition, no significant alteration was observed in the IL-10 concentration levels in epithelial cells stimulated with Der p and treated with ellagic acid. This effect is at odds with previous results from our group<sup>8</sup> and others<sup>22</sup> which demonstrated ellagic acid increased IL-10 production. These differences could be associated with the different experimental models employed. Distinct from ellagic acid, *L. pacari* extract increased the IL-10 concentration in cells stimulated by Der p. Aside from ellagic acid, other compounds, such as gallic acid,<sup>39</sup> rosmarinic acid<sup>38</sup> and chlorogenic acid,<sup>40</sup> we had identified during phytochemical analysis, was also shown to increase IL-10 production. As such, these compounds alone and/or in combination with other constituents could explain the effect of *L. pacari* extract on IL-10 production.

Many therapeutic effects of plant extracts and their substances have been demonstrated by their immunomodulatory (immunostimulant or immunosuppressant) effects on the immune system.<sup>41</sup> In experimental toxocariasis, *L. pacari* extract reduced cell recruitments below to control.<sup>7</sup> These results are in agreement with our results whose decreasing of cytokines in Der p-stimulated cells was below to control which it is associated to significant increase of IL-10 production (immunosuppressive cytokine). *Phyllanthus amarus* extract, like *L. pacari*, also contain ellagic acid, gallic acid and other substances, demonstrated also immunosuppressive effects (cell migrations, phagocytosis, cytokine production and cell proliferations) which it was comparable to immunosuppressive control drug (cyclophosphamide).<sup>41</sup> This effect could be associated to one or more compounds or same to synergic effect of several substances. Besides of ellagic acid did not increase the IL-10 production in our model, it also can present potential immunosuppressive effects.<sup>42,43</sup> So, *L. pacari* and ellagic acid could downregulate the immune system causing an immunosuppressive effect and this effect or other effects from them could be more evidenced in altered cells such as cancerigenous or same inflammatory cells than normal or non-stimulated cells.<sup>44,45</sup> Further studies are warranted to understand these underlying anti-inflammatory properties of *L. pacari*, ellagic acid and other substances, found in the *L. pacari* extract, separately.

## 5. Conclusion

In conclusion, ellagic acid gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid were found in the *L. pacari* stem bark

extract. In addition, *L. pacari* extract and ellagic acid decreased the Der p-induced activation of bronchial epithelial cells by reducing the IL-6, IL-8 and CCL2 production and downregulating the NF- $\kappa$ B and STAT3 signaling pathways. In addition, *L. pacari*, different from ellagic acid, demonstrated immunomodulatory potential by increasing IL-10 production suggesting effects of other compounds (e.g. gallic acid, ferulic acid, chlorogenic acid and rosmarinic acid). Together, these results demonstrated that *L. pacari* and ellagic acid have anti-inflammatory potential to dampen airway allergic inflammation.

## Funding

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (no. 475349/2010-5), Fundação de Apoio a Pesquisa do Estado de Minas Gerais (FAPEMIG) (APQ-01873-14), Rede de Pesquisa em Doenças Infeciosas Humanas e Animais do Estado de Minas Gerais and Universidade Federal do Triângulo Mineiro (UFTM), Brazil. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

## Declaration of Competing Interest

MU is an employee of AstraZeneca and holds share in the company.

All other authors declare that they have no conflict of interest.

## Appendix A. Supplementary data

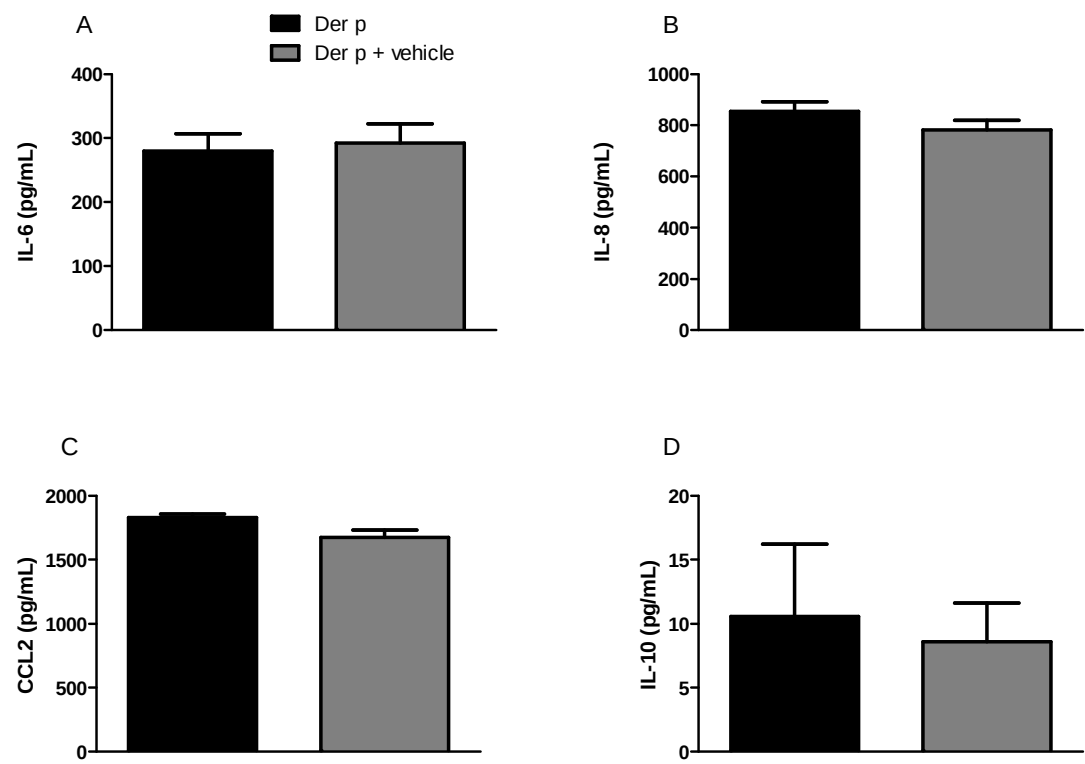
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphs.2020.01.004>.

## References

- Miller JD. The role of dust mites in allergy. *Clin Rev Allergy Immunol*. 2019 Dec;57(3):312–329. <https://doi.org/10.1007/s12016-018-8693-0>.
- Aghasafari P, George U, Pidaparti R. A review of inflammatory mechanism in airway diseases. *Inflamm Res*. 2019 Jan;68(1):59–74. <https://doi.org/10.1007/s00011-018-1191-2>.
- Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med*. 2012 May 4;18(5):684–692. <https://doi.org/10.1016/j.jaci.2012.07.050>.
- Yen CY, Chiang WD, Liu SY, Wang KT, Liao EC, Hsieh CL. Lithospermum erythrorhizon extract inhibits Der p2-induced inflammatory response through alleviation of thymic stromal lymphopoietin, nuclear factor Kappa B, and inflammasome expression in human bronchial epithelial cells. *J Ethnopharmacol*. 2017;201:1–8. <https://doi.org/10.1016/j.jep.2017.02.038>.
- Nieuwenhuizen NE, Kirstein F, Hoving JC, Brombacher F. House dust mite induced allergic airway disease is attenuated in CD11c<sup>cre</sup>IL-4R $\alpha$ <sup>flx</sup> mice. *Sci Rep*. 2018 Jan 17;8(1):885. <https://doi.org/10.1038/s41598-017-19060-9>. Erratum in: *Sci Rep*. 2018 Apr 11;8(1):6054. PMID: 29343807; PMCID: PMC5772663.
- Rogério AP, Sá-Nunes A, Faccioli LH. The activity of medicinal plants and secondary metabolites on eosinophilic inflammation. *Pharmacol Res*. 2010;62(4):298–307. <https://doi.org/10.1016/j.phrs.2010.04.005>.
- Rogério AP, Sá-Nunes A, Albuquerque DA, et al. *Lafloensia pacari* extract inhibits IL-5 production in toxocaríasis. *Parasite Immunol*. 2003;25(7):393–400. <https://doi.org/10.1046/j.1365-3024.2003.00648.x>.
- Favarin DC, Teixeira MM, Andrade EL, et al. Anti-inflammatory effects of ellagic acid on acute lung injury induced by acid in mice. *Mediat Inflamm*. 2013;13: Article ID 164202 <https://doi.org/10.1155/2013/164202>.
- Rogério AP, Fontanari C, Melo MC, et al. Anti-inflammatory, analgesic and anti-oedematous effects of *Lafloensia pacari* extract and ellagic acid. *J Pharm Pharmacol*. 2006;58:1265–1273. <https://doi.org/10.1211/jpp.58.9.0014>.
- De Freitas Alves C, Angeli GN, Favarin DC, et al. The effects of proresolution of ellagic acid in an experimental model of allergic airway inflammation. *Mediat Inflamm*. 2013;2013:863198. <https://doi.org/10.1155/2013/863198>.
- Oliveira JR, Silva PR, Rogério AP. AT-RvD1 modulates the activation of bronchial epithelial cells induced by lipopolysaccharide and *Dermatophagoides pteronyssinus*. *Eur J Pharmacol*. 2017;805:46–50. <https://doi.org/10.1016/j.ejphar.2017.03.029>.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1–2):55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).
- Barteneva NS, Fasler-Kan E, Vorobjev IA. Imaging flow cytometry: coping with heterogeneity in biological systems. *J Histochem Cytochem*. 2012 Oct;60(10):723–733. <https://doi.org/10.1369/0022155412453052>. Epub 2012 Jun 27. PMID: 22740345; PMCID: PMC3524563.
- Perez OD, Krutzik PO, Nolan GP. Flow cytometric analysis of kinase signaling cascades. *Methods Mol Biol*. 2004;263:67–94.
- Liang Ningjian, Kitts David D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients*. 2016;8(1):16. <https://doi.org/10.3390/nu8010016>.
- Sgarbossa A, Giacomazza D, Di Carlo M. Ferulic acid: a hope for Alzheimer's disease therapy from plants. *Nutrients*. 2015;7(7):5764–5782. <https://doi.org/10.3390/nu7075246>.
- Elufioye Taiwo O, Habtemariam Solomon. Hepatoprotective effects of rosmarinic acid: insight into its mechanisms of action. *Biomed Pharmacother*. 2019;112:108600. <https://doi.org/10.1016/j.biopha.2019.108600>.
- Kahkeshani N, Farzaei F, Fotouhi M, et al. Pharmacological effects of gallic acid in health and diseases: a mechanistic review. *Iran J Basic Med Sci*. 2019;22(3):225. <https://doi.org/10.22038/ijbms.2019.32806.7897>.
- Rios JL, Giner RN, Marin M, Recio MC. A pharmacological update of ellagic acid. *Planta Med*. 2018;84(15):1068–1093. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).
- Solon S, Lopes L, Teixeira de Sousa Jr P, Schmeda-Hirschmann G. Free radical scavenging activity of *Lafloensia pacari*. *J Ethnopharmacol*. 2000;72(1–2):173–178. [https://doi.org/10.1016/S0378-8741\(00\)00233-6](https://doi.org/10.1016/S0378-8741(00)00233-6).
- Reichert CL, da Silva DB, Carolllo CA, Weffort-Santos AM, de Moraes Santos CA. Metabolic profiling and correlation analysis for the determination of killer compounds of proliferating and clonogenic HRT-18 colon cancer cells from *Lafloensia pacari*. *J Ethnopharmacol*. 2018 Oct 5;224:541–552. <https://doi.org/10.1016/j.jep.2018.06.021>.
- Pereira LOM, Vilegas W, Tangerina MMP, et al. *Lafloensia pacari* A. St.–Hil: wound healing activity and mechanism of action of standardized hydro-ethanolic leaves extract. *J Ethnopharmacol*. 2018 Jun 12;219:337–350. <https://doi.org/10.1016/j.jep.2018.02.038>.
- Carneiro CC, da Costa Santos S, de Souza Lino Jr R, et al. Chemopreventive effect and angiogenic activity of punicalagin isolated from leaves of *Lafloensia pacari* A. St.–Hil. *Toxicol Appl Pharmacol*. 2016;310:1–8. <https://doi.org/10.1016/j.taap.2016.08.015>.
- Favarin DC, de Oliveira JR, de Oliveira CJ, Rogerio A de P. Potential effects of medicinal plants and secondary metabolites on acute lung injury. *BioMed Res Int*. 2013;2013:576479. <https://doi.org/10.1155/2013/576479>.
- Alves Cde F, Angeli GN, Favarin DC, et al. The effects of proresolution of ellagic acid in an experimental model of allergic airway inflammation. *Mediat Inflamm*. 2013;2013:863198. <https://doi.org/10.1155/2013/863198>.
- Uddin M, Lau LC, Seumois G, et al. EGF-induced bronchial epithelial cells drive neutrophil chemotactic and anti-apoptotic activity in asthma. *PLoS One*. 2013;8(9):e72502. <https://doi.org/10.1371/journal.pone.0072502>.
- Ruggieri S, Drago G, Longo V, et al. Sensitization to dust mite defines different phenotypes of asthma: a multicenter study. *Pediatr Allergy Immunol*. 2017 Nov;28(7):675–682. <https://doi.org/10.1111/pai.12768>.
- Tsoumakidou M, Tousse S, Semitekolou M, et al. Tolerogenic signaling by pulmonary CD1c+ dendritic cells induces regulatory T cells in patients with chronic obstructive pulmonary disease by IL-27/IL-10/inducible costimulator ligand. *J Allergy Clin Immunol*. 2014;134:944–954. e8 <https://doi.org/10.1016/j.jaci.2014.05.045>.
- Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol*. 2015;16(1):45. <https://doi.org/10.1038/ni.3049>.
- Rogério AP, Fontanari C, Borducchi É, et al. Anti-inflammatory effects of *Lafloensia pacari* and ellagic acid in a murine model of asthma. *Eur J Pharmacol*. 2008;580(1–2):262–270. <https://doi.org/10.1016/j.ejphar.2007.10.034>.
- Coutinho DS, Anjos-Valotta EA, do Nascimento CVMF, et al. 15-Deoxy-Delta-12,14-Prostaglandin J2 inhibits lung inflammation and remodeling in distinct murine models of asthma. *Front Immunol*. 2017;8:740. <https://doi.org/10.3389/fimmu.2017.00740>.
- Taniguchi K, Karin M. NF- $\kappa$ B, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol*. 2018;18(5):309. <https://doi.org/10.1038/nri.2017.142>.
- Nishihara M, Ogura H, Ueda N, et al. IL-6-gp130-STAT3 in T cells directs the development of IL-17+ Th with a minimum effect on that of Treg in the steady state. *Int Immunol*. 2007;19:695–702. <https://doi.org/10.1093/intimm/dxm045>.
- Gharav I, Nima M, et al. Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *J Biol Chem*. 2007;282(43):31460–31468. <https://doi.org/10.1074/jbc.M704267200>.
- Xue J, Ge X, Zhao W, et al. PIPK $\gamma$  regulates CCL2 expression in colorectal cancer by activating AKT-STAT3 signaling. *J Immunol Res*. 2019;12:3690561. <https://doi.org/10.1155/2019/3690561>.
- Wawrzyniak P, Akdis CA, Finkelman FD, Rothenberg ME. Advances and highlights in mechanisms of allergic disease in 2015. *J Allergy Clin Immunol*. 2016 Jun;137(6):1681–1696. <https://doi.org/10.1016/j.jaci.2016.02.010>.
- Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10:170–181. <https://doi.org/10.1038/nri2711>.
- Akdis CA, Blaser K. IL-10 induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. *FASEB J*. 1999;13:603–609. <https://doi.org/10.1096/fasebj.13.6.603>.
- Lima VN, Oliveira-Tintino CD, Santos ES, et al. Antimicrobial and enhancement of the antibiotic activity by phenolic compounds: gallic acid, caffeic acid and

- pyrogallol. *Microb Pathog.* 2016;99:56–61. <https://doi.org/10.1016/j.micpath.2016.08.004>.
40. Chkhikvishvili I, Sanikidze T, Gogia N, et al. Rosmarinic acid-rich extracts of summer savory (*Satureja hortensis* L.) protect Jurkat T cells against oxidative stress. *Oxid Med Cell Longev.* 2013;2013:456253. <https://doi.org/10.1155/2013/456253>.
  41. Ilangkovan M, Jantan I, Mesaik MA, Bukhari SN. Immunosuppressive effects of the standardized extract of *Phyllanthus amarus* on cellular immune responses in Wistar-Kyoto rats. *Drug Des Dev Ther.* 2015;9:4917. <https://doi.org/10.2147/DDDT.S88189>.
  42. Allam G, Abuelsaad AS, Alblihed MA, Alsulaimani AA. Ellagic acid reduces murine schistosomiasis mansoni immunopathology via up-regulation of IL-10 and down-modulation of pro-inflammatory cytokines production. *Immunopharmacol Immunotoxicol.* 2016 Jul 3;38(4):286–297. <https://doi.org/10.1080/08923973.2016.1189561>.
  43. Anderson KC, Teuber SS. Ellagic acid and polyphenolics present in walnut kernels inhibit in vitro human peripheral blood mononuclear cell proliferation and alter cytokine production. *Ann N Y Acad Sci.* 2010 Mar;1190(1):86–96. <https://doi.org/10.1111/j.1749-6632.2009.05259.x>.
  44. Zhang HM, Zhao L, Li H, Xu H, Chen WW, Tao L. Research progress on the anticarcinogenic actions and mechanisms of ellagic acid. *Cancer Biol Med.* 2014 Jun;11(2):92–100. <https://doi.org/10.7497/j.issn.2095-3941.2014.02.004>.
  45. da Silva Marcondes DB, Reichert CL, de Andrade LF, de Moraes Santos CA, Weffort-Santos AM. Cytotoxicity and apoptogenic effects of *Lafoensia pacari*. *J Ethnopharmacol.* 2014 Nov 18;157:243–250. <https://doi.org/10.1016/j.jep.2014.09.018>. Epub 2014 Oct 13.

Supplementary Figure 1



Supplementary Figure 2

