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The anti-inflammatory effect of *Ilex paraguariensis* A. St. Hil (Mate) in a murine model of pleurisy

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ABSTRACT

Ilex paraguariensis is a native plant from Southern America, where it is used as a beverage. In traditional medicine, it is used to treat many diseases including inflammation. However, we do not yet know precisely how this effect occurs. We therefore evaluated its anti-inflammatory effect in a murine model of pleurisy. The standardized CE, BF and ARF fractions, Caf, Rut and CGA were able to reduce leukocyte migration, exudate concentration, MPO and ADA activities and NOx levels. Moreover, *I. paraguariensis* also inhibited the release of Th1/Th17 pro-inflammatory cytokines, while increasing IL-10 production and improving the histological architecture of inflamed lungs. In addition, its major compounds decreased p65 NF-κB phosphorylation. Based on our results, we can conclude that *I. paraguariensis* exerts its anti-inflammatory action by attenuating the Th1/Th17 polarization in this model. This fact suggests that the use of this plant as a beverage can protect against Th1/Th17 inflammatory diseases.

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1. Introduction

Ilex paraguariensis A. St. Hil. (Aquifoliaceae), popularly known as yerba mate, is a native species from Southern Latin America, where it is used as a beverage and has great economic and social importance. In traditional medicine, it is used to treat arthritis, fatigue, obesity, and liver and intestinal affections [1]. The bioactive properties of *I. paraguariensis* are related to its phytochemical composition, especially methylxanthines [2], saponins and phenolic compounds [1]. In the last 15 years, several studies of *I. paraguariensis* have been performed, demonstrating interesting biological effects like anti-obesity [3], anti-diabetic [3], neuroprotective [4], antioxidant [5], antimicrobial [6], and anti-inflammatory [7] effects. However, as far as we know, there have been no studies to explore whether this anti-inflammatory effect is due to a possible effect on the cytokine environment involved in *in vivo* inflammatory process.

The non-resolving inflammatory process involves on a lack of immune system control and, in most cases, leads to chronic disease that require a long-term treatment and often result in loss of function of the affected organ. Notably, the disruption of normal balance of pro- and anti-inflammatory cytokines is a shared characteristic of these diseases *e.g.* neutrophilic asthma, rheumatoid arthritis, systemic erythematosus lupus, and many others [8,9]. In this context, the growing understanding about the immune modulation, Th1/Th2/Th17 balance and the intricate cell signaling related to these diseases had been leading researchers to take a different approach to deal with these conditions.

It is well known that adhesion to patient therapy in long-term treatments for non-self-resolving inflammatory process is very weak, which contributes to the high percentage of related disabilities and deaths [10]. For this reason, the discovery of natural source-derived compounds, specially from plants commonly used as food intake like *I. paraguariensis*, seems to have great potential in the treatment of these conditions [11,12].

In order to better clarify this point, in the present study, we focused on evaluating whether the anti-inflammatory property of this plant is related to its ability to change the cytokine environment. For this propose, *I. paraguariensis* standardized crude extract (CE), its related fractions – buthanolic (BF) and aqueous residue (ARF) fractions – and its major compounds caffeine (Caf), rutin (Rut) and chlorogenic acid (CGA) were tested in an *in vivo* model of pleurisy. We also investigated the effects on leukocyte migration and plasma exudation, myeloperoxidase (MPO)

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and adenosine deaminase (ADA) activities, oxide nitric metabolite (NOx) concentration, pro- and anti-inflammatory cytokine levels, histological lung parameters, and finally, the action of its major compounds on phosphorylation of the p65 subunit of NF-κB (p-p65 NF-κB).

2. Materials and methods

2.1. Plant material

Ilex paraguariensis A. St. Hil. leaves were harvested in October/2012 in Erechim (27°38′03″ S and 52°16′26″ W), in the State of Rio Grande do Sul, Brazil. A voucher specimen was identified by Dr. Branca Maria Severo and deposited in the Herbarium of Universidade de Passo Fundo (RSPF 11074). The leaves were immediately frozen, lyophilized, crushed and stored at −20 °C until required for the preparation of extracts.

2.2. Extract preparation

The *I. paraguariensis* extracts were prepared by turboextraction in an Ultra-Turrax. Briefly, 200 g of leaves were extracted for five minutes, with 1000 mL of ethanol 20° GL (Gay Lussac) as the liquid extractor (1:5 m/v), yielding the hydroethanolic extract. After filtration, the ethanol was eliminated under reduced pressure, the volume was adjusted to 800 mL with distilled water, and the preparation was separated into two fractions. One fraction was evaporated under reduced pressure to dryness to obtain the crude extract (CE). The second fraction was then partitioned with *n*-BuOH, yielding the *n*-BuOH fraction (BF) and aqueous residual fraction (ARF). The *n*-BuOH of BF was evaporated under reduced pressure and all the samples, CE, BF and ARF, were dried by lyophilization.

2.3. Chromatographic separation

Chromatographic separation was performed using an Acquity-UPLC™ (Waters, MA, USA) system equipped with a quaternary pump, degasser and autosampler. Detection was carried out using a photodiode array detector (PDA). The column used was a Waters BEH C18 column, 1.7 μm, 50 × 2.1 mm at 40 °C. The method used a gradient combining solvent A (formic acid/water, pH 2.5) and solvent B (acetonitrile), programmed as follows: 0–5 min, linear change from A-B (97:3 v/v) to A-B (90:10 v/v); 5–6 min, isocratic A-B (90:10 v/v); 6–9 min, linear change to A-B (80:20 v/v) and 9–10 min, linear change to A-B (10:90 v/v). The flow rate was kept constant at 0.3 mL min^{-1} and the injection volume was 5 μL. The peaks were characterized by comparing the retention time, UV spectra and by co-injection of the sample with the reference standards. Quantification was performed by external calibration, using their corresponding standards. Caffeine was quantified at 280 nm, while the phenolic compounds chlorogenic acid and rutin were quantified at 320 nm. All the analyses were performed in triplicate, and the peak area measured. The standard solutions were analyzed in different ranges: 0.05–50 μg mL^{-1} for the caffeine (Sigma-Aldrich®); 0.05–100 μg mL^{-1} for the chlorogenic acid (Fluka®) and 0.25–50 μg mL^{-1} for the rutin (Sigma-Aldrich®). Quantification was performed using six to eight-point regression curves (caffeine, $r^2 = 1$; chlorogenic acid, $r^2 = 0.9999$; rutin, $r^2 = 0.9999$). The regression equations were "$y = 39.320x + 2074.9$" for caffeine, "$y = 46.696x - 1896$" for chlorogenic acid, and "$y = 15.618x + 178.92$" for rutin. The extracts were analyzed at a concentration of 500 μg mL^{-1}. The results were expressed as milligrams per gram of extract (mg compound/g^{-1} E).

2.4. LC–MS analysis

In addition to LC/PDA analysis, the identification was carried out by liquid chromatography (UPLC, Waters Acquity mode) coupled to a high-resolution mass spectrometer (Xevo G2 QTof model), equipped with an electrospray ionization source and controlled by MassLynx v.4.1 software for data acquisition. The mass spectrometer parameters were set as follows: ionization mode, electrospray negative ion; capillary voltage, 2.0 kV; source block temperature, 90 °C; desolvation temperature, 350 °C; nebulizer nitrogen flow rate, 30 L h^{-1}; desolvation nitrogen gas flow, 600 L h^{-1}; and cone voltage, 40 V. The spectra were recorded by scanning the mass range from m/z 100 to 1000 with scan time of 0.5 s.

2.5. LC-PDA validation procedure

The LC-PDA quantification method was validated according to the ICH guidelines (2005) to comply with the requirements for specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of optical detection (LOD).

2.6. Animals

In this experimental protocol, we used female Swiss mice (18–22 g), housed under standardized and controlled conditions (20 ± 2 °C, 12 h light/dark periods) with free access to chow and water. All experiments were designed to minimize animal suffering and to use the minimum number of animals required to achieve a valid statistical evaluation. The experiments were performed according to the regulations of the Brazilian College of Animal Experimentation (COBEA) and are in accordance with the rules of the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (CEUA - PP00965).

2.7. Experimental design of the murine model of pleurisy

The pleurisy was performed as previously reported [13]. Briefly, the experimental protocol was divided into two steps. First, to establish a dose–response curve, animals were randomly divided in different groups ($n = 6$) and challenged with Evans Blue dye solution (25 mg/kg) administered by the intravenous route (i.v). After 10 min, different groups were treated with different doses of CE (10–50 mg/kg), BF (0.1–10 mg/kg), ARF (0.1–10 mg/kg), Caf (0.1–5 mg/kg), Rut (0.01–1 mg/kg), CGA (0.01–1 mg/kg) or dexamethasone (0.5 mg/kg) (Dex) by the oral route (p.o.). Shortly after 0.5 h, pleurisy was induced by a single injection of 0.1 mL of sterile saline containing λ-carrageenan 1% (w/v) (Cg) administered by the intra-pleural route (i.pl). The animals were euthanized after 4 h with an overdose of pentobarbital (120 mg/kg) administered by the intraperitoneal route (i.p.), and the pleural cavity was exposed and washed with 1.0 mL of sterile phosphate buffered saline (PBS, pH 7.2) (Laborclin, Pinhais, Paraná, Brazil) containing heparin (20 IU/mL). The pleural fluid was used to measure the primary inflammatory parameters: total and differential leukocyte count and exudate concentration. In order to determine the time-course response of the plant material, other groups of animals were pre-treated with the lowest effective dose of CE obtained above (25 mg/kg) at 0.5, 1 and 2 h before pleurisy induction.

In another set of experiments, previously selected doses were used to perform the MPO, ADA and NOx assays and quantification of pro- and anti-inflammatory cytokines in the pleural fluid. Also, p65 NF-κB phosphorylation and histological changes were performed on lung tissue samples. For these experiments, the animals were orally treated with CE (25 mg/kg), BF (1 mg/kg), ARF (1 mg/kg), Caf (5 mg/kg), Rut (1 mg/kg) or CGA (0.1 mg/kg), followed by induction of pleurisy by Cg 1% (i.pl.). In parallel with all the experiments, a group of animals was challenged with 0.1 mL of Cg 1% (i.pl.) only, and was named as a positive control group, while another group received 0.1 mL of saline (0.9% NaCl) (i.pl.), and was considered a negative control group.

2.8. Quantification of the leukocyte content

To quantify the leukocyte content, pleural fluid samples were submitted to a veterinarian automatic counter (BC-2800 Vet, Mindray, Nanshan, Shenzhen, China). For the differential leukocyte count, cytospin preparations from exudates were stained with May–Grünwald–Giemsa. The results were expressed as the total number of cells ($\times 10^6$ cells/mL).

2.9. Quantification of exudate concentration

The quantification of exudate concentration was performed indirectly by measuring the amount of Evans blue dye in the pleural cavity. The fluid sample was centrifuged (at 300 $\times g$ for 5 min) (SorvallTM ST 40, ThermoScientific®, Swedesboro, New Jersey, USA) and an aliquot (200 μL) of supernatant was transferred to a 96 well-ELISA plate. The amount of dye was estimated by colorimetric measurement at 620 nm, using an ELISA plate reader (Organon Teknika, Roseland, New Jersey, USA). The results were expressed in μg/mL by interpolation from an Evans blue dye standard curve ranging from 0.01 to 50 μg/mL.

2.10. Determination of myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities

To evaluate the effects on MPO and ADA activities, the collected pleural fluid samples were centrifuged (300 $\times g$ for 5 min) and in-house assays were performed according to the methods previously described [14,15]. With the support of an ELISA plate reader (Organon Teknika, Roseland, New Jersey, USA) colorimetric measurements were made at 450 nm and 620 nm, respectively. The MPO activity was estimated by an interpolation from a standard curve of MPO from human neutrophils (0.7–140 mU/mL), and the results were expressed in mU/mL. An ammonium sulfate solution, measured in triplicate as a standard point of 20 U/L, was used to estimate ADA activity expressed in U/L.

2.11. Measurement of nitric oxide products (NOx)

Nitric oxide was quantified using the Griess assay and vanadium chloride III, as previously reported [16]. After centrifugation of pleural fluid samples (300 $\times g$ for 5 min), the colorimetric measurement was determined at 540 nm using an ELISA plate reader (Organon Teknika, Roseland, New Jersey, USA). The NO amount was indirectly determined by interpolation from the nitrite standard curve (0–20 μM), and the results were expressed in μM.

2.12. Pro- and anti-inflammatory cytokine quantification

Cytokine quantification was done using the Mouse Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, California, USA) for the detection of IL-2, IL-4, IL-6, IL-10, IFN-γ and IL-17A. CBA analysis was performed as per the manufacturer's instructions. The calibration curves were drawn using a set of diluted cytokine standards (0–5000 pg/mL) to determine the protein concentrations. Briefly, a volume of 50 μL of capture bead mixture was added to 50 μL of sample and standards. To this mixture was added 50 μL of phycoerythrin-conjugated detection antibody followed by incubation for 2 h in the dark. The samples were washed with 1 mL of wash buffer, centrifuged (at 200 $\times g$ for 5 min), and the pellet resuspended in 300 μL of wash buffer. Samples were acquired using a BD FACSVerse flow cytometer (BD Bioscience, San Jose, California, USA) and analyzed by the software FCAP Array v3.0.

The quantification of TNF-α was performed using an ELISA kit with monoclonal specific antibody (eBioscience, Inc, San Diego, California, USA). The whole process was conducted according to the manufacturer's instructions. The cytokine concentration was estimated through an interpolation from a standard curve, and the colorimetric measurement was performed at 450 nm, supported by an ELISA plate reader (Organon Teknika, Roseland, New Jersey, USA).

2.13. Histological analysis

Mice lungs were removed 4 h after Cg injection, washed in PBS, and fixed in formalin solution 10% (v/v) for 48 h. The tissues were dehydrated in a series of ethanol solutions of increasing concentrations in graded series followed by xylene, then included in paraffin and sliced into 3-μm sections (LEICA-Instruments CM3050, Nussloch, Germany). The slices were stained with hematoxylin–eosin and analyzed under light microscopy (200×). The classification was performed using the scores reported in a previous study [17], based on the degree of parenchymal distortion in the alveolar tissue. The scores used were: 0 = normal; 1 = increased thickness in <50% of interalveolar septa (IAS) due to edema and/or neutrophil infiltration; 2 = increased thickness in >50% of IAS; 3 = increased thickness in >50% of IAS and the presence of neutrophils within the alveolar space; and 4 = consolidated infiltration of neutrophils with distortion of normal alveolar architecture. The mean score was reported for each microscope section.

2.14. Analysis of p65 phosphorylation (p-p65 NF-κB)

Mice lung tissues were collected, to evaluate the effects of *I. paraguariensis* major compounds on p65 NF-κB phosphorylation. The quantity of total proteins was measured by the Lowry assay, and the samples were adjusted to 60 μg protein/sample [18]. A commercial kit (PathScan® Phospo-NF-κB p65 Ser536, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA) was used in accordance with manufacturer's protocol. The colorimetric obtained in an ELISA plate reader (Organon Teknika, Roseland, New Jersey, USA) at 450 nm. The results were expressed as relative fold change in comparison with saline group, which represents the baseline p-p65 NF-κB level.

2.15. Reagents and drugs

The following chemicals used were obtained from: *i*) Sigma-Aldrich Co. (St. Louis, Missouri, USA): adenosine, carrageenan (degree IV), Evans blue dye, formalin, hydrogen peroxide, hexadecyltrimethyl ammonium bromide, human neutrophil myeloperoxidase, *o*-dianisidine •2HCl (3,3'dimethoxybenzidine), α-naphthylethylenediamide •2HCl, phenol, sodium azide, sodium dodecyl sulfate, sodium hypochlorite, sodium nitroprussiate, sulfanilamide, vanadium chloride (III) and xylene. *ii*) Synth (Diadema, São Paulo, Brazil): ethanol. *iii*) Aché Pharmacological Laboratories S.A (Guarulhos, São Paulo, Brazil): dexamethasone. *iv*) Vetec (Rio de Janeiro, Rio de Janeiro, Brazil): sodium hydrogen phosphate, zinc sulfate. *v*) BioTech (São Paulo, São Paulo, Brazil): hydrogen peroxide 30%. *vi*) Reagen (Rio de Janeiro, Rio de Janeiro, Brazil): sodium hydroxide. *vii*) Roche (São Paulo, São Paulo, Brazil): heparin. *viii*) Newprov (Pinhais, Paraná, Brazil): May-Grunwald dye. *ix*) Laborclin (Pinhais, Paraná, Brazil): Giemsa dye. *x*) BD Bioscience (San Jose, California, USA): Mouse Th1/Th2/Th17 CBA kit. *xi*) eBioscience Inc. (San Diego, California, USA.): ELISA kit Insta One Phospho-NF-κB p-65 (Ser536). The other reagents used were of analytical grade and were obtained from several commercial sources.

2.16. Data analysis

All data were expressed as mean ± SEM. Statistical differences between groups were tested by one-way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test. The results were analyzed using GraphPad Prism v5.0 software (GraphPad Software Inc., San Diego, California, USA) and the values of $P < 0.05$ were considered significant.

3. Results

3.1. Phytochemical characterization of bioactive compounds

In the *I. paraguariensis* extracts and its related fractions, nine major compounds were identified by using LC-PDA and LC–MS, which were reported as previously [19–22]. Theobromine (peak 1), chlorogenic acid (peak 3), caffeine (peak 4), 4-*O*-caffeoylquinic acid (peak 5) and rutin (peak 6) were identified based on theirs retention times, UV absorption spectra, and mass spectra, and by the comparison with authentic samples. Fig. 1 shows the comparison of LC-PDA chromatograms of *I. paraguariensis* CE, BF and ARF with detection at 280 nm. The retention time, maximum UV absorption and detected mass ions for each compound are summarized in Table 1 in Ref. [23]. Table 1 shows the different content of quantified compounds of *I. paraguariensis* extract and fractions. Caffeine and rutin showed higher concentration in the BF. On the other hand, the BF presented lower contents of chlorogenic acid.

3.2. LC-PDA validation procedure

The validation parameters data are summarized in Table 2 in Ref. [23].

3.3. Effects of I. paraguariensis on leukocytes influx and exudate concentration

When compared with the positive control group, the CE significantly decreased the leukocyte content in the pleural cavity (% inhibition: 10 mg/kg: 45.48 ± 4.28; 25 mg/kg: 49.15 ± 1.64 and 50 mg/kg: 52.26 ± 2.49) ($P < 0.01$) due to the inhibition of neutrophil migration (% inhibition: 10 mg/kg: 65.32 ± 4.66; 25 mg/kg: 62.29 ± 4.54 and 50 mg/kg: 60.25 ± 2.90) ($P < 0.01$). Furthermore, CE was also able to reduce the exudate concentration (% inhibition: 25 mg/kg: 26.73 ± 4.05 and 50 mg/kg: 29.27 ± 4.52) ($P < 0.01$) (Table 2).

Among the CE-related fractions, BF showed significant effect on leukocyte migration (% inhibition: 1 mg/kg: 54.80 ± 1.94 and 10 mg/kg: 55.37 ± 3.08) ($P < 0.01$), neutrophil influx (% inhibition: 0.1 mg/kg: 24.15 ± 6.26; 1 mg/kg: 61.39 ± 1.76 and 10 mg/kg: 61.91 ± 3.38) ($P < 0.01$) and also on exudate concentrations (% inhibition: 0.1 mg/kg: 20.59 ± 5.61; 1 mg/kg: 21.10 ± 3.96 and 10 mg/kg: 26.77 ± 2.90) ($P < 0.01$) (Table 2). Another fraction, ARF, was also effective in reducing leukocyte total content (% inhibition: 0.1 mg/kg: 46.89 ± 9.35; 1 mg/kg: 54.24 ± 8.84 and 10 mg/kg: 38.14 ± 5.77) ($P < 0.01$), neutrophil influx (% inhibition: 0.1 mg/kg: 57.78 ± 4.75; 1 mg/kg: 68.20 ± 2.64 and 10 mg/kg: 49.68 ± 2.97) ($P < 0.01$), and exudate concentration (% inhibition: 1 mg/kg: 31.21 ± 5.87 and 10 mg/kg: 25.62 ± 2.44) ($P < 0.01$) (Table 2).

Table 1
Contents of phenolic compounds and methylxanthines of *Ilex paraguariensis* extract and fractions.

Sample	Caffeine (mg/g)	Chlorogenic acid (mg/g)	Rutin (mg/g)
CE	29.3 ± 0.06[a]	67.5 ± 0.3[a]	12.4 ± 0.04[a]
BF	101.7 ± 0.4[b]	58.4 ± 0.1[b]	42.0 ± 0.09[b]
ARF	3.9 ± 0.02[c]	68.3 ± 0.1[c]	2.2 ± 0.01[c]

Caffeine (Caf), chlorogenic acid (CGA) and rutin (Rut) content were determined by CLAE-PDA and the data are mean ± S.D. values expressed as mg/g extract (N = 3). Different letters indicate significant differences (ANOVA and Tukey's post test, $P < 0.05$)

The major compounds of *I. paraguariensis* tested in this study also demonstrated potential anti-inflammatory properties. First, the major compound Caf was effective in inhibiting the leukocyte content in the pleural fluid (% inhibition: 1 mg/kg: 40.96 ± 5.86 and 5 mg/kg: 50.28 ± 3.76) ($P < 0.01$), due to its ability to decrease the migration of neutrophils (% inhibition: 0.1 mg/kg: 47.07 ± 1.03; 1 mg/kg: 55.38 ± 4.28 and 5 mg/kg: 67.78 ± 2.01) ($P < 0.01$). Moreover, Caf only showed a significant reduction in the exudate concentration at the dose of 5 mg/kg (% inhibition: 23.17 ± 8.74) ($P < 0.05$) (Table 2).

On the other hand, Rut significantly decreased the leukocyte influx (% inhibition: 0.01 mg/kg: 36.44 ± 4.19; 0.1 mg/kg: 38.42 ± 8.32 and 1 mg/kg: 46.61 ± 5.58) ($P < 0.01$) at the expense of neutrophil content (% inhibition: 0.01 mg/kg: 49.36 ± 3.47; 0.1 mg/kg: 62.10 ± 1.27 and 1 mg/kg: 55.37 ± 3.39) ($P < 0.01$), and showed an inhibitory profile upon the exudate concentration (% inhibition: 0.1 mg/kg: 17.17 ± 2.91 and 1 mg/kg: 28.77 ± 3.51) ($P < 0.05$) (Table 2).

Finally, CGA demonstrated significant inhibition in leukocyte content (% inhibition: 0.1 mg/kg: 48.59 ± 5.47 and 1 mg/kg: 48.14 ± 4.27) ($P < 0.01$), neutrophil content (% inhibition: 0.01 mg/kg:

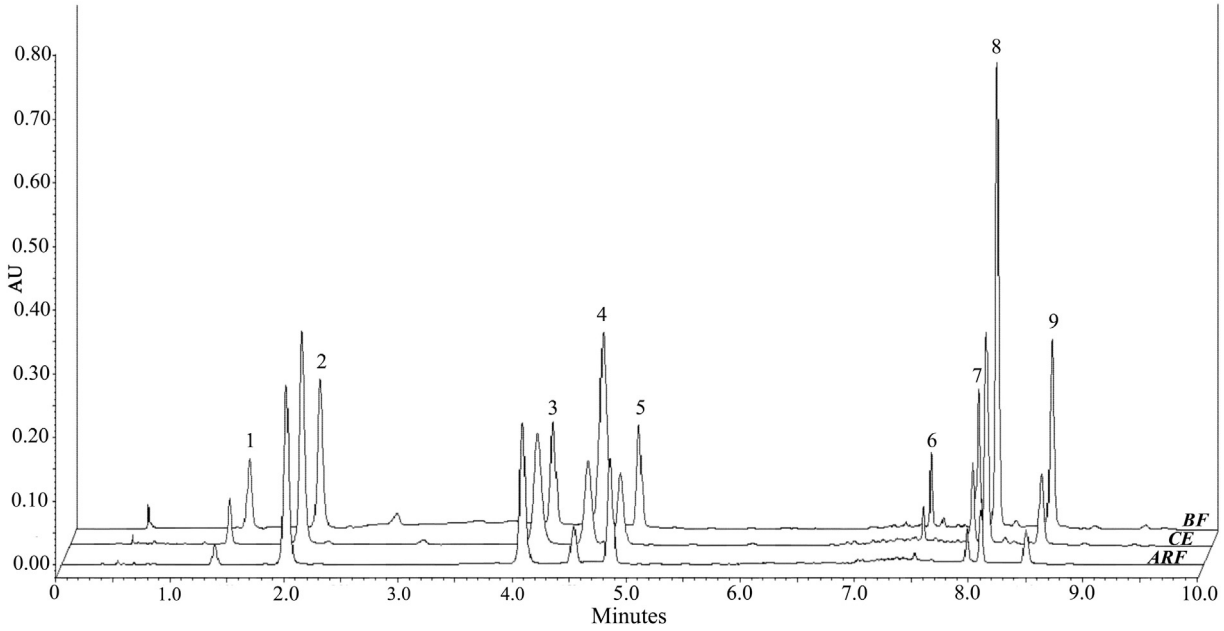

Fig. 1. UPLC-PDA chromatograms of *I. paraguariensis* extract and fractions with detection at 280 nm. 1, theobromine; 2, 3-*O*-caffeoylquinic acid; 3, 5-*O*-caffeoylquinic acid; 4, caffeine; 5, 4-*O*-caffeoylquinic acid; 6, rutin; 7, 3,4-dicafeoylquinic acid; 8, 3,5-dicafeoylquinic acid; 9, 4,5-dicafeoylquinic acid. ARF, aqueous residual fraction; CE, crude extract; BF, *n*-BuOH fraction.

Table 2
Effects of *Ilex paraguariensis* crude extract, its derived fractions and major compounds upon leukocyte migration, exudate concentration in the mouse model of pleurisy induced by carrageenan.

Groups (mg/kg)	Leukocytes ($\times 10^6$)	Neutrophils ($\times 10^6$)	Exudation (μg/mL)
Sal[a]	0.88 ± 0.09	0.46 ± 0.03	0.53 ± 0.15
Cg[a]	5.90 ± 0.33	5.18 ± 0.39	9.62 ± 0.74
Dex (0.5)[b]	2.48 ± 0.41**	2.08 ± 0.32**	5.04 ± 0.27**
CE (10)[b]	3.22 ± 0.25**	1.80 ± 0.24**	8.58 ± 0.65
CE (25)[b]	3.00 ± 0.10**	1.95 ± 0.23**	7.05 ± 0.39*
CE (50)[b]	2.82 ± 0.15**	2.06 ± 0.15**	6.80 ± 0.43*
BF (0.1)[b]	5.17 ± 0.40	3.93 ± 0.32**	7.64 ± 0.54**
BF (1)[b]	2.97 ± 0.22**	1.99 ± 0.09**	7.59 ± 0.38**
BF (10)[b]	2.63 ± 0.18**	2.10 ± 0.16**	7.05 ± 0.28**
ARF (0.1)[b]	3.13 ± 0.55**	2.19 ± 0.24**	9.61 ± 0.80
ARF (1)[b]	2.70 ± 0.52**	1.65 ± 0.14**	6.62 ± 0.56**
ARF (10)[b]	3.22 ± 0.24**	2.60 ± 0.15**	7.16 ± 0.23**
Caf (0.1)[b]	4.83 ± 0.50	2.74 ± 0.21**	12.70 ± 0.83
Caf (1)[b]	3.48 ± 0.34**	2.31 ± 0.22**	9.67 ± 0.67
Caf (5)[b]	2.93 ± 0.22**	1.67 ± 0.10**	7.39 ± 0.84*
Rut (0.01)[b]	3.75 ± 0.25**	2.62 ± 0.18**	8.51 ± 0.26
Rut (0.1)[b]	3.63 ± 0.49**	1.96 ± 0.06**	7.97 ± 0.28*
Rut (1)[b]	3.15 ± 0.33**	2.31 ± 0.17**	6.87 ± 0.34**
CGA (0.01)[b]	5.12 ± 0.23	2.60 ± 0.13**	8.32 ± 0.54*
CGA (0.1)[b]	3.03 ± 0.32**	1.75 ± 0.31**	7.35 ± 0.23**
CGA (1)[b]	3.06 ± 0.25**	1.78 ± 0.12**	7.48 ± 0.42**

Crude extract (CE: 50–10 mg/kg), buthanolic fraction (BF: 10–1 mg/kg), aqueous residue fraction (ARF: 10–1 mg/kg), caffeine (Caf: 5–0.1 mg/kg), rutin (Rut: 1–0.01 mg/kg) and chlorogenic acid (CGA: 1–0.01 mg/kg) were administered 0.5 h before carrageenan-induced pleurisy. Sal = negative control group treated only with sterile saline (0.9% NaCl); Cg = the positive control group treated only with carrageenan (1%); Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg) 0.5 h before pleurisy induction. Each group represents the mean ± SEM, N = 6 animals. [a]Administered by intra-pleural route (i.pl.). [b]Administered by oral route (p.o.). * $P < 0.05$; ** $P < 0.01$.

49.70 ± 2.45; 0.1 mg/kg: 66.29 ± 5.97 and 1 mg/kg: 65.53 ± 2.38) ($P < 0.01$), and pleural fluid leakage concentration (% inhibition: 0.1 mg/kg: 23.59 ± 2.37 and 1 mg/kg: 22.20 ± 4.35) ($P < 0.05$) (Table 2).

As expected, Dex presented anti-inflammatory effect by reducing the total leukocyte content in the pleural fluid (% inhibition: 57.91 ± 6.99) ($P < 0.01$), due to neutrophil inhibition (% inhibition: 59.82 ± 6.32) ($P < 0.01$), and also reduced the exudate concentration (% inhibition: 47.60 ± 2.82) ($P < 0.01$) (Table 2).

3.4. Effects of I. paraguariensis on MPO and ADA activities

The selected doses of CE (25 mg/kg), BF (1 mg/kg), ARF (1 mg/kg), Caf (5 mg/kg), Rut (1 mg/kg) and CGA (0.1 mg/kg), from the experiments described above, were used to perform the MPO and ADA assays, since these enzymes are closely linked to leukocyte activation and transmigration. All the treated groups showed a significant decrease in MPO activity (% inhibition of CE: 30.29 ± 4.58; BF: 28.73 ± 1.74; ARF: 24.94 ± 4.52; Caf: 37.21 ± 2.51; Rut: 29.01 ± 4.93 and CGA: 30.29 ± 4.78) ($P < 0.01$), likewise on the ADA activity (% inhibition of CE: 29.00 ± 11.97; BF: 41.93 ± 0.73; ARF: 49.56 ± 4.22; Caf: 38.11 ± 11.26; Rut: 51.25 ± 11.02 and CGA: 32.52 ± 8.70) ($P < 0.05$) (Table 3). Under the same experimental conditions, the reference drug Dex also inhibited MPO and ADA activities (% inhibition: 29.83 ± 4.96 and 60.40 ± 8.30, respectively) ($P < 0.01$) (Table 3).

3.5. Effects of I. paraguariensis on NOx concentration

Because exudation is closely related to nitric oxide levels, we performed the nitrite/nitrate dosage to confirm the hypothesis that this inhibition caused by *I. paraguariensis* on exudation, could be directly associated with a decrease in nitric oxide levels. A significant reduction in nitrate/nitrite levels was observed in all the tested groups (% inhibition of CE: 43.71 ± 3.40; BF: 61.11 ± 2.23; ARF: 23.26 ± 5.56; Caf: 45.30 ± 5.50; Rut: 36.18 ± 7.68 and CGA: 35.34 ± 4.36) ($P < 0.01$), as well in Dex group (% inhibition: 54.12 ± 2.54) ($P < 0.01$) (Table 3).

Table 3
Effects of crude extract, fractions and major compounds of *I. paraguariensis* on myeloperoxidase (MPO), adenosine-deaminase (ADA) and nitrate/nitrite levels (NOx) in the mouse model of pleurisy induced by carrageenan.

Groups (mg/kg)	MPO (mU/mL)	ADA (U/L)	NO_x (μM)
Sal[a]	64.74 ± 2.21	1.51 ± 0.03	8.44 ± 1.26
Cg[a]	133.8 ± 9.18	9.00 ± 1.25	28.80 ± 1.26
Dex (0.5)[b]	93.88 ± 6.64**	3.57 ± 0.75**	13.21 ± 0.73**
CE (25)[b]	93.27 ± 6.12**	6.39 ± 1.08*	16.21 ± 0.98**
BF (1)[b]	95.37 ± 2.32**	5.23 ± 0.07*	11.20 ± 0.64**
ARF (1)[b]	100.4 ± 6.05**	4.54 ± 0.38**	22.10 ± 1.52**
Caf (5)[b]	84.02 ± 3.36**	5.57 ± 1.02**	15.75 ± 1.58**
Rut (1)[b]	96.33 ± 6.59**	4.39 ± 0.99*	18.38 ± 2.21**
CGA (0.1)[b]	93.27 ± 6.40**	6.08 ± 0.78*	18.62 ± 1.26**

Crude extract (CE: 25 mg/kg), buthanolic fraction (BF: 1 mg/kg), aqueous residue fraction (ARFr: 1 mg/kg), caffeine (Caf: 5 mg/kg), rutin (Rut: 1 mg/kg) and chlorogenic acid (CGA: 0.1 mg/kg) were administered 0.5 h before carrageenan-induced pleurisy. Sal = negative control group treated only with sterile saline (0.9% NaCl); Cg = the positive control group treated only with carrageenan (1%); Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg) 0.5 h before pleurisy induction. Each group represents the mean ± SEM, N = 6 animals. * $P < 0.05$; ** $P < 0.01$. [a] Administered by intra-pleural route (i.pl.). [b] Administered by oral route (p.o).

3.6. Effects of I. paraguariensis on IL-6, IL-17 A, IFN-γ, TNF-α, IL-10, IL-2 and IL-4 concentrations

In addition to the anti-inflammatory effect associated with the decrease in leukocyte migration, exudate concentration, MPO and ADA activities and NOx levels, *I. paraguariensis* also caused significant changes in the production of some anti- and pro-inflammatory cytokines that are the keys of the immune system.

In our experiments, CE, BF and ARF showed significant inhibition on IL-6 (% inhibition of CE: 91.63 ± 1.05; BF: 76.10 ± 2.97 and ARF: 40.75 ± 0.95) ($P < 0.01$), on IL-17 A (% inhibition of CE: 66.57 ± 1.32; BF: 38.94 ± 3.13 and ARF: 46.44 ± 9.55) ($P < 0.01$), on IFN-γ (% inhibition of CE: 48.67 ± 1.30; BF: 47.94 ± 10.66 and ARF: 42.95 ± 2.85) ($P < 0.01$) and also upon TNF-α (% inhibition of CE: 23.20 ± 6.27; BF: 55.27 ± 9.26 and ARF: 17.25 ± 3.29) (Table 4). In addition, CE and ARF increased the production of anti-inflammatory cytokine IL-10 levels when compared with the inflamed group (% increase in CE: 42.38 ± 30.43; ARF: 45.61 ± 15.53) ($P < 0.05$); However, the BF fraction, was not able to increase the levels of IL-10 ($P > 0.05$) (Table 4).

The major compounds also showed important effects on the studied cytokines. Rut inhibited the IL-6 levels (% inhibition: 64.19 ± 1.59) ($P < 0.01$), and CGA caused a massive reduction of IL-6 levels, to values below the detection limit of the kit (<1.4 pg/mL) ($P < 0.01$). The exception was the isolated compound Caf, which was not able to reduce the IL-6 levels ($P > 0.05$) (Table 4). The concentrations of IL-17A, IFN-γ and TNF-α in the fluid leakage were significantly decreased by pretreatment with Caf (% of inhibition: IL-17A: 38.84 ± 5.42; IFN-γ: 45.01 ± 10.24 and TNF-α: 40.75 ± 6.90) ($P < 0.01$) and Rut (% of inhibition: IL-17A: 48.32 ± 4.74; IFN-γ: 45.54 ± 4.10 and TNF-α: 28.56 ± 2.19) ($P < 0.01$). However, the pretreatment with CGA was only able to decrease the levels of IL-17A and TNF-α (% inhibition IL-17A: 18.24 ± 3.85 and TNF-α: 39.24 ± 3.55) ($P < 0.05$) (Table 4).

Additionally, the pretreatments with Rut and Caf did not change the IL-10 levels ($P > 0.05$), while the pretreatment with CGA showed a positive effect on IL-10 concentration, increasing their levels (% increase: 358.60 ± 18.53) ($P < 0.05$) (Table 4).

As expected, Dex showed the expected profile through the inhibition of all pro-inflammatory cytokines (% inhibition on IL-6: 98.96 ± 0.17; IL-17A: 74.14 ± 1.52; IFN-γ: 58.52 ± 2.93 and TNF-α: 58.69 ± 6.48) ($P < 0.05$), and increased the levels of anti-inflammatory cytokine IL-10 (% of increase: 83.62 ± 19.97) ($P < 0.01$) (Table 4).

Table 4
Effects of *I. paraguariensis* crude extract, its derived fractions and major compounds on pro- and anti-inflammatory cytokine levels in the mouse model of pleurisy induced by carrageenan.

Cytokines	IL-6 (ng/mL)	IL-17A (pg/mL)	IFN-γ (pg/mL)	IL-10 (pg/mL)	TNF-α (pg/mL)
Sal[a]	0.01 ± 0.01	0.82 ± 0.29	1.21 ± 0.29	17.20 ± 3.59	85.05 ± 8.35
Cg[a]	544.3 ± 56.29	89.88 ± 3.55	11.82 ± 1.02	35.12 ± 3.79	898.10 ± 44.7
Dex (0.5)[b]	5.68 ± 0.94 **	23.35 ± 1.36 **	4.90 ± 0.35 **	72.03 ± 1.22 **	371.00 ± 58.24**
CE (25)[b]	45.57 ± 5.71 **	30.05 ± 1.18 **	6.07 ± 0.15 **	61.60 ± 9.45 *	689.70 ± 56.30*
BF (1)[b]	130.1 ± 16.19**	54.88 ± 2.81 **	6.15 ± 1.26 **	44.85 ± 2.16	401.7 ± 83.21**
ARF (1)[b]	322.5 ± 5.18 **	48.14 ± 8.59 **	6.74 ± 0.34 **	71.28 ± 5.64 **	743.10 ± 29.56*
Caf (5)[b]	443.7 ± 97.84	54.97 ± 4.87 **	6.50 ± 1.21 **	55.87 ± 4.93	532.10 ± 61.98**
Rut (1)[b]	194.9 ± 8.66 **	46.45 ± 4.26 **	6.44 ± 0.48 **	50.67 ± 6.49	641.60 ± 19.63**
CGA (0.1)[b]	<0.0014 Ψ	79.38 ± 5.13 *	10.45 ± 1.27	161.10 ± 6.51 **	545.70 ± 31.85**

Crude extract (CE: 25 mg/kg), buthanolic fraction (BF: 1 mg/kg), aqueous residue fraction (ARF: 1 mg/kg), caffeine (Caf: 5 mg/kg), rutin (Rut: 1 mg/kg) and chlorogenic acid (CGA: 0.1 mg/kg) were administered 0.5 h before carrageenan-induced pleurisy. Sal = negative control group treated only with sterile saline (0.9% NaCl); Cg = the positive control group treated only with carrageenan (1%); Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg) 0.5 h before pleurisy induction. Each group represents the mean ± SEM, N = 6 animals. * $P < 0.05$; ** $P < 0.01$; Ψ all tested samples showed values under the detection limit. [a] Administered by intra-pleural route (i.pl.). [b] Administered by oral route (p.o).

In our experiments, none of the tested groups, including the control group (Sal and Cg), were able to significantly change the levels of IL-2 and IL-4 cytokines to detectable levels (Data not shown).

3.7. Effect of I. paraguariensis on the histological architecture of the lungs

The induction of pleurisy by carrageenan provided a significant lung injury with massive infiltration of neutrophils into the alveolar space, and significant distortion of normal alveolar architecture. Treatment with CE, BF, ARF, Caf, Rut or even CGA, administrated 0.5 h before induction of pleurisy, significantly decreased the thickness of the interalveolar septa and neutrophil infiltration when compared with the positive control group (% inhibition of CE: 31.58 ± 6.45; BF: 36.84 ± 6.45; ARF: 36.84 ± 10.53; Caf: 31.58 ± 6.45; Rut: 42.11 ± 5.26 and CGA: 63.16 ± 6.45) ($P < 0.05$) (Fig. 2). Dex treatment also significantly reduced the histological parameters studied (% inhibition: 52.63 ± 9.85) ($P < 0.01$) (Fig. 2).

3.8. Effects of I. paraguariensis major compounds on p-p65 NF-κB

Since NF-κB plays a crucial role in the synthesis of pro- and anti-inflammatory mediators, we investigated the effects of Caf, Rut and CGA on p65 phosphorylation. All the studied compounds, and Dex, were able to reduce the phosphorylation of the p65 subunit when compared with the Cg control group, which represents the positive group of p-p65 NF-κB (% inhibition of Caf: 54.53 ± 1.55; Rut: 60.73 ± 1.26; CGA: 61.48 ± 1.40 and Dex: 55.85 ± 1.89) ($P < 0.01$) (Fig. 3).

4. Discussion

Although pleurisy induced by carrageenan is not an asthma model, the administration of carrageenan into the pleural cavity provides an inflammatory environment similar to that observed in patients with neutrophilic asthma phenotype, characterized by a Th1/Th17 polarization. The imbalance between pro- and anti-inflammatory cytokine production, pro-inflammatory enzyme activities, and histological changes in the lung tissue are the most notable similarities [13,24]. The actual approach to handling asthma, based on suppression of pro-inflammatory mediators with the use of corticosteroids, has not been producing good results, and is often inefficient. Thus, the search for compounds that act by reestablishing the natural balance of the immune system seems to be a good goal to treat this condition.

In view of the above, and considering the preliminary data obtained in our laboratory, we decided to study the anti-inflammatory efficacy of *I. paraguariensis* using the murine model of pleurisy. Our data demonstrated that pretreatment with *I. paraguariensis* CE, fractions (BF and

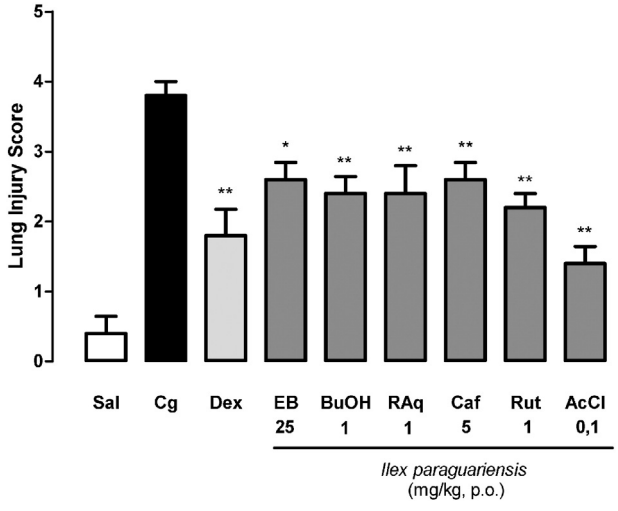

Fig. 2. Effects of *I. paraguariensis* on mice lung histology following induction of pleurisy by carrageenan. Graphic comparative analysis of histology scores. CE (25 mg/kg, p.o.); BF (1 mg/kg, p.o); ARF (1 mg/kg, p.o.); Caf (5 mg/kg, p.o.); Rut (1 mg/kg, p.o.) or CGA (0.1 mg/kg, p.o.) were administered 0.5 h before carrageenan-induced pleurisy. Sal = negative control group treated only with sterile saline (0.9% NaCl); Cg = positive control group treated only with carrageenan (1%); Dex = animals pre-treated with dexamethasone (0.5 mg/kg) 0.5 h before pleurisy induction. Each group represents the mean ± SEM. N = 6 animals. * $P < 0.05$; ** $P < 0.01$.

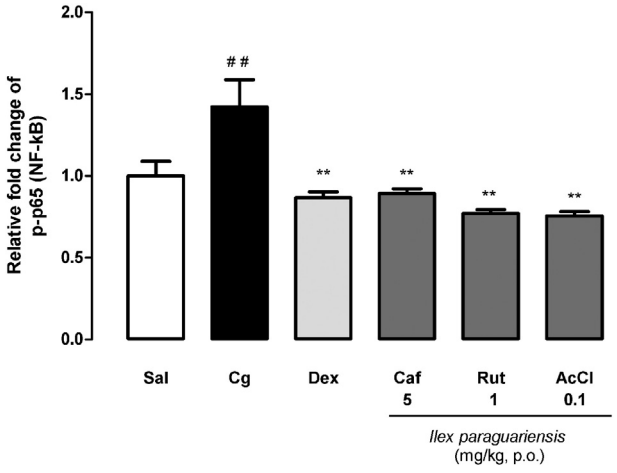

Fig. 3. Effects of *I. paraguariensis* major compounds on p65 phosphorylation (p-p65 NF-κB). Caffeine (Caf: 5 mg/kg, p.o.), rutin (Rut: 1 mg/kg, p.o.) and chlorogenic acid (CGA: 0.1 mg/kg, p.o.) were administered 0.5 h before carrageenan-induced pleurisy. Sal = group treated only with sterile saline (0.9% NaCl); Cg = group treated only with carrageenan (1%); Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg, p.o.) 0.5 h before pleurisy induction. The results were expressed as a relative fold change compared with the negative saline group which represents a p65 phosphorylation basal expression. N = 4 animals. ## $P < 0.01$ statistical comparative analyses between the Cg group and saline group. ** $P < 0.01$ statistical comparative analyses between the treated group and the Cg group.

ARF) and major compounds (Caf, Rut and CGA) were effective in inhibiting leukocyte migration to the inflammatory site, both in the analysis of fluid leakage and lung tissue, and this effect was due to ability of this plant to inhibit the neutrophil influx. Moreover, *I. paraguariensis* was also able to inhibit neutrophil activation in fluid leakage, since all the plant material tested decreased the activity of MPO, an enzyme that is located in the azurophilic granules of these cells, and released through its activation. Similar results for this effect on the leukocytes were obtained by Schinella and co-workers (2014), who analyzed the effect of *I. paraguariensis* on acute and chronic 12-*O*-tetradecanoylphorbol 13-acetate-induced mouse ear edema and acute carrageenan-induced mouse paw edema, and also showed an important decrease in leukocyte influx, also due to neutrophil inhibition [7].

According to our findings, one possible explanation for the effect of *I. paraguariensis* on neutrophil influx to the site of inflammation may be the ability of the plant material to suppress ADA activity. ADA is an enzyme responsible for inosine generation through adenosine deamination. Among many functions, adenosine, when it is available in the inflammatory environment, acts on the A_{2A} receptors of the neutrophils, downregulates the expression of adhesion proteins, and decreases their degranulation and oxidant activity [25–27].

The other signal of inflammation evaluated was the exudate concentration, which was also significantly reduced by treatment with yerba mate. The exudation levels are directly linked to the increase in the amount of NO produced by the activation of induced nitric oxide synthase (iNOS). The released NO plays an important role as a vasodilator and contributes to exudation [28]. This fact may explain the ability of *I. paraguariensis* to reduce these inflammatory parameters, as it significantly decreased exudate concentration and NO levels in the pleural cavity. In accordance with our results, it was recently demonstrated that yerba mate exerts its anti-inflammatory profile in carrageenan-induced paw edema in mouse through its ability to decrease the formation of edema, and this result was directly correlated with the reduction of COX-2 and iNOS expression [7].

The imbalance of pro- and anti-inflammatory cytokines plays a marked role in inflammatory diseases, particularly in non-self-resolved disease. Among the pro-inflammatory cytokines, IL-6, TNF-α, IFN-γ and IL-17 play an important role in the pleurisy model of inflammation induced by carrageenan [29]. The increase of these cytokines induced by carrageenan administration was significantly inhibited by all tested samples of *I. paraguariensis*. Similarly, other researchers have demonstrated the potential effect of yerba-mate on major pro-inflammatory cytokines. *E.g.* Lim and colleagues (2015), who showed, in a recent study, that yerba-mate is effective in decreasing IFN-γ and IL-6 levels in a spared nerve injury rat model [30]. In addition, a study by Carmo and co-workers (2013) showed that consumption of *I. paraguariensis* reduced IL-6 and TNF-α production by the bone marrow cells in rats fed a high-fat diet [31], and the positive impact of yerba-mate on TNF-α concentration was also reported in a cigarette smoke-exposed mouse model [32]. To our knowledge, there is no published study to date that reports the effect on this plant of IL-17A levels, a relevant cytokine involved in this model and also in the neutrophilic phenotype of asthma. This result needs to be highlighted, given that refractory and severe asthma produces a Th1/Th17 phenotype, in which IL-17 assumes an important role. Of note is the fact that Latin Americans who consume a higher amount of yerba-mate in the form beverages called “chimarrão” or “terere” exhibit lowest prevalence of severe asthma characterized by Th1/Th17 polarization, when compared with people who do not drink this beverage in large amounts [24,33].

The levels of two other pro-inflammatory cytokines, IL-2 and IL-4, were not detected in our experiments, given that these are not primordial and characteristic cytokines in the carrageenan model, which produces a massive polarization to Th1/Th17 axis. In contrast, *I. paraguariensis* produced an increase in IL-10 production, a Th2 cytokine with immunosuppressive characteristic responsible for leading the balance of Th1/Th2/Th17 to baseline levels, providing an immunoregulatory profile [34,35]. This outcome on IL-10 levels was due to CGA effect, and it is the same effect demonstrated by ARF and CE, where this compound was most available. Corroborating this result, the study conducted by Pimentel and colleagues (2013) shows an increase in IL-10/TNF-α ratio in rat liver and muscle due yerba-mate supplementation on a diet-induced obesity model [36].

The effects on cytokine levels involve a complex intracellular process. To summarize, Cg triggers the intracellular signaling pathways by Toll-like receptor 4 (TLR4) activation and oxygen reactive species (ROS) production [37]. NF-κB is therefore an important player in the control of cytokine expression. Its classical inducible form consists of the p65/p50 heterodimer, in which the p65 subunit phosphorylation activates this factor and culminates in the production of many mediators [38]. The NF-κB is the most important transcription factor involved in the inflammatory process, it regulates various gene encoding inflammatory mediators with highlight to pro-inflammatory cytokines [39,40]. This transcription factor is activated by stimuli such as carrageenan and LPS, through TLR4 activation which induce the IκB phosphorylation resulting in transmigration of NF-κB activated form to the nucleus, where gene expression of pro-inflammatory mediators is triggered [41]. Over-activation of this nuclear factor is present in all inflammatory diseases, and its inhibition has been of great interest to researchers [42,43]. In our study, *I. paraguariensis* major compounds were able to inhibit p65 phosphorylation. This corroborates with previously published data which demonstrates that Caf [44], Rut [16] and CGA [45] both attenuate NF-κB activation. As consequence, the plant produced an improvement in inflammatory environment, reducing neutrophil accumulation and edema in the pleural space and lung tissues, as demonstrated in our experiments.

In summary, our results allow us to conclude that *I. paraguariensis* exerts anti-inflammatory action through its ability in attenuating Th1/Th17 polarization. Furthermore, we can hypothesize that the high consumption of yerba mate among the Southern Latin America people may be a factor that explains the lower levels of neutrophilic asthma in this population.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

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