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Aqueous extracts from *Uncaria tomentosa* (Willd. ex Schult.) DC. reduce bronchial hyperresponsiveness and inflammation in a murine model of asthma

Bruna Cestari Azevedo[a], Lucas Junqueira Freitas Morel[a], Fábio Carmona[b], Thiago Mattar Cunha[b], Silvia Helena Taleb Contini[a], Piero Giuseppe Delprete[c], Fernando Silva Ramalho[b], Eduardo Crevelin[d], Bianca Waléria Bertoni[a], Suzelei Castro França[a], Marcos Carvalho Borges[b], Ana Maria Soares Pereira[a,*]

[a] Departamento de Biotecnologia em Plantas Medicinais, Universidade de Ribeirão Preto, Av. Costábile Romano 2201, 14096-900 Ribeirão Preto, SP, Brazil
[b] Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Monte Alegre, 14049-900 Ribeirão Preto, SP, Brazil
[c] Herbier de Guyane, Institut de Recherche pour le Développement, 275 Route de Montabo, BP 90165, 97323 Cayenne Cedex, French Guiana
[d] Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Monte Alegre, 14049-900 Ribeirão Preto, SP, Brazil

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mitraphylline (PubChem CID: 94160)
quinic acid (PubChem CID: 6508)
rutin (PubChem CID: 5280805)

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ABSTRACT

Ethnopharmacological relevance: *Uncaria tomentosa* (Willd. Ex Schult) DC is used by indigenous tribes in the Amazonian region of Central and South America to treat inflammation, allergies and asthma. The therapeutic properties of *U. tomentosa* have been attributed to the presence of tetracyclic and pentacyclic oxindole alkaloids and to phenolic acids.

Aims of the study: To characterize aqueous bark extracts (ABE) and aqueous leaf extracts (ALE) of *U. tomentosa* and to compare their anti-inflammatory effects.

Materials and methods: Constituents of the extracts were identified by ultra performance liquid chromatography-mass spectrometry. Anti-inflammatory activities were assessed *in vitro* by exposing lipopolysaccharide-stimulated macrophage cells (RAW264.7-Luc) to ABE, ALE and standard mitraphylline. *In vivo* assays were performed using a murine model of ovalbumin (OVA)-induced asthma. OVA-sensitized animals were treated with ABE or ALE while controls received dexamethasone or saline solution. Bronchial hyperresponsiveness, production of Th1 and Th2 cytokines, total and differential counts of inflammatory cells in the bronchoalveolar lavage (BAL) and lung tissue were determined.

Results: Mitraphylline, isomitraphylline, chlorogenic acid and quinic acid were detected in both extracts, while isorhyncophylline and rutin were detected only in ALE. ABE, ALE and mitraphylline inhibited the transcription of nuclear factor kappa-B in cell cultures, ALE and mitraphylline reduced the production of interleukin (IL) − 6, and mitraphylline reduced production of tumor necrosis factor-alpha. Treatment with ABE and ALE at 50 and 200 mg kg^{-1}, respectively, reduced respiratory elastance and tissue damping and elastance. ABE and ALE reduced the number of eosinophils in BAL, while ALE at 200 mg kg^{-1} reduced the levels of IL-4 and IL-5 in the lung homogenate. Peribronchial inflammation was significantly reduced by treatment with ABE and ALE at 50 and 100 mg kg^{-1} respectively.

Conclusion: The results clarify for the first time the anti-inflammatory activity of *U. tomentosa* in a murine model of asthma. Although ABE and ALE exhibited distinct chemical compositions, both extracts inhibited the production of pro-inflammatory cytokines *in vitro*. *In vivo* assays revealed that ABE was more effective in treating asthmatic inflammation while ALE was more successful in controlling respiratory mechanics. Both extracts may have promising applications in the phytotherapy of allergic asthma.

* Corresponding author.
E-mail address: apereira@unaerp.br (A.M.S. Pereira).

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1. Introduction

The tropical woody vine *Uncaria tomentosa* (Rubiaceae) is distributed widely in countries of Central and South America including Brazil, Bolivia, Colombia, Costa Rica, Ecuador, Guatemala, Guiana, Nicaragua, Panama, Peru and Venezuela. In Brazil, the species is found mainly in the Amazonian region, especially in the northern states of Acre, Amapá, Amazonas and Pará (Valente, 2013).

Ethnopharmacological reports have confirmed the use of *U. tomentosa* by a number of indigenous Amazonian tribes, including the Aguarina, Cashibo, Coribo, Shipido and Ashaninka, to treat asthma, cancer, inflammation, abscesses and allergies (Keplinger et al., 1999; Obregón Vilches, 1994; Taylor, 2002). Moreover, the anticancer, anti-inflammatory and immunostimulant activities of different *U. tomentosa* extracts have been confirmed by preclinical studies (Castilhos et al., 2015; Hardin, 2007; Keplinger et al., 1999; Kośmider et al., 2017), and the efficacy of extracts in the treatment of rheumatoid arthritis and the prevention of side effects of chemotherapy has been demonstrated (De Paula et al., 2015; Farias et al., 2012; Mur et al., 2002).

The most studied constituents of *U. tomentosa* are tetracyclic oxindole alkaloids (TOAs), such as corynoxeine, isocorynoxeine, *rhynchophylline and* iso*rhyncophylline* (Keplinger et al., 1999), *that act on the central nervous system (*Shi et al., 2003*;* Zhou and Zhou, 2012*), and* pentacyclic oxindole alkaloids (POAs), including *mitraphylline,* iso*mitraphylline,* pteropodine*,* isopteropodine*, speciophylline* and uncarine F (Keplinger et al., 1999), that act on the immune system (Lamm et al., 2001; Mur et al., 2002; Rojas-Duran et al., 2012; Winkler et al., 2004). However, the components of *U. tomentosa* responsible for the anti-inflammatory activity of the extracts remain questionable since some researchers attribute this property to POAs (Laus et al., 1997; Lopez-Avila et al., 1997) while others claim that the activity is associated with the presence of phenolics such as chlorogenic, quinic and quinovic acids (Akesson et al., 2005; Pavei et al., 2010).

The chemical profile of *U. tomentosa* tends to vary significantly, in both qualitative and quantitative aspects, among individual specimens in a population (Kaiser et al., 2016; Peñaloza et al., 2015) and between extracts derived from different parts of the same plant. However, the majority of studies reported so far have been performed with bark and root extracts, although collection of these materials causes severe lesions or death of the plants (Keplinger et al., 1999). Moreover, since *U. tomentosa* has not yet been domesticated, the bark and roots employed in the preparation of herbal remedies are obtained by predatory harvesting from natural populations, thereby rendering the species vulnerable to genetic erosion and threatening its very existence (Honório et al., 2017). While the collection of leaves would be far more sustainable, extracts derived from leaves have rarely been investigated (Barnes et al., 2007) owing primarily to the difficulty in obtaining material from the canopy of a tree that grows to a height of more than 15 m.

In light of the above, we were interested to test the hypothesis that leaf extracts, along with bark extracts, would have application in the phytotherapy of allergic asthma. With this aim in mind, we compared the anti-inflammatory effects of aqueous extracts from bark and leaves of *U. tomentosa* using a murine *model* of *ovalbumin (OVA)*-induced *asthma. The results presented herein substantiate the ethnopharmacological uses of U. tomentosa in the treatment of respiratory diseases and clarify the effects and mechanisms of action of the extracts and their constituents.*

2. Materials and methods

2.1. Ethical considerations

Details of the study were approved by the Ethics Committee on Animal Research of the Ribeirao Preto Medical School, University of Sao Paulo (FMRP-USP; protocol no. 221/2014). Procedures involving experimental animals were conducted in the Laboratory of Lung Pathophysiology and the Laboratory of Pathology at FMRP-USP, following guidelines established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Plant material

Bark and leaves of *Uncaria tomentosa* (Willd. ex Schult.) DC. (The Plant List, 2013), known as *unha de gato* or cat's claw, were collected at Fazenda São João, located in Bannach, PA, Brazil (07°34′26′ S; 50 °34′51′W; altitude 415 m). The material was identified by Dr. Pietro Giuseppe Delprete (Herbier de Guyane, Institut de Recherche pour le Développement, Cayenne, French Guiana) and a voucher specimen was deposited in the *Herbarium of Medicinal Plants* at UNAERP with voucher number HPMU-3133. The study was authorized by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) on behalf of Conselho de Gestão do Patrimônio Genético/Ministério do Meio Ambiente (protocol no. 010102/2015–9).

2.3. Preparation of extracts of U. tomentosa

Samples of bark and leaves were dried for 72 h at 45 °C in a circulating-air oven, pulverized and passed through a *40-mesh sieve. A portion (60 g) of powdered bark* was decocted with 3 L of distilled water for 30 min, filtered through filter paper, frozen and, subsequently freeze dried to yield 3.7 g of a crude aqueous bark extract (ABE). A sample (3.2 g) of crude aqueous leaf extract (ALE) was prepared in an exactly similar manner using 20 g of powdered leaves and 1 L of distilled water.

2.4. Analysis of oxindole alkaloids in bark and leaf extracts

The oxindole alkaloids present in the aqueous extracts of *U. tomentosa* were identified by ultra performance liquid chromatography-mass spectrometry (UPLC-MS) using a Waters (Milford, MA, USA) Acquity UPLC H-Class system equipped with a photodiode array detector and a Waters Xevo TQ-S tandem quadrupole mass spectrophotometer with the electrospray source operated in the positive ion mode. Samples containing 1 mg mL^{-1} of ABE or ALE in methanol were passed through 0.45 μm pore size syringe filters and aliquots (5 μL) injected onto a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm i.d., 3.5 μm particle size; Agilent, Santa Clara, CA, USA). Elution was performed using a mixture of ammonium acetate (0.2%) in water (solvent A) and acetonitrile (solvent B) supplied at a flow rate of 0.6 mL min^{-1}. The mobile phase was maintained at 35% B between 0 and 18 min, followed by a linear increase from 35% to 50% B between 18 and 32 min, and subsequently maintained at 50% B between 32 and 35 min before returning to 35% B until the end of analysis at 40 min. The *source* was maintained at 150 °C, the capillary voltage was 3.2 kV, the desolvation temperature was 350 °C, the desolvation gas (N_2) flow rate was 600 L h^{-1}, and the mass scan range was 100–600 *m/z* in the full-scan mode. Reference standards of *mitraphylline* and iso*mitraphylline were purchased from* ChromaDex (Irvine, CA, USA), while standard *isorincophylline* was kindly supplied by Dr. Adriana Lopes (UNAERP).

2.5. Quantification of mitraphylline in bark and leaf extracts

The concentrations of mitraphylline in aqueous extracts of *U. tomentosa* were determined by UPLC-MS/MS using the set up described in Section 2.4. operated in the multiple reaction monitoring (MRM) mode. Samples of ABE and ALE were accurately weighed, dissolved in HPLC grade methanol to yield stock solutions with concentrations of 1.0 mg mL^{-1} and passed through 0.45 μm pore size syringe filters. Working solutions with concentrations of 0.01, 0.1, 1, 10 and 50 μg mL^{-1} were prepared by serial dilution of stock solutions with methanol. A stock solution containing mitraphylline standard in methanol (1 mg mL^{-1}) was prepared in order to construct calibration curves with analyte concentrations in the range of 5–500 ng mL^{-1}.

Aliquots (10 μL) of ABE, ALE and mitraphylline standard were injected in triplicate onto an Ascentis Express C_{18} column (100 × 4.6 mm i.d.; 2.7 μm particle size; Sigma Aldrich, St Louis, MO, USA). Elution was performed using a mixture of formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in acetonitrile (solvent B) supplied at a flow rate of 0.5 mL min^{-1}. The mobile phase commenced at 30% B with a linear increase to 90% B within 3 min and was subsequently maintained at 90% B for 2 min before returning to 30% B within the following 5 min. MRM of mitraphylline (retention time 2.07 min) was performed with argon as the collision gas and the transition of precursor ion (*m/z* 369) to product ion (*m/z* 160) was followed under optimal conditions involving a declustering potential of 40 V and a collision energy of 25 eV. Data were acquired and processed using TargetLynx™ Application Manager software (Waters), and the concentrations of mitraphylline present in ABE and ALE samples expressed in μg mL^{-1}.

2.6. Analysis of phenolic acids and rutin in bark and leaf extracts

The presence of quinic acid, chlorogenic acid and rutin in aqueous extracts of *U. tomentosa* was confirmed by UPLC-MS analysis using the set up described in Section 2.4. with the electrospray source operated in the negative ion mode. Aliquots (5 μL) of ABE, ALE and reference standards (1 mg mL^{-1}) were injected in triplicate onto an Ascentis Express C_{18} column (100 × 4.6 mm i.d.; 2.7 μm particle size; Sigma Aldrich). Elution was performed using a mixture of formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in methanol (solvent B) supplied at a flow rate of 0.5 mL min^{-1}. The mobile phase was maintained at 3% B between 0 and 4 min, followed by linear increases from 3% to 60% B between 4 and 15 min and from 60% to 90% B between 15 and 19 min before returning to 3% B within the following 5 min. The *source* was maintained at 150 °C, the capillary voltage was 2.5 kV, the desolvation temperature was 300 °C, the desolvation gas (N_2) flow rate was 600 L h^{-1}, and the mass scan range was 100–700 *m/z* in the full-scan mode.

2.7. In vitro evaluation of the anti-inflammatory activities of bark and leaf extracts

The anti-inflammatory activities of ABE, ALE and mitraphylline standard were determined using the murine macrophage cell line RAW 264.7-Luc (ATCC®, TIB-71™; American Type Culture Collection, Manassas, VA, USA), which stably expresses luciferase on a nuclear factor kappa-B (NF-κB) responsive promoter (pNF-κB-Luc). Cultures of the cell line were maintained as described by Pinho-Ribeiro et al. (2016). Assays were performed by transferring 200 μL aliquots of a cell suspension (0.3×10^6 cells mL^{-1}) to each well of a 96-well microplate and incubating at 37 °C under an *atmosphere* with *5*% CO_2. After 24 h, test substances were added to individual wells as follows: ABE at 3, 10, 30, 100 and 300 μg/well; ALE at 1, 5, 15, 50 and 150 μg/well; and mitraphylline at 0.2205, 0.735, 2.205, 7.35 and 22.05 μg/well. Negative control wells, containing suspension cells without extracts or mitraphylline, were included in each assay. After 1 h, cells were incubated for 4 h with 10 μM mL^{-1} solution of lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in order to elicit pro-inflammatory pathways, following which the supernatants were removed from the wells and the remaining cells lysed with 30 μL of Tris-NaCl-Tween lysis buffer [10 mM Tris-HCl pH 8.5, 5 mM EDTA, 200 mM NaCl and 1% Triton] using an insulin syringe plunger for maceration. Cell lysates (10 μL) were transferred separately to new 96-well microplates and luciferase activities determined using a Victor X5 luminometer (PerkinElmer, Waltham, MA, USA) and the Promega (Fitchburg, WI, USA) Dual Luciferase Reporter assay system. Data were expressed as the ratio of relative luminescence units between test samples and control as described by Ruiz-Miyazawa et al. (2015). The supernatants were retained in order to measure the concentrations of the cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF-α) using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN, USA) following the manufacturer's instructions.

2.8. Evaluation of the cytotoxicity of bark and leaf extracts

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide tetrazolium (MTT) assay was employed to determine cell viability after exposure to ABE, ALE and mitraphylline. RAW 264.7-Luc suspension cells were incubated with aqueous extracts or mitraphylline in 96-well plates as described in Section 2.7., while suspension cells without extracts/mitraphylline were used as negative controls. Following exposure of cells to extracts or mitraphylline plus elicitation with LPS, the supernatants were removed from the wells and the remaining cells incubated with 200 μL of MTT in Roswell Park Memorial Institute (*RPMI*) *1640* medium (2 mg mL^{-1}) for 1 h. The insoluble formazan product generated in viable cells was solubilized by incubation with 150 μL of dimethyl sulfoxide for 10 min and absorbances were determined at 590 nm using a microplate spectrophotometer. The results were expressed as the percentage reduction in cell viability relative to controls (Silva et al., 2015).

2.9. Effects of bark and leaf extracts on the allergic response to ovalbumin (OVA) in a murine model of asthma

Male BALB/c mice, six to eight weeks-old and weighing between 20 and 30 g, were supplied by the animal breeding facility at FMRP-USP. Animals were maintained in isolation under controlled light and temperature conditions and received commercial chow and water *ad libitum*.

Mice were sensitized to OVA twice, with an interval of seven days between sensitizations, by intraperitoneal injection of 200 μL of isotonic saline solution containing 10 μg of OVA (Sigma-Aldrich) and 1 mg of aluminum hydroxide as adjuvant. One week after the second sensitization, animals were challenged on four alternate days by nasal instillation of 50 μL saline solution containing 10 μg of OVA while under light sedation induced by inhalation of isofluorane (Isoforine®; Cristália Produtos Químicos e Farmacêuticos, Itapira, SP, Brazil). Animals in the control group were challenged only with saline solution.

Treatments with bark and leaf extracts were applied *via* intraperitoneal injection over seven consecutive days starting at the same time as the first challenge. Experimental groups ($n = 6 - 8$ animals per group) were as follows: (i) OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; (ii) OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; (iii) OVA/ABE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ABE; (iv) OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; (v) OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; (vi) OVA/200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; (vii) OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone (Hipolabor, Sabará, MG, Brazil); and (viii) OVA/SAL group: challenged with OVA and treated with saline solution.

2.10. Evaluation of bronchial hyperresponsiveness (BHR)

BHR was assessed 24 h after the last OVA challenge by means of the methacholine challenge test (Hantos et al., 1992). Each animal was anesthetized with xylazine and ketamine (10 and 100 mg kg^{-1} i.p., respectively; Syntec, Cotia, SP, Brazil) and tracheostomy was performed in order to insert a metal cannula into the trachea. The cannula was connected to a ventilator for small animals (flexiVent®, SCIREQ, Montreal, Canada) and ventilation was adjusted to 150 breaths min^{-1} with a *positive end-expiratory pressure* (PEEP) of 3 cm H_2O. Complete muscle paralysis was induced by i.p. injection of pancuronium bromide (1.2 mg kg^{-1}) and respiratory measurements were performed under

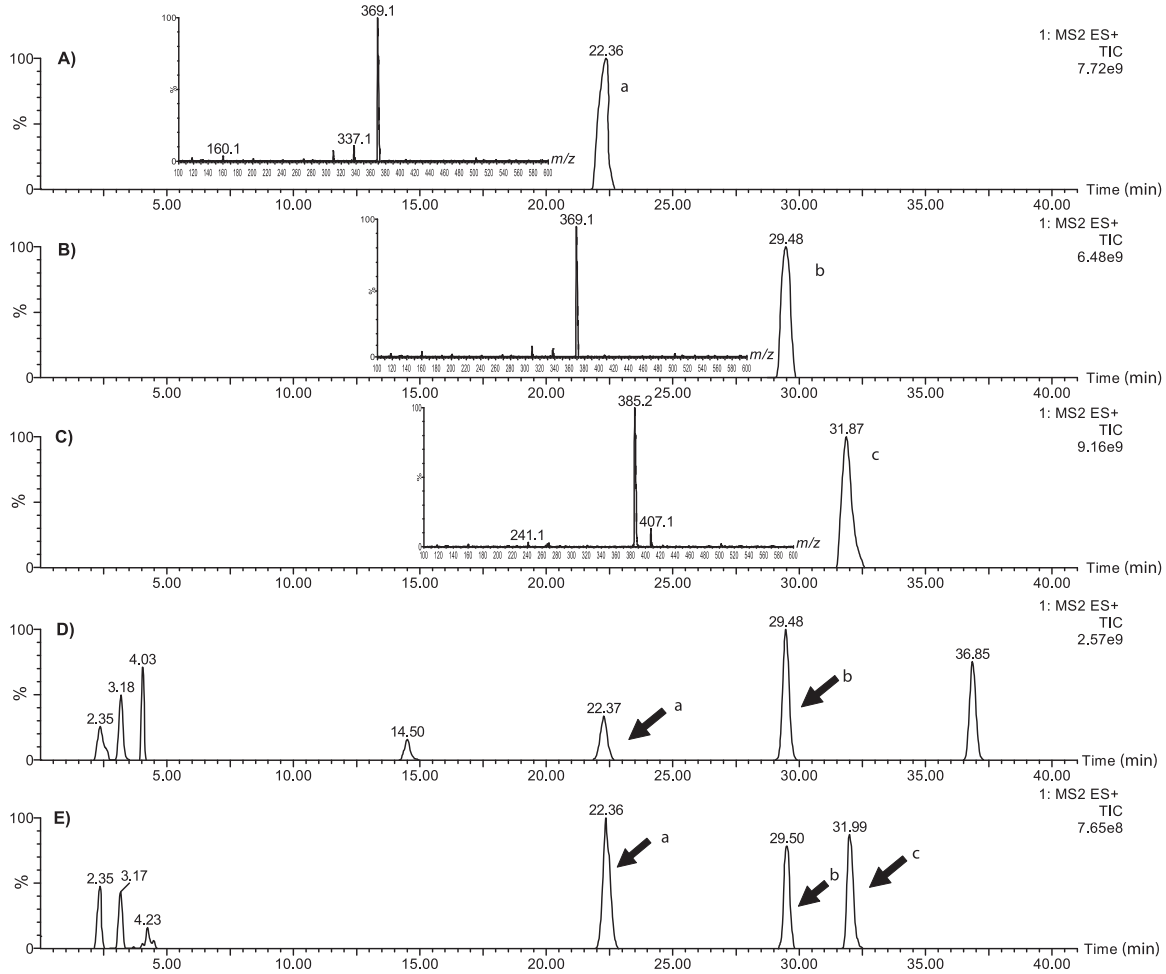

Fig. 1. UPLC-MS chromatograms of mitraphylline (**A**), isomitraphylline (**B**) and iso*rhyncophylline* (**C**) standards, and of aqueous bark extract (ABE; **D**) and aqueous leaf extract (ALE, **E**) from *U. tomentosa*. In panels **D** and **E**, peaks indicated by arrows bearing lower-case letters correspond to mitraphylline (**a**), isomitraphylline (**b**) and iso*rhyncophylline* (**c**).

basal conditions (Fonseca et al., 2017; Morel et al., 2017) and after exposure to increasing concentrations of methacholine (6.25, 12.5, 25 and 50 mg mL^{-1}) administered by an ultrasonic nebulizer (Hudson RCI, Temecula, CA, USA). The parameters assessed were respiratory system resistance (*Rrs*) and elastance (*Ers*), and tissue damping (*G*) and elastance (*H*). Values for these parameters were calculated from curves with coefficients of determination ≥ 0.9.

2.11. Analysis of bronchoalveolar lavage (BAL) samples

Inflammatory activities in airways were assessed in BAL, two samples of which were collected, after ventilation, from each animal by infusion of 1 mL of isotonic saline solution through the tracheal cannula and subsequent aspiration. The samples were centrifuged and the sediments resuspended separately in 500 µL of isotonic saline solution. An aliquot (100 µL) of this suspension was used to obtain a total cell count with the aid of a hemocytometer while the remaining portion was used in the preparation of Cytospin slides (Thermo Fisher Scientific, Waltham, MA, USA). Slides were stained with hematoxylin and eosin (H &E) for differential counting of inflammatory cells (eosinophils, neutrophils, macrophages and lymphocytes) in a total of 300 cells per animal.

2.12. Quantification of IgE

Following BAL sample collection, the rib cage of each animal was opened and blood was collected from the right ventricle by direct puncture. Blood was centrifuged and the serum separated and stored at −80 °C. Measurement of anti-OVA IgE was performed using ELISA BD OptEIA™ kits (BD Biosciences, San Diego, CA, USA) as previously described (Fonseca et al., 2017; Morel et al., 2017).

2.13. Histological analysis and quantification of cytokines in lung tissue

Saline solution (5 mL) was infused in the left ventricle with the aim of removing blood from the lungs. The right lung was manually extricated and stored in Ambion™ RNAlater™ (Thermo Fisher Scientific) stabilization solution until required for the preparation of homogenates (Fonseca et al., 2017; Morel et al., 2017) for the quantification of inflammatory cytokines. The left lung was insufflated for 25 min with 10% neutral buffered formalin (NBF) solution at a pressure of 25 cm H_2O, fixed in NBF for 24 h, embedded in paraffin, sectioned (5 µm) and stained with H&E for histological assessment.

Quantifications of IL-4, IL-5, IL-10, interferon-gamma (IFN-γ) and transforming growth factor-beta (TGF-β) in lung homogenates were performed using ELISA BD OptEIA™ kits (BD Biosciences), while IL-13 levels were determined using ELISA Ready-Set-Go! (eBioscience, San Diego, CA, USA), following the manufacturer's instructions.

Paraffin sections were examined under a Leica DM500 microscope (Leica Microsystems, Heerbrugg, Switzerland) and images recorded at a magnification of 400 × for quantification of peribronchial inflammation. For morphological analysis, four or five airways presenting intact epithelium were selected from each mouse and the areas corresponding to the basal membrane were delimited. The number of inflammatory

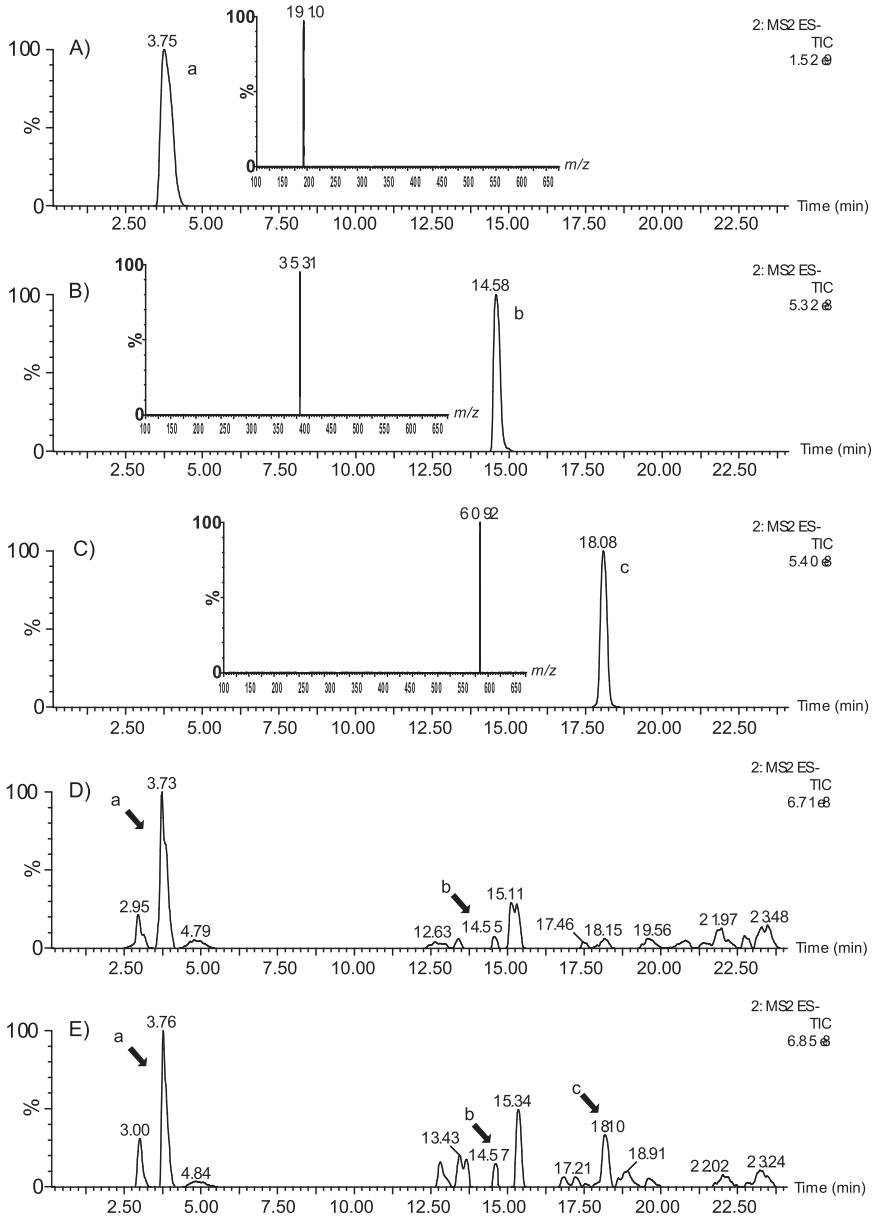

Fig. 2. UPLC-MS chromatograms of quinic acid (**A**), chlorogenic acid (**B**) and rutin (**C**) standards, and of aqueous bark extract (ABE; **D**) and aqueous leaf extract (ALE; **E**) from *U. tomentosa*. In panels **D** and **E**, peaks indicated by arrows bearing lower-case letters correspond to quinic acid (**a**), chlorogenic acid (**b**) and rutin (**c**).

cells was determined using a semi-quantitative method (Sur et al., 1999) and the degree of peribronchial inflammation was scored from 0 to 4 (corresponding to absence, mild, moderate, pronounced and severe inflammation, respectively) with 0.5 increments when inflammation was intermediate between two categories.

2.14. Statistical analysis

Two-way analysis of variance (ANOVA) and the Bonferroni post-test were used to compare groups challenged with different concentrations of methacholine, while one-way ANOVA and the Bonferroni post-test were employed in simple comparisons between experimental groups. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) with the level of statistical significance set at $P < 0.05$.

3. Results and discussion

3.1. Chemical characteristics of bark and leaf extracts from U. tomentosa

UPLC-MS analysis was highly efficient in the separation of constituents and revealed that *aqueous extracts of the bark and leaves of* U. tomentosa *differed both qualitatively and quantitatively with respect to the content of oxindole alkaloids. While the POAs mitraphylline (*Fig. 1*A) and isomitraphylline (*Fig. 1*B) were identified in both ABE and ALE, the TOA isorhyncophylline (*Fig. 1*C) was detected only in ALE (*Fig. 1*D and E). Generally,* U. tomentosa *exhibits considerable chemical heterogeneity in which variations in profile can be observed in individuals from different habitats as well as in different parts of a single plant (*Laus et al., 1997*; Montoro et al., 2004*). Since mitraphylline is considered the chemical marker of* U. tomentosa (Falkiewicz and Lukasiak, 2001; Laus et al., 1997; Luna-Palencia et al., 2013), we quantified this POA in the study samples and found that the concentration of *mitraphylline* in ALE was more than twice that of ABE (14.7 ± 0.8 and 6.6 ± 0.8 μg mL^{-1}, respectively). The quantitative difference in mitraphylline content of

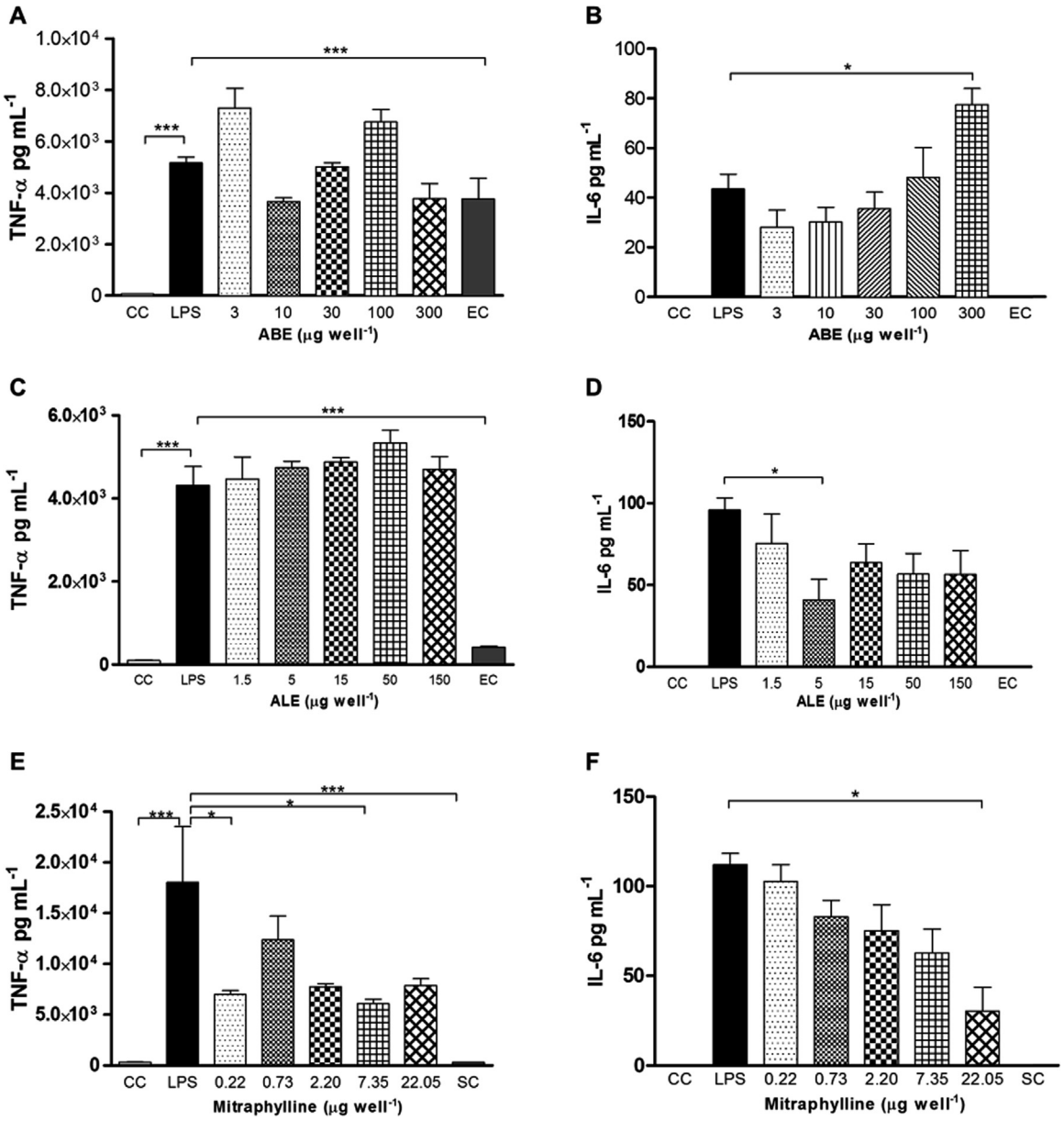

Fig. 3. Quantification of inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) as determined by enzyme-linked immunosorbent assay (ELISA) in lipopolysaccharide (LPS; 10 μM mL^{-1})-stimulated macrophage cell cultures (RAW 264.7-Luc) following treatment with aqueous bark extract (ABE; **A** and **B**), aqueous leaf extract (ALE; **C** and **D**) and standard mitraphylline (**E** and **F**). Comparisons between cells stimulated with different concentrations of extracts were performed using one-way analysis of variance followed by Bonferroni test, and the levels of statistical significance were set at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$(***). Controls were: CC (non-elicited, non-treated suspension cells), LPS (lipopolysaccharide-elicted, non-treated suspension cells), EC (extract control, non-elicited suspension cells, treated with *Uncaria tomentosa* extract) and SC (substance control, non-elicited suspension cells, treated with mitraphylline).

leaves and bark extracts from U. tomentosa *has been reported previously* (Navarro Hoyos et al., 2015; Peñaloza et al., 2015).

Three chemotypes of *U. tomentosa* have already been described (Kaiser et al., 2016; Peñaloza et al., 2015), namely: type I containing mainly POAs with a *cis* configuration such as *speciophylline,* pteropodine, isopteropodine and uncarine F; type II containing mainly POAs with a *trans* configuration such as *mitraphylline and* isomitraphylline; and type III containing mainly TOAs such as corynoxeine, isocorynoxeine, *rhyncophylline and* isorhyncophylline. *It would appear, therefore, that the specimens of* U. tomentosa *used in the present study were closely related to chemotype II. It is important to verify the chemical characteristics of the plants employed because POAs and TOAs have been shown to have opposing effects. For example, in an experiment involving human endothelial cells,* Wurm et al. (1998) *established that POAs stimulated* the release of a lymphocyte-proliferation-regulating factor while TOAs reduced such release in a dose-dependently manner. The antagonism between TOA and POA directly influences immunoregulatory activity and limits the potential application of *U. tomentosa* as a phytotherapeutic agent (Barnes et al., 2007).

Along with oxindole alkaloids, various phenolic compounds were also detected in aqueous extracts of *U. tomentosa*. Quinic and chlorogenic acids (Fig. 2A and B) were identified in ABE and ALE, with quinic acid being the major constituent in both extracts (Fig. 2D and E). According to previous studies, quinic acid is responsible for the anti-inflammatory activity of an alkaloid-free commercial extract of *U. tomentosa* (Akesson et al., 2005; Sheng et al., 2005). However, specimens of *U. tomentosa* exhibit considerable variation regarding the levels of quinic acid depending on habitat, germplasm source and plant part (Peñaloza et al., 2015). The flavonoid rutin (Fig. 2C) was detected in ALE and the anti-inflammatory activity of this substance has been

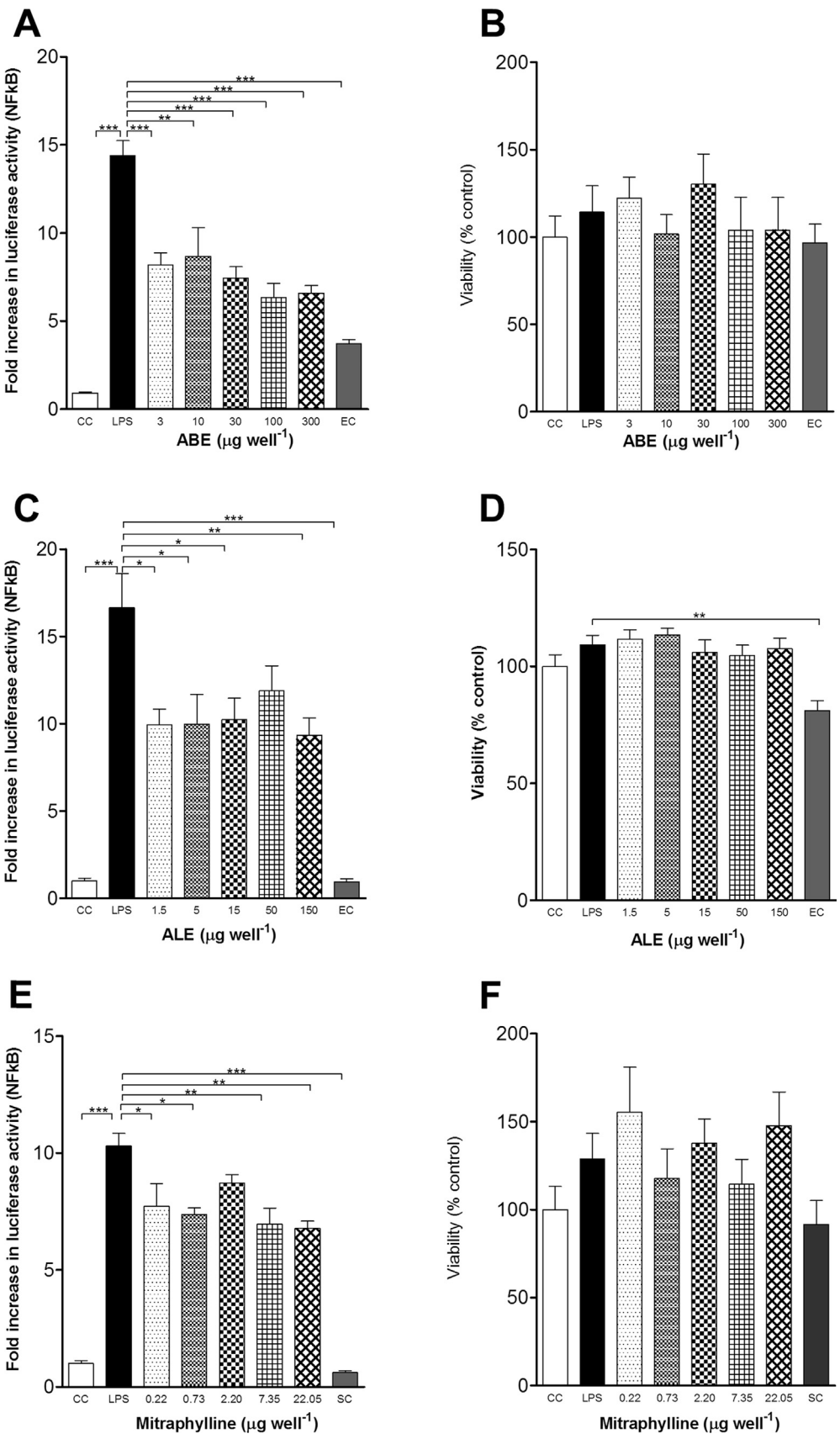

Fig. 4. Quantification of luciferase activity associated with the nuclear factor kappa (NF-κB) luciferase-responsive gene as determined by enzyme-linked immunosorbent assay (ELISA) in lipopolysaccharide (LPS; 10 μM mL^{-1})-stimulated macrophage cell cultures (RAW 264.7-Luc) following treatment with aqueous bark extract (ABE; **A** and **B**), aqueous leaf extract (ALE; **C** and **D**) and standard mitraphylline (**E** and **F**). Comparisons between cells stimulated with different concentrations of extracts were performed using one-way analysis of variance followed by Bonferroni test, and the levels of statistical significance were set at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$(***). Controls were: CC (non-elicited, non-treated suspension cells), LPS (lipopolysaccharide-elicted, non-treated suspension cells), EC (extract control, non-elicited suspension cells, treated with *Uncaria tomentosa* extract) and SC (substance control, non-elicited suspension cells, treated with mitraphylline).

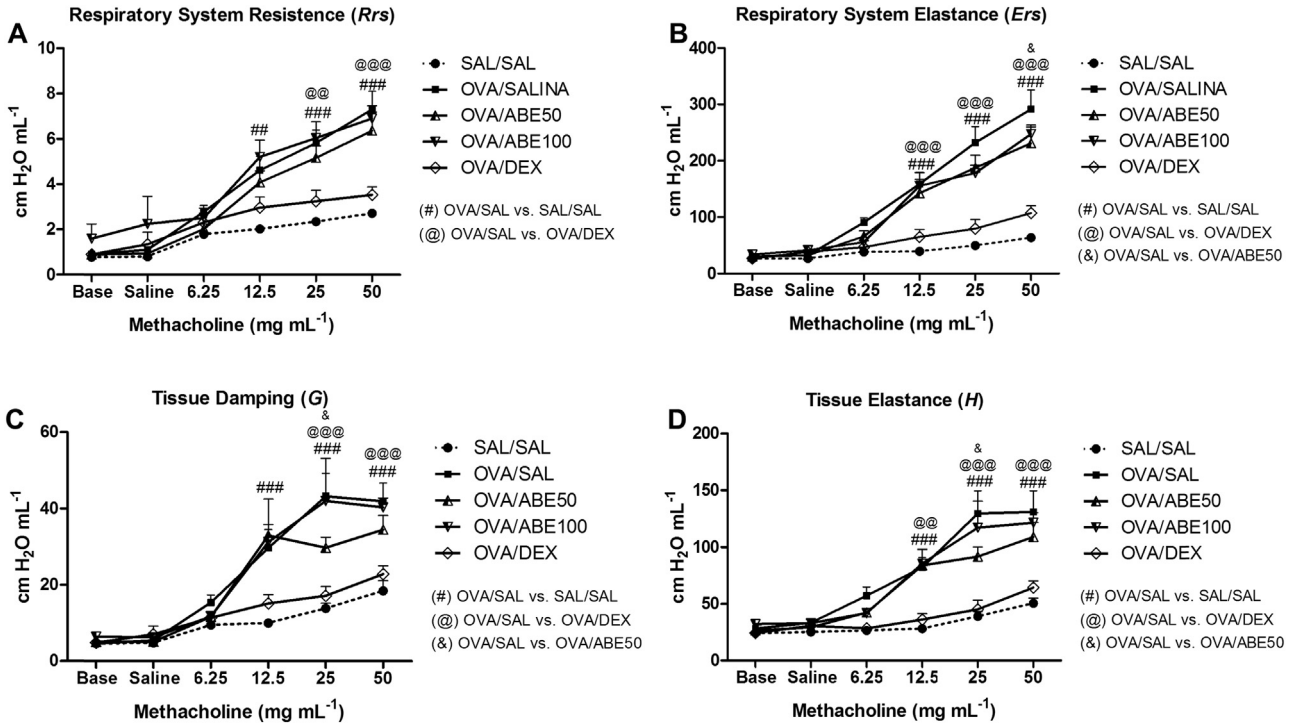

Fig. 5. Respiratory system resistance (*Rrs*; **A**), respiratory system elastance (*Ers*; **B**), tissue damping (*G*; **C**) and tissue elastance (*H*; **D**) determined by forced oscillation techniques and the methacholine challenge test (6.25–50 mg mL^{-1}) in experimental animals treated with aqueous bark extract (ABE) and control animals. Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution. Comparisons between groups were performed using two-way analysis of variance followed by Bonferroni test. The statistical significances of differences between groups are indicated as follows: OVA/SAL *vs* SAL/SAL - $P < 0.01$ ($^{\#\#}$) and $P < 0.001$ ($^{\#\#\#}$); OVA/SAL *vs* OVA/ABE50 - $P < 0.05$ ($^{\&}$); OVA/SAL *vs* OVA/DEX - $P < 0.01$ ($^{@@}$) and $P < 0.001$ ($^{@@@}$).

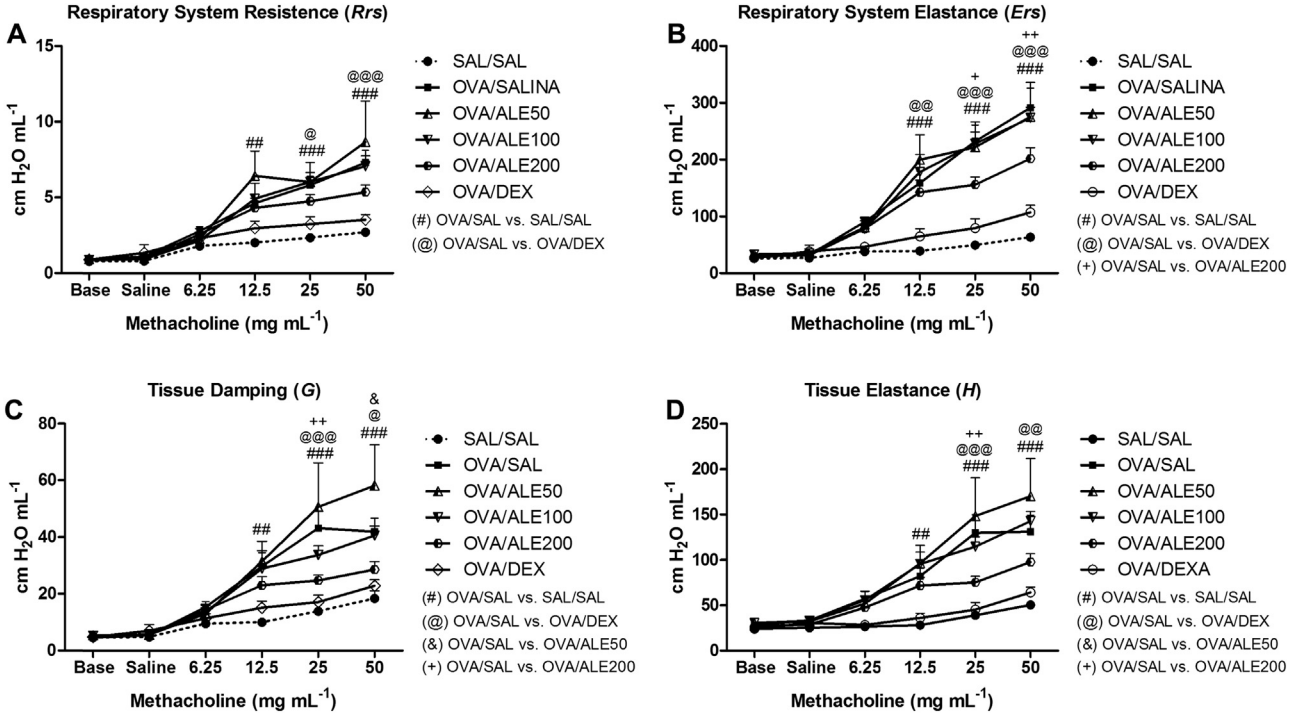

Fig. 6. Respiratory system resistance (*Rrs*; **A**), respiratory system elastance (*Ers*; **B**), tissue damping (*G*; **C**) and tissue elastance (*H*; **D**) determined by forced oscillation techniques and the methacholine challenge test (6.25–50 mg mL^{-1}) in experimental animals treated with aqueous leaf extract (ALE) and control animals. Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution. Comparisons between groups were performed using two-way analysis of variance followed by Bonferroni test. The statistical significances of differences between groups are indicated as follows: OVA/SAL *vs* SAL/SAL - $P < 0.01$ ($^{\#\#}$) and $P < 0.001$ ($^{\#\#\#}$); OVA/SAL *vs* OVA/ALE50 - $P < 0.05$ ($^{\&}$); OVA/SAL *vs* OVA/ALE200 - $P < 0.05$ ($^{+}$) and $P < 0.01$ ($^{++}$); OVA/SAL *vs* OVA/DEX - $P < 0.01$ ($^{@@}$) and $P < 0.001$ ($^{@@@}$).

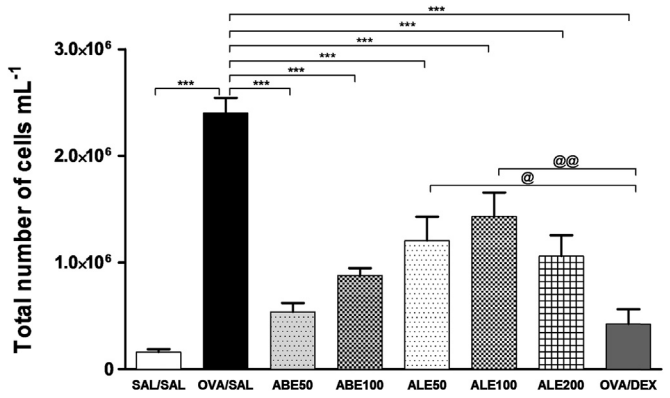

Fig. 7. Total counts of inflammatory cells in bronchoalveolar lavage samples from control animals and experimental animals treated with aqueous bark extract (ABE) aqueous leaf extract (ALE). Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution. Comparisons between groups were performed using one-way analysis of variance followed by Bonferroni test. The statistical significances of differences are indicated as follows: SAL/SAL or experimental groups *vs.* OVA/SAL $P < 0.001$ (***); experimental groups *vs* OVA/DEX - $P < 0.05$ ($^{@}$) and $P < 0.01$ ($^{@@}$).

recorded (Choi and Kim, 2013; Morimoto et al., 2011; Nikfarjam et al., 2017).

Considering that *U. tomentosa* presents a complex chemical profile and that the predominance of bioactive compounds is variable, it is important to standardize individual extracts in order to adjust them for specific therapeutical applications.

3.2. Anti-inflammatory activities of bark and leaf extracts

In vitro assays revealed that treatment of LPS-stimulated macrophages with ABE and ALE at the tested concentrations had no significant influence on the production of TNF-α (Fig. 3A and C). This finding contrasts with previous reports describing the effectiveness of aqueous and methanolic extracts of *U. tomentosa* as TNF-α inhibitors (Allen-Hall et al., 2007; Pantano et al., 2008; Sandoval et al., 2000). Such discrepancies reinforce the importance of standardizing *U. tomentosa extracts* since diverse chemotypes might present differential pharmacological activities (Kaiser et al., 2016; Peñaloza et al., 2015). In contrast, treatment of LPS-stimulated macrophages with ALE at 5 μg mL^{-1} led to a significant ($P < 0.05$) reduction in the production of IL-6, whereas treatment with ABE at 300 μg mL^{-1} significantly increased IL-6 production (Fig. 3 B and D).

Production of TNF-α was reduced significantly when LPS-stimulated macrophages were treated with 0.22 and 7.35 μg mL^{-1} of standard mitraphylline (Fig. 3E). In addition, the formation of IL-6 was reduced by this POA in a dose-dependent manner, although the decrease was statistically significant only at the highest concentration (22.05 μg mL^{-1}) (Fig. 3F). The inhibitory effect of mitraphylline on IL-6 production has been observed previously in cultures of human neutrophils (Montserrat-de la Paz et al., 2016).

Increased production of plasma TNF-α and IL-6 can be observed following infection or trauma stimuli, and excessive levels of such pro-inflammatory cytokines may exacerbate acute conditions such as sepsis and rheumatoid arthritis (Cohen and Sachar, 2017; Tanaka et al., 2016). Inhibition of TNF-α and IL-6 by mitraphylline demonstrates the potential of this drug in the treatment of inflammatory diseases, as it has been well documented (Montserrat de la Paz et al., 2016; Rojas-Duran et al., 2012), and reinforces the promising applicability of mitraphylline-enriched plant extracts.

The transcription of NF-kB was inhibited by ABE at all tested concentrations (*i.e.* 3–300 μg mL^{-1}; Fig. 4A), by ALE at 1.5, 5, 15 and 150 μg mL^{-1} (Fig. 4C), and by standard mitraphylline at 0.22, 0.73, 7.35 and 22.5 μg mL^{-1}(Fig. 4E). Although the inhibition of NF-kB by bark extracts from *U. tomentosa* has been reported previously (Akesson

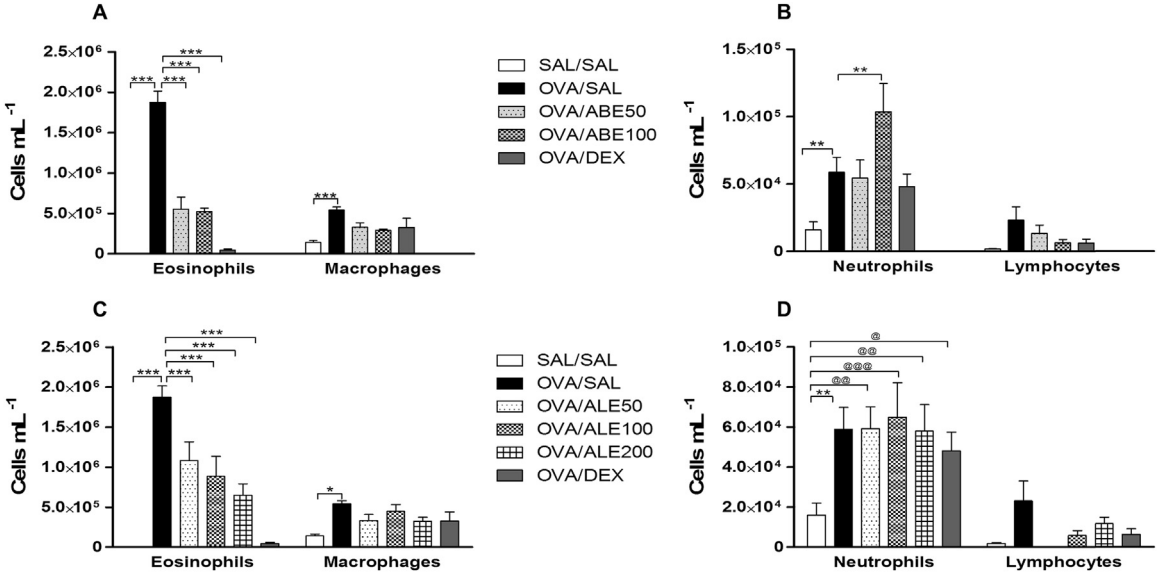

Fig. 8. Differential counts of inflammatory cells in control and experimental animals treated with aqueous bark extract (ABE; **A** and **B**) and aqueous leaf extract (ALE; **C** and **D**). Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution. Comparisons between groups were performed using one-way analysis of variance followed by Bonferroni test. The statistical significances of differences are indicated as follows: SAL/SAL or experimental groups *vs.* OVA/SAL $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***); experimental groups *vs.* SAL/SAL $P < 0.05$ (@), $P < 0.01$ (@@) and $P < 0.001$ (@@@).

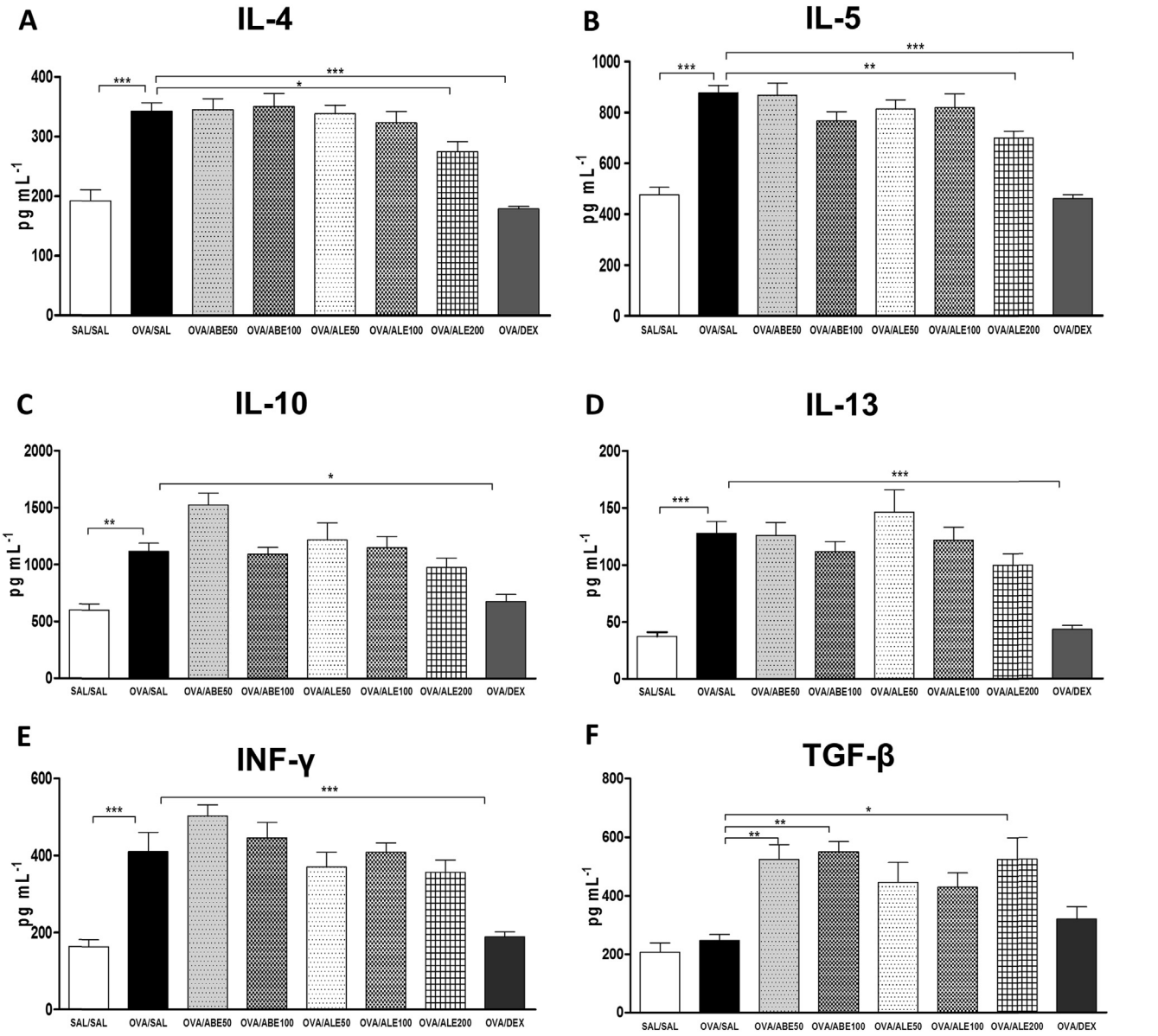

Fig. 9. Quantification of inflammatory cytokines by enzyme-linked immunosorbent assays performed on lung homogenates of control animals and experimental animals treated with aqueous bark extract (ABE) and aqueous leaf extract (ALE). Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution. Comparisons between groups were performed using one-way analysis of variance followed by Bonferroni test. The statistical significances of differences are indicated as follows: SAL/SAL, SAL/DEX or experimental groups *vs.* OVA/SAL $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

et al., 2003; Allen-Hall et al., 2010), this is the first report of such activity by *U. tomentosa* leaf extracts and mitraphylline. The NF-kB pathway is involved in the regulation of more than 500 pro-inflammatory genes and is directly implicated in the pathophysiology of various diseases including asthma, arthritis, diabetes and atherosclerosis (Hayden and Ghosh, 2008; Lentsch and Ward, 1999; Zhang et al., 2017). As shown in Fig. 4B, D and F, cell viability was not affected by treatments with ABE, ALE or mitraphylline.

3.3. Effects of bark and leaf extracts on BHR

Administration of ABE did not reduce *Rrs* in mice at methacholine concentrations in the range of 6.25–50 mg mL^{-1} (Fig. 5A). However, in comparison with the OVA/SAL control group, administration of ABE at 50 mg kg^{-1} gave rise to a significant ($P < 0.05$) decrease in *Ers* at a methacholine concentration of 50 mg mL^{-1} (Fig. 5B) and reduced *G* and *H* values at a methacholine concentration of 25 mg mL^{-1} (Fig. 5C and D). It is of interest to note that administration of ABE at 200 mg kg^{-1} induced death in all animals suggesting that the toxicity of bark extract at high concentrations should be further investigated.

Treatment of animals with ALE at 200 mg kg^{-1} led to significant decreases in *Ers* at methacholine concentrations of 25 and 50 mg mL^{-1} (Fig. 6B) and reduced *G* and *H* values at a methacholine concentration of 25 mg mL^{-1} (Fig. 6C and D) in comparison with the OVA/SAL group. Low doses of ALE (*i.e.* 50 mg kg^{-1}) opposed the effects of high doses (200 mg kg^{-1}) such that *G* values increased at a methacholine concentration of 50 mg mL^{-1} (Fig. 6C).

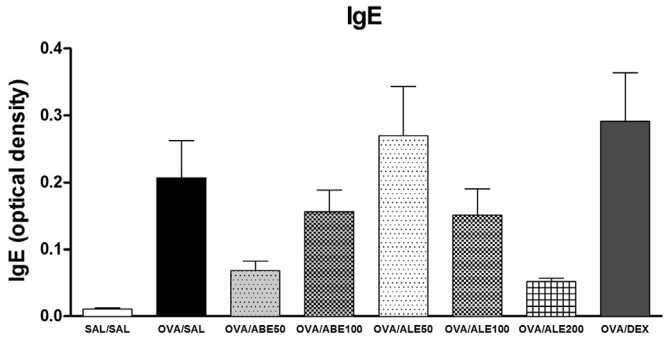

Fig. 10. Quantification of serum anti-OVA immunoglobulin E (anti OVA-IgE) complexes as determined by enzyme-linked immunosorbent assay in control animals and experimental animals treated with aqueous bark extract (ABE) and aqueous leaf extract (ALE). Experimental groups ($n = 6 - 8$ animals per group) were as follows: OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE; OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; OVA/SAL group: challenged with OVA and treated with saline solution; SAL/SAL group - challenged and treated with saline solution.

3.4. Effects of bark and leaf extracts on inflammatory cell counts in BAL samples

Administration of ABE and ALE at all concentrations tested induced significant ($P < 0.05$) reductions in the total number of inflammatory cells in BAL samples in comparison with the OVA/SAL group (Fig. 7). Thus, treatment with ABE at 50 and 100 mg kg^{-1} significantly reduced the number of eosinophils (Fig. 8A) in BAL samples, while the number of neutrophils increased significantly following administration of 100 mg kg^{-1} of bark extract (Fig. 8B). Additionally, treatment with ALE significantly reduced the number of eosinophils in BAL samples in a dose-dependent manner at concentrations in the range of 50–200 mg kg^{-1} (Fig. 8C), but increased significantly the number of neutrophils (Fig. 8D). The numbers of macrophages and lymphocytes in BAL samples were not altered significantly following treatment with bark or leaf extracts of *U. tomentosa*. ABE was more effective than ALE in reducing eosinophilia, probably because of the presence of mitraphylline and isomitraphylline and the absence of iso*rhyncophylline. These findings corroborate those of* Wurm et al. (1998) who demonstrated the antagonistic anti-inflammatory effects of TOAs and POAs in *in vitro* experiments.

3.5. Effects of bark and leaf extracts on cytokine levels in lung homogenates and serum IgE

Th2-type cytokines play important roles in the pathophysiology of allergic inflammation, including asthma. For example, IL-4 amplifies Th2 cell response while IL-5 is involved in the recruitment of eosinophils (Woolnough and Wardlaw, 2015; Xu et al., 2007). Our results showed that, in comparison with the OVA/SAL group, treatment of OVA-sensitized animals with ALE at 200 mg kg^{-1} reduced significantly ($P < 0.05$) the production of IL-4 and IL-5 by Th2 cells (Fig. 9A and B). However, no alterations in the levels of these two cytokines were observed in ABE-treated animals or in the concentrations of IL-10, IL-13 and IFN-γ following treatment with ALE or ABE (Fig. 9C, D and E). In contrast, treatment with ALE at 200 mg kg^{-1} or ABE at 50 and 100 mg mL^{-1} increased the concentration of TGF-β significantly (Fig. 9F). TGF-β is associated with structural changes observed in asthmatic airways, and it has been suggested that this factor has a dual role in that it can function as a pro- or anti-inflammatory cytokine. Nevertheless, there is evidence that TGF-β may participate of the inflammatory process in asthma (Duvernelle et al., 2003).

Previous studies involving murine *models* of *OVA*-induced *asthma (*Chen et al., 2014*;* Choi and Kim, 2013*)* have demonstrated that the flavonoid glycoside rutin *is able to reduce the concentrations of* IL-4, IL-5 and IL-13. In light of the above, we propose that mitraphylline and rutin are responsible for the reduction in Th2-type cytokines observed in OVA-sensitized animals treated with ALE because: (i) rutin was present in ALE but absent in ABE, (ii) the concentration of mitraphylline in ALE was more than two-fold higher than that in ABE, and (iii) no reduction in Th2 cytokines was observed in animals treated with ABE.

Allergen-specific IgE, an immunoglobulin that participates in the initiation and propagation of allergic diseases, is produced mainly by cells of the respiratory mucosa through contact with allergens (Eckl-Dorna and Niederberger, 2013). Thus, anti-IgE therapy represents an alternative treatment for asthma, allergic rhinitis and food allergy and in the prevention of associated symptoms (Brownell and Casale, 2004). However, OVA-specific IgE levels were not significantly altered by any of the treatments with ABE or ALE (Fig. 10).

3.6. Effects of ABE and ALE on peribronchial inflammation

*Microscopic analysis of lung tissue showed that, in comparison with the OVA/SAL group (*Fig. 11*B), treatment with ABE at 50* mg kg^{-1} and ALE at *100* mg kg^{-1} (Fig. 11D, G and I) significantly ($P < 0.001$ and 0.05, respectively) reduced inflammation of lung tissues. We propose that ABE has an anti-inflammatory effect in asthma since treatment with this extract reduced the density of inflammatory cells in lung tissues together with the total number of these cells, particularly granulocytes (eosinophils), in BAL samples. However, higher doses of ABE (100 mg kg^{-1}) did not reduce inflammation (Fig. 11E and I) or bronchoconstriction.

The anti-inflammatory activities of the extracts are probably associated with the presence of mitraphylline, isomitraphylline and, more especially, with chlorogenic and quinic acids, the properties of which are well documented. It has been shown that chlorogenic acid lowers LPS-induced inflammation in intestinal epithelial cells (Palócz et al., 2016) and down-regulates cyclooxygenase-2 expression in renal tissue, which is associated with oxidative stress and inflammation in many chronic diseases (Ye et al., 2017).

Moreover, the ability to inhibit the activation of NF-kB has been associated with the mechanism of action of *U. tomentosa* extracts (Allen-Hall et al., 2010) and of its constituents, quinic acid (Akesson et al., 2005; Zeng et al., 2009), chlorogenic acid (Feng et al., 2005) and rutin (Lee et al., 2012; Yeh et al., 2014). It has been shown that this transcription factor triggers inflammatory processes in the lung epithelium in response to different stimuli such as cytokines, allergens, infections and microbial substances (Poynter et al., 2003; Sheller et al., 2009). NF-kB is activated by inhibition of kappa light chain gene enhancer alpha (*IκBα*), a cytoplasmic protein complex responsible for the transcriptional regulation of pro-inflammatory genes. In response to stimuli, the lκB-α subunit undergoes phosphorylation and decomposition with subsequent migration of the p50 and p65 dimers from the cytoplasm to the nucleus, where they regulate the transcription of genes coding for cytokines, chemokines and other inflammatory mediators, thereby amplifying the process (Sethi et al., 2008). We have demonstrated for the first time that the activation of NF-kB is inhibited by mitraphylline, although the inhibition of LPS-mediated inflammation had been demonstrated previously by Montserrat de la Paz et al. (2016).

Surprisingly, a higher dose of ALE (200 mg kg^{-1}) did not reduce inflammation in lung tissue (Fig. 11H and I), although it diminished methacholine-induced bronchoconstriction, suggesting a *bronchodilator activity*. The observation that *high doses of ALE did not reduce the density of inflammatory cells in lung tissue but increased the number of neutrophils in BAL samples may be explained by the fact that ALE, unlike ABE,* contained higher concentrations of TOAs (as demonstrated by the presence of iso*rhyncophylline) and that* TOAs and POAs have antagonist effects.

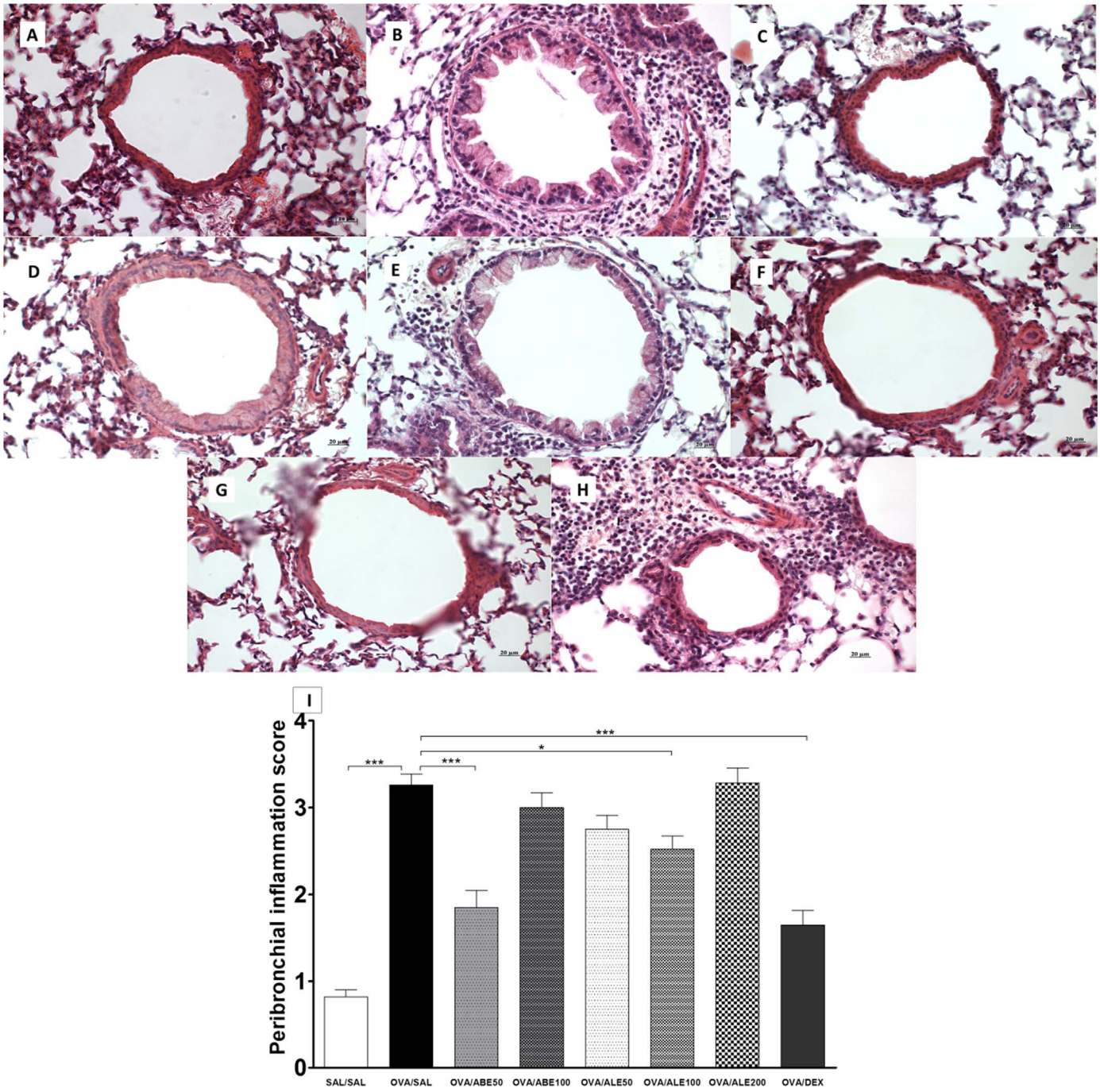

Fig. 11. Quantification of peribronchial inflammation, as determined by histological examination (hematoxylin/eosin stain; 400× magnification), in control animals and experimental animals treated with aqueous bark extract (ABE) and aqueous leaf extract (ALE). Experimental groups ($n = 6 - 8$ animals per group) were as follows: **A**) SAL/SAL group - challenged and treated with saline solution; **B**) OVA/SAL group: challenged with OVA and treated with saline solution; **C**) OVA/DEX group: challenged with OVA and treated with 2 mg kg^{-1} of dexamethasone; **D**) OVA/ABE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ABE; **E**) OVA/ABE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ABE; **F**) OVA/ALE50 group: challenged with OVA and treated with 50 mg kg^{-1} of ALE; **G**) OVA/ALE100 group: challenged with OVA and treated with 100 mg kg^{-1} of ALE; **H**) OVA/ALE200 group: challenged with OVA and treated with 200 mg kg^{-1} of ALE. For morphological analysis, 4–5 airways presenting intact epithelium were selected from each mouse and the areas corresponding to the basal membrane were delimited. Peribronchial inflammation was scored as: 0 - no inflammation, 1 - mild, 2 - moderate, 3- pronounced and 4 - severe inflammation, with 0.5 increments when inflammation was intermediate between two categories (Sur et al., 1999). Comparisons between groups were performed using one-way analysis of variance followed by Bonferroni test. The statistical significances of differences are indicated as follows: SAL/SAL, SAL/DEX or experimental groups *vs.* OVA/SAL $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

4. Conclusions

Our results clarify for the first time the *anti-inflammatory* activity of *U. tomentosa* in a murine model of asthma, thus supporting the ethnopharmacological uses of the plant. The aqueous extracts from bark and leaf of *U. tomentosa* have distinct chemical compositions and, consequently, their pharmacological activities are dissimilar. *In vitro* tests revealed that, although both extracts inhibited the production of Th1 pro-inflammatory cytokines (IL-6 and TNF-α) and NF-kB activation, the bark extract was more effective in treating asthmatic inflammation while the leaf extract was more successful in controlling respiratory mechanics, *i.e.* relaxing bronchial muscle and expanding

bronchial airways. Both extracts may have promising applications in the phytotherapy of allergic asthma.

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