



Hydroethanolic extract from *Echinodorus scaber* Rataj leaves inhibits inflammation in ovalbumin-induced allergic asthma



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A B S T R A C T

Ethnopharmacological relevance: *Echinodorus scaber*, Alismataceae, is popularly known in Brazil as “chapéu-de-couro”. The plant leaves are used by the population as decoction, infusion, or maceration in bottled spirits, to treat inflammatory respiratory diseases.

Aim of the study: To investigate the anti-inflammatory mechanism of the hydroethanolic extract of leaves of *Echinodorus scaber* (HEEs) in allergic asthma. A phytochemical analysis of the extract was performed as well. **Materials and methods:** The leaves of *Echinodorus scaber* were prepared by maceration in 75% ethanol. Preliminary phytochemical analysis was carried out using basic classical methods, and the secondary metabolites detected in HEEs were analyzed and confirmed by high-performance liquid chromatography (HPLC). The *in vivo* anti-inflammatory activity of HEEs was evaluated in Swiss male albino mice sensitized and challenged by OVA. The HEEs (1, 5 and 30 mg/kg, p.o.) was administered to mice twice a day, 1 h before the challenge, from days 19 through 24. The mechanism of action of HEEs was studied by evaluating the levels of T_H2 cytokines (IL-4, IL-5 and IL-13) in bronchoalveolar lavage fluid (BALF) and IgE production in blood plasma. Histopathological changes triggered by OVA-sensitization/challenge in the lung tissue were also investigated.

Results: HEEs reduced total leukocyte, eosinophil, neutrophil, and mononuclear cell counts at all doses tested, with maximum effect at 30 mg/kg (73.9%, 75.9%, 75.5%, and 65.2% reduction, $p < 0.001$, respectively). Increases in T_H2 cytokine secretion (IL-4, IL-5 and IL-13) and in IgE levels were also attenuated by HEEs. Preliminary phytochemical screening seems to indicated the presence of phenolic compounds, flavonoids and alkaloids. HPLC analyses evidenced the presence of phenolic compounds, such as gallic acid, rutin and vitexin.

Conclusion: Our findings provided pharmacological preclinical evidence for the popular use of the leaves of *Echinodorus scaber* in allergic inflammation. Its anti-inflammatory effect was dependent on the decrease in migratory inflammatory cells, and both T_H2 cytokines and IgE levels. It is suggested that vitexin, gallic acid and rutin, known anti-inflammatory compounds, may participate in the anti-asthmatic effect of the HEEs, by acting jointly along with other components present in the extract.

1. Introduction

According to the Global Initiative for Asthma (GINA, 2016), asthma

affects about 300 million people worldwide and is regarded as one of the most important public health issues around the globe (Reddel et al., 2015). Furthermore, asthma also has significant economic impact,

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since it is a frequent cause of hospitalization and absenteeism (Costa et al., 2013).

Allergic asthma results from an exacerbated response to allergens, and is clinically characterized by hyper-responsiveness of airways, airway inflammation, and high production of allergen-specific immunoglobulin E (IgE) (Hall and Agrawal, 2014). The inflammation that results from allergic asthma involves a complex network of interactions between innate and adaptive immune systems as well as with the respiratory epithelium.

Its clinical evolution is associated with the activation of T lymphocytes and the release of T helper type 2 (T_H2) cytokines (Fajt and Wenzel, 2015). Initially, after exposure to an allergen, the local release of mediators by resident cells in the lung promotes the recruitment and activation of inflammatory cells, such as eosinophils, neutrophils, and lymphocytes into the bronchial mucosa (Ishmael, 2011). The most important mediators involved in the allergenic immune response are the interleukins IL-4, IL-5 and IL-13 that drive the amplification of the T_H2 response (Chung, 2015), and triggers allergen-specific IgE antibody production by B lymphocytes (Wu and Scheerens, 2014).

This enhanced allergen-specific IgE secretion competes, with other IgE molecules non-associated with the allergen, for Fcε receptors expressed on basophils and mast cells. The cross-linking between proximal Fcε receptors, caused by the recognition of the allergen by the specific IgE attached, results in the release of histamine, chemokines, leukotrienes, and other mediators responsible for the amplification of the inflammatory response (Ishmael, 2011).

However, current treatments used in the control of asthma symptoms are predominantly based on the use of inhaled glucocorticoids (GIs), due to their vast immunosuppressive effect, and of agonists of β₂-adrenergic receptors, which are both effective in preventing and reducing bronchospasm. Although widely used in the treatment of asthma, they have limited effectiveness in some types of asthma, and can cause side effects during prolonged use, thus contributing to non-compliance with treatment and to therapeutic failures (Olaguibel et al., 2012; Stoloff, 2008). Therefore, new agents such as anti-leukotrienes and anti-IgE drugs, which act on more specific asthma targets, have been emerged as treatment options (Brand, 2011; Chini et al., 2014). In addition, clinical trials with immunobiological agents, such as anti-IgE (NCT00546143), anti-IL-4 (NCT01312961), anti-IL-5 (NCT01287039) and anti-IL-13 (NCT01987492) have been undertaken, thus demonstrating the importance of seeking for more effective and safer new pharmacological agents.

In this context, medicinal plants commonly employed to treat asthma, could constitute an important source for bioprospecting for new molecules and for the development of herbal medicines (Newman and Cragg, 2012). An important matter about medicinal plants is that, with its diversity on secondary metabolites, they can have a superior effectiveness, given that its constituents could have synergistic or additive effects, and could also act on multiple pharmacological targets (Koeberle and Werz, 2014).

Among the plants popularly employed in the treatment of asthma, *Echinodorus scaber* Rataj (Alismataceae), a heterotypic synonym for *Echinodorus macrophyllus* (Kunth) Micheli, deserves special attention. It is a sub-aquatic herbaceous plant native to Brazil (Leite et al., 2007), locally known as "chapéu-de-couro", found in the regions of the Amazon and Pantanal, particularly at rivers banks and swampy areas (Matias et al., 2015). In traditional medicine, the leaves of *E. scaber* are used by inhabitants of rural and urban communities, in the form of infusion or decoction, or in maceration in bottled alcoholic beverages, to treat allergies, inflammation, kidney infections, rheumatism and respiratory diseases (Bieski et al., 2012; De la Cruz, 2008; Messias et al., 2015). Also, the powdered leaves of *E. scaber* are sold as plant-drugs in supermarkets and health food stores in Brazil, in the form of sachet for preparing tea-like infusions, and are used for the combat and prevention of inflammatory disorders, and as diuretic (Nunes et al., 2003).

As a corroborative evidence of its popular use, pharmacological studies with *E. scaber* have demonstrated its antinociceptive (Coelho et al., 2013), immunosuppressant (Pinto et al., 2007), nephroprotective (Portella et al., 2012), and particularly as shown by our group, its anti-inflammatory activity (Tanus-Rangel et al., 2010). Also, da Silva et al. (2016) reported that a flavonoid enriched fraction of *E. scaber* had higher anti-inflammatory activity even than the aqueous extract of leaves, by reducing NO and LTB₄ levels, vasodilatation and neutrophil migration. The polar extracts of *E. scaber* leaves were shown not to be genotoxic in *in vivo* models, after treatment with the same recommended doses for human subjects (Da Costa Lopes et al., 2000). At present, there are no reports over important adverse reactions from this plant registered at the National Health Agency of Brazil (ANVISA) (Brasil, 2011).

Echinodorus scaber appears in the 1st and 2nd editions of the Brazilian National Pharmacopoeia (Brasil, 1959; Silva, 1929). Also, preparations of this plant are part of the list of herbal medicines authorized by ANVISA to be used as over-the-counter medicines, as a diuretic and as anti-inflammatory (Brasil, 2010). In light of this, it becomes necessary to investigate the anti-asthmatic effect of this plant, and this was our aim in the present work, by studying the effect of the hydroethanolic extract of the leaves of *E. scaber* (HEEs) in an allergic inflammatory murine model induced by ovalbumin (OVA), and to elucidate its likely action mechanisms. Furthermore, in order to standardize the HEEs, a preliminary phytochemical screening, followed by HPLC fingerprint was carried out.

2. Materials and methods

2.1. Drugs and reagents

Aluminium hydroxide [Al (OH)₃], rutin hydrate (PubChem CID: 24899379), gallic acid monohydrate (PubChem CID: 24856708), dexamethasone acetate (Dexa), hematoxylin solution, eosin Y solution, ovalbumin (OVA, grade II and V), and Tween 20, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vitexin (PubChem CID: 5280441), was from Fluka (St. Louis, Missouri, USA). Mouse IL-4, IL-5 and IL-13, and IgE ELISA detection kits were purchased from eBioscience (San Diego, CA, USA).

2.2. Plant material

The leaves of *E. scaber* were harvested from Poconé City, Mato Grosso, Brazil (GPS coordinates: 15°50'060" S and 54°24'804" W) in April 2013. In accordance to Brazilian laws, accesses to the associated traditional knowledge and to the genetic patrimony for research purposes, was authorized by the Council on Genetic Patrimony of the Ministry of Environment (CGEN/MMA, No. 045/2009). Botanical identification was done at Herbário UFMT (Universidade Federal de Mato Grosso) and a voucher specimen (No. 40740) was deposited in place. *Echinodorus scaber* is not actually included in the list of endangered Brazilian plants (Brasil, 2014).

2.2.1. Plant extract preparation

Three kilograms (3 kg) of fresh leaves of *E. scaber* were cleaned, dried at room temperature, passed through an electric mill (model TE-625 Tecnal, SP, Brazil), and thereafter sieved (mesh size No. 40). The dried powder (430 g) obtained was macerated in a 75% hydroethanolic solution (1:3 w/v) for 7 days, filtered and concentrated in a rotary evaporator (Marconi MA 120, São Paulo, Brazil) under reduced pressure of 600 mm Hg at 40 °C. The residual solvent was eliminated in an oven at 45 °C for 24 h, obtaining HEEs (with a yield of 11.4% w/w), which was stored in amber bottles and kept at 2–8 °C. At the time of use, HEEs was dissolved in 2% Tween 20 in distilled water.

2.3. Phytochemical analysis

2.3.1. Preliminary phytochemical screening

Preliminary basic phytochemical analyses were performed according to the methods described by Matos (2009), based on chemical reactions involving coloration shifts, precipitation and foam formation.

2.3.2. Quantitative analysis of secondary metabolites

2.3.2.1. Total phenolic content. The quantification was performed using the Folin–Ciocalteu method described by Amorim et al. (2008) as modified by Balogun et al. (2014), using gallic acid as a standard. Total phenols were determined by interpolation of the absorbance of samples against a calibration curve of gallic acid, and then expressed as mg of gallic acid equivalents (GAE) per gram of dry extract (GAE mg/g HEEs). All experiments were performed in triplicate.

2.3.2.2. Total flavonoid content. The quantification was performed according to the description of Peixoto Sobrinho et al. (2008) as modified by Balogun et al. (2014), using rutin as standard. Total flavonoids content was determined by interpolating the absorbance of the samples against a calibration curve of rutin, and expressed as mg of rutin equivalents (RE) per gram of dry extract (mg RE/g HEEs). All experiments were performed in triplicate.

2.3.3. High performance liquid chromatography (HPLC) analysis

HPLC was developed in a Shimadzu® chromatograph (LC-10 Avp series, Japan) equipped with a LC-10 AD pump, DGU-14A degasser, UV–vis (SPD-0A) detector, column oven (CTO-10A), manual injector Rheodyne (loop 20 µL) and CLAS (LC-10A) integrator. The compounds were evidenced and quantified by comparing the sample retention times with those of authentic standards, such as gallic acid, vitexin and rutin. Extract and standard solutions were prepared by dilution in 0.1% phosphoric acid in Milli-Q water, and filtered through a 0.22 µm Millipore® membrane.

2.4. Animals

Male Swiss-Webster mice (25–30 g), from the Central Animal House of UFMT, were maintained in propylene cages at 25 ± 1 °C with a 12 h light/dark cycle and having free access to standard pellet chow and water. The adopted diet regime for animals during treatments consisted of feed restriction within the periods of 8:00–9:00 h and of 16:00–17:00 h. The experimental protocol followed the International Guiding Principles for Biomedical Research Involving Animals (CIOMS/ICLAS) and was approved by the Ethics Committee on Use of Animals in Research of our institution (CEUA/UFMT), under protocol No. 23108.028454/12-2.

2.5. In vivo anti-inflammatory evaluation

2.5.1. Induction of allergic asthma with ovalbumin

The classical OVA-induced immediate hypersensitivity mouse model described by Nader et al. (2012) was used, with minimal modifications (Fig. 1). On days 1 and 10, mice were sensitized by intraperitoneal injection (200 µL i.p.) of a suspension containing OVA (grade V; 100 µg/mL) and Al (OH)₃ (10 mg/mL) in 0.9% saline. From days 19 through 24, after the initial sensitization, animals were challenged with aerosolized OVA (grade II; 3% in 0.9% saline) for 20 min daily in an enclosed acrylic chamber (25×13×13 cm) connected to a nebulizer (NE-U0003 soniclear; SP, Brazil). Animals (n=6/group) sensitized and challenged with OVA, were treated orally by gavage (p.o.) 2 times daily with either vehicle (2% Tween 20), HEEs (1, 5, and 30 mg/kg), or Dexamethasone

as the standard drug (0.5 mg/kg). Animals in sham group (n=6) were sensitized/challenged and treated with 0.9% saline.

2.5.2. Determination of IgE

Total blood plasma IgE level was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions, using blood plasma samples collected 24 h after the last OVA challenge. For the blood collection, after anesthesia (ketamine/xylazine, 40/50 mg/kg i.p.), the inferior vena cava of each animal was punctured, and the collected blood centrifuged at 12,000×g for 5 min at 4 °C for cell clearing. The samples were stored at –20 °C until the test run.

2.5.3. Collection of bronchoalveolar lavage fluid (BALF)

Twenty-four hours after the last challenge, and after blood collection, animals were killed with overdose of the anesthetic agent (ketamine 200 mg/kg and xylazine 50 mg/kg i.p.), tracheotomized and cannulated (polyethylene children's urethral catheter tube No. 06) for BALF collection, obtained after two consecutive washes, 1 mL each, with PBS buffer (pH 7.4) plus EDTA 1 M. Both washes were unified and homogenized as a single sample. From the volume recovered, 20 µL was added to 380 µL of Turk's solution in order to count the total number of leukocytes in a Neubauer chamber. Also, slides for differential counts were prepared from 20 µL aliquots of the cell suspension, and stained with InstantProv® (NewProv; PR, Brazil). Cells numbers were determined with the aid of an optical microscope (1000x magnification) (Nikon, Tokyo, Japan), by counting at least 100 cells per slide, and differentiating cell types (neutrophils, eosinophils, basophils and mononuclear cells).

2.5.4. Histopathological analysis

After BALF collection, the lungs of mice were infused via tracheal intubation with 1 mL of 10% neutral formalin, removed and immersed in the same fixative for at least 24 h. Tissues were paraffinized, and 6 µm sections were cut and stained with hematoxylin and eosin (HE) for examining cell infiltration under light microscopy. The severity of inflammation was evaluated by peribronchial and perivascular cell infiltration counting, and by edema determination, using a modified score system (see footnotes on Table 1) described by Myou et al. (2003).

2.5.5. Cytokines quantification

BALF samples were stored in –80 °C freezer until the time of assay. Concentrations of mouse IL-4, IL-5 and IL-13 were determined with ELISA kits, in accordance to manufacturer's instructions.

2.6. Data analysis

The results were expressed as mean ± standard error of the mean (χ ± SEM). Comparisons between means were determined by one-way analysis of variance (ANOVA), and when significant, it was followed by the Student-Newman-Keuls test for multiple comparisons. Non-parametric data were expressed as median and interquartile range (Q₁–Q₃), analyzed by Kruskal-Wallis test and the statistical significance among groups determined by the Dunn's test. All analyses were performed using GraphPad Prism® 5.0 for Windows (San Diego, CA, USA). Values of *p* < 0.05 were considered significant.

3. Results

3.1. Phytochemical analysis

The preliminary phytochemical analysis of the HEEs seems to reveal the presence of compounds of phenolic, flavonoid and alkaloid classes. The content of total phenolics and flavonoids were 88.88 ± 0.64 mg EGA/g and 79.11 ± 0.20 mg ER/g, respectively. Analysis by

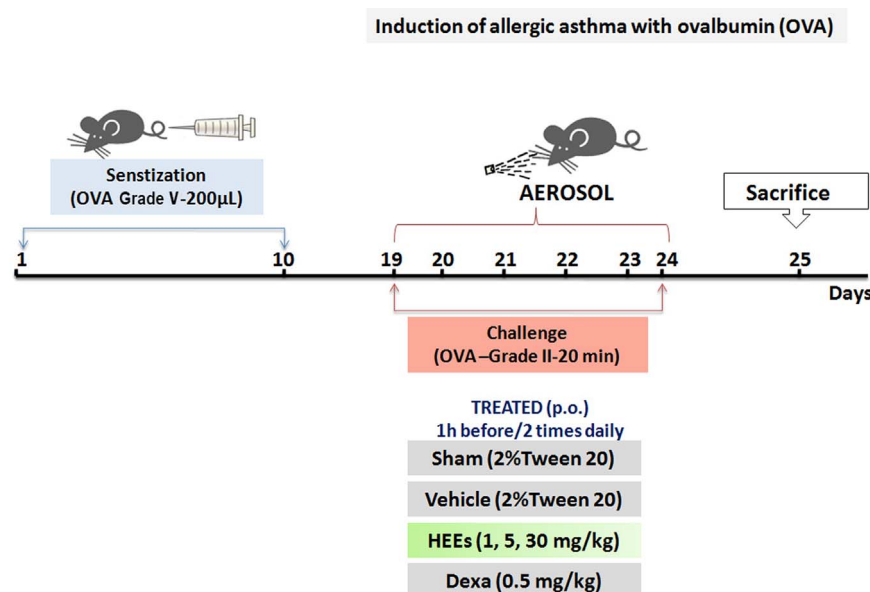


Fig. 1. Mouse model of airway inflammation and treatment with hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs).

Table 1

Effect of oral administration of vehicle (2% Tween 20), hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs 1, 5 and 30 mg/kg) and dexamethasone (Dexa 0.5 mg/kg) on histopathologic scoring of lung tissues of mice subjected to allergic reaction induced by ovalbumin (OVA).

Group (mg/kg, p.o.)	Edema	Peribronchial infiltrate	Perivascular infiltrate
Sham	1.0 (1.0 – 2.0)	1.0 (1.0 – 1.2)	1.0 (1.0 – 1.2)
Vehicle	4.0 (3.0 – 4.0) ^{††}	4.0 (3.0 – 4.0) [†]	4.0 (3.7 – 4.0) ^{†††}
HEEs 1	3.5 (3.0 – 4.0)	4.0 (3.0 – 4.0)	4.0 (3.0 – 4.0)
HEEs 5	3.0 (2.0 – 3.0)	4.0 (3.0 – 4.0)	2.0 (2.0 – 3.0)
HEEs 30	2.0 (1.0 – 2.0) [*]	3.5 (2.7 – 4.0)	2.0 (1.7 – 3.0)
Dexa 0.5	1.0 (1.0 – 1.3) ^{**}	1.0 (1.0 – 1.2) [*]	1.5 (1.0 – 2.0) ^{**}

The values were expressed as median – interquartile range. Hematoxylin and eosin staining was performed to examine the pathologic alteration in lung tissues. The scoring system for edema was as follow: 0 – absent; 1 – mild, presence of disperse erythrocytes in the interstitial space; 2 – mild to moderate, presence of several erythrocytes in the interstitium; 3 – moderate, increase of capillary lumen and of the interstitial space, high concentration of erythrocytes; 4 – severe, destruction of vessel walls and high concentration of erythrocytes. The scoring system on severity of inflammation was: 1 – a few cells; 2 – a ring of cells 1 deep cell layer; 3 – a ring of cells 2–4 deep cell layers; 4 – a ring of cells more than 4 deep cell layers. Kruskal-Wallis test, followed by post-test of Dunn. [†] $p < 0.05$, ^{††} $p < 0.01$, ^{†††} $p < 0.001$ vs sham; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs vehicle.

HPLC confirmed the presence of phenolic compounds, as detected by preliminary phytochemical analysis and indirectly quantified spectrophotometrically. In the HPLC chromatogram, were evidenced the presence of gallic acid (time: 19.0 min), vitexin (time: 43.7 min) and rutin (time: 44.1 min), with the following proportions in the HEEs: gallic acid 3.00 µg/mg, vitexin 15.04 µg/mg, and rutin 7.47 µg/mg (Fig. 2).

3.2. HEEs alleviated airway inflammation in BALF and lung tissues

To evaluate the inhibitory effect of HEEs in the asthma model of pulmonary inflammation induced by OVA in mice, we assessed leukocyte infiltration into the airway by measuring the total number of leukocytes, eosinophils, neutrophils and mononuclear cells present in the collected BALF (Fig. 3).

Sensitization and challenge with OVA promoted sharp increases (p

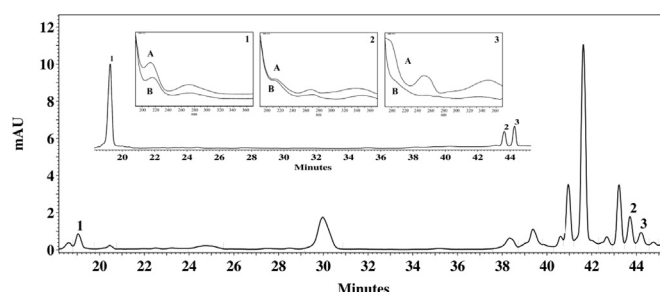


Fig. 2. High-performance liquid chromatography (HPLC) fingerprint of hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs) detected at 280 nm. Peak 1: gallic acid, 2: vitexin and 3: rutin. Insert HPLC of authentic standards of phenolic compounds and UV absorption spectrum of each standard compound (A) and HEEs (B).

< 0.001) in the total number of cells, eosinophils, neutrophils and mononuclear cells present in the BALF collected from animals in the vehicle treated group (OVA/OVA) by 7, 129, 8 and 6 folds, respectively, when compared to animals in sham group, with 11.77 ± 2.14 , 0.17 ± 0.05 , 6.24 ± 1.10 , and 2.35 ± 0.42 ($\times 10^6$) cells, respectively.

The HEEs (1, 5, and 30 mg/kg) reduced ($p < 0.001$) the number of total leukocytes (71.4%, 50.2% and 73.9%), eosinophils (72.1%, 54.1% and 75.9%), neutrophils (74.1%, 54.0% and 75.5%), and mononuclear cells (60.9%, 30.8% and 65.2%) in BALF, when compared to the vehicle group. In a similar way, Dexa 0.5 mg/kg, the standard drug, also reduced ($p < 0.001$) the total number of cells (83.7%); eosinophils (86.8%); neutrophils (83.7%) and mononuclear cells (79.0%) in the BALF.

Histopathological analysis of the collected lungs tissues (Fig. 4 and Table 1) showed that sensitization and challenge with OVA promoted intense edema ($p < 0.01$), and peribronchial ($p < 0.05$) and perivascular ($p < 0.001$) infiltration, compared to the sham group. Treatment with the HEEs (30 mg/kg) was significantly effective only in reducing edema (50%, $p < 0.05$). Nevertheless, Dexa at the dose of 0.5 mg/kg, was more effective by reducing all the investigated inflammatory parameters ($p < 0.01$ for edema, $p < 0.05$ for peribronchial infiltrates, and $p < 0.01$ for perivascular infiltrates).

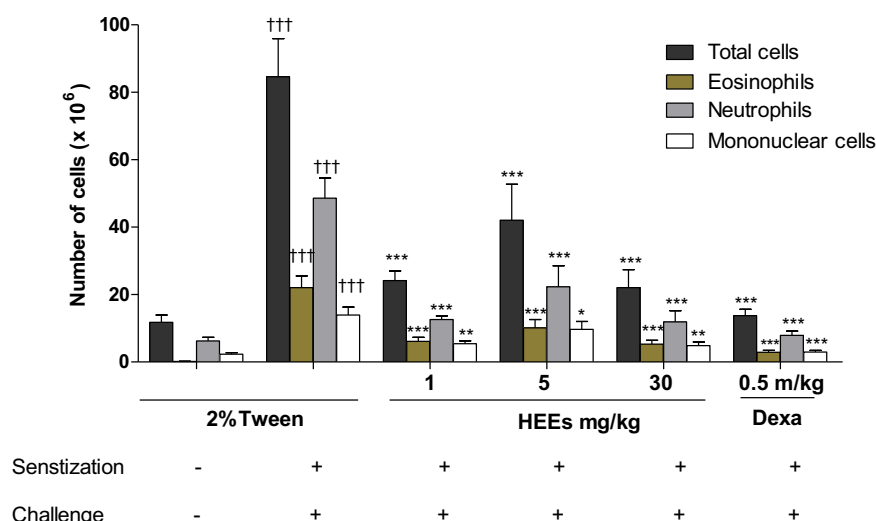


Fig. 3. Effect of oral treatments (2 times daily) with the vehicle (2% Tween 20) hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs, 1, 5 and 30 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on total cells, eosinophils, neutrophils, and mononuclear cells present in the bronchoalveolar lavage fluid (BALF) of mice sensitized (1st and 10th) with ovalbumin (OVA) grade V and aluminium hydroxide (0.1 mg/mL and 10 mg/mL in 0.9% saline ip., 0.2 mL/animal) and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulization, 20 min daily). The sham group was treated with the vehicle (2% Tween 20, 0.1 mL/10 g, p.o) and sensitized and challenged with 0.9% saline. The results were expressed as Mean $\pm$ SEM for 6 animals. One way ANOVA, followed by Student-Newman-Keuls. ⁺⁺⁺ $p < 0.001$ vs. Sham; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle.

3.3. HEEs inhibited the release of total IgE into serum

Sensitization and challenge with OVA promoted a huge increase ($p < 0.001$) by about 85 folds in the concentration of IgE in the blood plasma of the vehicle animal group, when compared to animals in the sham group (9.85 ± 1.10 pg/mL). Treatment with 1, 5 and 30 mg/kg of HEEs reduced levels of IgE, compared to the vehicle group, attaining inhibitory effects of 46.6%, 58.0% and 59.4% ($p < 0.05$), respectively. The animals treated with Dexa (0.5 mg/kg) also attenuated ($p < 0.05$) the elevated plasma concentration of IgE by 42.2%, compared to the vehicle group (Fig. 5).

3.4. HEEs inhibited the release of IL-4, IL-5 and IL-13 in the BALF

The effects of HEEs treatments on the concentrations of IL-4, IL-5 and IL-13 in BALF of mice with lung inflammation are shown in Fig. 6A–C. Sensitization and challenge with OVA promoted an increase ($p < 0.001$) of IL-4 (~14 folds), IL-5 (~19 folds) and IL-13 (~11 folds) in the BALF of the animal vehicle group, when compared the sham group (0.37 ± 0.28 pg/mL, 0.23 ± 0.10 pg/mL and 2.63 ± 0.60 pg/mL, respectively).

Treatment with HEEs (1, 5 and 30 mg/kg) reduced the concentrations of IL-4 and IL-13, attaining a maximal inhibitory effect of 39.7%

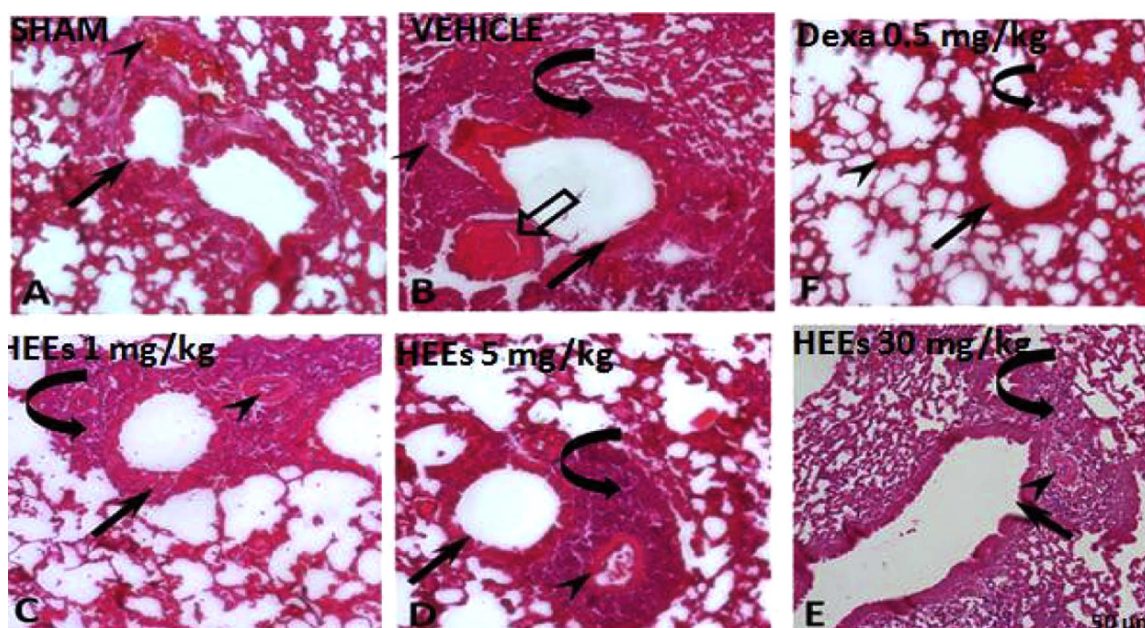


Fig. 4. Photomicrographs show morphology of lung tissues of mice subjected to allergic reaction induced by ovalbumin (OVA). (A) Control animals (sham) showing absence of cellular infiltration in the peribronchial region (arrow) and perivascular (arrowhead). (B) sensitized and challenged with OVA animal and treated only with 2% Tween 20 (vehicle) showing intense cellular infiltrate (curved arrow) in the peribronchial region (arrow) and perivascular (arrowhead). Also, there is large deposition accumulation, presumably from mucus in the bronchial lumen (open arrow). (C–E) Animals treated with HEEs, challenged and sensitized with OVA showed a reduced cell infiltrate (curved arrow) in the peribronchial region (arrow) and perivascular (arrowhead). (F) Animals treated with dexamethasone (0.5 mg/kg), challenged and sensitized with OVA showed few infiltrated leukocytes (curved arrow) in the peribronchial region (arrow) and perivascular (arrowhead). Hematoxylin-eosin stain; Bar=50 μ m.

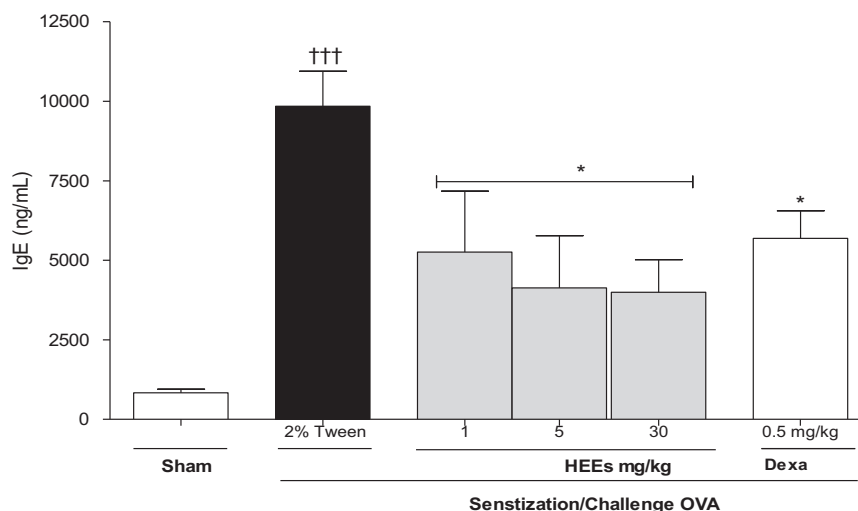


Fig. 5. Effect of oral administration of vehicle (2% Tween 20), hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs, 1, 5 and 30 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on the concentrations of IgE present in the bronchoalveolar lavage fluid (BALF) of mice sensitized (1st and 10th) with ovalbumin (OVA) grade V and aluminium hydroxide (0.1 mg/mL and 10 mg/mL in 0.9% saline ip., 0.2 mL/animal) and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulization, 20 min daily). The sham group was treated with the vehicle (2% Tween 20, 0.1 mL/10 g, p.o.) and sensitized and challenged with 0.9% saline. The results were expressed as Mean $\pm$ SEM for 6 animals. One-way ANOVA, followed by Student-Newman-Keuls. ††† $p < 0.001$ vs. Sham; * $p < 0.05$ vs. vehicle.

($p < 0.001$) and 82.5% ($p < 0.001$), respectively, at the dose of 1 mg/kg, and IL-5, with a maximum inhibition of 96.1% ($p < 0.001$) at the dose of 30 mg/kg, when compared to the vehicle group. Dexa (0.5 mg/kg) reduced the IL-4, IL-5, and IL-13 by 35.8% ($p < 0.01$), 93.0% ($p < 0.001$), and 98.0% ($p < 0.001$), respectively.

4. Discussion

In order to support the popular use of the leaves of *E. scaber* in the treatment of allergic inflammation, the allergic asthma murine model induced by OVA was chosen, because of its resemblance with the observed cardinal hypersensitivity hallmarks in the human acute allergic asthma (Kumar et al., 2008). In the same way, as observed in human allergic asthma, the OVA-induced lung inflammation in mice results in an enhanced T_H2 response, which develops towards a marked increase of infiltrated leukocytes in the lung tissue, particularly eosinophils and neutrophils. Polymorphonuclear cells recruitment is accompanied by high levels of anti-OVA IgE, mucus secretion and production of T_H2 -like cytokines, especially IL-4, IL-5 and IL-13 (Kumar et al., 2008).

In this study, we employed a 75% hydroethanolic solution as the extracting solvent. This type of preparation, along to water, is used by the local population for the traditional medicinal use of *E. scaber*. The hydroethanolic extraction was chosen because, even being its polarity close to that of water, it allows for better conservation of the material being extracted and is more efficient for acquisition of active pharmacological constituents (Tomsone et al., 2012). Furthermore, hydroethanolic solutions in the preparation of plants extracts is widespread used in Brazil and constitutes one of the different forms recognized in the Brazilian Pharmacopeia (Brasil, 2011).

The doses of the HEEs were chosen based on asthma murine pilot experiments of our lab, and from previous literature reports on inflammation (Tanus-Rangel et al., 2010).

The presence of polymorphonuclear cells in large quantities in the BALF (Just et al., 2002) and in the lung tissues of patients with asthma (Laviolette et al., 2013), is a characteristic parameter of the outcome of the disease. In this sense, in our study, the treatment with HEEs was able to prevent the migration of both neutrophils and eosinophils in the BALF (Fig. 2). Histopathological analyses of the lung also demonstrated that there were reductions in edema (Fig. 4 and Table 1). Our data are in accordance with others who demonstrated that polar extracts of *E. scaber* leaves also interfered in the leukocyte trafficking

and with the prevention of edema on other inflammatory models, such as in the carrageenan-induced pleurisy (Tanus-Rangel et al., 2010), and in the sheep red blood cells (SRBC)-induced delayed type hypersensitivity (Pinto et al., 2007). In our results, we cannot rule out for the possible inhibition, observed by others (da Silva et al., 2016; Rosa et al., 2016), of LTB_4 (a potent chemoattractant for neutrophils) and NO (a modulator of leukocyte recruitment, and a potent vasodilator), in the reduction of vasodilation, and in the cell migration in the lung and BALF of mice treated with HEEs.

In OVA-challenge, airways inflammation, including the recruitment of polymorphonuclear cells, is closely related to the upregulation of T_H2 -like cytokines (Kumar et al., 2008). So, in asthmatic mouse, a primary secretion of IL-4 by basophils or by naïve $CD4^+$ T lymphocytes primed by dendritic cells, induces the differentiation of T helper lymphocytes toward T_H2 cells, which in turn secrete more IL-4 that will rise the T_H2 -differentiation (Khodoun et al., 2004; Liu et al., 1998), showing its crucial role in *in vivo* allergic asthma. Furthermore, IL-4 is also required for the primary synthesis of IgE by B lymphocytes (Finkelman et al., 1991). In fact, it has been reported that anti-IL-4 or anti-IL-4R α (receptor) blocks IgE synthesis in OVA-sensitized mice, as well as that there is not asthma-associated IgE production in IL-4 $-/-$ BALB/c mice (Grunewald et al., 1998).

In the same way, the role of IL-5 in allergic inflammation is similarly important, since many studies have highlighted an association between IL-5 and asthma severity: IL-5 induces eosinophilia both systemically (from bone-marrow) and locally (infiltration to lungs) in mice sensitized/challenged by OVA (Hogan et al., 2000). Clinical studies with anti-IL-5 biological drugs (mepolizumab) demonstrated effectiveness in subgroups of patients with asthma, suggesting that blockade of this cytokine contributes to the restriction of the allergic reactions caused by eosinophilia (Pavord et al., 2012). Also, IL-13 is highlighted as another central asthmatic cytokine, since it is a potent inducer of eotaxin expression by airway epithelial cells and mucus secretion by goblet cells in the airways, among others effects (Gour and Wills-Karp, 2015).

Our data showed that treatment with HEEs can alleviate type I hypersensitivity induced by OVA *in vivo*, since it downregulates IL-4, IL-5 and IL-13 cytokines in the BALF (Fig. 6), reduces the release of IgE in blood plasma (Fig. 5), and relieves lung edema (Table 1). These data are also in accordance with other authors who have shown that the aqueous extract of *E. scaber* leaves reduced T cell-dependent antibody production in a mice model of SRBC-induced delayed type hypersensi-

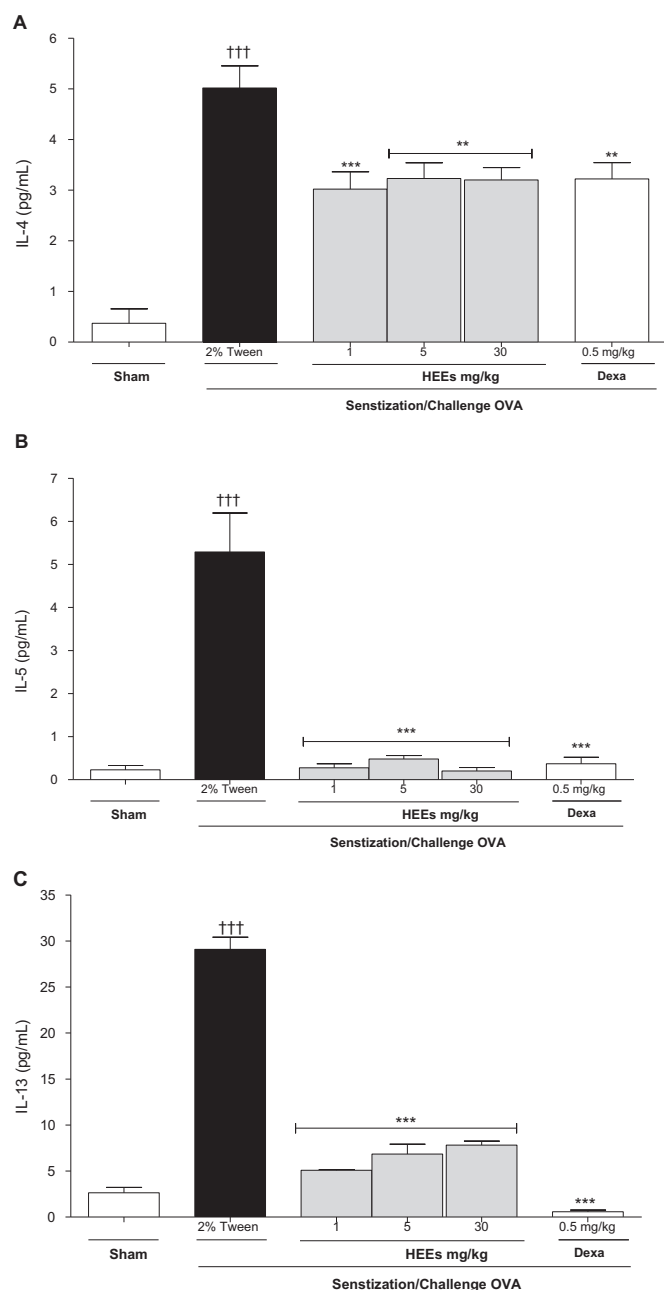


Fig. 6. Effect of oral administration of vehicle (2% Tween 20), hydroethanolic extract of the leaves of *Echinodorus scaber* (HEEs, 1, 5 and 30 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on the concentrations of IL-4 (A), IL-5 (B) and IL-13 (C) present in the bronchoalveolar lavage fluid (BALF) of mice sensitized (1st and 10th) with ovalbumin (OVA) grade V and aluminium hydroxide (0.1 mg/mL and 10 mg/mL in 0.9% saline ip., 0.2 mL/animal) and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulization, 20 min daily). The sham group was treated with the vehicle (2% Tween 20, 0.1 mL/10 g, p.o.) and sensitized and challenged with 0.9% saline. The results were expressed as Mean $\pm$ SEM for 6 animals. One-way ANOVA, followed by Student-Newman-Keuls. $^{+++} p < 0.001$ vs. Sham; $^{*} p < 0.05$ vs. vehicle.

tivity (Pinto et al., 2007). Based on these findings, it is possible that the HEEs might be acting directly or indirectly on B cells by reducing IgE synthesis, and/or on T cells, therefore by decreasing the levels of T_H2 -like cytokines responsible for IgE synthesis and airway eosinophilia. Furthermore, the possibility that the HEEs could interfere with other pathways involved in the inflammatory process, such as down-regulation of Janus kinase (JAK)/STAT pathways, or regulation of transcriptional factors, such as GATA-3, NF- κ B, NF-AT, and AP-1, all associated with the differentiation towards the T_H2 response (Mowen and

Glimcher, 2004), cannot be discarded. Further studies will be necessary to elucidate these points.

Preliminary phytochemical analysis revealed the presence of phenolic compounds, flavonoids and alkaloids. Unfortunately, with the authentic standards at our disposal we can only confirm, through HPLC analysis, the presence of some of these compounds, including gallic acid, rutin, and vitexin as components of the HEEs (Fig. 2). Other peaks in the HPLC chromatogram reveals the significant presence of some unidentified compounds that could have also an important contribution on the HEEs anti-asthmatic effect. Future studies should take into account for this possibility and clearly identify and isolate these compounds.

As for as the phenolic compounds identified in our work, previous studies showed that extracts containing gallic acid (Dorsch et al., 1992; Kim et al., 2006), rutin (Jung et al., 2007) or vitexin (Tong et al., 2013) are effective for asthmatic relief. Also, rutin and gallic acid has been shown to suppress thymic stromal lymphopoietin production in mast cells (Moon and Kim, 2012), in addition to attenuate production of T_H2 cytokines and its associated allergic effects, in both *in vitro* and *in vivo* models (Ahad et al., 2015; Moon and Kim, 2012; Nafees et al., 2015; Shen et al., 2012; Vo et al., 2012). We also recently report the potent anti-inflammatory action of vitexin and its inhibitory effects on inflammatory mediators, although using a murine model of acute inflammation (Rosa et al., 2016).

Taking into account that those compounds have separated anti-asthmatic effects, it could be believed that they could act jointly to bring about its anti-asthmatic effect, along with others unidentified compounds present in the HEEs (da Silva et al., 2016; Silva et al., 2012).

5. Conclusion

Based on the results, we may conclude that the anti-asthmatic activity displayed by the HEEs depends on the decrease of the cell migration, which in turn may reduce T_H2 -cytokines and IgE. This effect may probably be due to the joint action of vitexin, rutin, and gallic acid with other components present in the HEEs.

Conflict of interest

The authors declare that there are no conflicts of interest.

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