Biomedicine & Pharmacotherapy 97 (2018) 143–151

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Vitexin inhibits inflammation in murine ovalbumin-induced allergic asthma

Cláudio Luís Venturini[a], Antonio Macho[a], Karuppusamy Arunachalam[a], Danielle Ayr Tavares de Almeida[a], Suellen Iara Guirra Rosa[a], Eduarda Pavan[a], Sikiru Olaitan Balogun[a,c], Amílcar Sabino Damazo[b], Domingos Tabajara de Oliveira Martins[a,*]

[a] Área de Farmacologia, Departamento de Ciências Básicas em Saúde, Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil
[b] Área de Histologia e Biologia Celular, Departamento de Ciências Básicas em Saúde, Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil
[c] Curso de Farmácia, Faculdade Noroeste do Mato Grosso, Associação Juinense de Ensino Superior (AJES), Juína, Mato Grosso, Brazil

ARTICLE INFO

Keywords:
Vitexin
Allergic asthma
Ovalbumin
Anti-inflammatory

ABSTRACT

Vitexin is an important component of various medicinal plants frequently used to treat asthma, such as *Crataegus* spp., *Vitex* spp., *Passiflora* spp., and *Echinodorus* spp. However, there is no information about the vitexin potential as anti-asthmatic. The aim of the present work was to evaluate the anti-hypersensitive activity of vitexin in a murine ovalbumin (OVA)-induced allergic asthma model. Mice were sensitized to OVA by i.p. injection on days 1st and 10th, followed by a daily challenge with OVA using a nebulizer, from days 19th to 24th. Vitexin or dexamethasone were orally administered 1 h before each OVA challenge. Vitexin attenuates migration induced by OVA-hypersensitivity of eosinophil, neutrophil, and mononuclear cells in bronchoalveolar lavage fluid (BALF). Histological analysis of the lungs shows that vitexin suppressed leukocyte infiltration, mucus production and pulmonary edema. Increases in Th2 cytokines in BALF in OVA-induced asthma is also attenuated by vitexin, as well as plasma levels of IgE. Overall, these results suggest that vitexin can suppress OVA-induced allergic inflammation in mice and provide a strong rationale for further developing vitexin as a candidate treatment for allergic hypersensitivity. These data corroborate the popular use of vitexin-rich plants for asthma treatment.

1. Introduction

Asthma can be triggered as a result of exposure to allergens, viruses, air pollutants, oxidants, certain drugs, chemicals, changes in temperature and pressure of the environment, emotional disturbances (panic attacks), hyperventilation and hypocapnia (laughter, crying, screaming), exercise, gastroesophageal reflux, food additives and endocrine factors (menstrual cycle, pregnancy, thyroid disease) [1].

The process of developing asthma encompasses a complex network of inflammatory mediators involved in the recruitment and activation of a variety of cells of the immune system (innate and adaptive), as well as the trafficking of inflammatory cells to the lungs. Morphological changes in the epithelium (goblet cell metaplasia, hyperplasia, and increased mucin stores), submucosa (myofibroblast hyperplasia, subepithelial fibrosis and increased glandular cell volume), smooth muscle cells (hypertrophy and hyperplasia) and an increase of respiratory blood vessels, referred to as airway remodeling, occur in individuals susceptible to exaggerated response to allergens [2].

Experimental evidence suggests that T cells are the main mediators of allergic chronic inflammatory response, particularly Th2 cells, where the primary signals that activate these cells in asthma involve cytokines release by the airway epithelial cells, which in turn promote the release of other Th2-specific cytokines that expand the inflammatory response [1,3]. Thus, the cytokines IL-4, IL-5, IL-9 and IL-13, mainly of Th2 origin, have an important role in the pathogenesis of asthma, being responsible for the most striking features of this disease [1,2].

The actual basic strategy for the treatment of bronchial asthma is to combine inhaled drugs that promote the rapid symptomatic relief of asthma exacerbation, reducing bronchoconstriction (using long-acting β2-adrenergic agonists, LABAs), and airway inflammation (via inhaled corticosteroid anti-inflammatory drugs, ICs). Unfortunately, 5–10% of patients do not respond to this therapy combination, and others have low adherence or undesirable side effects upon continued use. Modern therapies include the use of anti-leukotrienes (for mild persistent asthma), alone or associated with ICs or LABAs, monoclonal anti-IgE antibodies (for moderate to severe asthma), and anti-IL-5 (for severe asthma and eosinophilic phenotype) [1,2,4].

Nevertheless, given the nature of the asthma disease and the poor implementation and effectiveness of current preventive and curative treatments, asthmatic patients are increasingly seeking after

* Corresponding author at: Av. Fernando Correa da Costa, no 2367, CEP: 78060-900, Cuiabá, Mato Grosso, Brazil.
E-mail address: taba@terra.com.br (D.T.d.O. Martins).

http://dx.doi.org/10.1016/j.biopha.2017.10.073
Received 2 July 2017; Received in revised form 13 October 2017; Accepted 16 October 2017

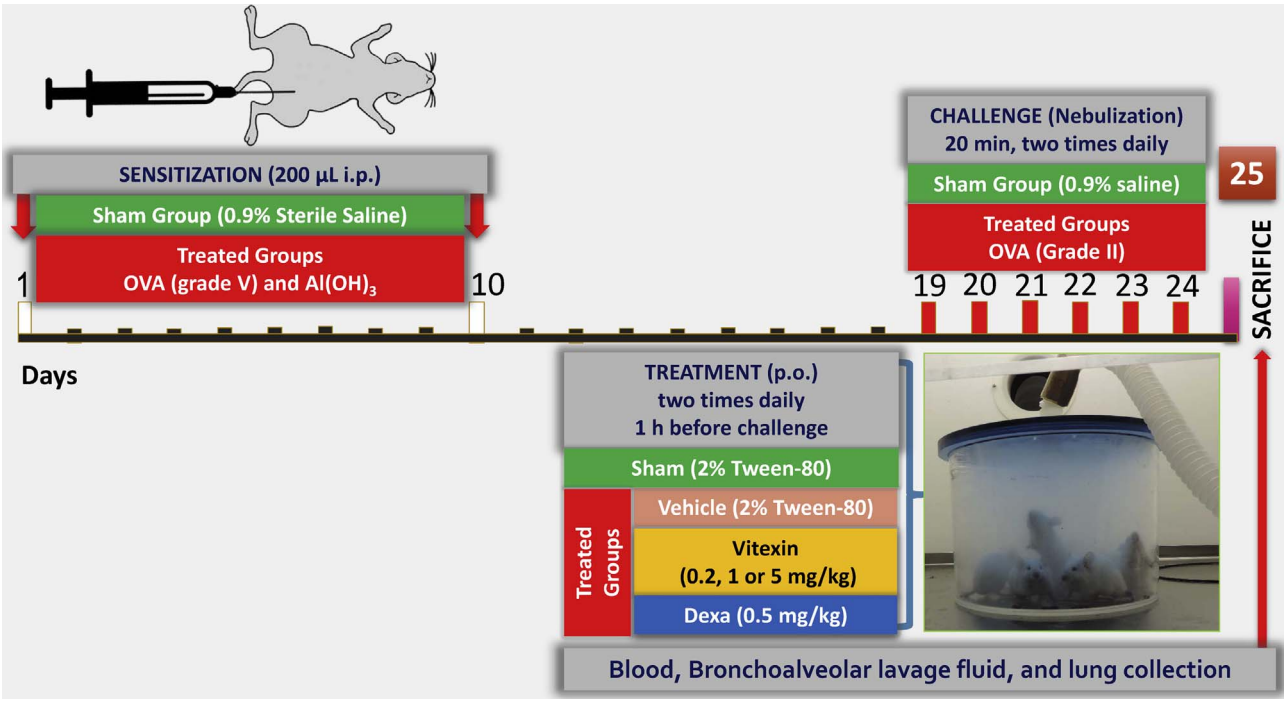

Fig. 1. Schematic representation of the protocol for ovalbumin-induced airway inflammation in mice, and treatment with vitexin and dexamethasone (Dexa).

complementary and alternative medicine (CAM). The patients' demand for these practices are on the increase, among which is phytotherapy [4]. In fact, the plant kingdom, which produces a wide and diverse array of secondary metabolites that play important roles in adapting plants to their environments, represents an important source of pharmacologically active substances. Among these, flavonoids have been reported to have antiallergic, anti-inflammatory and antioxidant activities [5,6].

In Brazil, the infusion, decoction or maceration, in water or alcohol, of *Echinodorus scaber* Rataj. (Alismataceae) leaves, synonymous with *Echinodorus macrophyllus* (Kunth.) Micheli, is widely used by traditional populations to treat allergies, respiratory diseases, inflammation, rheumatism and kidney disorders [7,8]. Also, in the 1st edition of the Phytotherapic Form of the 5th edition of the Brazilian Pharmacopoeia, infusion of leaves (1 g in 150 mL of water) of *E. scaber* is indicated as a light anti-inflammatory and diuretic [9].

Among other substances, our group has previously isolated isovitexin (9.07 µg/mg) from the hydroethanolic extract of *Echinodorus scaber*, [10], and identified vitexin (15.04 µg/mg) [11], both in a relatively high yield. Vitexin is a C-glycosylated flavone first isolated by Perkin [12] from *Vitex lucens* Kirk (Verbenaceae). The structure of vitexin was first described by Briggs and Cambie [13], and is also known as apigenin-8-C-β-D-glycopyranoside or 5,7,4-trihydroxyflavone-8-glucoside. Vitexin is widely distributed in the plant kingdom, especially in *Crataegus* spp., *Vitex* spp., and *Passiflora* spp. [14], many of which, like *E. scaber*, are commonly used around the world to treat asthma and other conditions [15–19].

Vitexin has received great attention from the scientific community for presenting a wide variety of pharmacological activities, including anti-inflammatory [20–22], antioxidant [23,24], antispasmodic [25,26], and antinociceptive [27–29], as well as antitumoral, antihypertensive, antidepressant, neuroprotective and cardioprotective (extensively reviewed by He et al. [14]). Also, vitexin is one of the active components of various herbal medicines used for the treatment of gastrointestinal, cardiac, menopausal and premenstrual, anxiolytic and hypnotic symptoms [14].

Nevertheless, although there are a few studies demonstrating the anti-inflammatory activity and mechanism of action of vitexin using *in vivo* and *in vitro* experimental models, none of these investigated its potential for allergic asthma. The present study is necessary to strengthen the phytotherapeutic use of vitexin-containing plants in the treatment of asthma. Thus, the aim of the present work was to investigate the possible anti-hypersensitive allergic activity of vitexin in an ovalbumin-induced model of asthma in mice.

2. Materials and methods

2.1. Drugs and reagents

Aluminum hydroxide [$Al(OH)_3$], dexamethasone acetate (Dexa), hematoxylin and eosin (H & E), periodic acid-Schiff (PAS), ovalbumin (OVA, grade II and grade V) and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-4, IL-5, IL-13 and IgE quantitative ELISA kits were purchased from eBioscience (San Diego, CA, USA). Vitexin (PubChem CID: 5280441), was procured from Fluka (St. Louis, Missouri, USA). For preparative solutions, vitexin was dissolved in 2% Tween-80 in water.

2.2. Animals

Male Swiss-Webster mice, 8–12 weeks old, 18–22 g, from the Central Animal House of the Federal University of Mato Grosso (UFMT), were used. The animals were maintained in propylene cages (30 × 20 × 13 cm) at 25 ± 1 °C with a 12 h light/dark cycle and free access to standard pellet chow and water. The diet regime for the animals consisted of feed restriction from the period of 9:30 to 10:30 h and 16:00 to 17:00 h. In all experiments, six animals were used for each experimental treatment. 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 process no. 23108.028454/12-2.

2.3. Induction of allergic asthma with ovalbumin (OVA)

For the induction of immediate hypersensitivity in mouse airways,

the protocol described by Nader et al. [30] was employed, with minor modifications (Fig. 1). On days 1 and 10, mice were sensitized by intraperitoneal injection (200 µL) with a suspension containing OVA (grade V, 100 µg/mL) and $Al(OH)_3$ (10 mg/mL) in sterile saline solution (0.9%). On days 19th–24th, after the first sensitization, animals were challenged two-times (2×) daily with OVA (grade II, 3% in 0.9% saline) for 20 min in a closed acrylic chamber (25 × 13 × 13 cm) connected to a nebulizer (NEU0003 Soniclear; SP, Brazil), under a continuous flow of aerosol. Animals (n = 6/group) which where sensitized and challenged with OVA were treated by gavage (p.o.), 2× daily, 1 h before each challenge, with either vehicle (2% Tween-80), vitexin (0.2, 1 or 5 mg/kg), or dexamethasone (0.5 mg/kg) as the standard drug. The animals in the sham group (n = 6) were sensitized and challenged with only 0.9% saline and then treated with the vehicle (2% Tween-80) at the same time intervals. At the end, on the 25th day, the followings were collected: bronchoalveolar lavage fluid (BALF) for leukocyte counts and cytokine determinations, blood (plasma) for total immunoglobulin E (IgE) dosage, and the lungs for histopathological analysis.

2.4. Determination of IgE

Twenty-four hours after the last challenge (on the 25th day after the 1st sensitization), and after anesthesia (ketamine/xylazine, 40/50 mg/kg i.p.), the inferior vena cava of each animal was punctured, and the blood collected was centrifuged until cell sedimentation at 4 °C. Total IgE levels in plasma were determined by enzyme-linked immuno-sorbent assay (ELISA), following the manufacturer's instructions.

2.5. Collection of bronchoalveolar lavage fluid (BALF) and leukocyte counts

Immediately after the blood collection, the animals were euthanized with overdose of the anesthetic agent (ketamine 200 mg/kg and xylazine 50 mg/kg i.p.). Following an adaptation of the method described by Nader et al. [30], animals were tracheostomized and cannulated (Children urethral catheter no. 6) to collect BALF, obtained with two consecutive washes (1 mL, each) with phosphate buffered saline (PBS, pH 7.4) plus EDTA 1 M. Both collected lavages were pooled and unified as a single sample. From the volume recovered, 0.01 mL was added to 0.39 mL of Turk solution (0.5% methylene blue in 30% acetic acid) for total leukocyte count in a Neubauer chamber. The results were expressed as the number of total leukocytes (× 10^6/mL BALF) for each group. Slides for differential counts were prepared with 0.02 mL aliquots of the cell suspension and stained with InstantProv® (NewProv, São Paulo, Brazil). The absolute number of each cell type (neutrophils, eosinophils, and mononuclear cells, including macrophages and lymphocytes), was determined with the aid of an optical microscope (Nikon, Chiyoda, Tokyo, Japan) using a 1000× magnification and under immersion oil, from the counting of at least 100 cells per slide, so that the percentage of each cell type could be inferred.

The rest of the BALF was centrifuged to clear it of cells, and was then aliquoted and kept at −80 °C until used for cytokine determination.

2.6. Cytokines quantification

Th2 cells secreting IL-4, IL-5 and IL-13 in BALF were determined using ELISA kits, according to the manufacturer's instructions.

2.7. Histopathological analysis

After the collection of BALF, mice lungs were perfused with PBS buffer solution (pH 7.4) containing 1 M EDTA at 4 °C, removed and fixed in 4% paraformaldehyde in phosphate buffer for 18 h at 4 °C. After fixation, they were washed in PBS, dehydrated in increasing solutions of ethanol, diaphonized with xylol, impregnated and embedded in paraffin blocks. Afterwards, the material was cut into 3 µm sections in a microtome (Hirax M60, Carl Zeiss, Germany), packed in histological glass slides and stained with hematoxylin and eosin (H & E) for analysis of leukocyte infiltrate and edema; 0.25% toluidine blue and 0.25% sodium borate, for analysis of mast cell degranulation; and periodic acid–Schiff (PAS) for mucus staining and evaluation. Morphometric histological analyses were performed under light microscopy (Axio Scope A1, Carl Zeiss, Germany) using AxioVision Software v4.8.1 (Carl Zeiss, Germany). Morphological alterations were evaluated by assigning scores, according to Duan et al. [31], with adaptations: the H & E staining method was performed to evaluate edema, perivascular and peribronchiolar infiltrate using the following scores: 0 – no inflammatory cells, 1 – few cells, 2 – a ring of cells one depth cell layer, 3 – a ring of cells two to four depth cell layers, 4 – a ring of cells with more than four depth cell layers. For the analysis of mucus, PAS staining method was used, applying the scores: 0 – without evident mucus; 1 – discontinuous presence of mucus on the epithelial surface; 2 – presence of a thin and continuous strip of mucus; 3 – presence of a thick layer of mucus; 4 – total obstruction of the bronchiolar lumen. For analysis of mast cell degranulation, toluidine blue/sodium borate staining was used, and the following scores: 0–inactivated; 1 – 25% activated; 2 – 50% activated; 3 – 75% activated; 4 – 100% activated.

2.8. Data analysis

Parametric values were expressed as mean ± standard error of the mean ($\overline{X}$ ± SEM), and the non-parametric as median (md) and interquartile intervals (Q_1;Q_3). Comparisons between means were analyzed by one-way analysis of variance (ANOVA), and when significant, this was followed by the Student–Newman–Keuls test for multiple comparisons. Non-parametric data were analyzed by Kruskal–Wallis test and the statistical significance among groups determined by Dunn's test. Values of $p < 0.05$ or $p > 0.95$ were considered significant for null (different mean values, H_0: $\overline{X}1 - \overline{X}2 \neq 0$) and alternative hypothesis (equal mean values, Ha: $\overline{X}1 - \overline{X}2 = 0$), respectively. All analyses were performed using GraphPad Prism® (v7.00 for Windows, San Diego, CA, USA).

3. Results and discussion

The anti-asthmatic activity of vitexin was investigated in a model of inflammation (acute allergic asthma) induced by OVA sensitization in mice. In this model, animals present molecular, cellular and histologic hypersensitivity hallmarks similar to that seen in acute human allergic asthma, both being characterized by pathophysiological changes in the lower airways, mainly triggered by the activity of leukocyte infiltrates (particularly eosinophils and neutrophils), as well as by mucus hypersecretion, and by high production of allergen-specific IgE and Th2-type cytokines [32].

The doses of vitexin (0.2, 1 and 5 mg/kg p.o.) employed in this study were based on a previous anti-inflammatory study by Rosa et al. [22] who reported good improvement in a mouse model of lipopolysaccharide (LPS)-induced acute peritonitis. The selected doses are relatively low, considering the work of Choo et al. [33] showing that doses up to 2 g/kg p.o. of this flavone did not cause gross signs and symptoms of acute toxicity in mice.

3.1. Vitexin reduced airway inflammation in bronchoalveolar lavage fluid (BALF) and lung tissues

The number of white blood cells, eosinophils, neutrophils, and mononuclear cells present in the BALF of the sham group were 6.67 ± 0.42 × 10^6, 1.31 ± 0.18 × 10^6, 0.87 ± 0.08 × 10^6 and 4.49 ± 0.22 × 10^6 cells, respectively. Sensitization and challenge

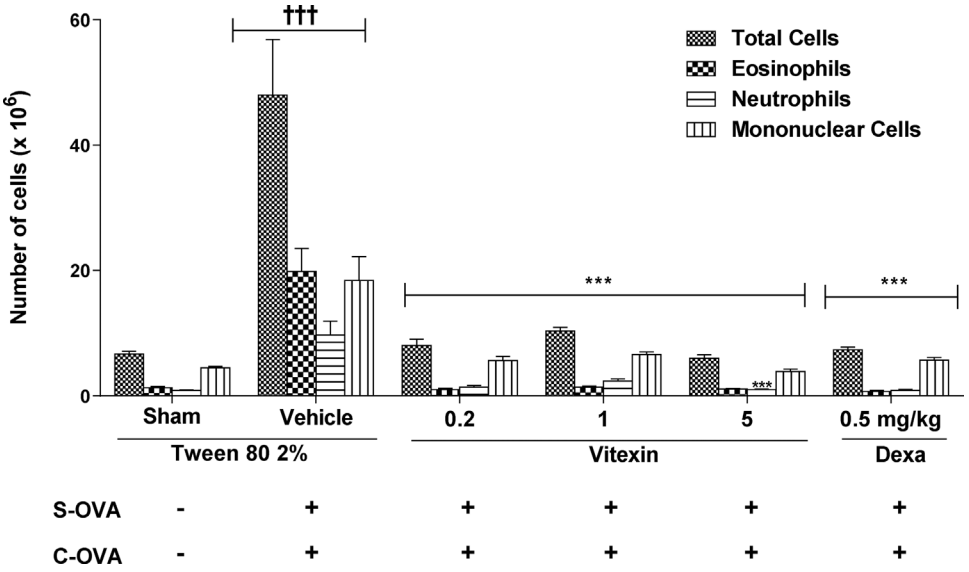

Fig. 2. Vitexin inhibits inflammatory cell influx on bronchoalveolar fluid. Effect of oral treatments (2 times daily) with the vehicle (2% Tween-80), vitexin (0.2, 1 or 5 mg/kg), or dexamethasone (Dexa, 0.5 mg/kg) on total leukocytes, eosinophils, neutrophils, and mononuclear cells present in the bronchoalveolar lavage fluid (BALF). Mice were previously sensitized (on 1st and 10th days) with ovalbumin (OVA) grade V (0.1 mg/mL) and aluminum hydroxide (10 mg/mL) in 0.9% saline i.p., 0.2 mL/animal, and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulized, 20 min daily). The sham group was intraperitoneally injected and challenged with sterile 0.9% saline instead of OVA, and received orally 2% Tween-80 as treatment instead of vitexin. The results were expressed as Mean ± SEM of 6 animals per group. One way ANOVA, followed by Student–Newman–Keuls. $^{†††}p < 0.001$ vs. Sham; $^{***}p < 0.001$ vs. vehicle. S-OVA: Sensitization with OVA, C-OVA: Challenge with OVA.

with OVA promoted an increase ($p < 0.001$) of about 7×, 15×, 11× and 4× for each cell type, respectively (Fig. 2), indicating a satisfactory level of sensitization to the allergen.

Treatment with vitexin (0.2, 1 or 5 mg/kg) reduced leukocyte infiltration at all doses ($p < 0.001$) (Fig. 2), reaching its highest apparent effect at 5 mg/kg. Vitexin-induced reductions, relative to vehicle for each cell type, were as follows: 87% for total leukocytes, 95% for eosinophils, 89% for neutrophils and 79% for mononuclear cells respectively. Results for the standard, dexamethasone (0.5 mg/kg), were: 85% for total leukocytes, 96% for eosinophils, 91% for neutrophils and 69% for the mononuclear cells respectively, relative to the vehicle group ($p < 0.001$). By comparing differences among vitexin treated groups, and between vitexin groups and dexamethasone, the effects were considered of equal statistical significance (always $p > 0.95$) in all cases.

These results are in agreement with other authors who have demonstrated that vitexin is effective in the prevention of leukocyte migration, independent of the phlogistic agent or of the site of inflammation [22,34,35].

In patients with severe asthma, leukocyte migration, and especially eosinophils, correlates with the production of peroxidases, cysteinyl leukotrienes and reactive oxygen species by these cells, which leads to bronchoconstriction and mucus secretion, with bronchial hyperresponsiveness and severe airway obstruction [36]. In this way, histopathological analyses of pulmonary tissue sections (Fig. 3 and Table 1) demonstrated that the sensitization and challenge with OVA promoted a significant ($p < 0.001$) increase in edema (3.00 [3.00;4.00]), perivascular (4.00 [4.00;4.00]) and in peribronchial (4.00 [3.00;4.00]) infiltrates, when analyzed by H & E staining. Also, mucus determination (4.00 [3.00;4.00]) by PAS staining, and degranulation of mast cells (4.00 [3.00;4.00]) by toluidine blue staining, were all significantly ($p < 0.001$) increased when compared to the sham group (0.00 [0.00;0.00]). In the treatments with vitexin (0.2, 1 and 5 mg/kg), significant reductions were observed only at the highest dose (5 mg/kg) for edema (1.00 [0.75;2.00], $p < 0.05$), perivascular and peribronchial infiltrates (1.00 [1.00;1.25], $p < 0.01$), mucus production (1.00 [1.00;1.25], $p < 0.05$) and, in a 50%, for mast cell degranulation (2.00 [1.75;2.00], $p < 0.01$), when compared to the vehicle. Dexamethasone (0.5 mg/kg), the standard drug used in all experiments, had similar values to the highest dose of vitexin, being more efficient in the reduction of mucus (0.00 [0.00;1.00], $p < 0.001$) but not statistically efficient in the prevention of edema (2.00 [2.00;2.00], not significant) (Table 1). Although inhibition of leukocyte migration was evident at all concentrations for both, vitexin and dexamethasone, relief of lung damage was unexpectedly more moderate, being only significant for the higher dose of vitexin, and partially for dexamethasone. Nevertheless, it should be highlighted that vitexin and dexamethasone were administered orally and not directly by inhalation, so their effect on lung tissues may be delayed. Therefore, it can be assumed that a large part of the lung damage induced by OVA could be explained in part by the rapid deleterious effect caused by resident pulmonary inflammatory cells, which are less accessible for orally administered drugs, rather than by the migrated leukocytes. Even so, our results suggest a good interrelationship between leukocyte migration in BALF and lung histopathological effects in both, vitexin and dexamethasone treatments.

3.2. Vitexin inhibits the release of IL-4, IL-5 and IL-13 in the BALF

An important point in the OVA-induced asthma model in mice is that the increase in transmigration of leukocytes into the airways, particularly eosinophils, is a process coordinated by specific chemokines [32], such as eotaxin, in combination with adhesion molecules such as VCAM-1 and VLA-4, and that this increase is strongly controlled by IL-4, IL-5, and IL-13 cytokines, released by Th2 lymphocytes [37]. In effect, it is widely reported that the IL-5 cytokine is responsible for the development of eosinophils in the bone marrow and their recruitment into the lung and interstitial mucosa, due to the production of chemokines. IL-4 potentiates this effect since it promotes the differentiation of T lymphocytes into Th2-type cells and the chemoattraction of leukocytes to the inflammatory focus. Also, IL-13, homologous to IL-4, has been reported to be the most potent inducer of eotaxin expression by airway epithelial cells on the respiratory tract, besides its participation in mucus production in the lung tissue [2].

It is important to note that new therapies for asthma, which are under development, are targeting Th2-cytokines, including IL-4 receptor α-chain antibody (e.g. Dupilumab®, Regeneron Pharmaceuticals), recombinant soluble IL-4 receptor antagonist (Altrakincept®, Aerovance Incorporation; Pitrakinra, Amgen), IL-13 blockade (Lebrikizumab®, Genentech) and antibody to the IL-5 receptor (Benralizumab®, AstraZeneca/MedImmune) [2,38–40].

In this context, we investigated whether the anti-allergic mechanism of vitexin involves the reduction of Th2-cytokines in the BALF of mice sensitized and challenged by OVA, since the reduction of the leukocyte infiltrate in the airways is strongly related to the regulation of Th2-cytokines release [32]. The concentrations of IL-4, IL-5 and IL-13 present in the BALF of the sham group were of 1.50 ± 0.57, 0.18 ± 0.10 and 2.63 ± 0.60 pg/mL, respectively. Sensitization and challenge with OVA promoted an increase ($p < 0.001$) by about 7×, 29× and 5× in

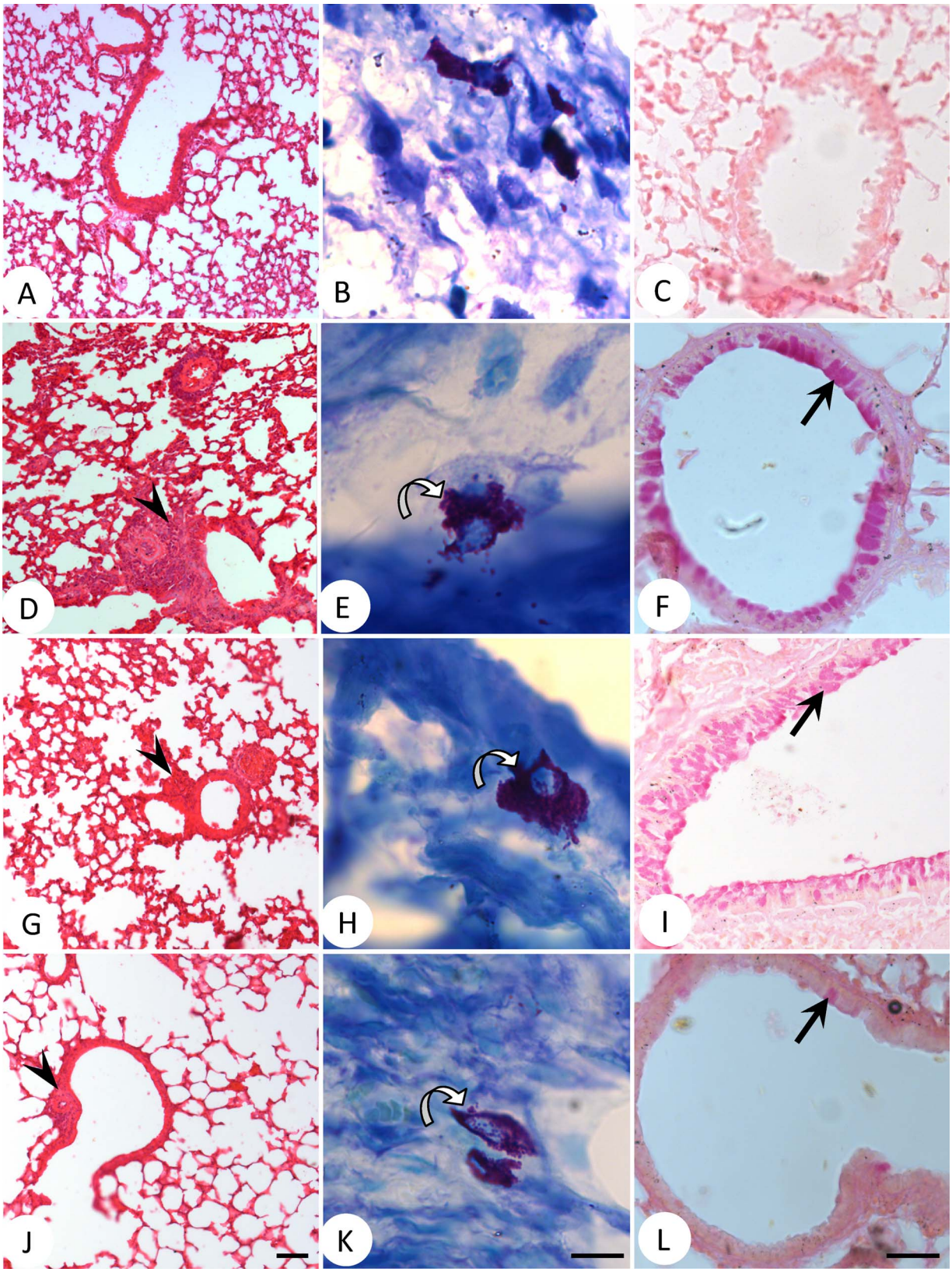

Fig. 3. Vitexin attenuates OVA-induced airway inflammation in bronchoalveolar lavage fluid (BALF) and lung tissues. Sham group (A–C) having interstitial lung tissue without trans-migrated leukocytes (A), intact mast cells (B), and absence of mucus production (C) by the respiratory epithelium. Vehicle group (D–F), OVA sensitized and challenged, presents intense cellular infiltration in the perivascular and peribronchial region (D, arrowhead), degranulated mast cells (E, curved arrow) and excessive production of mucus by the respiratory epithelium (F, arrow). The OVA-sensitized/challenged group, treated with vitexin (5 mg/kg) (G–I) presents reduced cellular infiltration (G, arrowhead), lesser degranulation of mast cells (H, curved arrow) and lower mucus production by the respiratory epithelial cells (I, arrow). The dexamethasone treated (0.5 mg/kg) control group (J–L) presents a significant reduction of the cellular infiltrate (J, arrowhead) and inhibition of the production of mucus by the respiratory epithelium (L, arrow). However, although it seemingly reduced mast cell degranulation, there is a presence of many extruded granules (K, curved arrow). (A, D, G, J) Coloration: H & E, Bar = 100 μm; (B, E, H, K) toluidine blue, Bar = 20 μm; (C, F, I, L) PAS, Bar = 50 μm.

Table 1
Histopathological analysis of lung tissues of mice subjected to asthma induced by ovalbumin (OVA) and treated orally (p.o.) with 0.9% saline (vehicle), vitexin (0.2, 1.0 or 5.0 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg).

Group (mg/kg p.o.)		Edema Hematoxylin and eosin staining	Perivascular infiltrate Hematoxylin and eosin staining	Peribronchial infiltrate Hematoxylin and eosin staining	Mast cells degranulation Toluidine blue staining	Mucus PAS staining
Sham		0.00 (0.00;0.00)	0.00 (0.00;0.00)	0.00 (0.00;0.00)	0.00 (0.00;0.00)	0.00 (0.00;0.00)
Vehicle		3.00 (2.75;3.00)†††	4.00 (4.00;4.00)†††	4.00 (3.00;4.00)†††	4.00 (3.00;4.00)†††	4.00 (3.00;4.00)†††
Vitexin	0.2	2.00 (1.00;2.25)	2.50 (2.00;3.00)	2.00 (2.00;2.00)	3.00 (2.75;3.00)	2.50 (2.00;3.00)
	1.0	1.50 (1.00;3.00)	2.50 (2.00;3.00)	1.50 (1.00;2.00)	2.00 (2.00;3.00)	3.00 (2.00;3.00)
	5.0	1.00 (0.75;2.00)*	1.00 (1.00;1.25)**	1.00 (1.00;1.25)**	2.00 (1.75;2.00)**	1.00 (1.00;1.25)*
Dexa	0.5	2.00 (2.00;2.00)	1.00 (1.00;1.25)**	1.00 (1.00;1.25)**	2.00 (2.00;2.25)*	0.00 (0.00;1.00)***

PAS: periodic acid-Schiff; Morphological alterations were evaluated by assigning scores: 0 – no inflammatory cells, 1 – few cells, 2 – a ring of cells one depth cell layer, 3 – a ring of cells two to four depth cell layers, 4 – a ring of cells with more than four depth cell layers. For the analysis of mucus, PAS staining method was used, applying the scores: 0 – without evident mucus; 1 – discontinuous presence of mucus on the epithelial surface; 2 – presence of a thin and continuous strip of mucus; 3 – presence of a thick layer of mucus; 4 – total obstruction of the bronchiolar lumen. For analysis of mast cell degranulation, toluidine blue/sodium borate staining was used, and the following scores: 0–inactivated; 1 – 25% activated; 2 – 50% activated; 3 – 75% activated; 4 – 100% activated. Values were expressed as median and interquartile range (Q_1;Q_3) of 6 animals per group. Analysis of variance (ANOVA) of Kruskal–Wallis, followed by Dunn's test: $^{\dagger\dagger\dagger}p < 0.001$ vs. sham (not sensitized group); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. vehicle (group sensitized and challenged with OVA).

the concentrations of IL-4, IL-5 and IL-13, respectively (Fig. 4A–C). Treatment with vitexin (0.2, 1 and 5 mg/kg) reduced the concentrations of the three cytokines at all doses ($p < 0.01$), with maximum apparent effect ($p < 0.001$) at 1 mg/kg for IL-4 (64%), 0.2 mg/kg for IL-5 (96%) and 1 mg/kg for IL-13 (65%), values comparable to those obtained with 0.5 mg/kg of dexamethasone (73% inhibition for IL-4, 96% for IL-5, and 65% for IL-13, relative to the vehicle group, $p < 0.001$). As in the case of leukocyte infiltration, apparent differences among vitexin treated groups, and between vitexin groups and dexamethasone were not significant ($p > 0.05$), and were even statistically equal ($p > 0.95$) in the case of the IL-5, so we can consider similar cytokine inhibition for the three doses of vitexin employed.

Vitexin promoted reductions in the release of the IL-4, IL-5 and IL-13 cytokines in BALF of mice sensitized and challenged with OVA, thereby negatively modulating the development of allergic inflammatory response in the lungs. These data could be considered to be in agreement with findings by Borghi et al. [27] and Rosa et al. [22] who demonstrated that vitexin reduces the production of proinflammatory cytokines (TNF, IL-1β, IL-6 and IL-33) in various acute *in vivo* inflammation models and in an LPS-induced *in vitro* model. We have previously shown that inhibition of these cytokines by vitexin seems to be due to a direct inhibition of p38, ERK1/2 and JNK signaling pathways in RAW-264.7 mouse cells [22]. According to the current understanding, an enhanced p38/JNK and depressed ERK pathways lead to a Th1-type deviated inflammation, whereas an elevated ERK and decreased p38/JNK pathways lead to a Th2-type deviated inflammation [41]. Therefore, it could be suggested that by inhibiting all the three pathways, vitexin attenuates both acute and immediate hypersensitive inflammations, by inhibiting both, Th1 and Th2 cell differentiation. In addition, these "inflammatory pathways" seem to be necessary for leukocyte migration [42,43] and, consequently, its inhibition might explain the reduced cellular infiltrate in the BALF with vitexin (Fig. 2).

An important literature finding is that vitexin seems to target and inhibit the hyperalgesic action of transient receptor potential vanilloid type 1 (TPRV1 or VR1) channel [27]. This calcium channel is actually known, not only for its hyperalgesic effect in neurons during inflammation [44] but also for its important immunomodulatory effect over early and late events of TCR-induction on $CD4^+$ T-lymphocytes [45,46]. Thus, different pathways could be targeted by vitexin to accomplish the inhibition of asthma-associated cytokines.

3.3. Vitexin inhibit the release of total IgE on blood plasma

An elevation of IgE in plasma of asthmatic patients is strongly related to the increase in Th2-cytokines and hence to the worsening in clinical features of the disease [47]. Particularly, the IL-4 cytokine is directly involved in the lymphocyte-B differentiation towards IgE synthesis. This emphasizes the role of IgE as a central mediator and therapeutic target in asthma, given that free allergen-specific IgE binds to its high-affinity receptor (FcεRI) present in mast cells, basophils and dendritic cells. New contacts, between those IgE-FcεRI complexes and the allergen, induce cellular activation, inflammation, and the production of mediators, including more Th2-type cytokines [2,48].

Taking this into account, the concentration of IgE present in the blood plasma was analyzed in the different groups of treated mice (Fig. 5). In the sham group, the IgE concentration was 47 ± 7 ng/mL, whereas sensitization and challenge with OVA promoted a huge 675-fold increase ($p < 0.001$). Treatment with vitexin (0.2, 1 and 5 mg/kg) reduced ($p < 0.001$) the plasma IgE concentration at all doses, being apparently more effective at the lower dose of 0.2 mg/kg (79%), whereas Dexa at 0.5 mg/kg (with 12,784 ± 3558 ng/mL, $p < 0.001$) was less effective, resulting in a reduction by 59%. Thus, vitexin clearly reduced IgE levels and seem to be more effective than dexamethasone at the standard dose used.

Recently, various authors [1,38] have claimed that, as steroids have multiple side effects when administered for long time, the advent of more specific inhibitors of the Th2-response will provide better benefits to patients with asthma, without any adverse effects. Between these treatments, neutralizing IgE monoclonal antibodies (anti-IgE) have been included as a treatment for moderate patients with severe symptoms and poorly controlled therapy, where the main effect is related to a decreased plasma IgE level that leads to a decrease in asthma exacerbations [4]. In this context, phase 4 clinical studies (NCT00546143) of the anti-IgE antibody Omalizumab® have demonstrated robust anti-asthmatic effects, reinforcing the role of IgE in allergic asthma [49]. Therefore, the great effect of vitexin in alleviating IgE levels, as well as IgE inducting cytokines, could justify its potential therapeutic use in the future.

3.4. Vitexin is an effective and potent anti-asthmatic flavonoid

Flavonoids are widely reported in the literature, *in vitro* and *in vivo*, due to their powerful anti-allergic, anti-inflammatory, immunomodulatory, and anti-oxidant activities; therefore, these may play an important role in the prevention and management of allergic asthma [5,6]. Nevertheless, it is noteworthy to note that when administered orally *in vivo*, a high dose of flavonoids is always required to accomplish a significant alleviation of asthma (generally, 10–20 mg/kg) [6,50,51]. These doses are generally reduced when administered ip., as is the case for fisetin (0.3 mg/kg) or apigenin (5–10 mg/kg) [52,53]. To the best of our knowledge, only luteolin has been reported to have a good response when administered p.o. at low doses of 1 mg/kg for curative purposes (administered concomitantly to the OVA challenge) and 0.1 mg/kg for

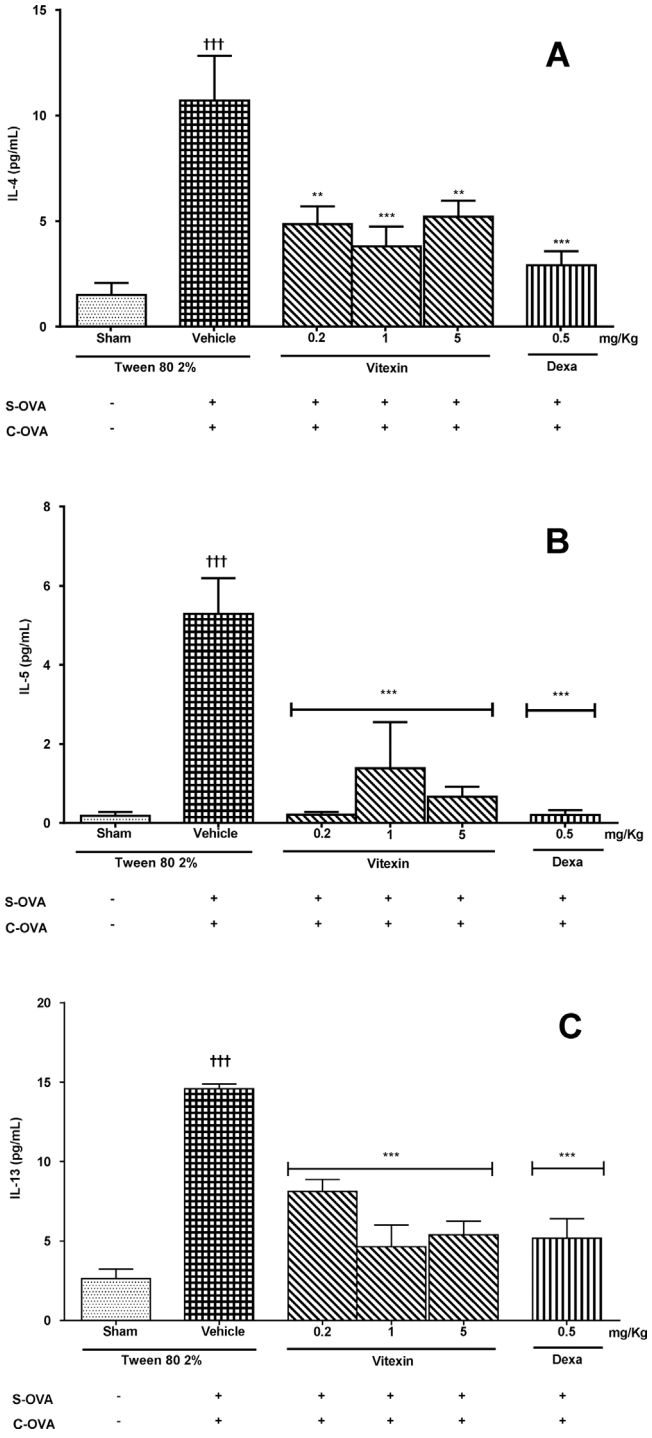

Fig. 4. Vitexin inhibits the secretion of pro-inflammatory Th2-specific cytokines on bronchoalveolar fluid. Effect of oral administration of vehicle (2% Tween-80), vitexin (0.2, 1 or 5 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on the concentrations of IL-4 (A), IL-5 (B) and IL-13 (C) present in the bronchoalveolar lavage fluid (BALF). Mice were previously sensitized (on 1st and 10th days) with ovalbumin (OVA) grade V (0.1 mg/mL) and aluminum hydroxide (10 mg/mL) in 0.9% saline i.p., 0.2 mL/animal, and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulized, 20 min daily). The sham group was intraperitoneally injected and challenged with sterile 0.9% instead of OVA, and received orally 2% Tween-80 as treatment instead of vitexin. The results were expressed as Mean ± SEM for 6 animals per group. One-way ANOVA, followed by Student–Newman–Keuls. $^{†††}p < 0.001$ vs. Sham; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle. S-OVA: Sensitization with ovalbumin, C-OVA: Challenge with ovalbumin.

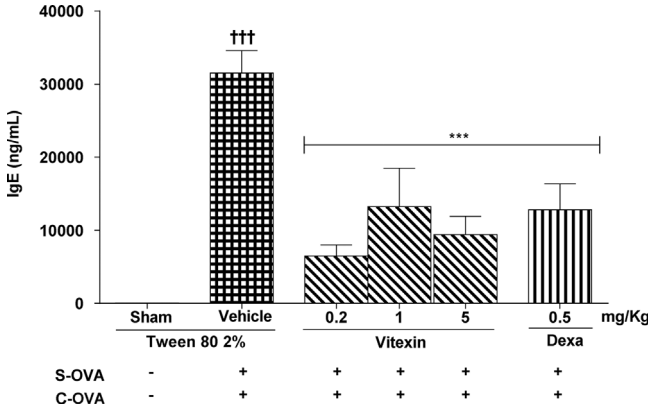

Fig. 5. Vitexin inhibits the secretion of OVA-induced IgE on blood plasma. Effect of oral administration of vehicle (2% Tween-80), vitexin (0.2, 1 or 5 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on the concentrations of immunoglobulin E (IgE) present in blood plasma. Mice were previously sensitized (on 1st and 10th days) with ovalbumin (OVA) grade V (0.1 mg/mL) and aluminum hydroxide (10 mg/mL) in 0.9% saline i.p., 0.2 mL/animal, and challenged (19th to 24th days) with OVA grade II (3% in saline, nebulized, 20 min daily). The sham group was intraperitoneally injected and challenged with sterile 0.9% instead of OVA, and received orally 2% Tween-80 as treatment instead of vitexin. The results were expressed as mean ± SEM for 6 animals per group. One-way ANOVA, followed by Student–Newman–Keuls. $^{†††}p < 0.001$ vs. Sham; $^{***}p < 0.001$ vs. vehicle. S-OVA: Sensitization with ovalbumin, C-OVA: Challenge with ovalbumin.

preventive purposes (administered previously or throughout the duration of the experiment) [54].

An important fact of our work, together with the first description of its anti-inflammatory effect in a murine model of allergic asthma, is that the flavonoid vitexin seems to be highly potent for curative purposes, taking into consideration that doses as low as 0.2 mg/kg p.o. showed no difference or presented statistically similar results to that of dexamethasone at the dose of 0.5 mg/kg ($p > 0.95$), in general for leukocyte migration and $p > 0.995$ for IL-5. It is expected that even lower doses of vitexin may also have a beneficial effect in asthma.

4. Conclusion

All of these results show for the first time the anti-inflammatory activity of vitexin in OVA-induced allergic asthma. Its anti-inflammatory effect involves a reduction of leukocyte influx and a decrease in Th2 cytokines and allergen-induced IgE productions, resulting in reductions of the cell infiltrates, mucus secretion and edema in the lung tissues.

Our work addressed the basic understanding of vitexin as a potential treatment for asthma and will support further studies on vitexin in this sense. Also, and not the least, this reinforces and validates the current popular use of vitexin-rich medicinal plants in asthma treatment.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgments

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Bionorte, Process n° 551737/2010-7), Fundação de Amparo à Pesquisa do Estado de Mato Grosso (FAPEMAT, Process n° 205978/2011) and Instituto Nacional de Ciência e Tecnologia em Áreas Úmidas (INAU/CNPq/MCTI, Process n° 704792/2009) for the financial support of the present study. We also thank the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) and to INAU/CNPq/MCTI for provide postdoctoral fellowship to Dr A. Macho (PNPD) and Prof. Dr S. Olaitan Balogun (PDJ and DTI 1A), respectively.

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