

Inhaled *Cissampelos sympodialis* Down-Regulates Airway Allergic Reaction by Reducing Lung CD3⁺T Cells

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Cissampelos sympodialis Eichl. (Menispermaceae) root infusion is used in Northeast Brazil to treat allergic asthma. We have previously shown that oral use of the plant extract reduces eosinophil infiltration into the lung of ovalbumin (OVA)-sensitized mice. However, drugs taken by inhalation route to treat asthma achieve better outcomes. Thereby, in this study, we evaluated the inhaled *C. sympodialis* alcoholic extract as a therapeutic treatment in OVA-sensitized BALB/c mice. The parameters which were analyzed consisted of leukocyte recruitment to the airway cavity, tissue remodeling and cell profile. The inhaled extract inhibited mainly eosinophil recruitment to the pleural cavity, bronchoalveolar lavage and peripheral blood. This treatment reduced the OVA-specific IgE serum titer and leukocyte infiltration in the peribronchiolar and pulmonary perivascular areas as well as mucus production. In addition, we also tested isolated alkaloids from the plant extract. The flow cytometric analysis showed that methylwarifteine (MW) and, mainly, the inhaled extract reduced the number of CD3⁺T cells and eosinophil-like cells. Therefore, inhaled *C. sympodialis* extract and MW lead to down-regulation of inflammatory cell infiltration with remarkable decrease in the number of T cells in an experimental model of respiratory allergy, suggesting that the plant can be delivered via inhalation route to treat allergic asthma. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: *Cissampelos sympodialis*; methylwarifteine; inhalation route; down-regulation; CD3⁺T cells; airway allergic reaction.

INTRODUCTION

Respiratory diseases such as asthma, bronchitis, rhinitis and chronic obstructive pulmonary disease are chronic inflammatory allergic illnesses characterized by variable airflow obstruction, airway hyperresponsiveness and tissue remodeling in association with airway inflammation (Umetsu and Dekruyff, 2006; Wang *et al.*, 2011).

The use of inhaled medications to treat respiratory diseases has been of great importance to medicine for decades, providing high drug concentration, rapid onset of action, low systemic exposure, thereby, reducing side effects (Tronde *et al.*, 2007). However, most drugs used to treat respiratory diseases as corticosteroids, β_2 -adrenergic agonists, phosphodiesterase (PDE) inhibitors and leukotriene receptor antagonists such as montelukast and zafirlukast control the illness symptoms but also induce long-term side effects (Kemp, 2003; Holgate and Polosa, 2008; Polosa, 2008). Therefore, classical pharmacotherapy limitations to treat asthma and other allergic conditions open a research field to find alternative drugs to control these diseases.

The root infusion of *Cissampelos sympodialis* Eichl. (Menispermaceae) is usually used in folk medicine in

Northeast Brazil to treat inflammatory illnesses including asthma, bronchitis, colds and rheumatism (Correa, 1984; Barbosa-Filho *et al.*, 1997). Menispermaceae family is well known for producing different types of alkaloids (Barbosa-Filho *et al.*, 2000), and phytochemical studies showed the presence of bisbenzylisoquinoline alkaloids (warifteine (WAF) and methylwarifteine (MW)), morphinandienoni alkaloids (milonine (MIL)), aporfinic alkaloids (laurifoline) (Barbosa-Filho *et al.*, 1997), bisbenzyltretaquinoline alkaloids (roraimine) and oxaporfinic alkaloids (liriodenine) (De Lira *et al.*, 2002). In general, alkaloids present a variety of biological activities such as inhibition of apoptosis (Teh *et al.*, 1991), decrease of NF κ B signaling in macrophages (Chen *et al.*, 1997) and anti-inflammatory and analgesic effects (Costa *et al.*, 2008).

Pharmacological studies showed that alcoholic fractions of roots or leaves (AFL) of *C. sympodialis* presented relaxant effect on tracheal smooth muscle, inhibited synthesis of nucleotide enzymes cyclic PDE IV and V and increased cyclic adenosine monophosphate (cAMP) levels in alveolar leukocytes of guinea pigs (Thomas *et al.*, 1995, 1997b, 1997a). AFL also inhibited human neutrophil degranulation induced by N-formyl-Met-Phe associated with an increased cAMP-dependent protein kinase A activity (Thomas *et al.*, 1999) and decreased histamine-induced spasm in normal guinea pigs and antigen-induced anaphylactic response in ovalbumin (OVA)-sensitized guinea pigs (Thomas *et al.*, 1997a).

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The immunomodulatory effect of AFL was also demonstrated. Spleen cells of BALB/c mice stimulated with concanavalin-A and different concentrations of AFL increased the production of IL-10 associated with T-cell proliferative response inhibition (Piuvezam *et al.*, 1999). Furthermore, it was observed that AFL increased the production of IL-10 in *Trypanosoma cruzi* infected macrophages and inhibition nitric oxide production (Alexandre-Moreira *et al.*, 2003a). Moreover, AFL inhibited proliferative response of B cells with Toll-like receptor stimuli and production of IgM and IgG by increasing the intracellular cAMP levels suggesting a therapeutic use of this extract in diseases associated with high production of immunoglobulin (Alexandre-Moreira *et al.*, 2003b; Rocha *et al.*, 2010).

In vivo studies demonstrated that oral treatment with AFL suppressed the inflammatory process induced by phlogistic agents (Batista-Lima *et al.*, 2001), inhibited the production of IgE in experimental models of allergy and was associated with a decrease of IL-5 and IL-13, a Th2 cytokine profile (Bezerra-Santos *et al.*, 2004; Cerqueira-Lima *et al.* 2010), but also with increase of IFN- γ (Bezerra-Santos *et al.*, 2004; Bezerra-Santos *et al.*, 2005). These data clearly demonstrated the induction of a Th1 skewed cytokine profile by the extract in allergic experimental models corroborating with previous data showing its immunomodulatory effect.

Alkaloid WAF showed an immunomodulatory effect. WAF inhibited eosinophil and neutrophil migration into the pleural cavity and bronchoalveolar lavage (BAL) of OVA-sensitized BALB/c mice (Bezerra-Santos *et al.*, 2006), and it also reduced eotaxin levels, cytoplasm lipid body formation and cis-leukotriene generation in human eosinophils. WAF also inhibited hyperalgesia reaction in passively sensitized or chemically stimulated rats, spleen cell proliferation mediated by antigen or mitogen and reduced the antigen-specific-IgE-induced mast cell degranulation (Costa *et al.*, 2008). Together, these data provide strong support for the proposition that the plant extract, as well as WAF, induces a modulatory effect on allergic and inflammatory reactions.

Based on the available information on *C. sympodialis*, we studied the effect of the plant extract (AFL) and its alkaloids by inhalation as a therapeutic protocol in an experimental model of respiratory allergy.

EXPERIMENTAL PROCEDURES

Animals

Isogenic female BALB/c mice (7–8-week-old), weighing between 25 and 30 g, and female Wistar rats, weighing on an average 200 g, were used in the experiments. The animals were kept in cages at a temperature of $25 \pm 2^\circ\text{C}$ and subjected to 12/12-h light–dark cycle with free access to water and a controlled diet, based on pellets, throughout the trial period. Animals were supplied by the Animal Facility of the Federal University of Paraíba, Brazil. Animal manipulation was performed according to animal care guide (Sherwin *et al.*, 2003), and the experimental protocols were approved by the Committee for Experimentation on Animal Research of UFPB (CEPA N° 0602/05). Animals were euthanized

with 10% ketamine hydrochloride. Groups of 5 to 10 animals were used for the experiments.

Alcoholic extract of leaves of *Cissampelos sympodialis*

Cissampelos sympodialis was obtained at the Botanical Garden of UFPB/Brazil (voucher species Agra 1456). Approximately 3 kg of fresh leaves was collected, dried at 50°C and pulverized. The leaves were powdered and subjected to three successive alcohol extractions in a percolator at room temperature ($25\text{--}30^\circ\text{C}$). The AFL was obtained with a mixture of water and ethanol (30/70 v/v). Then solvent was removed by using rotavapor at a temperature of 60°C under reduced pressure, and the dry weight of the extract was 79.9% based on the present solid waste. The extract showed 34% of solubility in water. The AFL was prepared in sterile saline (Piuvezam *et al.*, 1999) to posterior standardization using the alkaloid WAF as a pattern (Cerqueira-Lima *et al.*, 2010).

Alkaloid extraction and purification

The *C. sympodialis* extract was dissolved in 3% HCl and extracted with CHCl_3 . Aqueous fraction was basified with NH_4OH to pH 9 and again extracted with CHCl_3 . The CHCl_3 extract was washed with H_2O and dried with MgSO_4 . The solvent was evaporated, and the total tertiary alkaloid fraction was subjected to chromatography column over alumina, eluting with hexane containing increasing amounts of $\text{CHCl}_3/\text{MeOH}$. Fractions eluted with $\text{CHCl}_3\text{--MeOH}$ (49:1) were further purified by thin layer column (1.0 mm layer) for isolation of WAF, MW and MIL alkaloids (Melo *et al.*, 2003; Cerqueira-Lima *et al.*, 2010). The isolated alkaloid solutions were prepared using 1 mg of the crystal in 50 μL of HCl 1 N and 800 μL of distilled water. The pH was adjusted to 7.0 with NaOH 1 N. Volume was completed to 1000 μL , and the dilutions were made in distilled water for the *in vivo* assay and in PBS for the *in vitro* assay.

Inhaled treatment

Five groups ($n=5\text{--}10$) of female BALB/c mice were used in all experimental protocols, and they were divided in: saline group (non-sensitized animals), OVA group (OVA-sensitized animals and treated with saline), AFL 10 or AFL 100 groups (OVA-sensitized animals and treated with 10 or 100 mg/mL of AFL, respectively), Dexa group (OVA-sensitized animals and treated with 0.1 mg/mL of dexamethasone). For the histological analysis, a naive animal group was added to demonstrate influence of the inhalation procedure.

Allergic pleurisy

Allergic pleurisy was achieved after mice were subcutaneously (s.c.) sensitized with 0.2 mL of OVA at 50 μg (grade V SIGMA) and 5 mg of aluminum hydroxide dissolved in sterile saline on the first and seventh day of the experimental protocol. After 14 days of OVA-sensitization, the animals were treated with

inhaled AFL, saline or Dexamethasone 1 h before the challenge procedure (0.1 mL of OVA 12 µg/cavity dissolved in sterile saline). Pleural lavage was obtained after OVA challenge (8 and 24 h) with an injection of HBSS⁻ (calcium and magnesium free) solution at pH 7.2 and was kept on ice to maintain cell viability. Pleural lavage was diluted in Turk's solution (1:40), and cells were directly counted in a Neubauer chamber using optic microscope after diluting samples of the pleural lavage (1:40). For differential counts, samples were centrifuged at 1000 rpm, at 4 °C for 10 min. Cytospin slides from each BAL were stained with May-Grünwald - Giemsa. For each slide 100 cells were counted, using a 100× oil-immersion objective (Bezerra-Santos *et al.*, 2006).

Allergic pulmonary inflammation

Female BALB/c mice ($n=5$) were sensitized intraperitoneally (i.p.) with 0.2 mL OVA at 10 µg (Grade V Sigma) and 2.0 mg of aluminum hydroxide on the 1st and 10th day of the experimental protocol. The animals were challenged with aerosolized OVA (1% in 0.9% saline) from 19th to 26th day for 20 min in a closed chamber (Lloyd *et al.*, 2001). One hour before each OVA challenge, animals were treated with inhaled AFL (10 or 100 mg/mL) or intranasal (i.n) application of the alkaloids (2.0 mg/kg). The dose of 2.0 mg/kg of each alkaloid was chosen due to its effect on reducing inflammatory cells in the BAL (Lloyd *et al.*, 2001). Twenty-four hours after last OVA challenge, animals were euthanized with ketamine hydrochloride, and the tracheas were isolated to obtain the BAL. The total cell counts were performed in a Neubauer chamber, using an aliquot of the diluted sample (1:40) in Turk solution in an optic microscope (40× objective). For differential counts, samples were centrifuged at 1000 rpm, at 4 °C for 10 min. Cytospin slides from each BAL were stained with May-Grünwald-Giemsa. Differential cell counts were performed by light microscopy. Each slide was analyzed until the count of 100 cells was reached using oil-immersion objective.

Flow cytometric analysis

In a heterogeneous cell population analyzed by flow cytometric analysis, the forward-scattered light (FSC) and side-scattered light (SSC) measurements allow us to distinguish one cell population from another by cell size (diffraction of light) and granularity (light diffusion), respectively. The analysis shows that granularity of lymphocytes is identified as FSC^{lo}/SSC^{lo} and granularity such as those of eosinophils as SSC^{hi} (Van Rijt *et al.*, 2004). To determine the lung cell population of different animal groups (CTR: non-sensitized animals; OVA: OVA-sensitized animals and treated with saline; AFL: OVA-sensitized animals and treated with 40 mg/kg of AFL, Dexa: OVA-sensitized animals and treated with 2 mg/kg of dexamethasone; MW, MIL or WAR: OVA-sensitized animals and treated with 2 mg/kg of each alkaloid), bronchoalveolar cells (see section 2.6) were analyzed using the FSC and SSC parameters as well as the CD3 expression. For these studies, 1×10^6 bronchoalveolar cells/mL were incubated with anti-mouse CD3 FITC conjugated (R&D Systems,

USA) for 30 min at 4 °C. Cells were washed twice with cold PBS and resuspended in PBS. 10000 events were acquired using a Becton Dickinson FACScan, and the data were analyzed using WinMIDI software.

OVA-specific IgE titer

The OVA-specific IgE titer was determined using the passive cutaneous anaphylaxis (PCA) test. Sensitized and treated BALB/c mice had intracardiac bleeding to obtain the sera. The sera were aliquoted, labeled and frozen at -20 °C until it was used for the PCA assay. The PCA reaction was performed as follows: 50 µL of serum from each BALB/c mice group was injected by dermal route on the shaved back of Wistar rats. After 48 h, all rats were anesthetized with hydrochloride (270 mg/kg i.p.), and their tails were washed with water and, into the vein, 0.5 mL of a solution containing the Evans Blue dye (1 % VETECO) and OVA (2.0 mg/rat - Grade II - SIGMA) was injected. After 30 min, animals were euthanized, and the OVA-specific IgE titer was measured. The highest serum dilution giving a 5-mm diameter flare or bluing reaction was taken as the PCA titer (Holt *et al.*, 1981).

Lung histology

Lungs were inflated by injecting 1.0 mL of 10 % buffered formalin using a catheter. Lungs were then removed, fixed in the same solution and embedded in paraffin. Five-µm thick sections of paraffin-embedded lungs were stained with hematoxylin-eosin (H&E), Golmori's trichrome and periodic acid-Schiff (PAS) according to standard protocols and examined under light microscopy. Digital photographs of at least 10 broncho-vascular bundles per tissue section (with bronchioles cross-sectional diameters ranging from 120 to 250 µm) were obtained under light microscopy A.T.x100 or x400 magnifications. For the analysis of inflammatory cell infiltration, H&E stained sections were used (A.T.x100). Mucus secretion from goblet cells of the bronchial epithelium was analyzed by the staining of sections with PAS. Goblet cells were recognized by the intense dark red staining of their mucus content with PAS, and their characteristic distended lateral border and basal nucleus. The Golmori's trichrome dye was used for peribronchiolar collagen fibers. Density of collagen fibers was defined by the positively stained area in relation to total tissue in the area of the bronchovascular bundles per millimeter square, at a magnification of A.T. x400.

Statistical analysis

All data were expressed as mean ± standard deviation and statistically analyzed by applying the Student *t* test (unpaired), in which *P* values <0.05 were considered significant. *** *P* < 0.001 were considered significant when OVA group was compared with saline group. +*P* < 0.05, ++*P* < 0.001 and +++*P* < 0.0001 were considered significant when the AFL (10 or 100) or Dexa groups were compared with the OVA group. All data were analyzed by GraphPad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Effect of the inhaled AFL on the cell recruitment into the pleural cavity and in the BAL

Inhaled AFL inhibited the inflammatory cell migration into the pleural cavity (Fig. 1A) and into the BAL (Fig. 1B) 24 h after OVA challenge. The dose of 10 mg/mL or 100 mg/mL induced a significant ($P < 0.05$ and $P < 0.001$, respectively) decrease in the total number of cells into the pleural cavity and into the BAL when compared to the OVA group. Influx of mononuclear cells into the pleural cavity and into the BAL was also significantly ($P < 0.05$) reduced in both AFL and dexamethasone

treatments (Fig. 1A and 1B). Neutrophils were absent in the pleural cavity and significantly ($P < 0.001$) reduced into the BAL in AFL-treated animals. AFL or dexamethasone treatments also showed a significant ($P < 0.001$ or $P < 0.05$) reduction in the number of eosinophils into the pleural cavity as well as into the BAL (Fig. 1A and B).

Effect of the inhaled AFL and alkaloids on T cell recruitment into the lung

The present study demonstrated that inhaled AFL (100 mg/mL) provoked approximate 4-fold reduction in the percentage of CD3⁺T cells and a 10-fold one in the cells with high concentration of granules, indicating

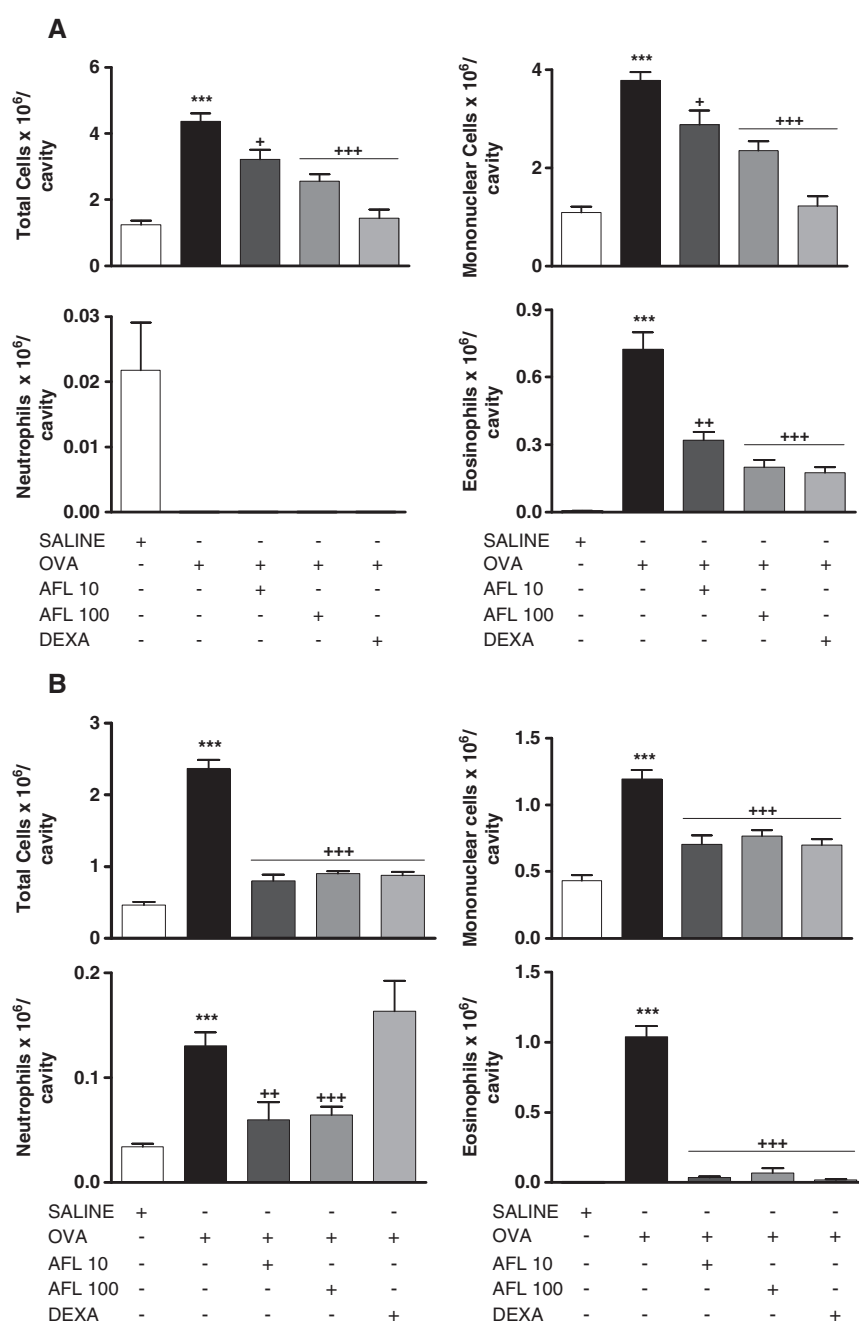


Figure 1. Effect of inhaled AFL on the cell recruitment into the pleural cavity and into the bronchoalveolar lavage. A. cells from the pleural cavity of the different groups of animals after 24 h of OVA challenge. B. cells from the bronchoalveolar lavage (BAL) of different groups of animals after 24 h of OVA challenge. Results are expressed as the mean $\pm$ S.E.M. from at least six animals. ***Significantly different from saline group ($p \leq 0.001$). +/+ +/+ +/+ significantly different from OVA group ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively). The data are representative of two experiments ($n = 6-10$).

eosinophils (Fig. 2A and B) which was similar to what was achieved with the injection of the immunosuppressant drug, dexamethasone (Fig. 2A and B). Additionally, the alkaloids isolated from the extract, that is, MW, MIL and WAR, were also tested, and it is important to note that only the alkaloid MW was capable of lowering the CD3⁺T cells and eosinophils in higher levels (2-fold decrease *vs.* the non-treated group). These data indicated that MW and mainly AFL efficiently reduced the CD3⁺T cell and eosinophil counts.

Effect of the inhaled AFL on the OVA-specific IgE serum titer

Inhaled AFL at 100 mg/mL significantly decreased OVA-specific IgE serum titer in OVA-sensitized animals. However, AFL at 10 mg/mL and dexamethasone did not change the levels of OVA-specific IgE (Fig. 3). Furthermore, our data demonstrated, for the first time that inhaled AFL has a systemic effect in modulating B cells.

Effect of inhaled AFL in the lung histology

For the histological analysis of six lung sections of each animal group, saline (A); OVA (B); AFL 10 (C); AFL 100 (D); naive animal (E) and dexta (F), H&E (Fig. 4), Gomori's trichrome (Fig. 5) and PAS (Fig. 6) stains were used. The H&E staining allowed us to observe the morphology of blood vessels, bronchioles, cell infiltration and lung parenchyma. The OVA group (Fig. 4B) showed a massive cell infiltration in the peribronchiolar and pulmonary perivascular regions, but absence of cell infiltration in alveoli and vein/venular peribronchiolar congestion, characterizing the pulmonary inflammatory process. The saline and naive groups (Fig. 4A and E) did not present changes in the lung histology. The treatment with 10 mg/mL of AFL (Fig. 4C) did not inhibit cell migration. On the other hand, treatment with 100 mg/mL of AFL (Fig. 4D) or dexta (Fig. 4F) decreased cell infiltration into the peribronchiolar and perivascular regions. The Gomori's Trichrome staining allowed us to observe the extracellular matrix components such as collagen, elastic fibers and reticular fibers. The OVA group (Fig. 5B) showed collagen fiber deposition in the peribronchiolar and perivascular regions, but not in the pulmonary alveoli (Fig. 5A and E). These observations confirm the inflammatory process installation in this animal group. The animal groups to which 100 mg/mL of AFL or dexamethasone were delivered (Fig. 5D and F) showed inhibition of collagen fibers. However, the group receiving 10 mg/mL of AFL (Fig. 5C) did not present effects on this parameter.

The PAS reaction is useful to demonstrate the presence of mucopolysaccharides in lung sections. The OVA group (Fig. 6B) showed mucus production by the unicellular exocrine glands (goblet cells) in the primary, terminal and respiratory bronchioles. The saline group (Fig. 6A) showed no mucus producing cells. Only AFL 100 (Fig. 6D) inhibited the mucus production in the terminal and respiratory bronchioles, similar to what was observed in the dexta group (Fig. 6F).

DISCUSSION

Respiratory allergic diseases are a health problem worldwide, resulting from a complex interaction between genetic and environmental factors. They are chronic inflammatory disorders characterized by airway changes such as mucus production, eosinophilic inflammation, bronchial hyperresponsiveness, fibrosis and tissue remodeling (Lee *et al.*, 2006; Tronde *et al.*, 2007; Yan *et al.*, 2011).

One of the goals of therapies for respiratory diseases is to reduce the local inflammatory response by decreasing the number of inflammatory cells, as well as their activity. To reach these goals, the drugs are usually delivered by inhalation achieving high local drug concentration and few systemic side effects (Lee *et al.*, 2006; Yan *et al.*, 2011).

Several types of oral or i.n. antihistamines, short-acting and long-acting β -2-agonists and steroids have been used to treat respiratory allergies. Corticosteroids and long-action β -2-agonists are the first choice for pharmacotherapy in respiratory allergies. However, oral long-term treatment with these drugs promotes appearance of several side effects as well as diminishing of the desirable effect (Karaman, *et al.*, 2011; Meltzer, 2011; Yan *et al.*, 2011). Thus, new effective therapeutic modalities to treat these disorders, presenting fewer side effects, need to be available urgently.

With this in mind, the present study evaluated the effect of the inhaled AFL of *Cissampelos sympodialis* and its isolated alkaloids in an experimental model of respiratory allergy.

In this study, inhaled AFL inhibited inflammatory cell recruitment into the pleural cavity and lung, as well as peripheral blood (data not shown), IgE serum titer and decreased the mucus and collagen productions on lung tissue. Both, inhaled AFL and MW, but neither WAF nor MIL, inhibited CD3⁺T cell and eosinophil infiltrates in the lung tissue of OVA-sensitized animals.

Inflammatory cell migration into the pleural cavity and BAL of OVA-sensitized BALB/c mice, mostly eosinophils, was inhibited by inhaled AFL 8 h (data not shown) and 24 h after OVA challenge, similar to what was achieved in the dexamethasone treatment. In addition, inhaled AFL treatment slightly decreased the number of leukocytes into the peripheral blood with a slightly increase of lymphocyte number (data not shown). Data suggest that the inhaled AFL acts mainly in the lung, but also presents systemic effects.

Previous studies of our group demonstrated that oral treatment with AFL in an experimental model of allergy did not cause any systemic toxicity (Melo-Diniz, *et al.*, 2002; Bezerra-Santos, *et al.*, 2006), and both AFL and WAF have a profound effect on eosinophil function, decrease of eotaxin levels, cysteinyl-leukotriene generation and cytoplasmic lipid body formation (Bezerra-Santos *et al.*, 2006). It is important to mention that other plant extracts also have similar effects (El Gazzar *et al.*, 2006; Jin *et al.*, 2006; Roh *et al.*, 2008).

Even though there are a lot of studies with oral or intraperitoneal injections of medicinal plants or isolated compounds in experimental models of allergies, scanty information is available on inhalation route.

Inhaled AFL or methylwarifene was effective in reducing the number of CD3⁺T cells and eosinophils

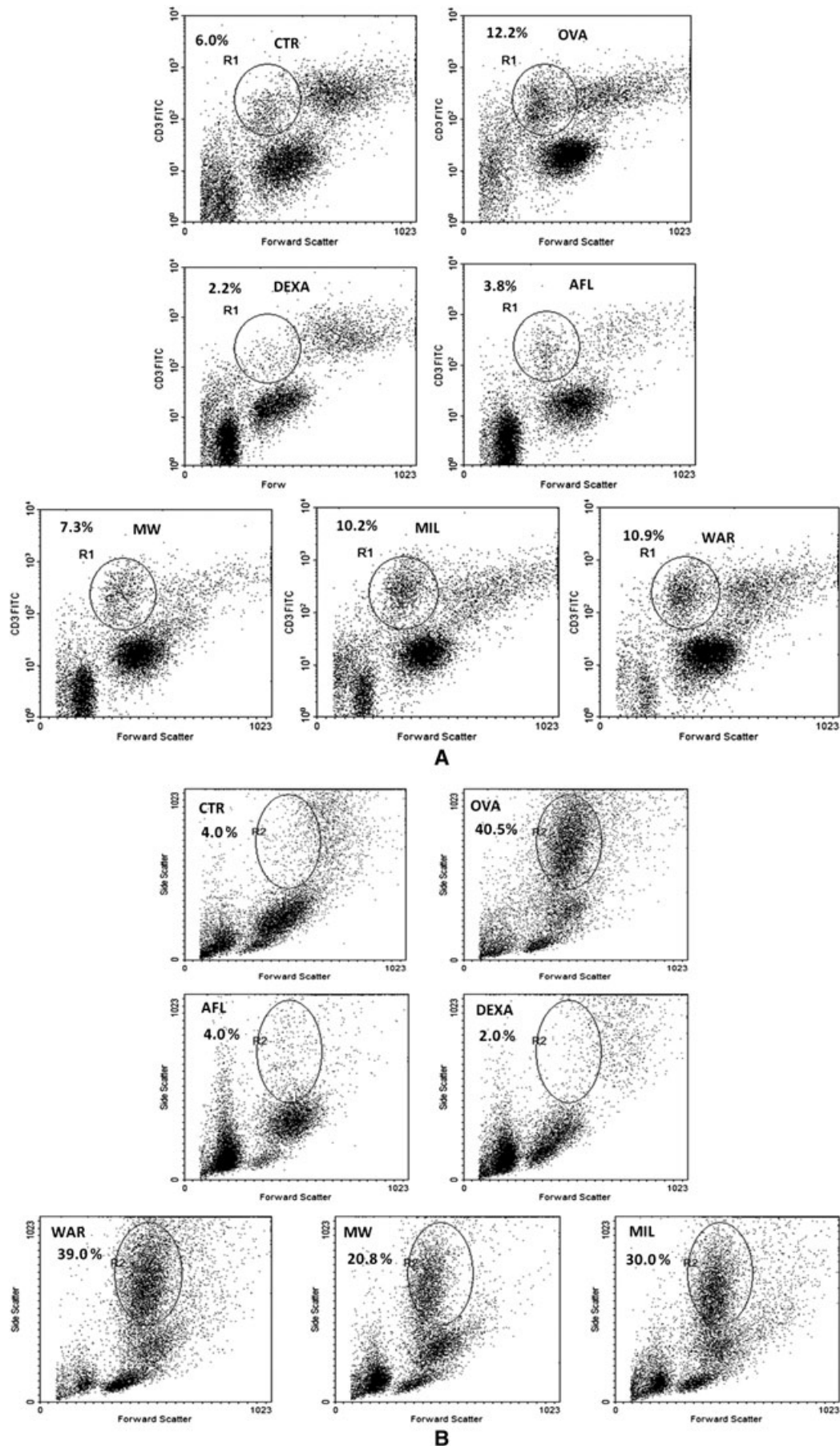


Figure 2. Effect of AFL and its alkaloids on the percentage of airway CD3⁺ T cells and eosinophil-like cells. CD3 expression was analyzed by flow cytometry using anti-CD3 antibody, and granularity of cells was analyzed according to forward scatter parameters. Panel A represents the percentage of CD3⁺ cells, and panel B represents the percentage of eosinophil-like cells. CTR: non-sensitized animals; OVA: OVA-sensitized animals and treated with saline; DEXA: OVA-sensitized animals and treated with 2 mg/kg of dexamethasone; AFL: OVA-sensitized animals and treated with 40 mg/kg of AFL; MW, MIL or WAR: OVA-sensitized animals and treated with 2 mg/kg of each alkaloid. The results are representative of three separated experiments.

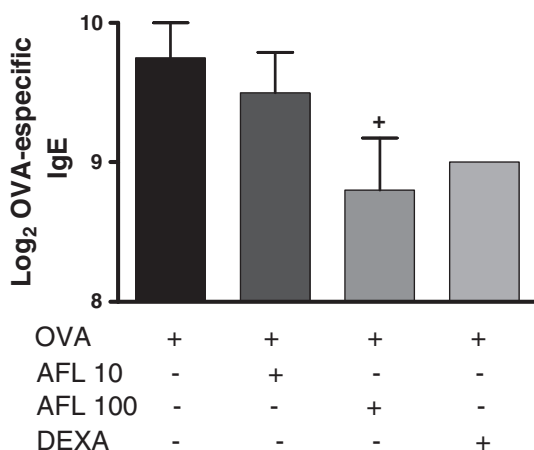


Figure 3. Effect of inhaled AFL on the IgE OVA-specific titre in BALB/c mice sensitized with OVA. Sensitized mice were treated with saline, AFL 10 group, AFL 100 group or dexamethasone group 1 h before each challenge. + Significantly different from OVA group ($p \leq 0.05$). The data are representative of three separated experiments ($n = 6$).

into BAL. Surprisingly, neither MIL nor warfarin when delivered by inhalation was able to achieve the same results. In contrast, oral treatment of OVA-sensitized mice with WAF inhibited eosinophil migration to the pleural cavity and to the BAL (Bezerra-Santos *et al.*, 2006). The fact that MW, a physiological structural modification of WAF, was more effective than the latter in modulating the number of inflammatory cells when locally delivered opens new possibilities for the research in this field, regarding the

structure and the activity of these alkaloids and their anti-inflammatory and anti-allergic properties.

T cells and eosinophils are the main cells involved in the pathogenesis of asthma and rhinitis. In respiratory allergies, the presence of T_H2 cells (CD3⁺T cells) is usually observed in the lungs. Allergen exposure of airways induces a T_H2 dominant cytokine profile with IL-4, IL-5 and IL-13 production, as well as recruitment and activation of inflammatory cells, including eosinophils, mast cells and neutrophils (Bisset and Schmid-Grendelmeier, 2005). Thereby, regulation of T_H2 cells in the lungs of allergic individuals is vital for the approach to symptoms control, justifying an increasing interest in this research field (Keynan *et al.*, 2008). According to this perspective, a previous study by Bezerra-Santos and col. (2004) demonstrated that spleen cells from oral AFL-treated OVA-sensitized mice produced high amounts of IFN- γ , a Th1 cytokine profile and low serum IgE titer in this experimental model, due to its Th2-type cells down-regulation potential.

Furthermore, the present study demonstrated that inhaled AFL has a systemic effect decreasing OVA-specific IgE serum titer. IgE is produced by antigen-specific B cells and is a hallmark of respiratory allergic diseases. Its production in allergies is related with the Th2 cytokine profile. Rocha and col. (2010) demonstrated that B cells in the presence of AFL and stimulated with several B cell stimuli did not present proliferate response and IgM production (Alexandre-Moreira *et al.*, 2003b). These effects were due to increasing levels of intracellular cAMP.

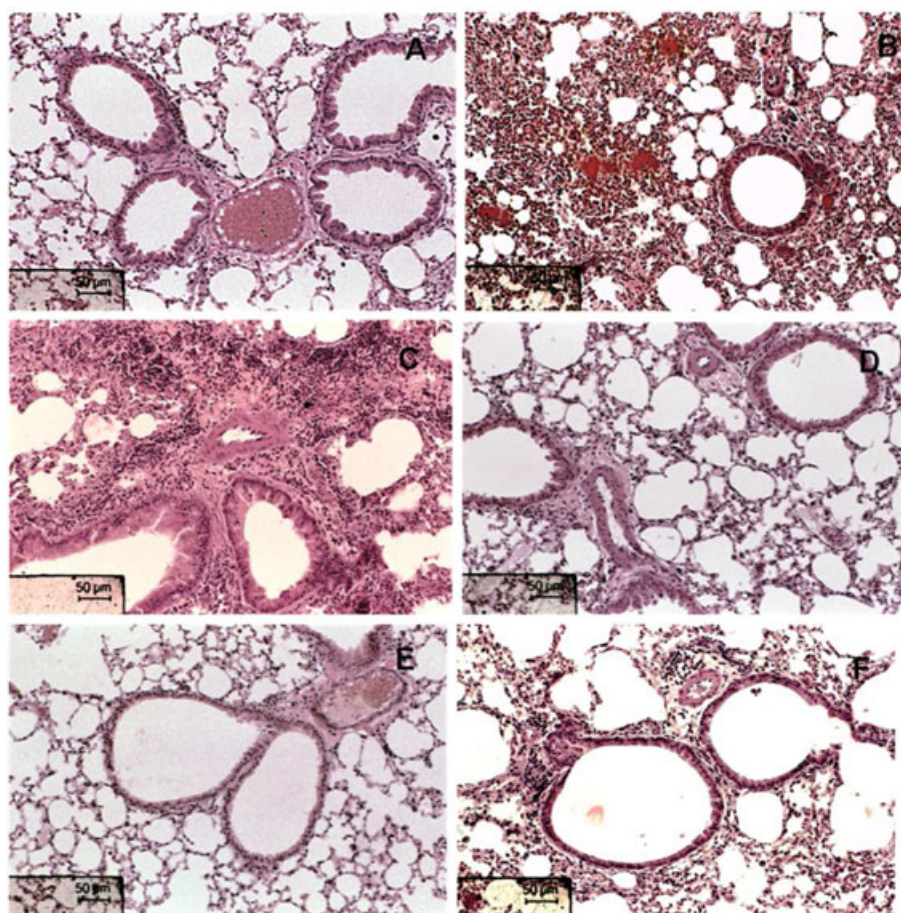


Figure 4. Photomicrograph representative of the airways and lung parenchyma stained with H-E AT $\times 100$. Saline (A), OVA (B), AFL 10 group (C), AFL 100 group (D), naive animal (E) dexamethasone group (F). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

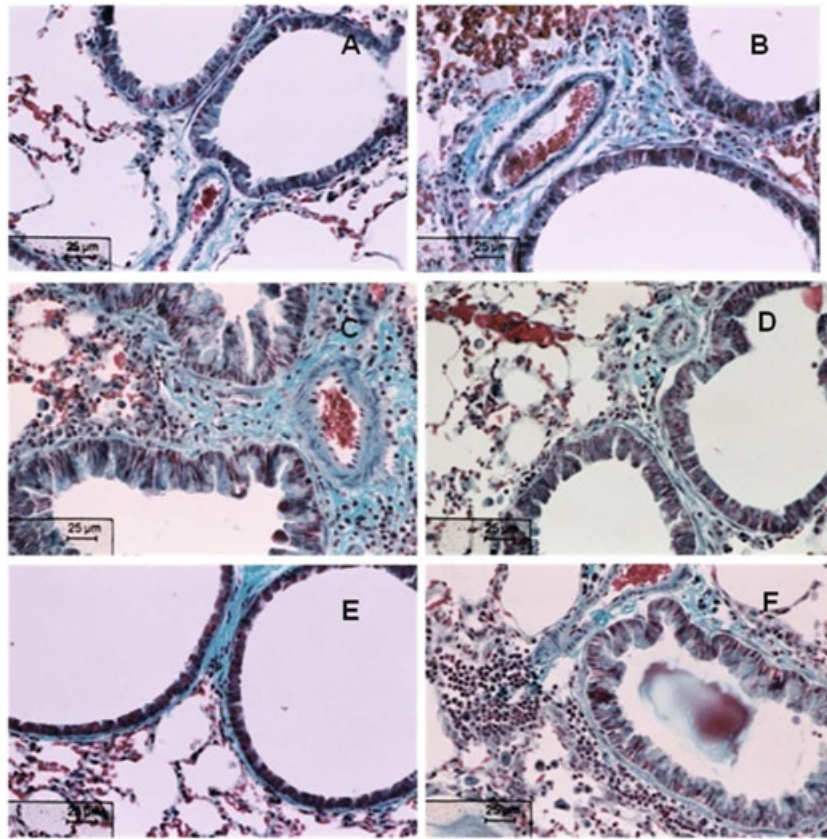


Figure 5. Photomicrograph representative of the airways and lung parenchyma stained with GomoriTrichrome AT 400x. Saline group (A), OVA group (B), AFL 10 group (C), AFL 100 group (D), naive animal (E) dexa group (F). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

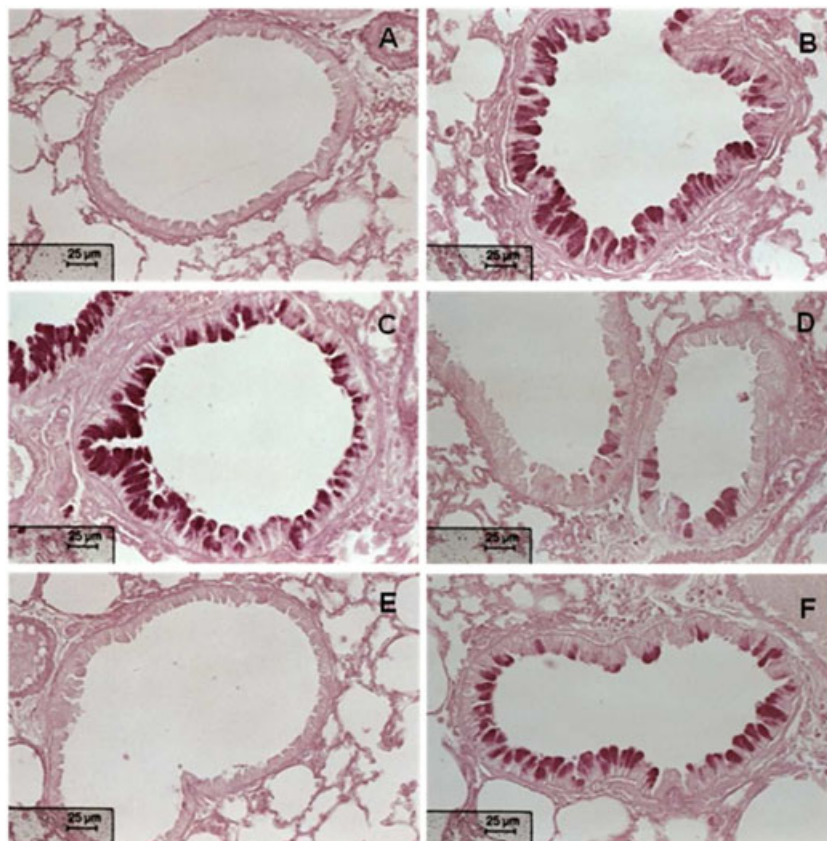


Figure 6. Photomicrograph representative of the airways and lung parenchyma stained with periodic acid Schiff (PAS) in AT 400x. Saline group (A), OVA group (B), AFL 10 group (C), AFL 100 group (D), naive animal (E) dexa group (F). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Moreover, *in vivo* treatment with WAF decreased the production of IgM and IgG showing clearly the immunomodulatory property of the plant in B cells (Rocha *et al.*, 2010).

Histological analysis of lungs from inhaled AFL-treated OVA-sensitized BALB/c mice showed a reduced number of inflammatory cells into the peribronchiolar and perivascular areas, reduction in collagen and mucus production, resulting from reduction of goblet cell hyperplasia in the respiratory epithelium. These results were very similar to those observed in dexamethasone-treated animals. Comparative histological analysis in this study clearly showed that the inflammatory process was properly installed and reversed by inhaled the AFL. Therefore, these data clearly demonstrate the local effect of the plant.

Airway remodeling is an important feature of asthma characterized by thickening of bronchial wall due to a variety of structural changes including: epithelial injury and epithelial cell turnover (Laitinen *et al.*, 1985; Jeffery *et al.*, 1989), subepithelial fibrosis (Roche *et al.*, 1989), deposition of extracellular matrix proteins as laminin, tenascin, decorin, versican and collagen (types I, III and IV) (Bousquet *et al.*, 1996; Carroll *et al.*, 2000), increase of small blood vessel area (Carroll *et al.*, 1997), hypertrophy and hyperplasia of goblet cells and mucous glands (Dunnill *et al.*, 1969) and an increase airway smooth muscle mass in both central and peripheral airways resulting from smooth muscle cell hypertrophy and/or hyperplasia (Carroll *et al.*, 1993). Therefore, inhaled AFL ameliorated the structural aspect of the

pulmonary epithelium in this model of asthma pointing to the possible production a phytotherapy medicine based on inhaled *C. sympodialis* extract to treat respiratory illnesses.

This study demonstrated that inhaled *C. sympodialis* treatment of animals with allergic airway inflammation is as effective as the oral treatment in controlling lung inflammatory response and the production of IgE. In addition, T cell immunomodulatory effect of the isolated alkaloid MW and the *C. sympodialis* extract clearly provides the scientific evidence to support its medicinal efficacy in the management of allergic inflammatory diseases orchestrated by T cells. In addition, the results allowed us to suggest that the alcoholic extract of the leaves of *Cissampelos sympodialis* can be the plant material for a phytotherapy medicine to be delivered by inhalation to prevent airway remodeling.

Acknowledgements

This work was supported by Brazilian agencies: CNPq and CAPES. The authors are indebted to Dr. José Maria Barbosa Filho for alkaloid supply, Jose Crispin Duarte for technical assistance and Fernando Antônio Cavalcanti Viana for *Cissampelos sympodialis* collect.

Conflict of Interest

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

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