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► **To cite this version:**

Lais Costa Agra, Marvin Paulo Lins, Patrícia Da Silva Marques, Salette Smaniotto, Christianne Bandeira De Melo, et al.. Uvaol attenuates pleuritis and eosinophilic inflammation in ovalbumin-induced allergy in mice. *European Journal of Pharmacology*, 2016, 780, pp.232-242. 10.1016/j.ejphar.2016.03.056 . hal-01295649

**HAL Id: hal-01295649**

**<https://univ-rennes.hal.science/hal-01295649>**

Submitted on 10 Jun 2016

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## **Uvaol attenuates pleuritis and eosinophilic inflammation in ovalbumin-induced allergy in mice**

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### **ABSTRACT**

Uvaol, a triterpene present in olives and virgin olive oil, has been shown to possess anti-inflammatory properties and antioxidant effects. However, until now, no studies have demonstrated its potential effects on allergic inflammation. The aim of this study was to evaluate the anti-inflammatory effects of uvaol in a mouse model of allergy characterized by eosinophil-dominant inflammation in actively sensitized mice. The anti-inflammatory effect of uvaol was analyzed in two murine models of allergic inflammation (pleurisy and asthma). In these models, Swiss mice were sensitized and challenged with ovalbumin (OVA). In the pleurisy model, the pleural eosinophilic

inflammation and IL-5 concentrations were examined 24 h after the OVA challenge, while in the asthma model were examined the airway inflammation via bronchoalveolar lavage (BAL) fluid cytology and lung histopathology analyses. Our results showed that uvaol decreased the accumulation of eosinophils and the concentration of IL-5 in pleural effluent. Uvaol also demonstrated important anti-inflammatory activity by inhibiting production of IL-5 and influx of leukocytes, mainly of eosinophils, in BAL fluid, but without interfering with levels of reactive oxygen species in leukocytes. Moreover, the eosinophil infiltration, mucus production, number of alveoli that collapsed, and IL-5 levels in the lung were clearly decreased by uvaol treatment. These findings indicate that uvaol can be a good candidate for the treatment of allergic inflammation by inhibiting eosinophil influx and IL-5 production in ovalbumin-induced allergy.

*Keywords:* Uvaol; Triterpene; Eosinophil; IL-5; Inflammation.

*Chemical compound studied in this article:* Uvaol (PubChem CID: 92802)

## **1. Introduction**

Epidemiological studies report that the prevalence of allergic diseases has increased dramatically worldwide (Sole et al., 2014) in both industrialized and developing countries. The World Health Organization estimates that about 700 million people worldwide have some type of allergic disease (Rutkowski et al., 2014), which affects the quality of life of these individuals and their families, thereby negatively influencing the socioeconomic welfare of society.

In allergic reactions that require specific-allergen sensitization, the re-exposure to the antigen causes activation and secretion of a wide spectrum of mediators in target cells that directly damage the surrounding tissue and induce leukocyte infiltration,

contributing to exacerbation of the inflammatory response (Barnes, 2011). This leads the antigen-induced accumulation of eosinophils into the tissue, which contributes significantly to tissue damage at sites of allergic inflammation (Nauta et al., 2008; Rose et al., 2010). Indeed, eosinophil-derived inflammatory mediators can be measured in the sputum, in bronchoalveolar lavage, and around areas of damaged epithelium of asthmatics (Brightling et al., 2003). In addition, eosinophilic infiltrate has been correlated clinically with the airway hyperresponsiveness (Siddiqui et al., 2007).

Interleukin-5 (IL-5) has been implicated as a key factor in eosinophil function associated with allergic conditions by promoting recruitment, activation, and survival at inflammatory sites and differentiation and maturation in the bone marrow (Barnes, 2011; Corren, 2012). Therefore, because of the importance of eosinophils in allergy and other associated disorders, IL-5 has been proposed as a potential target in the treatment of these diseases (Corren, 2011; Wechsler, 2008). In fact, the use of monoclonal antibody against IL-5, mepolizumab (Liu et al., 2013) or reslizumab (Kips et al., 2003), reduces the risk of exacerbations and inhibits the development of pulmonary eosinophilia, but not improvement the lung function. The first choice therapy to prevent the clinic manifestations associated with allergies is the use of corticosteroids. However, after extended periods of high-dose treatment, corticosteroids can have substantial side effects (Rizzo and Sole, 2006). Thus, the development of efficient alternative agents and therapeutics for allergic conditions is urgently needed.

Several plant-derived secondary metabolites that reduce the production and/or activity of pro-inflammatory mediators have been proposed as alternative therapeutic agents (Calixto et al., 2004). This therapeutic potential has enabled the development of new drugs, such as Acheflan<sup>®</sup>, from natural products (Calixto, 2005), for the treatment

of various inflammatory conditions. Thus, the natural biological compounds continue to contribute to the commercial drugs being manufactured currently.

Pentacyclic triterpenes are widespread in the plant kingdom and are present in the fruits, leaves, and barks of medicinal plants (Hill and Connolly, 2013). These secondary plant metabolites are attracting increasing interest due to their beneficial anti-inflammatory (de Oliveira et al., 2015), anti-diabetic (Sheng and Sun, 2011), and antibacterial (Gilabert et al., 2015) effects. Recently, studies have also reported its effects on immune regulation (Martin et al., 2012a), regulation of blood sugar (de Melo et al., 2010), lowering of blood pressure (Somova et al., 2003), skin inflammation (Passos et al., 2013), and antitumor activity (Zhang et al., 2014). Thus, considering the wide range of biological activities of triterpenes, there has been increased interest in using them for pharmacological studies and for prospective new drug development.

Although several studies have shown the biological effects of a large number of triterpenoids, studies reporting the *in vivo* anti-inflammatory activity of uvaol are still scarce. Uvaol (Urs-12-ene-3,28-diol) is a biologically active molecule present in several foods as well as in plants used in folk medicine for their antioxidant (Allouche et al., 2011) and antibacterial effects (Martins et al., 2011). Despite its known pharmacological effects, the actions of uvaol on allergic inflammatory response are not yet elucidated. Moreover, there have been any studies on the anti-asthmatic or anti-inflammatory effects of uvaol in a murine model of allergy. Here, we evaluated the effect of uvaol on the eosinophilic response triggered by an allergen in two models of allergic inflammation in actively sensitized mice.

## 2. Materials and methods

### 2.1. Animals

Male Swiss mice weighing 25–30 g were obtained from the breeding colonies of the Federal University of Alagoas (UFAL). Animals were maintained with free access to food and water and were kept at  $22 \pm 2^\circ\text{C}$  with a controlled 12-h light–dark cycle in an animal housing facility at the Institute of Biological and Health Sciences.

Experiments were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were used only once. All experimental procedures were performed in accordance with the guidelines for the ethical use of conscious animals in pain research published by the International Association for the Study of Pain (Zimmermann, 1983).

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian Society of Laboratory Animals Science (SBCAL). The protocol was approved by the Committee on the Ethics of Animal Experiments at the Federal University of Alagoas (Comissão de Ética no Uso de Animais – CEUA, License nº 9244/2011-45). All efforts were made to minimize the suffering of the animals.

### 2.2. Reagents

The following substances, purchased from Sigma Chemical Co. (St. Louis, MO, USA), were used: uvaol (Urs-12-ene-3,28-diol,  $\geq 95\%$  purity) (Fig. 1), ovalbumin (OVA), 2',7'-dichlorofluorescein diacetate (DCF-DA), protease inhibitor cocktail (MDL number MFCD00677817), Tween-20, eosin, hematoxylin, periodic acid-Schiff (PAS) kit and elastic stain kit, and phosphate-buffered saline (PBS). Aluminum hydroxide was from Alfa Aesar (Ward Hill, MA, USA), dexamethasone (DEXA; Decadron<sup>®</sup>) from

Teuto-Brasileiro (Goiânia, GO, BRA), xylazine (Anasedan<sup>®</sup>) and ketamine (Dopalen<sup>®</sup>) from Ceva (Paulínia, SP, BRA), ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) from Synth (Diadema, SP, BRA), and May-Grunwald-Giemsa from Merck (São Paulo, SP, BRA). All drugs were dissolved in sterile 0.9% (w/v) NaCl (saline). Commercially available enzyme-linked immunosorbent assays (ELISA Ready-Set-Go<sup>®</sup> eBioscience, San Diego, CA, USA) were used to measure IL-5 levels in the bronchoalveolar lavage (BAL) and lung, according to the manufacturer's instructions.

The uvaol was dissolved in 2% DMSO, and the drugs were dissolved to concentrations so as to allow for administration of a constant volume of 10  $\mu$ l/g in accordance with the average weight of animals. Control animals received similar volumes of the vehicle only. Oral pre-treatments (p.o.) were always administered 60 min before injection of the inflammatory stimuli with OVA.

### 2.3. Allergic pleurisy

Mice were immunized via subcutaneous (s.c.) injection on days 0 and 7 with 0.2 mL of a solution containing 50  $\mu$ g of OVA adsorbed to 5 mg of aluminum hydroxide. At day 14, sensitized mice were then challenged intrapleurally with OVA (12.5  $\mu$ g/cavity) dissolved in a final volume of 50  $\mu$ l with sterile saline. Groups of mice were treated with vehicle (2% DMSO in sterile saline) or uvaol (100, 200, or 500  $\mu$ mol/kg) orally 60 min prior to the allergen challenge. Control animals were pre-treated (60 min) with dexamethasone (10  $\mu$ mol/kg) via intraperitoneal (i.p.) injection. Mice were euthanized 24 h later and the thoracic cavity was washed with 1 ml of PBS containing EDTA (10 mM). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. Total cell counts were performed in a Neubauer chamber, and differential

cell counts (100 cells total) were performed on cytocentrifuge slides stained with May-Grunwald-Giemsa dye. The IL-5 concentrations in the supernatant of the centrifuged exudate (400 *xg*, 10 min, 4°C) were assayed by ELISA kits (BD-Bioscience Pharmingen) according to the manufacturer's protocol.

#### 2.4. Model of allergic asthma

Animals were immunized via s.c. injection on day 0 and boosted by i.p. injection 14 days later with 50 µg OVA adsorbed to 5 mg of aluminium hydroxide in 200 µl of sterile saline (0.9% NaCl). Intranasal OVA challenges (25 µg/25 µl saline) were administered on days 21, 22, and 23 under xylazine (5 mg/kg, i.p.) and ketamine (35 mg/kg, i.p.) anesthesia. Sensitized control mice were challenged only with the vehicle. Mice were then treated with uvaol (100, 200, and 500 µmol/kg) or vehicle (2% DMSO in sterile saline) by gavage 1 h prior to each allergen provocation. As a positive control, the mice were treated with dexamethasone (10 µmol/kg, i.p.).

#### 2.5. Cell and fluid recovery from the airway lumen

Forty-eight hours after the last antigenic challenge, the mice were killed by anesthetic overdose (sodium pentobarbital, 500 mg/kg, i.p.). Cells were recovered from the airway lumen through BAL. Airways were washed twice with 1 ml PBS containing 10 mM EDTA via a tracheal cannula. BAL fluid was centrifuged (400 *xg*, 10 min, 4°C). Cell pellets were re-suspended in 0.25 ml PBS for further enumeration of leukocytes in a Neubauer chamber by means of a light microscope after dilution in Türk solution. Differential cell counts were performed on May–Grunwald–Giemsa-stained cytopsin preparations under an oil immersion objective to determine the percentage of mononuclear cells, eosinophils, and neutrophils. Levels of IL-5 in the cell-free BAL

were measured according to manufacturer's instructions by ELISA kits (BD-Bioscience Pharmingen) according to the manufacturer's protocol.

To measure IL-5 in lung tissue, a solution containing Tween-20 (0.05%) and protease inhibitor cocktail (1%) in PBS was added to every 100 mg of lung tissue. The tissue was then homogenized with a tissue homogenizer (ULTRA 80-I<sup>®</sup>) for 7 min at 4°C. After homogenization, the suspension was centrifuged at 500 *xg* for 20 min at 4°C, and the concentrations of IL-5 in the lung were determined by ELISA kits (BD-Bioscience Pharmingen) according to the manufacturer's protocol.

#### 2.6. 2',7'-Dichlorofluorescein (DCF) fluorescence assay

Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein (DCF-DA), which is converted to the highly fluorescent DCF by cellular peroxides such as hydrogen peroxide. Cells from BAL were washed with PBS; then, the total number of cells was counted and the cells were treated with 5  $\mu$ M DCF-DA for 30 min at 37 °C. To measure intracellular reactive oxygen species activity, fluorescence was determined at 488 nm excitation and 525 nm emission by flow cytometry (FACSCanto™ II – BD Biosciences, San Jose, CA). The flow cytometry results for intracellular reactive oxygen species activity in total cells from BAL were analyzed using the WinMDI 2.9 program and expressed as percentages.

#### 2.7. Lung tissue histopathology

The lungs from each animal where BAL was not performed were resected for histological analysis. Lung tissues were fixed in 10% (v/v) neutral buffered formalin for 6 h. Tissues were subsequently embedded in paraffin, sectioned at 5  $\mu$ m thickness, and

stained with H&E solution and PAS kit to estimate mucus production. All images were digitalized using a color digital video camera DP25 (Olympus, Tokyo, Japan) adapted to a BX41 microscope (Olympus, Tokyo, Japan). For each lung, 10 fields (200×) were analyzed per section and data were used to calculate the mean inflammation score. Sections were stained with H&E solution for quantification of inflammatory cells by optical microscopy. The severity of peribronchial inflammation was graded semi-quantitatively as described by Myou et al. (Myou et al., 2003): 0, normal; 1, few cells; 2, a ring of inflammatory cells that was 1 cell layer deep; 3, a ring of inflammatory cells that was 2–4 cells deep; 4, a ring of inflammatory cells that was 4 cells deep. To evaluate the level of mucus expression in the airway, for each lung, 10 fields (400×) were analyzed per section using the ImageJ<sup>®</sup> 1.47 program.

## 2.8. Lung morphometric analyses

Morphometric analysis of the lung architecture was performed using an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines of known length, coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The volume fractions of collapsed and normal pulmonary areas were determined by the point-counting technique at a magnification of 200X across 10 random, non-coincident, microscopic fields.

The areas of airway lumen, smooth muscle layer, and airway epithelium were computed by counting the points falling on these tissues. The perimeter of the airways was estimated by counting the line interceptions of the integrating eyepiece with the epithelial basal membrane. The areas of smooth muscle and airway epithelium were corrected for airway perimeter by dividing their values by the number of line interceptions with the epithelial basal membrane of the corresponding airway. Because

the number of line interceptions (NI) with the epithelial basal membrane is proportional to the airway perimeter and the number of points (NP) falling on the airway lumen is proportional to airway area, the magnitude of bronchoconstriction [contraction index (CI)] was computed using the following equation:  $CI = NI/\sqrt{NP}$  (Nagase et al., 1992).

### 2.9. Statistical analysis

Data are reported as mean  $\pm$  standard error of the mean (S.E.M.) and were analyzed using GraphPad Prism® software, version 5.0 (San Diego, CA, USA). Comparisons between the experimental groups were performed either by one-way ANOVA followed by Tukey's test or two-way ANOVA followed by the Bonferroni *post hoc* test. P values less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Effect of uvaol on allergic pleurisy

Antigenic stimulation (OVA, 12.5 µg/cavity) into the pleural cavity of sensitized mice induced an increase in total leukocyte counts, which was characterized by an increase in the number of eosinophils, neutrophils, and mononuclear cells 24 h after challenge (Fig. 2 A-D). Treatment with uvaol (100, 200, and 500 µmol/kg) via oral administration caused a significant decrease in total leukocyte accumulation when it was administered 1 h before antigenic challenge (Fig. 2A). This reduction may have been attributed mainly to the effects on the eosinophil influx affecting other cells, such as neutrophils (Fig. 2C) or mononuclear cells (Fig. 2D). As expected, dexamethasone (10 µmol/kg, i.p.), used as the reference drug, caused significant inhibition of total leukocyte counts (64%), eosinophils (89%), mononuclear cells (44%), and neutrophils (52%).

As shown in Fig. 2E, the intrapleural injection of OVA (12.5 µg/cavity) induced an increase in IL-5 levels in the pleural effluent of sensitized mice 24 h after challenge. The oral pre-treatment of mice with uvaol (100, 200, and 500 µmol/kg) induced significant reduction in the levels of this cytokine ( $302.51 \pm 32.22$  to  $167.60 \pm 29.32$ ,  $128.91 \pm 13.49$ , and  $51.70 \pm 7.3$  pg/ml IL-5, respectively). As expected, dexamethasone treatment (10 µmol/kg, i.p.) reduced the IL-5 levels to  $37.28 \pm 7.70$  pg/ml.

#### 3.2. Effect of uvaol on airway inflammation induced by allergen

Administration of OVA into the airway of sensitized mice induced an increase in total leukocyte counts characterized by a significant increase in eosinophils and lymphocytes, but not by monocytes at 48 h post-challenge (Fig. 3A). Treatment with uvaol, only at doses of 200 and 500 µmol/kg (p.o.), caused a significant decrease in total

leukocyte (Fig. 3A) and eosinophil (Fig. 3B) counts when it was administrated 1 h before antigenic challenge. Although not statically significant, there was a reduction in monocytes and lymphocytes counts after treatment with uvaol. In fact, in this model, we also noted an increase in the amount of IL-5 in the BAL fluid from sensitized animals challenged with OVA compared to saline-injected mice ( $35.77 \pm 9.75$  to  $205.4 \pm 23.66$  pg/ml) (Fig. 3D). Uvaol (100, 200, and 500  $\mu\text{mol/kg}$ , p.o.) significantly decreased the IL-5 levels in BAL fluid ( $63.64 \pm 4.98$  pg/ml,  $120.5 \pm 23.61$  pg/ml,  $88.79 \pm 17.47$  pg/ml, respectively) (Fig. 3D). As expected, treatment with dexamethasone (10  $\mu\text{mol/kg}$ , i.p) decreased the total leukocyte (in 68%) and eosinophil counts (90%) in the allergic mice.

As shown in Fig. 4, the intracellular reactive oxygen species levels in the leukocytes from BAL were examined by flow cytometry. The reactive oxygen species generation in cells from BAL was significantly higher at 48 h after OVA challenge than after saline stimulus. The uvaol treatment did not prevent the increase in reactive oxygen species levels in BAL fluid cells at 48 h post challenge (Fig. 4).

### 3.3. Effect of uvaol on allergen-induced lung inflammation

We then examined the anti-inflammatory effects of uvaol in lung tissue after OVA-challenge. Histological examination of lung tissue was performed 48 h after the last OVA challenge. In OVA-induced asthmatic mice, we observed an intense leukocyte infiltration into the perivascular and peribronchiolar areas of the lung (Fig. 5B), as compared to the saline-challenged group (Fig. 5A). This inflammatory response was inhibited only in animals pretreated with uvaol (100, 200, and 500  $\mu\text{mol/kg}$ , p.o.) (Fig. 5C, 5D, and 5E, respectively). Fig. 5F shows the severity of peribronchial inflammation.

To determine whether uvaol suppressed mucus overproduction induced by OVA, we stained the lung sections with PAS. In OVA-challenged mice, mucus overproduction was clearly observed as violet color in the bronchial airways (Fig. 6B) compared to the saline-challenged group (Fig. 6A). In contrast, the extent of mucus staining was markedly diminished in OVA-challenged mice treated with uvaol (100, 200, and 500  $\mu\text{mol/kg}$ , p.o.) (Fig. 6C, 6D, and 6E, respectively). Fig. 6F represents the semi-quantitative evaluation on mucus production. Thus, our data show that uvaol significantly reduced the mucus hyper-secretion, a characteristic pathological feature of asthma.

We examined the effect of uvaol on the allergen-induced increase of pivotal pro-inflammatory cytokines in the lung of sensitized mice. As expected, OVA challenge led to a significant increase in the IL-5 levels compared to those in saline-challenged mice (Fig. 7).

The lung morphometric examination in the allergic animals demonstrated that the percentage area with alveolar collapse was significantly higher in the OVA-challenged mice (Fig. 8B) than the saline-challenged group (Fig. 8B). Uvaol treatment, in all doses (100, 200, and 500  $\mu\text{mol/kg}$ , p.o.), led to a significant reduction in alveolar collapse in comparison with that in OVA-challenged mice (Fig. 8C, 8D, and 8E, respectively). Dexamethasone treatment (10  $\mu\text{mol/kg}$ , i.p.) in the allergic mice provoked a slight reduction ( $21.4\% \pm 4.59\%$ ) in the alveolar collapse. Fig. 8F graphically represents the alveolar collapse.

#### 4. Discussion

Uvaol, which is a bioactive triterpene pentacyclic found in a wide range of plants, has been reported to exhibit anti-inflammatory and anti-oxidant effects (Allouche et al., 2011; Marquez-Martin et al., 2006). However, the potential anti-inflammatory activity of uvaol in allergen-triggered inflammation has yet to be studied. Thus, in this study, we evaluated the effect of uvaol on the allergic inflammatory responses in distinct models, that is, for pleurisy and asthma. These models reproduce the eosinophilic inflammatory response identified as a key alteration in the pathogenesis of allergic diseases. We present findings related to the anti-inflammatory properties of uvaol involving inhibition of both eosinophil infiltration and the IL-5 concentrations after allergic challenge.

The allergen-induced pleurisy model is characterized mainly by eosinophilic influx after 24 h post-induction, which allows the analysis of inflammatory mediators and cells; this model can also be used to evaluate the therapeutic efficacy of various drugs and phytochemical compounds for acute inflammation (Penido et al., 2005). Concerning the rise in the number of eosinophils in the pleural exudate, which is an important hallmark of allergic inflammatory response, we examined the interference of uvaol on the inflammatory cell infiltration. As expected, intrapleural injection of OVA in sensitized mice provoked an inflammatory reaction, characterized by intense migration of total leukocytes in the pleural cavity, while uvaol-treated mice showed suppressed eosinophil infiltration into the pleural cavity after OVA challenge.

It is widely accepted that IL-5 plays an important role in eosinophil infiltration in allergic inflammation. To gain insights into the mechanism of action of uvaol, its role in IL-5 production was analyzed. In our study, the IL-5 levels in the pleural exudate after allergenic stimulation in sensitized mice were significantly suppressed after uvaol

treatment. Interestingly, in strains of genetically IL-5-deficient mice, suppression of eosinophil infiltration was reported after allergen challenge in the sensitized state (Foster et al., 1996). In addition, the use of antibodies that neutralize IL-5 inhibits both allergen-induced blood eosinophilia and the recruitment of eosinophils into the lungs of murine models of asthma (Foster et al., 2002; Weltman and Karim, 2000). These findings indicate that uvaol may mediate an inhibitory effect on eosinophil accumulation at the inflammatory site in part by inhibiting the amount of IL-5 after antigenic challenge. Considering that inhibition of eosinophil accumulation in tissue has been shown to be a therapeutically useful strategy in the treatment of allergic diseases (Fulkerson and Rothenberg, 2013), we can infer that uvaol might represent a new anti-allergic agent.

In the murine model of asthma, where clinical and pathological features are similar to those of human allergic asthma, we verified that intranasal provocation with allergen in sensitized mice induced an increase in inflammatory cell migration into the BAL, mostly eosinophils. Our present findings show that uvaol inhibited eosinophil infiltration into the airways, as shown by a significant decrease in total cell counts and eosinophil counts in BAL fluid. In addition, the lung infiltration by eosinophils and the mucus overproduction were also attenuated, as revealed by significant reduction in inflammatory cell infiltration as well as PAS-positive cells.

Eosinophil transmigration into the inflamed tissue and mucus hypersecretion are orchestrated by Th2 cytokines such as IL-5, derived predominately from the airway leukocyte infiltrate (Foster et al., 2002; Lee et al., 1997). However, recent investigations have shown that apart from Th2 cells other cell types, including epithelial cells (Wu et al., 2010), goblet cells (Tanabe and Rubin, 2015) and innate lymphoid cells (Kim et al., 2013a), can contribute with the production of IL-5, or cooperate with Th2 cells to

produce IL-5 and induce airway eosinophilia. Our results showed that the amount of IL-5 in the BAL fluid and lungs from uvaol-treated mice substantially decreased. These results coincide with previous studies that reported the anti-allergic effect of other triterpenes, such as ursolic acid (Kim et al., 2013b), lupeol (Vasconcelos et al., 2008), and astilbic acid (Yuk et al., 2011), that were also able to significantly inhibit antigen-induced IL-5 production. Th2 cells orchestrate atopic asthma through the production of cytokines and other mediators, which contribute to establishing airway inflammation and remodeling. Thus, these observations support the notion that uvaol may affect IL-5-producing cells in OVA-challenged mice, once that infiltrating leukocytes and tissue cells can produce IL-5. However, future studies are required to determine which cell types producing IL-5 are sensitive to treatment with uvaol.

Somewhat surprisingly, uvaol exhibited anti-inflammatory activity, despite not having an effect on reactive oxygen species levels in leukocytes obtained from BAL of asthmatic mice. These observations show that uvaol has different effects on the production of cytokines and generation of reactive oxygen species, which may inhibit the recruitment of inflammatory cells by a mechanism (s) reactive oxygen species-independent. Moreover, in line with this evidence, an absence of correlation between anti-inflammatory activity and reactive oxygen species inhibition was also described with other natural compounds, such as apocynin and methoxyphenols (Houser et al., 2012).

Previous studies reported that the inhibition of the Th2 pathway using antibodies against IL-5 can be beneficial for patients with uncontrolled eosinophilic asthma (Nair et al., 2009; Wenzel et al., 2013). The exact mechanism by which uvaol reduces the allergen-induced inflammation remains unclear, although uvaol in human mononuclear cells mediates a decrease in IL-1 $\beta$  secretion (Marquez-Martin et al., 2006), a cytokine

important to expression of the adhesion molecule in eosinophils. Interestingly, Hakonarson et al. (Hakonarson et al., 1999) reported an autocrine interaction between IL-5 and IL-1 $\beta$ , where inhibition of IL-5 provokes the subsequent downregulation of IL-1 $\beta$ . Therefore, these results regarding IL-5 suggest that oral treatment with uvaol affects eosinophils by suppressing IL-5 production, a strategy that can be applied to relieve inflammation in allergic asthma. Additionally, it has been reported that inhibitors of phosphorylation of ERK1/2 are capable of suppressing the inflammatory response in an asthma model by reducing inflammation, remodeling, and mucus production in the airways (Duan et al., 2004). In addition, the ERK pathway is crucial for the release of Th2 cytokines, including IL-5, in PMBCs under allergic conditions (Liu et al., 2010). In addition, Martín and co-workers demonstrated that uvaol was able to reduce the phosphorylation of ERK1/2 in myofibroblasts *in vitro* (Martin et al., 2012b). Therefore, although speculative, the idea of uvaol having inhibitory effects on allergic reactions by these pathways cannot be dismissed. Further studies are necessary to investigate this concept.

In individuals with asthma, airway inflammation is associated with cellular and structural changes that result in thickening of the airway wall and airflow restriction (Bai, 2010). The count of collapsed alveoli as a bronchoconstriction index has been reported by others (Antunes et al., 2010). Likewise, in the current study, the histological assessment revealed that the allergic challenge in the asthmatic mice provoked changes in lung parenchyma noted as an increase in the number of collapsed alveoli. Our results demonstrated a dramatic reduction in alveolar collapse in the asthmatic mice treated with uvaol as compared to control. Studies in patients have shown that anti-IL-5 therapy improves the features of airway remodeling in asthmatic conditions (Huang et al., 2014; Sampson, 2001). Moreover, Martín et al. (Martin et al., 2012b) demonstrated that uvaol

could reduce the growth of myofibroblasts and decrease the perivascular fibrosis *in vivo*. Therefore, we may speculate that the reduction observed in the number of collapsed alveoli under uvaol treatment may be influenced by reduction in the expression of uvaol-induced IL-5. Collectively, the results herein provide new evidence demonstrating that treatment with uvaol might be a useful tool for preventing tissue changes associated with allergic inflammation. Thus, uvaol may contribute to attenuating the progression and chronicity of asthma.

Overall, to our knowledge, this study is the first to demonstrate that uvaol attenuates eosinophilic allergic inflammation, mucus secretion, and alveolar collapse in OVA-sensitized/challenged mice, a phenomenon that seems to involve the reduction of IL-5 concentration. These effects indicate that uvaol may have pharmacological effects that would be useful in the treatment of allergic diseases. However, further investigation is required to determine the specific mechanism by which uvaol accomplishes its effects.

## Acknowledgments

This project was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Programa de Cooperação Acadêmica/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and the Fundação de Amparo à Pesquisa do Estado de Alagoas (FAPEAL) (Brazil).

## Conflict of interest statement

The authors declare no conflict of interest.

## Reference

- Allouche, Y., Warleta, F., Campos, M., Sanchez-Quesada, C., Uceda, M., Beltran, G., Gaforio, J.J., 2011. Antioxidant, antiproliferative, and pro-apoptotic capacities of pentacyclic triterpenes found in the skin of olives on MCF-7 human breast cancer cells and their effects on DNA damage. *Journal of agricultural and food chemistry* 59, 121-130.
- Antunes, M.A., Abreu, S.C., Silva, A.L., Parra-Cuentas, E.R., Ab'Saber, A.M., Capelozzi, V.L., Ferreira, T.P., Martins, M.A., Silva, P.M., Rocco, P.R., 2010. Sex-specific lung remodeling and inflammation changes in experimental allergic asthma. *Journal of applied physiology* 109, 855-863.
- Bai, T.R., 2010. Evidence for airway remodeling in chronic asthma. *Current opinion in allergy and clinical immunology* 10, 82-86.
- Barnes, P.J., 2011. Pathophysiology of allergic inflammation. *Immunological reviews* 242, 31-50.
- Brightling, C.E., Symon, F.A., Birring, S.S., Bradding, P., Wardlaw, A.J., Pavord, I.D., 2003. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. *Thorax* 58, 528-532.
- Calixto, J.B., 2005. Twenty-five years of research on medicinal plants in Latin America: a personal view. *Journal of ethnopharmacology* 100, 131-134.
- Calixto, J.B., Campos, M.M., Otuki, M.F., Santos, A.R., 2004. Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Planta Med* 70, 93-103.
- Corren, J., 2011. Anti-interleukin-5 antibody therapy in asthma and allergies. *Current opinion in allergy and clinical immunology* 11, 565-570.

- Corren, J., 2012. Inhibition of interleukin-5 for the treatment of eosinophilic diseases. *Discovery medicine* 13, 305-312.
- de Melo, C.L., Queiroz, M.G., Fonseca, S.G., Bizerra, A.M., Lemos, T.L., Melo, T.S., Santos, F.A., Rao, V.S., 2010. Oleanolic acid, a natural triterpenoid improves blood glucose tolerance in normal mice and ameliorates visceral obesity in mice fed a high-fat diet. *Chemico-biological interactions* 185, 59-65.
- de Oliveira, A.M., de Araujo, A.F., Lyra Lemos, R.P., Conserva, L.M., de Souza Ferro, J.N., Barreto, E., 2015. Antinociceptive and anti-inflammatory activity of the siaresinolic acid, a triterpene isolated from the leaves of *Sabicea grisea* Cham. & Schltdl. var. *grisea*. *Journal of natural medicines* 69, 232-240.
- Duan, W., Chan, J.H., Wong, C.H., Leung, B.P., Wong, W.S., 2004. Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *Journal of immunology* 172, 7053-7059.
- Foster, P.S., Hogan, S.P., Ramsay, A.J., Matthaei, K.I., Young, I.G., 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *The Journal of experimental medicine* 183, 195-201.
- Foster, P.S., Hogan, S.P., Yang, M., Mattes, J., Young, I.G., Matthaei, K.I., Kumar, R.K., Mahalingam, S., Webb, D.C., 2002. Interleukin-5 and eosinophils as therapeutic targets for asthma. *Trends in molecular medicine* 8, 162-167.
- Fulkerson, P.C., Rothenberg, M.E., 2013. Targeting eosinophils in allergy, inflammation and beyond. *Nature reviews. Drug discovery* 12, 117-129.
- Gilabert, M., Marcinkevicius, K., Andujar, S., Schiavone, M., Arena, M.E., Bardon, A., 2015. Sesqui- and triterpenoids from the liverwort *Lepidozia chordulifera* inhibitors of bacterial biofilm and elastase activity of human pathogenic bacteria. *Phytomedicine : international journal of phytotherapy and phytopharmacology* 22, 77-85.
- Hakonarson, H., Maskeri, N., Carter, C., Chuang, S., Grunstein, M.M., 1999. Autocrine interaction between IL-5 and IL-1beta mediates altered responsiveness of atopic asthmatic sensitized airway smooth muscle. *The Journal of clinical investigation* 104, 657-667.
- Hill, R.A., Connolly, J.D., 2013. Triterpenoids. *Natural product reports* 30, 1028-1065.
- Houser, K.R., Johnson, D.K., Ishmael, F.T., 2012. Anti-inflammatory effects of methoxyphenolic compounds on human airway cells. *Journal of inflammation* 9, 6.
- Huang, W.C., Chan, C.C., Wu, S.J., Chen, L.C., Shen, J.J., Kuo, M.L., Chen, M.C., Liou, C.J., 2014. Matrine attenuates allergic airway inflammation and eosinophil infiltration by suppressing eotaxin and Th2 cytokine production in asthmatic mice. *Journal of ethnopharmacology* 151, 470-477.
- Kim, B.S., Wojno, E.D., Artis, D., 2013a. Innate lymphoid cells and allergic inflammation. *Current opinion in immunology* 25, 738-744.
- Kim, S.H., Hong, J.H., Lee, Y.C., 2013b. Ursolic acid, a potential PPARgamma agonist, suppresses ovalbumin-induced airway inflammation and Penh by down-regulating IL-5, IL-13, and IL-17 in a mouse model of allergic asthma. *European journal of pharmacology* 701, 131-143.
- Kips, J.C., O'Connor, B.J., Langley, S.J., Woodcock, A., Kerstjens, H.A., Postma, D.S., Danzig, M., Cuss, F., Pauwels, R.A., 2003. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *American journal of respiratory and critical care medicine* 167, 1655-1659.
- Lee, J.J., McGarry, M.P., Farmer, S.C., Denzler, K.L., Larson, K.A., Carrigan, P.E., Brenneise, I.E., Horton, M.A., Haczku, A., Gelfand, E.W., Leikauf, G.D., Lee, N.A., 1997. Interleukin-5 expression in the lung epithelium of transgenic mice leads to

- pulmonary changes pathognomonic of asthma. *The Journal of experimental medicine* 185, 2143-2156.
- Liu, J., Liu, L., Cui, Y., Zhang, J., Jiang, H., 2010. p38 MAPK regulates Th2 cytokines release in PBMCs in allergic rhinitis rats. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban* 30, 222-225.
- Liu, Y., Zhang, S., Li, D.W., Jiang, S.J., 2013. Efficacy of anti-interleukin-5 therapy with mepolizumab in patients with asthma: a meta-analysis of randomized placebo-controlled trials. *PloS one* 8, e59872.
- Marquez-Martin, A., De La Puerta, R., Fernandez-Arche, A., Ruiz-Gutierrez, V., Yaqoob, P., 2006. Modulation of cytokine secretion by pentacyclic triterpenes from olive pomace oil in human mononuclear cells. *Cytokine* 36, 211-217.
- Martin, R., Hernandez, M., Cordova, C., Nieto, M.L., 2012a. Natural triterpenes modulate immune-inflammatory markers of experimental autoimmune encephalomyelitis: therapeutic implications for multiple sclerosis. *British journal of pharmacology* 166, 1708-1723.
- Martin, R., Miana, M., Jurado-Lopez, R., Martinez-Martinez, E., Gomez-Hurtado, N., Delgado, C., Bartolome, M.V., San Roman, J.A., Cordova, C., Lahera, V., Nieto, M.L., Cachofeiro, V., 2012b. DIOL triterpenes block profibrotic effects of angiotensin II and protect from cardiac hypertrophy. *PloS one* 7, e41545.
- Martins, A., Vasas, A., Viveiros, M., Molnar, J., Hohmann, J., Amaral, L., 2011. Antibacterial properties of compounds isolated from *Carpobrotus edulis*. *International journal of antimicrobial agents* 37, 438-444.
- Myou, S., Leff, A.R., Myo, S., Boetticher, E., Tong, J., Meliton, A.Y., Liu, J., Munoz, N.M., Zhu, X., 2003. Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT. *The Journal of experimental medicine* 198, 1573-1582.
- Nagase, T., Lei, M., Robatto, F.M., Eidelman, D.H., Ludwig, M.S., 1992. Tissue viscance during induced constriction in rabbit lungs: morphological-physiological correlations. *J Appl Physiol (1985)* 73, 1900-1907.
- Nair, P., Pizzichini, M.M., Kjarsgaard, M., Inman, M.D., Efthimiadis, A., Pizzichini, E., Hargreave, F.E., O'Byrne, P.M., 2009. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *The New England journal of medicine* 360, 985-993.
- Nauta, A.J., Engels, F., Knippels, L.M., Garssen, J., Nijkamp, F.P., Redegeld, F.A., 2008. Mechanisms of allergy and asthma. *European journal of pharmacology* 585, 354-360.
- Passos, G.F., Medeiros, R., Marcon, R., Nascimento, A.F., Calixto, J.B., Pianowski, L.F., 2013. The role of PKC/ERK1/2 signaling in the anti-inflammatory effect of tetracyclic triterpene euphol on TPA-induced skin inflammation in mice. *European journal of pharmacology* 698, 413-420.
- Penido, C., Costa, K.A., Pennaforte, R.J., Costa, M.F., Pereira, J.F., Siani, A.C., Henriques, M.G., 2005. Anti-allergic effects of natural tetranortriterpenoids isolated from *Carapa guianensis* Aublet on allergen-induced vascular permeability and hyperalgesia. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* 54, 295-303.
- Rizzo, M.C., Sole, D., 2006. Inhaled corticosteroids in the treatment of respiratory allergy: safety vs. efficacy. *Jornal de pediatria* 82, S198-205.

- Rose, C.E., Jr., Lannigan, J.A., Kim, P., Lee, J.J., Fu, S.M., Sung, S.S., 2010. Murine lung eosinophil activation and chemokine production in allergic airway inflammation. *Cellular & molecular immunology* 7, 361-374.
- Rutkowski, K., Sowa, P., Rutkowska-Talipska, J., Sulkowski, S., Rutkowski, R., 2014. Allergic diseases: the price of civilisational progress. *Postepy dermatologii i alergologii* 31, 77-83.
- Sampson, A.P., 2001. IL-5 priming of eosinophil function in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 31, 513-517.
- Sheng, H., Sun, H., 2011. Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases. *Natural product reports* 28, 543-593.
- Siddiqui, S., Hollins, F., Saha, S., Brightling, C.E., 2007. Inflammatory cell microlocalisation and airway dysfunction: cause and effect? *The European respiratory journal* 30, 1043-1056.
- Sole, D., Filho, N.A., Sarinho, E.S., Camelo-Nunes, I.C., Barreto, B.A., Medeiros, M.L., Franco, J.M., Camargos, P.A., Mallol, J., Gurgel, R., de Andrade, D.M., Furlan, F.P., Silva, A.R., Cardozo, C., Andrade, C., 2014. Prevalence of asthma and allergic diseases in adolescents: nine-year follow-up study (2003-2012). *Jornal de pediatria*.
- Somova, L.O., Nadar, A., Rammanan, P., Shode, F.O., 2003. Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine : international journal of phytotherapy and phytopharmacology* 10, 115-121.
- Tanabe, T., Rubin, B.K., 2015. Airway goblet cells secrete pro-inflammatory cytokines, chemokines and growth factors. *Chest*.
- Vasconcelos, J.F., Teixeira, M.M., Barbosa-Filho, J.M., Lucio, A.S., Almeida, J.R., de Queiroz, L.P., Ribeiro-Dos-Santos, R., Soares, M.B., 2008. The triterpenoid lupeol attenuates allergic airway inflammation in a murine model. *Int Immunopharmacol* 8, 1216-1221.
- Wechsler, M.E., 2008. Combating the eosinophil with anti-interleukin-5 therapy. *The New England journal of medicine* 358, 1293-1294.
- Weltman, J.K., Karim, A.S., 2000. IL-5: biology and potential therapeutic applications. *Expert opinion on investigational drugs* 9, 491-496.
- Wenzel, S., Ford, L., Pearlman, D., Spector, S., Sher, L., Skobieranda, F., Wang, L., Kirkesseli, S., Rocklin, R., Bock, B., Hamilton, J., Ming, J.E., Radin, A., Stahl, N., Yancopoulos, G.D., Graham, N., Pirozzi, G., 2013. Dupilumab in persistent asthma with elevated eosinophil levels. *The New England journal of medicine* 368, 2455-2466.
- Wu, C.A., Peluso, J.J., Zhu, L., Lingenheld, E.G., Walker, S.T., Puddington, L., 2010. Bronchial epithelial cells produce IL-5: implications for local immune responses in the airways. *Cellular immunology* 264, 32-41.
- Yuk, J.E., Lee, M.Y., Kwon, O.K., Cai, X.F., Jang, H.Y., Oh, S.R., Lee, H.K., Ahn, K.S., 2011. Effects of astilbic acid on airway hyperresponsiveness and inflammation in a mouse model of allergic asthma. *Int Immunopharmacol* 11, 266-273.
- Zhang, W., Men, X., Lei, P., 2014. Review on anti-tumor effect of triterpene acid compounds. *Journal of cancer research and therapeutics* 10 Suppl 1, 14-19.
- Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16, 109-110.

**Fig. 1.** Chemical structure of uvaol.

**Fig. 2.** Effect of uvaol on total leucocytes (A), eosinophils (B), mononuclear cells (C), neutrophils (D) and IL-5 levels (E) induced by allergen into the pleural cavity from sensitized mice. Dexamethasone (Dex, 10  $\mu\text{mol/kg}$ ) was administered i.p. 1 h before the challenge. Each bar represents the mean  $\pm$  S.E.M. of six animals. +++  $P < 0.001$ ; ++  $P < 0.01$  and +  $P < 0.05$  as compared with the saline-challenge sensitized animals; \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  and \*  $P < 0.05$  as compared with the OVA-challenge sensitized animals.

**Fig. 3.** Effect of uvaol on airway inflammation. Dexamethasone (Dex, 10  $\mu\text{mol/kg}$ ) was administered i.p. 1 h before the challenge. Each bar represents the mean  $\pm$  S.E.M. of six animals. +++  $P < 0.001$  as compared with the saline-challenge sensitized animals; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$  as compared with the OVA-challenge sensitized animals.

**Fig. 4.** Effect of uvaol on intracellular reactive oxygen species levels generated by bronchoalveolar cells recovered from allergen-challenged mice. Columns indicate the mean  $\pm$  S.E.M. of 6 animals. +++  $P < 0.001$  as compared to sham-challenged group.

**Fig. 5.** Effect of uvaol on lung tissue inflammatory cell infiltration in mice. Panels show photomicrographs of lung preparations stained with hematoxylin and eosin from the saline-challenged (A), OVA-challenged (B), uvaol-treated (100  $\mu\text{mol/kg}$ ) OVA-challenged mice (C), uvaol-treated (200  $\mu\text{mol/kg}$ ) OVA-challenged mice (D), uvaol-treated (500  $\mu\text{mol/kg}$ ) OVA-challenged mice (E). Inflammatory score were shown in

panel (F). Data are expressed as mean  $\pm$  S.E.M. of 6 mice. ++  $P < 0.01$  as compared to the saline-challenged group. \*  $P < 0.05$  as compared to the OVA-challenged group.

**Fig. 6.** Effect of uvaol on mucus production in OVA-challenge mice. Panels show photomicrographs of lung preparations stained with Periodic Acid-Schiff (PAS), from the saline-challenged (A), OVA-challenged (B), uvaol-treated (100  $\mu\text{mol/kg}$ ) OVA-challenged mice (C), uvaol-treated (200  $\mu\text{mol/kg}$ ) OVA-challenged mice (D), uvaol-treated (500  $\mu\text{mol/kg}$ ) OVA-challenged mice (E). PAS-positive cells are indicated by arrows. Quantitative of mucus production was shown in panel (F). Data are expressed as mean  $\pm$  S.E.M. of 6 mice. ++  $P < 0.01$  as compared to the saline-challenged group. \*  $P < 0.05$  and \*\*  $P < 0.01$  as compared to the OVA-challenged group.

**Fig. 7.** Effects of uvaol on the IL-5 levels triggered by OVA challenge in the lung of mice. Each bar represents the mean  $\pm$  S.E.M. of 6 animals. +++  $P < 0.001$  as compared with the saline-challenge sensitized animals, \*\*\*  $P < 0.001$  as compared to the OVA-challenged group.

**Fig. 8.** Area of alveolar collapse in morphometric analysis. Panels show photomicrographs of lung preparations stained with H&E from the saline-challenged (A), OVA-challenged (B), uvaol-treated (100  $\mu\text{mol/kg}$ ) OVA-challenged mice (C), uvaol-treated (200  $\mu\text{mol/kg}$ ) OVA-challenged mice (D), uvaol-treated (500  $\mu\text{mol/kg}$ ) OVA-challenged mice (E). Percentage of alveolar collapse was shown in panel (F). \* indicates alveolar collapse. Data are expressed as mean  $\pm$  S.E.M. of 6 mice. ++  $P < 0.01$  as compared to the saline-challenged group. \*  $P < 0.05$  and \*\*  $P < 0.01$  as compared to the OVA-challenged group.









