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The triterpenoid lupeol attenuates allergic airway inflammation in a murine model

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Abstract

Asthma is a chronic inflammatory disease of the airways associated with a Th2 immune response. Despite their side effects, corticosteroids are the most used and effective drugs for treatment of asthma. In this work we investigated the efficacy of lupeol, a triterpenoid isolated from *Diplotropis ferruginea* Benth. (Fabaceae), in the treatment of bronchial asthma in BALB/c mice immunized with ovalbumin. Administration of lupeol caused the reduction of cellularity and eosinophils in the bronchoalveolar lavage fluid. Treatment with lupeol also reduced the production of mucus and overall inflammation in the lung. Levels of Type II cytokines IL-4, IL-5 and IL-13 were significantly reduced in mice treated with lupeol, an effect that was similar to that observed in dexamethasone-treated mice. In contrast, IgE production was not significantly altered after treatment with lupeol. In conclusion, our results demonstrate that lupeol attenuates the alterations' characteristics of allergic airway inflammation. The investigation of the mechanisms of action of this molecule may contribute for the development of new drugs for the treatment of asthma.

1. Introduction

The incidence of allergic diseases has increased considerably in recent decades, particularly in developed countries [1]. Asthma is an allergic disease associated with a wide range of signs and symptoms, including wheezing, cough, chest

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tightness, and shortness of breath [2,3]. The medical cost for treating asthmatic patients is high and on the increase [4]. Th2 cells are involved in the asthmatic response by releasing cytokines that are involved in the production of IgE [5–7]. While the exact etiology of asthma is still unknown, it is a clearly multifactorial pathology involving possible genetic predisposition coupled with exposure to certain environmental triggers [1]. The symptoms manifest as a result of airway inflammation, and exacerbations of bronchial asthma involve both episodic airway obstruction and marked airway inflammation [2,8]. All this together leads to marked airway oedema, impaired mucociliary function, and further impaired movement of airflow. If the latter changes persist, the inflammation associated with asthma can damage the respiratory epithelium and lead to remodeling of the airways [9–11].

Corticosteroids are the most commonly used drugs and have measurable effects on symptoms, lung function, bronchial responsiveness, and inflammation associated with asthma [12–14]. Side effects of chronic corticosteroid use include osteopenia, poor wound healing, hyperglycemia, hypertension and cataracts, and limit systemic administration [15,16]. Thus, the finding of substances with similar ability to limit inflammation associated with asthma but with no or lower toxicity is of great interest [17–19].

Medicinal plants are alternative options to conventional therapies to many diseases, and Brazil with its enormous biodiversity can contribute to the search of new natural products and bioactive molecules. *Diplotropis ferruginea* Benth. (Fabaceae) is a species found in the Atlantic rain forest of eastern Brazil. A decoction of *D. ferruginea* is drunk largely in the indigenous and folk medicine to treat inflammations and vaginal ulcers. It is also used in baths and washes external ulcers [20]. Previous chemical investigation has resulted in the isolation of flavonoids [21], benzenoids and a triterpene lupeol [22]. The flavonoid diplotropin isolated from *D. ferruginea* showed spasmolytic effect in guinea-pig ileum [23].

Several plant-derived secondary metabolites have been shown to interfere directly with molecules and mechanisms, such as the mediation of inflammatory responses and production/activity of second messengers, as well as with the expression of transcription factors and key pro-inflammatory molecules [24]. Triterpenes are regarded to be important as a potential natural source for medical compounds because of their wide range of biological activities [25]. Lupeol, a pentacyclic triterpene (Fig. 1), is a biologically active molecule that has received much attention due to its wide spectrum of medicinal properties

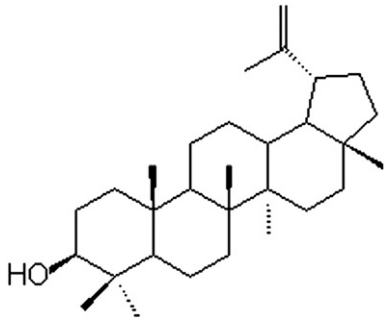

Figure 1 Chemical structure of lupeol.

that include strong antioxidant, antimutagenic, anti-inflammatory and antiartheritic effects, both in vitro and in vivo [26–30]. In this paper we explored the activity of lupeol isolated from *D. ferruginea* in an animal model of allergic airway inflammation.

2. Materials and methods

2.1. Plant material

The stem-bark of *D. ferruginea* Benth. was collected in September 2005 near the city of Caraúbas in the state of Rio Grande do Norte, Brazil and identified by the botanist, Dr. Maria de Fátima Agra, from the Laboratory of Pharmaceutic Technology of the Federal University of Paraíba, PB, Brazil. A voucher specimen (Agra 5559) was authenticated and deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Department of Systematics and Ecology, Federal University of Paraíba, PB, Brazil.

2.2. Extraction and isolation of lupeol

The crude ethanol extract (CEE) was obtained according to Almeida et al. (2003). The dried and powdered stem-barks (10 kg) of *D. ferruginea* were extracted with EtOH (95%), yielding, 413 g of crude ethanolic extract (CEE). The CEE was suspended in H_2O and partitioned with hexane and $CHCl_3$. The hexane extract (55.6 g) was dissolved in hot MeOH and left in a freezer for 24 h, yielding a precipitate which, after recrystallization from hexane-methanol, yielded pure lupeol (1.5 g).

2.3. Animals

Male BALB/c mice, 4–6 weeks old, were used in the experiments. All animals were raised and maintained at the animal facilities of the Gonçalo Moniz Research Center, FIOCRUZ, BA, in rooms with controlled temperature (22±2 °C) and humidity (55±10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (6 am–6 pm) and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

2.4. Sensitization and challenge with ovalbumin and treatment

Groups of seven mice received systemic immunization by subcutaneous injection of 10 μg of chicken egg ovalbumin (OVA; grade V, >98% pure; Sigma, St Louis, MO) diluted in 2 mg/ml alum (AlumImject; Pierce, Rockford, IL, USA) followed by a booster injection at day 14. A nasal challenge was performed starting at day 28, by inhalational exposure to aerosolised ovalbumin for 15 min/day, on five consecutives days. Exposures were carried out in an acrylic box. A solution of 1% ovalbumin in saline was aerosolised by delivery of compressed air to a sidestream jet nebuliser (RespiraMax, NS, Brazil). Two hours before each aerosol delivery, mice were treated orally with lupeol (60 mg/kg), dexamethasone (30 mg/kg) or vehicle (10% DMSO in saline).

2.5. Collection of blood and bronchoalveolar lavage

Twenty four hours after the last inhalational exposure, mice were anesthetized and bled via the brachial plexus for collection of blood samples used to estimate the IgE production. Bronchoalveolar lavage (BAL) was performed twice by intratracheal instillation of 1 ml of

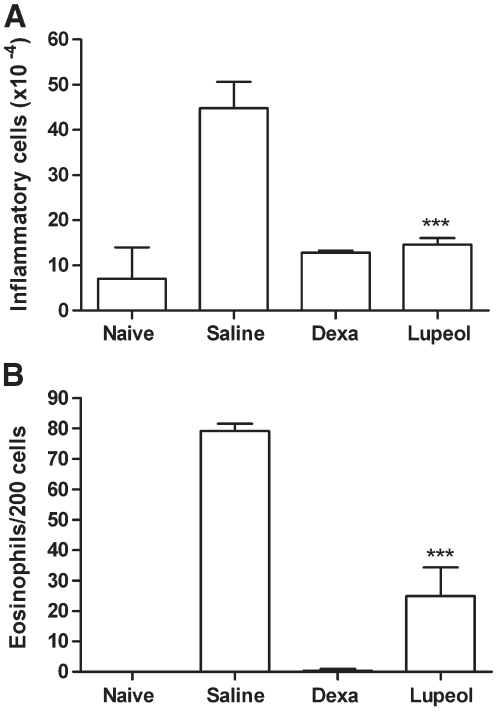

Figure 2 Leukocyte quantification in BAL samples obtained from mice submitted to different treatments. Mice were sacrificed 24 h after the last challenge with OVA. The cellularity in BAL fluid from normal or OVA-challenged mice treated with saline, dexamethasone (dexa) or lupeol was evaluated. (A) Total cell counts. (B) Number of eosinophils in 200 cells. Values are expressed as means ± SEM of 6–7 mice per group, in one of two experiments performed.

PBS. The first lavage fluid was centrifuged, and aliquots of the supernatant were kept at 70 °C until use for cytokine measurements. The second lavage fluid was centrifuged and the two cell pellets were resuspended in a PBS final volume of 1 ml. The number of total leukocytes in BAL fluid was estimated in a Neubauer chamber. Cytospin slides were prepared from BAL fluid and stained with Giemsa stain. At least 200 cells were counted per slide under light microscopy and they were differentiated according to standard morphological criteria.

2.6. Histopathological and morphometric analyses

The right lobe of the lungs from each animal was removed for histological analysis. The lung was inflated via the tracheal cannula with 4% buffered formalin, fixed in the same solution, and embedded in paraffin. Sections were stained with hematoxylin and eosin for quantification of inflammatory cells by optical microscopy. For each lung 10 fields (400×) were analyzed per section, and the data used to calculate the mean number of cells per mm^2. Mucus production was analyzed in periodic acid-Schiff (PAS)-stained sections. All images were digitalized using a color digital video camera (CoolSnap cf) adapted to a BX41 microscope (Olympus, Tokyo, Japan) calibrated with a reference measurement slide and were analyzed using Image Pro image program (version 6.1; Media Cybernetics, San Diego, CA, USA).

2.7. OVA-specific antibody levels and cytokine production

Immunoglobulin antibody levels to ovalbumin in sera samples from individual animals were quantified using an enzyme immunoassay modified from Jungsuwadee et al. (2004). For the measurement of OVA-specific IgG and IgG1, ELISA plates were coated with OVA at 10 μg/ml overnight at 4 °C. The plates were washed and blocked with PBS 5% non-fat milk for 1 h at room temperature. Sera diluted 1:100 were incubated for 2 h at room temperature. After washing, biotinylated anti-IgG1 or anti-IgG detection antibodies (American Qualex, San Clemente, CA, USA) were added and plates were incubated for 1 h. After incubation with streptoavidin–peroxidase conjugate, the reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate and read at 450 nm. OVA-specific IgE titers were determined by coating plates with anti-

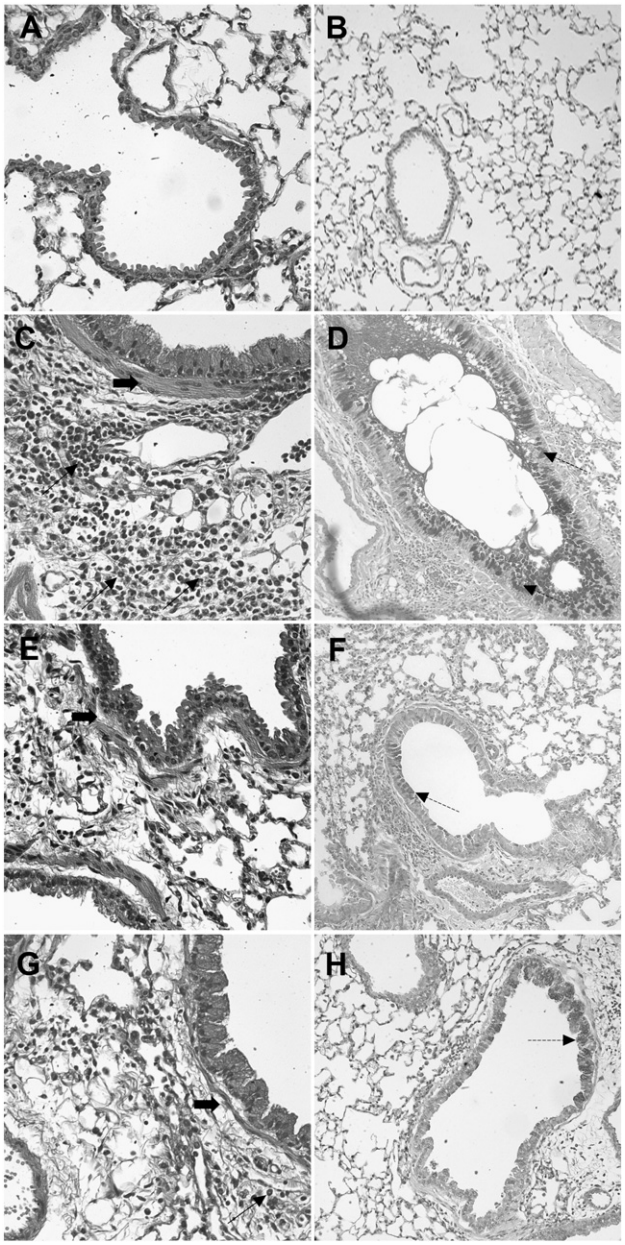

Figure 3 Histopathology of lungs from naïve and asthmatic mice. Lung sections of naïve (A, B) and asthmatic mice treated with vehicle (C, D), dexamethasone (E, F), and lupeol (G, H). Narrow arrows indicate eosinophils; large arrows indicate subepithelial fibrosis and prominent smooth muscle; dotted arrows indicate areas of PAS$^+$ cells. A, C, E, and G: H&E, 400×. B, D, F, and H: PAS, 200×.

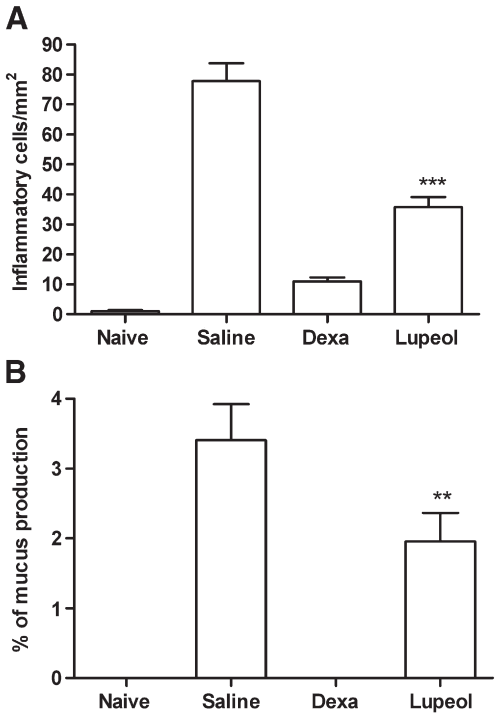

Figure 4 Quantification of inflammation and fibrosis in lungs of mice. (A) Intensity of inflammation in lungs of mice treated with saline, dexamethasone (dexa) or lupeol compared to naive animals. The number of inflammatory cells was evaluated on H&E-stained sections. (B) Analysis of mucus production on PAS-stained lung sections. The area of PAS staining was estimated by morphometric analysis. Data are expressed as means ± SEM of 7 mice per group, in one of two experiments performed.

mouse IgE (Pharmingem, San Diego, CA, USA) and detected with biotinylated-OVA (Fitzgerald, MA, USA). Concentrations of interleukin (IL)-4, IL-5 and IL-13 in BAL fluid were also determined by ELISA using specific antibody kits (R&D System, Minneapolis, MN, USA), according to manufacturer's instructions.

2.8. Statistical analysis

Results were expressed as means ± SEM of 7 mice per group. Statistical comparisons between groups were performed by analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test, using GraphPad InStat program (Software Inc., San Diego, CA, USA). Results were considered to be statistically significant when $P<0.05$.

3. Results

3.1. Treatment with lupeol reduces lung inflammation

To determine whether there were differences in lung inflammation between mice treated with lupeol and vehicle, BAL cytology was evaluated. The number of total BAL cells, which reflects the intensity of airway inflammation, was significantly reduced after lupeol treatment compared to vehicle-treated group (Fig. 2A). This inhibition was not statistically different from that caused by dexamethasone (about 3-fold of vehicle-treated controls). Lupeol (60 mg/kg, p.o.) treatment also decreased significantly the number of eosinophils in the BAL, although the number of eosinophils in BAL from mice treated with dexamethasone was statistically lower than that of lupeol-treated mice (Fig. 2B).

3.2. Histological evaluation of lungs from lupeol-treated mice

To characterize further the changes in lung pathology caused by antigen challenge of immunized mice, we examined lung sections stained with H&E. OVA challenge caused intense cell infiltrate containing many lymphocytes, macrophages and eosinophils (Fig. 3C). Mice treated with lupeol had reduced inflammation, particularly reduced eosinophil infiltration, although some less dense inflammatory foci remained (Figs. 3G and 4A). Dexamethasone treatment almost completely eliminated the inflammatory infiltrate, in particular eosinophils (Figs. 3E and 4A).

Lung sections were stained with PAS to analyze mucus overproduction. Lungs from naïve mice revealed rare PAS^+ cell in the respiratory epithelium compared to vehicle-treated

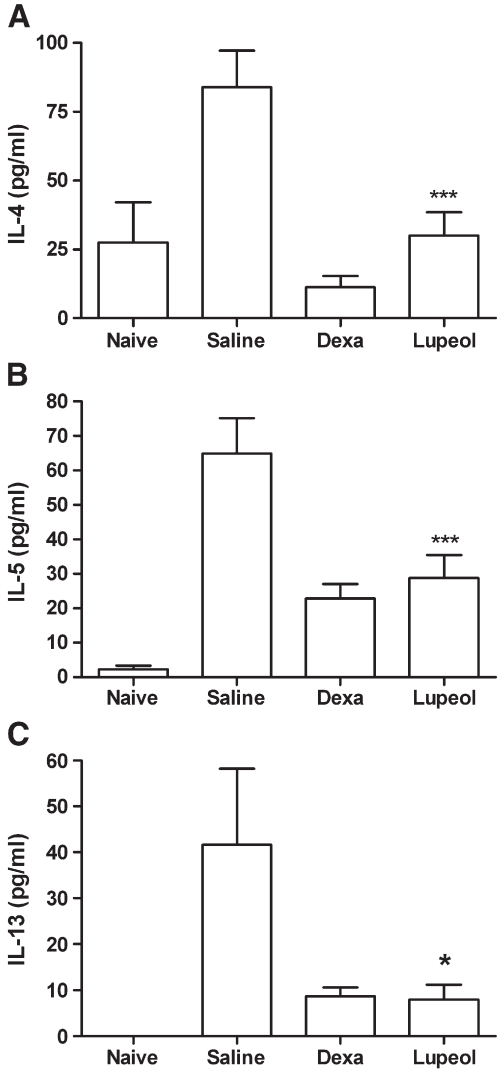

Figure 5 Decreased Type II cytokine production in lupeol-treated mice. IL-4 (A), IL-5 (B) and IL-13 (C) levels in individual mice from each experimental group were determined in BAL samples by ELISA. Data are expressed as means ± SEM of 6–7 mice per group. *$P<0.05$ and ***$P<0.01$.

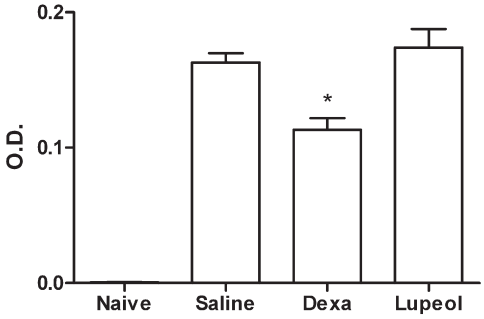

Figure 6 OVA-specific IgE antibodies in asthmatic mice. The levels of anti-OVA IgE antibodies were determined in serum samples from individual mice, by ELISA. The data are expressed as means ± SEM of 7 mice per group, in one of two experiments performed.

group, which had high PAS^+ staining (Figs. 3B and D; 4). Similar to dexamethasone, lupeol treatment was capable of modulating mucus production, decreasing the PAS^+ staining observed in the vehicle-treated group by 44% (Figs. 3 F and H; 4).

3.3. Treatment with lupeol modulated T-helper type 2 cytokine response

The production of cytokines in response to antigen challenge was studied in BAL fluid of individual mice from each group. As expected, the concentrations of Th2-associated cytokines IL-4, IL-5, and IL-13 were increased in OVA-immunized mice (Fig. 5). However, the levels of these cytokines were lower in lupeol-treated mice, compared to saline-treated group (two-fold for IL-5, three-fold for IL-4 and four-fold for IL-13). Levels of cytokines in lupeol-treated animals were similar to those of dexamethasone-treated mice (Fig. 5).

Ova-immunized mice treated with vehicle had high serum levels of anti-OVA IgE antibodies (Fig. 6). Whereas a statistically significant reduction in OVA-specific IgE antibodies was observed in mice treated with dexamethasone, no difference was found in lupeol-treated mice as compared to saline-treated controls (Fig. 6). Levels of total IgG or IgG1 OVA-specific were similar in asthmatic mice treated with vehicle, dexamethasone or lupeol (data not shown).

4. Discussion

Lupeol is a triterpenoid purified from many plant species used in popular medicine, as well as in a great variety of fruits and vegetables [30]. It has been demonstrated that lupeol has anti-inflammatory properties in experimental model of arthritis and it suppressed superoxide generation by human neutrophils induced by aracdonic acid [27–29]. However, the anti-inflammatory activity of lupeol has never been studied in a model of allergic airway inflammation.

In the present study we demonstrated that lupeol had potent anti-inflammatory activity in an allergic airway inflammation model induced by OVA administration in mice. This was evidenced by a marked reduction in eosinophil numbers in BAL fluid and in the lung, in Th2-associated cytokine levels and in mucus production.

In our study no signs of toxicity were observed in lupeol-treated mice. Previous studies also found that this triterpenoid did not cause any side effects even when administered in higher doses [27]. The fact that lupeol is present in a variety of edible fruits and vegetables also reinforces the potential safety of this substance for human use.

The effects of lupeol were similar to those of dexamethasone, a synthetic glucocorticoid commonly used as a gold standard anti-inflammatory drug. Glucocorticoids have a myriad of effects initiated by binding to their cytosolic receptors, translocating to the nucleus, and altering the regulation of inflammatory cytokine gene expression [16,31]. In allergic asthma, this results in the inhibition of macrophages, T-lymphocytes, eosinophils, and epithelial cells, reduced numbers of airway mast cells, reduced numbers of circulating and airway eosinophils, inhibit airway mucus secretion, and reduce histamine- and methacholine-induced airway responsiveness [13].

Lupeol reduced the production of PGE_2, TNF-α, and IL-1β in vitro [32], ear oedema induced by TPA in mice and paw swelling in an adjuvant arthritis model in rats [27]. The fact that lupeol does not show antinociceptive, anti-pyretic, and ulcerogenic effects indicate that this triterpene does not seem to act mainly by an inhibitory effect on PG synthetase, suggesting that the mechanism of anti-inflammatory action of lupeol is distinct from classical non-steroidal anti-inflammatory drugs [27].

In our study we observed effects similar to those of a glucocorticoid in an animal model of asthma. The demonstration that lupeol inhibits PGE_2, but not leukotriene C4 production, from activated macrophages [32] suggests that lupeol acts by a mechanism distinct of glucocorticoids, which strongly inhibit leukotriene C4 production [33]. Thus, the identification of the mechanisms of lupeol action may be of relevance, since glucocorticoids, the most commonly used class of drugs in asthma treatment, have a number of side effects.

The inhibition of Type II cytokine production by lupeol suggests that this substance acts inhibiting T cells. In fact, lupeol inhibits mitogen-induced lymphocyte proliferation (our unpublished results). Since IL-5 promotes the differentiation to and activation of eosinophils, lupeol may cause the reduction of eosinophil infiltrates found in lupeol-treated asthmatic mice.

The results of the present study show that lupeol attenuates inflammation in a murine model of asthma. The effects described herein, as well as those observed by other investigators, together with the broad spectrum of the biological effects of this substance, strongly suggest that lupeol has therapeutic potential for the treatment of asthma and other allergic diseases.

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