Ocimum gratissimum Linn. and rosmarinic acid, attenuate eosinophilic airway inflammation in an experimental model of respiratory allergy to Blomia tropicalis

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ABSTRACT

Allergic asthma has emerged as an important public health problem of urban populations in developed countries. Very often herbal medicine is used to treat this widespread disease, due to the lack of efficacy and the important side effects related to the classical drugs in use. Along this line, Ocimum gratissimum (Og) is a plant widely used in Brazilian folk medicine to treat inflammatory disorders, such as asthma.

In the present study we evaluated the immunomodulatory effects of Og and rosmarinic acid (RA, a polyphenolic compound) in a murine model of respiratory allergy induced by the Blomia tropicalis (Bt) mite. The respiratory allergy was induced in A/J mice by administration of Bt extract and the treatment was done using 25, 50 or 100 mg/kg of an Og methanolic extract or using 2, 20 or 200 mg/kg of RA. We then evaluated the changes induced by these drugs on immunological parameters related to the allergic process, which are up-regulated in this allergic model. The treatment of animals with 100 mg/Kg Og and 200 mg/Kg RA led to a significant reduction in the numbers of leukocytes/eosinophils in bronchoalveolar lavage (BAL); eosinophil peroxidase activity in BAL; presence of mucus in respiratory tract, histopathological changes in the lung, and IL-4 in BAL. These results suggest that the methanolic extract of Og and the polyphenol RA have therapeutic potential in this murine model of respiratory allergy to a clinically relevant human sensitizer allergen.

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1. Introduction

Asthma is now one of the commonest chronic diseases in the world, affecting over 300 million people, and its prevalence is rising, particularly in developing countries [1]. The prevalence of asthma in Brazil, where antigens from the Blomia tropicalis house dust mite are important sensitizing agents [2], is the 8th highest in the world [3]. Approximately 5–10% of patients have uncontrolled disease, despite taking inhaled therapy. These patients use a disproportionate amount of healthcare resources, as they are admitted to hospital, consume costly medication, and miss working days [4].

Although there are wide variations in the reported use of complementary and alternative medicine (CAM), a reasonable estimate is that up to 30% of adults and 60% of children with asthma in the USA are currently using some form of CAM to treat their condition [5].

Historically, herbal medicine has a great importance in the treatment of asthma. Various derivatives from medicinal plants were identified as antiasthmatic medicines, and some of their mechanisms of action were very well studied, such as those of α2 agonists, anticholinergics, methylxanthines and chromones [6]. The understanding of the chronic inflammatory scenario found in the airways of asthmatic patients led to the glycocorticoids being the gold standard drug in the treatment of allergic asthma [7]. The main disadvantage of these drugs is their undesirable side effects.

Based on the lack of an effective drug for asthma treatment without significant side effects, an ethnopharmacological survey was conducted by our research group in the city of Salvador, Bahia, Brazil, in order to find out the main natural products administered for the treatment of asthma in children, aiming at identifying plant species that could be the object of future studies as source of anti-asthmatic drugs [8].

One of this species was Ocimum gratissimum Linn (Labiatae), which is widely distributed in the tropics, is commonly used in folk medicine and has scientifically confirmed biological properties, such as antinociceptive [9], spasmolytic [10] and antibacterial [11] activities. Phytochemical studies revealed that O. gratissimum (Og) is rich in polyphenols, such as rosmarinic acid (RA) [12], which has

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recently been shown to have immunomodulatory activity, by suppressing T-cell receptor (TCR) signaling [13]. The rosmarinic acid from another plant species, *Perilla frutescens*, was able to prevent an eosinophilic airway inflammation in mice. These effects were associated with inhibition of the local expression of Th2 cytokines and chemokines [14].

Based on that, the objective of the present study, therefore, was to evaluate the effect of Og and the polyphenolic phytochemical RA in a murine model of respiratory allergy to *B. tropicalis* mite extract (Bt), and to investigate some of the immunological phenomena modulated by Og and RA, in order to elucidate the mechanism by which they may be exerting their effect on experimental allergy.

2. Materials and methods

2.1. Animals

Male AJ mice (25–30 g) were used throughout the study. Animals were maintained with free access to food and water. They were obtained from the animal facilities of the Fundação Oswaldo Cruz, Bahia, Brazil. Groups of 5 animals were used in each experiment. All the experimental procedures were approved by the Ethical Committee for Use of Experimental Animals of the Faculdade de Odontologia, Universidade Federal da Bahia, Brazil (protocol number: 02/09) and conducted according to international standards (http://grants.nih.gov/grants/olaw/GuideBook.pdf;http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

2.2. B. tropicalis extract

The *B. tropicalis* mites were cultivated in a fish food-containing standardized environment, purified with saturated NaCl and lysed in 0.15 M phosphate-buffered saline, pH 7.4 (PBS), in a blender (51BL30; Waring Commercial, Torrington, CO, USA). After several centrifugations with ether (9000 g for 10 min), for removal of lipids, the protein content was determined by Lowry's method [15] and the extract was stored at $-20\ °C$ until use. The *B. tropicalis* extract (Bt) was standardized by determining the Blo t 5 allergen concentration using a commercial capture ELISA (INDOOR Biotechnologies, Charlottesville, VI, USA). All used Bt batches contained 30–40 ng of this allergen per μg of protein.

2.3. O. gratissimum Linn. and its polyphenolic phytochemical rosmarinic acid

The methanolic (Og) and hexanic (OgHE) extracts of Og were prepared according to a technique previously described by Estrada-Soto and colleagues [16]. Og leaves were obtained at the Laboratório de Tecnologia Farmacêutica (LTF), Federal University of Paraíba, Brazil, and kept in a cool and airy environment for fifteen days for drying. The dry plant material was pulverized and crude extract were prepared by successive maceration process using methanol and hexane (3 times for 72 h at room temperature). After filtration, extracts were concentrated in vacuum at 40 °C. Rosmarinic acid (RA) or [(R)-O-(3,4-dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl) lactic acid 3,4-dihydroxycinnamic acid (R)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester-C18H16O8] was purchased from Sigma-Aldrich (catalog # 536954).

2.4. Standardization of O. gratissimum Linn. extract

The Og extract was standardized in terms of RA concentration by high performance liquid chromatography, with ultraviolet light detection at 330 nm, using a C18 column (250×4.6 mm ID, 5 μm particle size) and a C-18 pre-column (Phenomenex, Torrance, USA).

The mobile phase consisted of water acidified to pH 3.2 with formic acid (A) and acetonitrile (B) at a flow rate of 0.8 mL/min. The following gradient elution method was used for separation: 85% to 75% of A in 18 min, 75% to 45% of A in 7 min, 45% to 15% of A in 5 min, 15% to 85% of A in 5 min. A 20 μL sample was injected and the detection of RA was performed using light with a wavelength of 330 nm [12]. A control RA solution was injected at a concentration of 10.4 μg/mL.

2.5. Sensitization and challenge with B. tropicalis antigen

The murine model of respiratory allergy was induced as we previously described [17]. Briefly, A/J mice (n = 5) were initially sensitized with two subcutaneous injections (day 0 and day 7) of Bt (10 μg of protein), adsorbed to 4 mg/mL of Al(OH)$_3$ in saline (Fig. 1). Twenty-four hours after the last subcutaneous injection, the animals received three intranasal immunization boosters/challenges with Bt (10 μg/instillation) every other day, and, two days after the last immunization booster/challenge, they received a final intranasal challenge with 10 μg of Bt (Fig. 1). A negative control group received saline in both sensitization and challenge procedures. Twenty-four hours after the last challenge, the animals were euthanized with intraperitoneal injections of xilazine and ketamine (40 mg/kg/body weight).

2.6. Treatment with O. gratissimum Linn. and rosmarinic acid

The different groups were treated daily from the 8th to the 14th day of the experimental protocol, one hour after the intranasal challenges in the 8th, 10th, 12th, and 14th days (Fig. 1). The animals were treated orally with 25, 50 or 100 mg/kg of Og and, intraperitoneally, with 2, 20 or 200 mg/kg of RA [18] or orally/intraperitoneally with 3 mg/kg of Dexametazone (Dex). The RA was not administered orally due to the high rates of hydrolysis (up to 99%) held by the intestinal flora [19]. The groups of animals were named as: Control, non-sensitized and saline-treated mice; Bt, Bt-sensitized mice; Bt/Og$_{25}$; Bt/Og$_{50}$; Bt/Og$_{100}$, Bt-sensitized and 25, 50 or 100 mg/kg of Og-treated mice, respectively; Bt/RA$_2$; Bt/RA$_{20}$; Bt/RA$_{200}$, Bt-sensitized and 2, 20 or 200 of RA-treated mice; Bt/Dex, Bt-sensitized and Dex-treated mice.
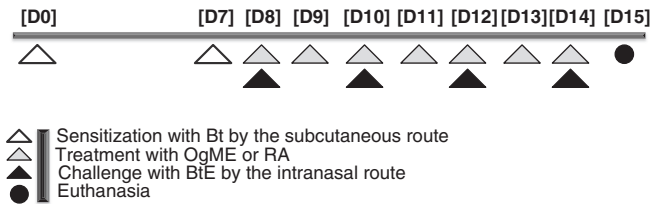
2.7. Bronchoalveolar lavage (BAL)

The trachea was cannulated and the lungs were carefully washed three times with 0.5 mL of PBS containing 1% of bovine serum albumin (BSA). The total number of leukocytes in the BAL was immediately determined in a hemocytometer, using Trypan blue. Differential cell counts were obtained by using May–Grunwald–Giemsa-stained cytospin preparations. A differential count of at least 100 cells was made in a blind fashion in accordance with standard morphologic criteria.

2.8. Eosinophil peroxidase (EPO) activity

The EPO activity in the cells obtained from the BAL was measured according to a previously described method [20]. Briefly, cell suspensions were frozen and thawed three times in liquid nitrogen. After centrifugation at 4 °C for 10 min at 1000 g, the cell lysates were placed into wells of 96-well plates (75 μL/well), followed by the addition of 150 μL of the chromogen and substrate solution (1.5 mmol/L of o-phenylenediamine and 6.6 mmol/L of H$_2$O$_2$ in 0.05 mol/L Tris–HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped with the addition of 75 μL of 0.2 mol/L citric acid, and the absorbance of the samples determined at 492 nm in an ELISA reader.

2.9. Levels of IL-4 in the bronchoalveolar lavage

The concentrations of IL-4 in the BAL were quantified by a standard ELISA, as recommended by the manufacturer (BD Pharmingen, USA).

[D0]   [D7] [D8] [D9] [D10] [D11] [D12] [D13] [D14] [D15]

△ Sensitization with Bt by the subcutaneous route
△ Treatment with OgME or RA
▲ Challenge with BtE by the intranasal route
● Euthanasia

Fig. 1. Experimental protocol for induction of respiratory allergy using aluminum hydroxide-adsorbed *Blomia tropicalis* extract (Bt) and assessment of the treatment with *O. gratissimum* methanolic extract (Og, 25, 50 or 100 mg/kg, orally) and rosmarinic acid (RA, 2, 20 or 200 mg/kg, intraperitoneally). [D0] to [D15], days 0 to 15 of experiments.

2.10. Histopathological analysis

The degree of peribronchiolar and perivascular inflammation was evaluated as described previously [14]. Briefly, lung tissues were fixed by inflation with freshly prepared 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin, for the assessment of cellular infiltration under optical microscopy with 200× magnification. The data on quantification of lung inflammation were acquired using the software Image-Pro Plus Version 6.1 (Media Cybernetics, San Diego, CA, USA) using the inflammatory area index.
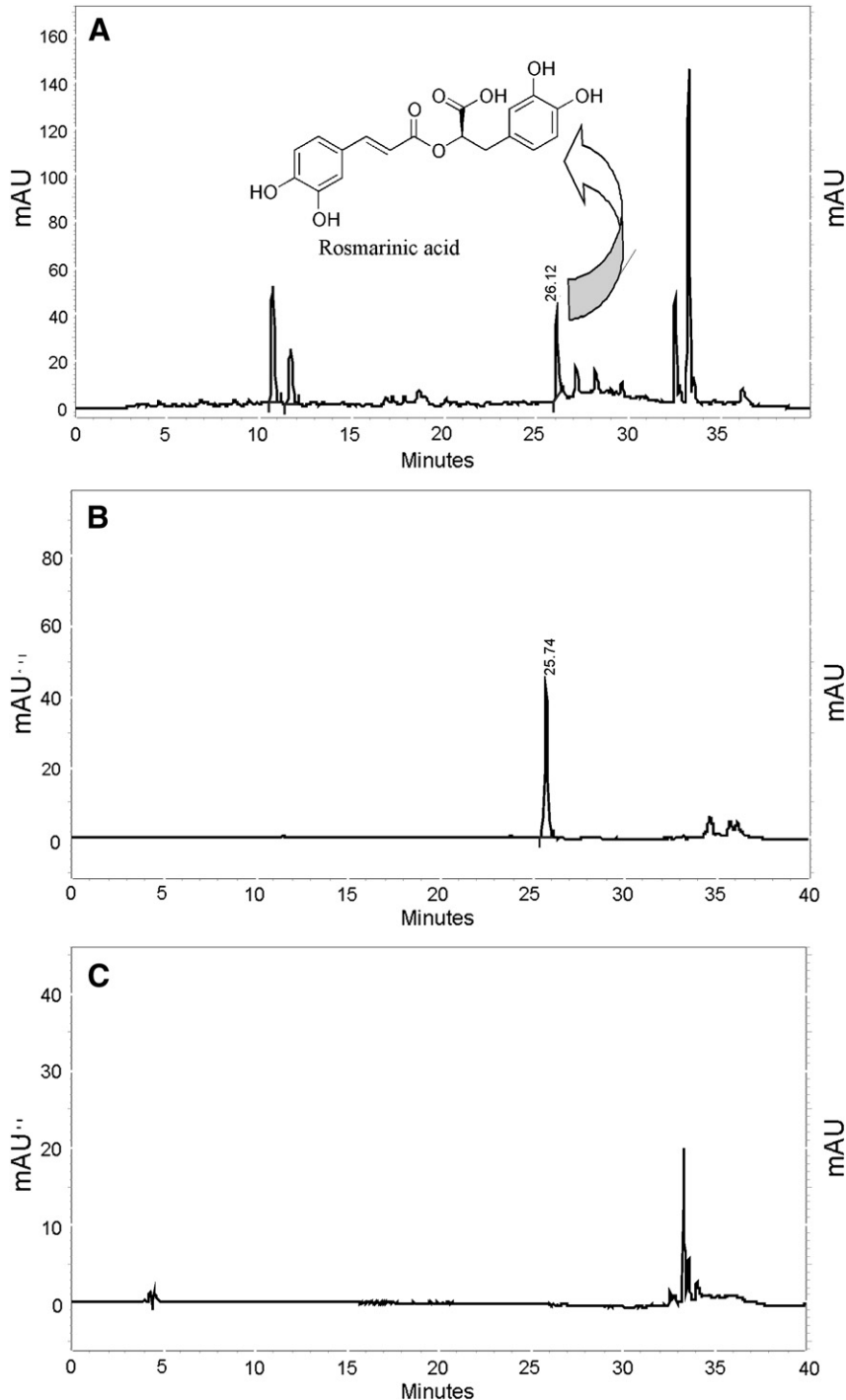
Fig. 2. Chromatogram of samples subjected to high performance liquid chromatography. Absorbance at 330 nm is shown at the Y axis. (A) Chromatogram of *O. gratissimum* methanol extract. (B) Chromatogram of rosmarinic acid. (C) Chromatogram of *O. gratissimum* hexane extract. Retention times are shown above peaks in (A) and (B).

Afterwards, we used the average from each animal/slide. Additionally, tissue sections were stained with periodic acid Schiff to assess mucus presence. A quantitative digital morphometric analysis was performed as described previously [17].

2.11. Measurement of anti-Bt IgE antibody levels in the BAL

Antibody levels were determined by ELISA using samples collected 24 h after the last Bt-challenge. In brief, wells of a 96-well microtitre high-binding plate (Costar) were coated with Bt (100 µg/mL) overnight, at 4 °C. The wells were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and blocked during 1 h with PBS-T containing 10% fetal calf serum (FCS) at room temperature (RT). After several washes with PBS-T, the mouse sera were added and incubated overnight at 4 °C. After this incubation period and washes, a biotin-conjugated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) was added in each well and incubated during 1 h at RT. A solution of avidin-horseradish peroxidase was then added to each well for 30 min. After washing, a solution containing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide was added and incubated during 30 min at RT and the reaction was stopped with 4 M sulfuric acid.

2.12. Statistical analysis

The one-way analysis of variance (ANOVA) and Tukey's post-test (for data with normal distribution) were used to determine the statistical significance between the experimental groups. Differences in p values ≤ 0.05 were considered statistically significant. Each experiment was repeated at least two times.

3. Results

3.1. Rosmarinic acid is present in the methanolic extract of O. gratissimum leaves

Fig. 2 shows the chromatogram of the methanolic extract of Og leaves (Fig. 2A), a RA solution (Fig. 2B) and an hexane extract of Og leaves (Fig. 2C), demonstrating the separation of a compound in the methanolic extract with the same retention time of RA in the standard sample (Fig. 2A and B). The estimated percentage of RA in the Og methanolic extract was 0.21% (based on peak area). On the other hand, the chromatogram of the hexane extract of Og (Fig. 2C) showed no RA characteristic peak, indicating the absence of the compound in that extract. Due to that, the immunopharmacological parameters were performed using the methanolic extract of *Ocimum gratissimum* leaves (coded thereafter as just Og).

3.2. Treatment with O. gratissimum methanolic extract and rosmarinic acid reduces the Bt-induced BAL eosinophilia

To assess the effects of Og and RA on the eosinophilic exudate in BAL of the Bt-sensitized and challenged mice, the presence of cells in the BAL was assessed 24 h after the last challenge. Bt-challenged mice displayed a significant increase of both total cells and eosinophils in relation to the control group ($p < 0.001$; $p < 0.05$, respectively; Fig. 3A and B). Oral administration of 100 mg/kg of Og, daily and 1 h after the Bt challenges, significantly suppressed the number of eosinophils and total inflammatory cells, in relation to the untreated Bt-immunized and challenged mice ($p < 0.01$; Fig. 3A and $p < 0.05$; Fig. 3B, respectively). Oral administration of 50 mg/kg of Og suppressed the number of eosinophils ($p < 0.05$; Fig. 3B), but did not modify the number of total inflammatory cells. The intraperitoneal
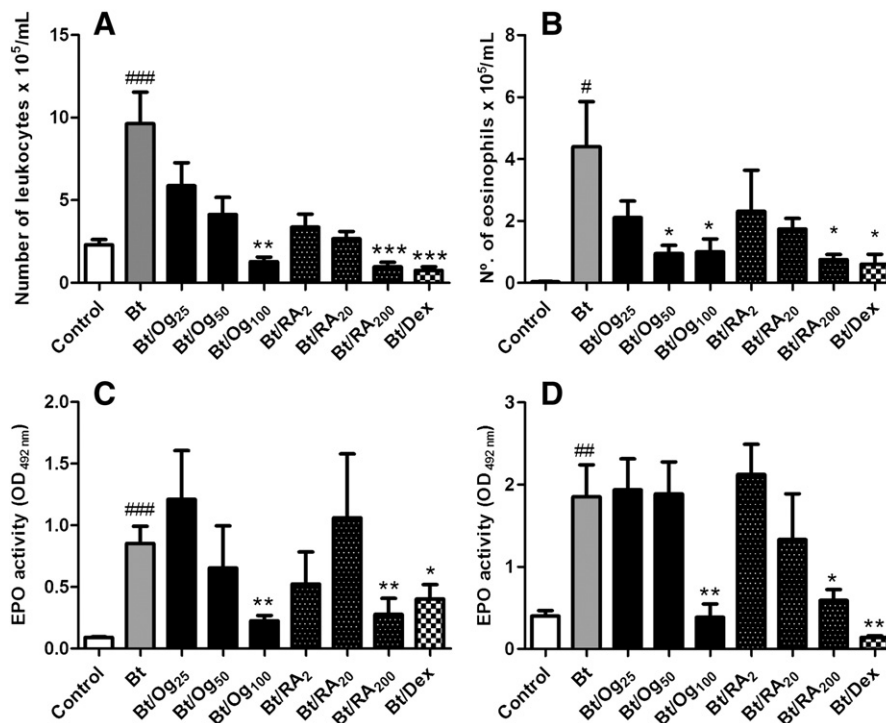
Fig. 3. Effect of treatment with *O. gratissimum* methanolic extract (Og) and with rosmarinic acid (RA) in the numbers of inflammatory cells, mainly eosinophils, in the airways of *Blomia tropicalis* extract (Bt)-immunized and challenged mice. (A) number of leukocytes in the bronchoalveolar lavage (BAL); (B) number of eosinophils in the BAL; (C) levels of EPO in the BAL; (D) levels of EPO in lung homogenates. Groups: Control, vehicle-treated animals; Bt, Bt-sensitized and challenged, and vehicle-treated mice; Bt/Og$_{25}$; Bt/Og$_{50}$; Bt/Og$_{100}$, Bt-sensitized and 25, 50 or 100 mg/kg of Og-treated mice, respectively; Bt/RA$_2$; Bt/RA$_{20}$; Bt/RA$_{200}$, Bt-sensitized and 2, 20 or 200 mg/kg of RA-treated mice, respectively; Bt/Dex, Bt-sensitized and challenged, and Dex-treated mice. Columns represent the mean values of the results obtained from six animals, and error bars represent the standard error from the means. # $p < 0.05$ vs control; ### $p < 0.001$ vs control; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs Bt group. ANOVA–Tukey.

administration of 200 mg/kg of RA was also able to significantly suppress the number of total inflammatory cells (p<0.001; Fig. 3A) and eosinophils (p<0.05; Fig. 3B). The groups Bt/Og$_{25}$, Bt/RA$_2$ and Bt/RA$_{20}$ did not show any significant change in cellular levels in the BAL. As expected, the administration of 3 mg/kg of Dex significantly suppressed the number of eosinophils and total inflammatory cells (p<0.01; Fig. 3A and B).

3.3. Treatment with O. gratissimum methanolic extract and rosmarinic acid reduces eosinophil peroxidase levels in BAL and lungs

The sensitization of the animals with Bt produced a significant increase of EPO activity in the BAL (p<0.001) and in the lungs (p<0.01) when compared to the control group (Fig. 3C and D). Treatment with O. gratissimum methanolic extract only at a dose of 100 mg/kg or with 200 mg/kg of RA decreased EPO activity in both BAL (p<0.01) and lung tissue (p<0.05) of Bt-immunized and challenged mice (Fig. 3C and D). As expected, the treatment with 3 mg/kg of Dex decreased EPO activity in both BAL (p<0.05) and lung tissue (p<0.001) of Bt-immunized and challenged mice (Fig. 3C and D).

3.4. Treatment with O. gratissimum methanolic extract and rosmarinic acid ameliorates the pathological changes of Bt-immunized animals

Histological evaluation of lung tissue revealed typical pathologic features of allergic asthma in the Bt-immunized mice, characterized by numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles (Fig. 4B). Treatment with 100 mg/kg of O. gratissimum methanolic extract (Fig. 4C), 200 mg/kg of RA (Fig. 4D) and Dex (Fig. 4E) markedly reduced the inflammatory cell infiltration within the peribronchiolar and perivascular regions. The reduction of inflammation was confirmed by quantification of inflammatory area (p<0.001; Fig. 4F).

3.5. Treatment with O. gratissimum methanolic extract and rosmarinic acid reduces the amount of mucus in the airways

To evaluate airway hypersecretion of mucus and goblet-cell hyperplasia, lung sections were stained with PAS. Mucus production was significantly induced in the airway of Bt-immunized and challenged mice (Fig. 5B). Treatment with 100 mg/kg of O. gratissimum methanolic extract (Fig. 5C), 200 mg/kg of rosmarinic acid (Fig. 5D)
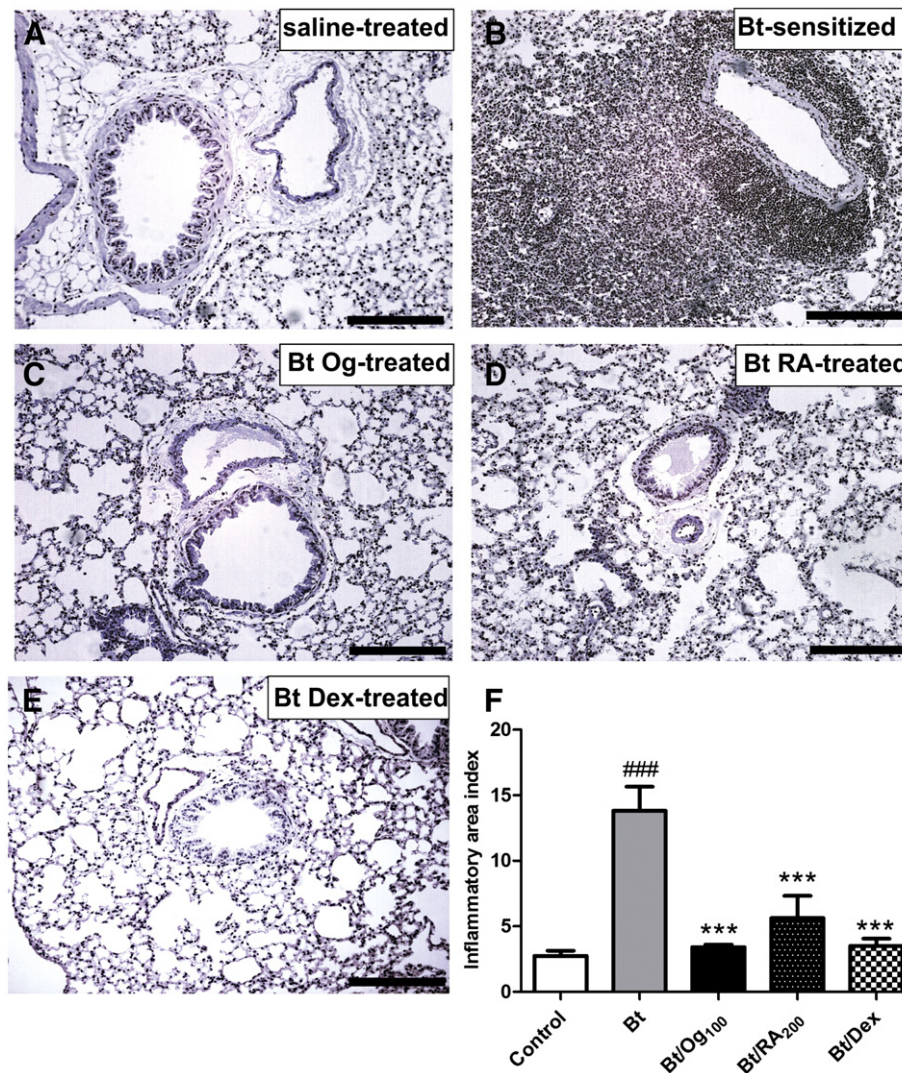
Fig. 4. Effect of treatment with *O. gratissimum* methanolic extract and rosmarinic acid (RA) on the production of mucus in the lung tissue of mice sensitized with Bt antigen. Sections were stained with periodic acid-Schiff (magnification×200). (A) Lung section from a control, saline-treated mice; (B) Lung section from a Bt-immunized and challenged, saline-treated mice; (C) Lung section from a Bt-immunized and challenged, *O. gratissimum* methanolic extract-treated mice; (D) Lung section from a Bt-immunized and challenged, RA-treated mice; (E) Lung section from a Bt-immunized and challenged, Dex-treated mice. (F) Quantification of lung inflammation. Scale bar, 100 μm. Each column represents the mean of inflammatory area index of 6 mice, and the vertical bars represent the standard deviation of the mean. ###p<0.001 vs control; ***p<0.001 vs Bt group, ANOVA–Tukey.
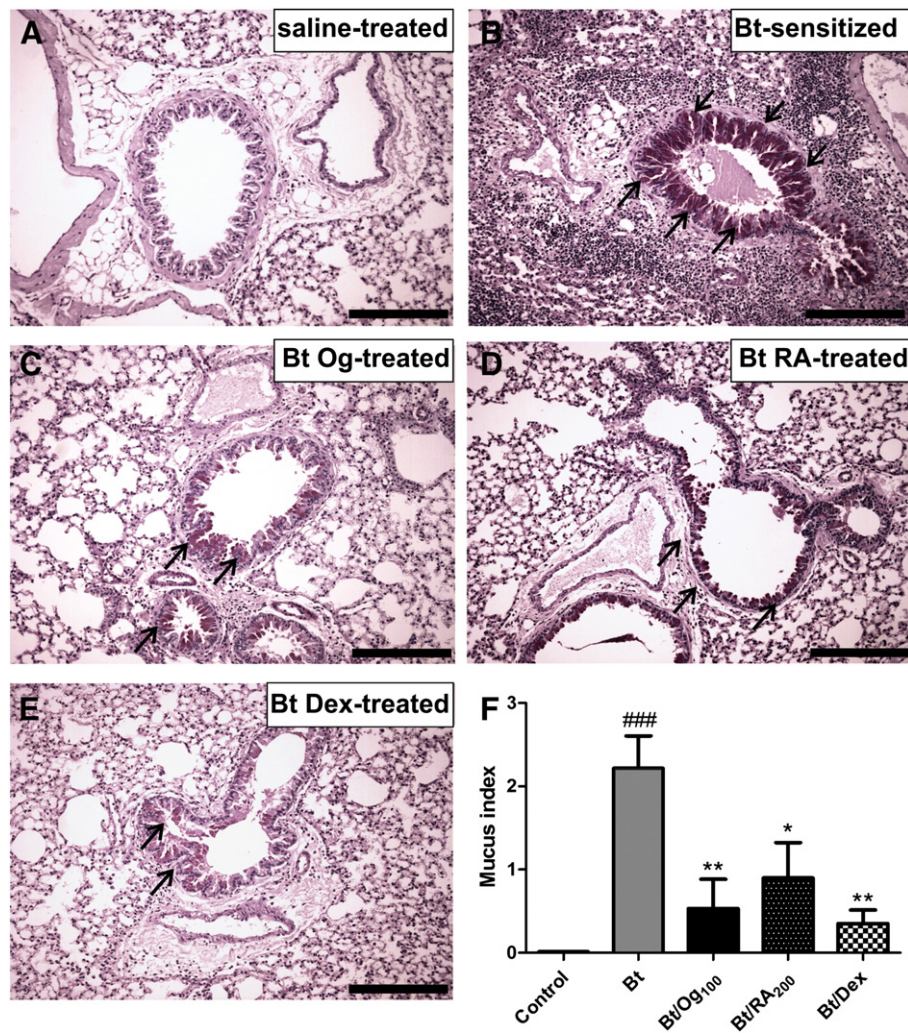
Fig. 5. Effect of treatment with *O. gratissimum* methanolic extract and rosmarinic acid (RA) on the production of mucus in the lung tissue of mice sensitized with Bt antigen. Sections were stained with periodic acid-Schiff (magnification×200). (A) Lung section from a control, saline-treated mice; (B) Lung section from a Bt-immunized and challenged, saline-treated mice; (C) Lung section from a Bt-immunized and challenged, 100 mg/kg of *O. gratissimum* methanolic extract-treated mice; (D) Lung section from a Bt-immunized and challenged, 200 mg/kg of RA-treated mice; (E) Lung section from a Bt-immunized and challenged, Dex-treated mice. (F) Mucus Index in the bronchi. Scale bar, 100 μm. Each column represents the mean of the mucus indexes of 6 mice, and the vertical bars represent the standard deviation of the mean. ###$p < 0.001$ vs control; *$p < 0.05$, **$p < 0.01$ vs Bt group, ANOVA. Arrows indicate the presence of mucus.

and Dex (Fig.5E) markedly suppressed mucus secretion in the lung tissue. The reduction of mucus production was confirmed by quantification of mucus (Bt/RA$_{200}$, $p < 0.05$; Bt/Og$_{100}$ and Bt/Dex, $p < 0.01$; Fig. 5F).

3.6. Treatment with rosmarinic acid and with O. gratissimum methanolic extract does not change the levels of Bt-specific IgE antibodies in the sera of Bt-immunized mice

Fig. 6 shows the levels of anti-Bt IgE antibodies in the sera of *O. gratissimum* methanolic extract- and RA-treated, Bt-immunized mice. Bt-immunized mice produced higher levels of specific IgE antibodies than control ($p < 0.01$). However, treatment with Og or RA, at tested doses, as well as the Dex did not reduce the IgE antibody levels.

3.7. Treatment with O. gratissimum methanolic extract and rosmarinic acid reduces levels of IL-4 in the BAL of Bt-immunized mice

To determine the possible mechanisms associated with the Og and RA effects in airway inflammation, levels of the T-helper type 2 (Th2) cytokine, IL-4 were evaluated. Levels of IL-4 in the BAL were higher in Bt-immunized and challenged mice than in the control group

($p < 0.05$). The treatment with 50 or 100 mg/kg of *O. gratissimum* methanolic extract ($p < 0.05$), 20 mg/kg ($p < 0.01$) or 200 mg/kg ($p < 0.001$) of rosmarinic acid and Dex ($p < 0.001$) led to significant reductions in levels of this Th2 cytokine in the BAL of Bt-immunized animals in relation to those of untreated, Bt-immunized animals (Fig. 7).

4. Discussion

The inflammatory response to allergens in the asthmatic lung is a consequence of infiltration of the airway wall by inflammatory cells, especially eosinophils and is associated with the increased expression of several inflammatory proteins in lung tissue, including cytokines, such as IL-4 [21]. The resolution of inflammation is an essential process for the establishment of appropriate host responses and the return to homeostasis [22].

The present study was conducted using a murine model of allergic airway disease induced by the sensitization to a common allergen, the *B. tropicalis* mite, which was previously characterized by our research group as leading to an increased number of eosinophils in the BAL fluid, to a marked influx of inflammatory cells into the lung around blood vessels and airways, and to airway luminal narrowing especially in A/J mice, the most sensitive to Bt amongst the
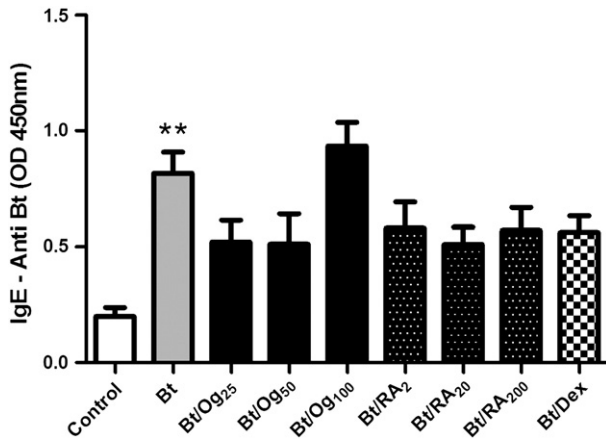
Fig. 6. Levels of anti-*Blomia tropicalis* extract (Bt) IgE antibodies in Bt-immunized mice and treated with methanolic extract of *O. gratissimum* and rosmarinic acid. Antibody levels were measured by indirect ELISA. Control (sensitized and treated animals with vehicle); Bt (Bt-challenged mice and treated with vehicle), Bt/Og$_{25}$; Bt/Og$_{50}$; Bt/Og$_{100}$, Bt-sensitized and 25, 50 or 100 mg/kg of Og-treated mice, respectively; Bt/RA$_{2}$; Bt/RA$_{20}$; Bt/RA$_{200}$, Bt-sensitized and 2, 20 or 200 mg/kg of RA-treated mice, respectively; Bt/Dex, Bt-sensitized and challenged, and Dex-treated mice. Columns represent the mean values of the results obtained from six animals, and error bars represent the standard error from the means. $^{\#\#}p<0.01$ vs control. ANOVA–Tukey.
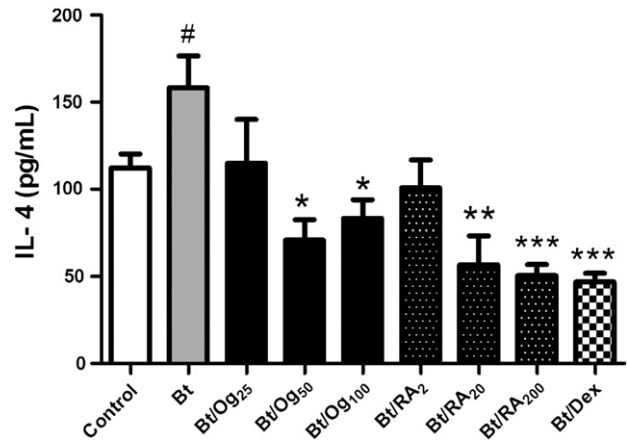
Fig. 7. Effect of the treatment with Og methanolic extract and rosmarinic acid on the levels of IL-4 in the BAL of Bt-challenged mice A/J. IL-4 quantification was done by sandwich ELISA. Control (vehicle-treated animals); Bt Bt-sensitized and challenged, and vehicle-treated mice;, Bt/Og$_{25}$; Bt/Og$_{50}$; Bt/Og$_{100}$, Bt-sensitized and 25, 50 or 100 mg/kg of Og-treated mice, respectively; Bt/RA$_{2}$; Bt/RA$_{20}$; Bt/RA$_{200}$, Bt-sensitized and 2, 20 or 200 mg/kg of RA-treated mice, respectively; Bt/Dex, Bt-sensitized and challenged, and Dex-treated mice. Columns represent the mean values of the results obtained from six animals, and error bars represent the standard error from the means. $^{\#}p<0.05$ vs control. * $p<0.05$, $^{**}p<0.01$ and *** $p<0.001$ vs Bt group. ANOVA–Tukey.

tested strains [17]. This allowed us to investigate the potential anti-allergic effect of an *O. gratissimum* extract, and the polyphenolic compound rosmarinic acid, in an experimental model of airway and lung inflammation induced by a clinically relevant aeroallergen.

O. gratissimum extracts have been shown to contain large amounts of polyphenolic compounds (flavonoids, stilbenes, phenolic acids and others), including RA [12,23]. Polyphenols have been shown to exert antiallergic, antiinflammatory, and bronchodilatory effects, by reducing the levels of inflammatory cytokines, chemokines, eosinophils and anti-allergen antibodies [24–26]. To assess the presence and the amount of RA in the Og used in this study, we analyzed the extract by HPLC, which allowed us to confirm the presence of RA in the Og (methanolic extract), but not in the hexane extract. The estimated amount of RA in the methanol extract was 0.2%, corroborating a previously published study [12].

An ethnopharmacology survey conducted by our research group, describing plant species used in the folk medicine to treat allergies [7], identified *O. gratissimum* as one of these plants. To date, however, no scientific study has confirmed this activity. Some biological activities exerted by *O. gratissimum* are attributed to its polyphenols that are present in high quantities in the plant [12]. In the present study, the treatment with Og and RA in Bt-sensitized and challenged mice resulted in a significant inhibition of airway and lung stroma inflammation, characterized by reduction in: (i) numbers of total inflammatory cells and eosinophils in BAL and lung; (ii) inflammatory cell infiltration in the peribronchiolar and perivascular pulmonary region; (iii) presence of mucus inside airways; (iv) levels of IL-4 in the BAL.

The administration of RA orally (100 mg/Kg) was not able to inhibit the inflammatory process (data not shown). This can be explained by the fact that 99% of this compound is degraded by intestinal bacterial flora [19]. Thus, the Og anti-inflammatory activity observed in this study is not attributed to RA exclusively, but in fact we believe that other compounds that may act synergistically could be responsible for its observed antiallergic effects.

The anti-inflammatory and immunomodulatory activities of RA has been ascribed to its inhibition of the lipoxygenase and cyclooxygenase pathways, interference with the complement cascade [27] and, mainly, the suppression of T-cell antigen receptor signaling [28]. These activities may explain, at least in part, the airway antiallergic activity of RA observed in this study.

Eosinophilia is a relevant pathological feature of allergic diseases, contributing to airway damage through the release of several cytotoxic mediators including EPO, eosinophil-derived major basic protein, eosinophil cationic protein and bronchoconstrictor mediators, such as leukotriene C4 (LTC4) [29]. Accordingly, the increased presence of eosinophils and their secreted products in the asthmatic lungs often correlates with severity and exacerbation of disease [30]. Additionally, the eosinophils have been shown to be a source of cytokines that are directly involved in the development of type I hypersensitivity, including IL-4, IL-5, and IL-13, suggesting that they have important roles in the immunopathology of allergic asthma [31,32].

Several studies attribute the antiallergic property of natural products to their ability to reduce the eosinophilic inflammatory process [29,33,34]. For example, an extract of *P. frutescens*, a species belonging to the same taxonomical family of *O. gratissimum*, attenuates allergic airway inflammation by inhibiting Th2 cytokines and eosinophil infiltration into the airways. This activity was attributed to the RA that was present in that extract despite the fact that the effect of pure rosmarinic acid was not evaluated and thus the contribution of other extract constituents cannot be ruled out [26]. Different mechanisms have been proposed to explain the reduction in lung eosinophilia induced by plant-derived products, such as the suppression of the synthesis and inhibition of the effects of eosinophil survival factors, and the direct induction of eosinophil apoptosis [35].

In order to explore the mechanism whereby Og and RA modulated eosinophils infiltration and activation we investigate the effect of these drugs on IL-4 production. Eosinophils activates cytokines and chemokines such as IL-4, IL-5 and eotaxin [36] which are involved in events related with airway infiltration and eosinophil activation, IgE production, and mucus secretion [37,38].

Increased mucus production by goblet cells in the airway epithelium is associated with airway inflammation and asthma. The data presented here demonstrated that Og and RA reduced the amount of mucus present in the airways in the Bt-induced experimental model of airway inflammation. IL-13 and IL-4 play an important role in the production of mucus [39]. Thus, the decrease in mucus in the airways of mice treated with Og and RA may be due to the inhibition of Th2 cytokines by these agents, which is supported by the reduced IL-4 levels that were found in the BAL of the treated mice. IL-4 promotes

the recruitment of eosinophils and stimulates B lymphocytes to synthesize IgE [40] thus, the reduction of this cytokine may exert an important antiasthmatic effect. High anti-allergen IgE antibody levels in the serum or BAL have been associated with airway hyper-responsiveness in both adults and children with asthma as well as contributing to the severity of the disease [41]. These antibodies activate events related to eosinophil and mast cell degranulation [42]. In this study, treatment with Og and RA did not significantly reduced the levels of *B. tropicalis*-specific IgE and this might be explained by the fact that although the active inflammatory response and cell migration are modulated, the IgE levels are already up-regulated in allergic animals. Thus, due to the half-life of IgE antibody its detection is not possible in a short-term protocol like ours, even when animals were treated with Dex. A possible effect of these drugs in the production of *B. tropicalis*-specific IgE antibodies should be assessed in a chronic experimental model of *B. tropicalis*-induced respiratory allergy that has a longer duration of treatment and evaluation time points.

In addition, based on the fact that RA is more active in reducing EPO activity in BAL rather than in lungs, we speculate that besides IL-4, RA may also modulate other cytokines and/or chemokines that can regulate the eosinophil migration from the lung tissue to the alveoli. This might reflect less eosinophils in the bronchoalveolar lavage and consequently less EPO.

The main limitation of this study was using only one mice strain and one common allergen for the sensitization protocol. We believe that other strains (not yet evaluated) or even other common sensitizing agent such as *Dermatophagoides pteronissinus* could be useful in future studies to investigate action and mechanisms of anti-allergic drug candidates. Another limitation of the study was related to the lung inflation protocol used as fixed-volume inflation rather than fixed-pressure inflation.

However, we believe that the results presented herein, obtained using an experimental model clinically relevant to human beings strongly support the potential usefulness of Og and RA as anti-inflammatory agents for the treatment of allergic asthma. In addition, these findings justify the need of further experiments to elucidate the molecular mechanisms underlying the Og and RA immunomodulatory effects.

Abbreviations

BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
Bt	*Blomia tropicalis*
CAM	Complementary and alternative medicine
ELISA	Enzyme-linked Immunosorbent Assay
EPO	Eosinophil peroxidase
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
i.n.	Intranasal route
i.p	Intraperitoneal route
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
LTC4	Leukotriene C4
MBP	Eosinophil-derived major basic protein
MeOH	Methanol
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NFIL3	Nuclear factor, interleukin 3 regulated
Og	*Ocimum gratissimum* Linn.
RA	Rosmarinic acid
RPM	Rotations per minute
s.c.	Subcutaneous route
STAT6	Signal transducer and activator of transcription 6
TCR	T-cell antigen receptor
v.o.	Oral route

Conflict of interest

All authors declare they have no competing financial interests.

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