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Antiasthmatic Effect of Eugenol (4-Allyl-2-Methoxyophenol) Mediated by Both Bronchodilator and Immunomodulatory Properties

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Abstract: Eugenol, an aromatic product, exhibits anti-inflammatory properties, however, little is known about its effect in allergic inflammation mediated by Th2-type cells and cytokines. We examined the pharmacological potential of this compound in a murine model of respiratory allergy to Bt (*Blomia tropicalis*). For this, AJ mice were sensitized (100 μg/animal s.c.) and challenged (10 μg/animal i.n.) with Bt mite extract. Sensitized animals were treated or not with eugenol (20, 40 or 80 mg/kg) and the following parameters were analyzed: number of total cells in BAL (bronchoalveolar lavage); EPO (eosinophil peroxidase activity) and histopathological changes in the lungs; serum level of specific IgE, IgG1 and IgG2a and; concentration of Th2 cytokines on BAL and spleen cultures. In addition, the capability of eugenol in relaxing tracheal smooth muscle was also evaluated. Our results showed that treatment with eugenol significantly reduced the airway inflammation, decreasing the cellular infiltrate, EPO and mucus in the lungs, as well as the production of Th2 cytokines. It was also demonstrated a dilator effect of eugenol observed by the relaxation of normal and hyper-reactivity isolated trachea upon carbachol stimulation. These results suggest that eugenol has potential as an anti-asthmatic drug by both bronchodilator and immunomodulatory properties.

Key words: Asthma; *Blomia tropicalis*, natural products, eugenol.

List of Abbreviations

BAL: Broncho alveolar lavage
BK: BradykininCch, carbachol
ELISA: Enzyme immunoassay test
Eug: Eugenol
EPO: Eosinophil peroxidase
Emax: Maximum relaxation
BHR: Bronchial hyper-reactivity
HBSS: Hanks' balanced salt solution
IL: Interleukin
i.n.: Intranasal
IgE: Immunoglobulin E-type
LPS: Lipopolysaccharide
NF-Kb: nuclear factor of activated B cells potentiating
OVA: Ovalbumin
PAS: Periodic Acid Schiff
PBS: Phosphate buffered solution bisódica
PWM: Pokweed (mitogen)
RPM: Revolutions per minute
s.c.: Subcutaneous
Th2: T helper lymphocyte type 2
v.o.: Oral

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1. Introduction

Atopic asthma is a chronic inflammatory disorder associated with hyperresponsiveness of the lower airways and variable airflow limitation [1-3]. It is characterized by a Th2-dominant response where interleukin-4 (IL-4), IL-5, and IL-13 are involved in the coordination, amplification and perpetuation of the inflammatory response by attracting additional inflammatory cells, mainly eosinophils, increasing mucus production and serum IgE (immunoglobulin E) Ab (antibody) levels [4-6].

Although current treatments are able to control symptoms and improve lung function in most patients, in severe asthma, acute exacerbations still occur and contribute significantly to morbidity and mortality of asthma in all age groups [2, 7]. Historically, herbal medicine has a great importance on asthma treatment. Four fifths classes of drugs currently are in use to treat asthma-namely, α2 agonists, anticholinergics, methyl xanthines and cromones-have herbal origins [8, 9].

Eugenol (4-allyl-2-methoxyphenol) is an aromatic compound, a member of the phenyl propanoids compounds. It is found in several species, especially in cloves, nutmeg [10] and *Ocimum gratissimum* whose immunomodulatory property was recently demonstrated in an experimental model of allergic asthma [11]. Eugenol is commonly used as a flavoring agent in cosmetics and food products and, in particular, in dentistry in zinc oxide-eugenol chelating cement. Its antioxidant and anti-inflammatory activities have been already investigated. Several studies have pointed out the anti-inflammatory effects of eugenol, reporting that this oil was able to modulate inflammatory markers such nitric oxide, iNOS, prostaglandin E2, mast cell degranulation and the transcription nuclear factor Kappa B (NF-kB) [12-18]. Also, it has been shown that phenolic compounds have a relaxing effect on tracheal smooth muscle from guinea pigs and may have bronchodilator effect [19]. However, little is known about the effect of eugenol in allergic inflammation mediated by cells and Th2-type cytokine.

Since eugenol exhibit anti-inflammatory properties and a possible relaxing effect on smooth muscle, we examined the pharmacological potential of eugenol in a murine model of respiratory allergy to Bt (*Blomia tropicalis*) mite.

2. Materials and Methods

2.1 Animals

Females AJ mice (20-25g) were obtained in the animal facilities from the Fundação Oswaldo Cruz, Bahia, Brazil and were used throughout the study. Animals were maintained with free access to food and water. 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).

2.2 Experimental Groups and Eugenol Oral Treatment

To investigate the anti-allergic effect of eugenol, we performed an experimental model of allergy to *Blomia tropicalis* dust mite as previously described [20]. As positive control, A/J mice (n = 5) were sensitized with two subcutaneous injections (at day 0 and day 7) of Bt (100 μg of protein), adsorbed to 4 mg/mL of $Al(OH)_3$ in saline. Twenty-four hours after the second subcutaneous injection, the animals received four intranasal boosters/challenges with Bt (10 μg/instilation) every other day. One day after the last challenge, the animals were euthanized with intraperitoneal injections of xylazine and ketamine (40 mg/kg/body weight). Mice sensitized and challenged with $Al(OH)_3$ in saline alone (vehicle) were considered negative controls. The tested groups were animals sensitized as the positive control and daily treated orally with 20, 40 or 80mg/kg of eugenol, obtained commercially from Sigma-Aldrich® (St. Louis, MA, USA), dissolved in 1% of Tween 20 in saline from the 8th to the 14th day of the experimental

protocol and one hour after the intranasal challenges with Bt. The groups of animals were defined as: Non-sensitized and vehicle-treated mice (control group); Bt, Bt-sensitized mice and vehicle-treated mice (positive control group); Bt/Eug 20, Bt-sensitized and eugenol 20mg/kg treated mice; Bt/Eug 40, Bt-sensitized and eugenol 40mg/kg treated mice; Bt/Eug 80, Bt-sensitized and eugenol 80mg/kg treated mice (tested groups). To determine the *in vivo* doses of eugenol, pilot studies were carried out using doses between 80 mg/kg and 160 mg/kg (this last dose was based in previous studies evaluating anti-inflammatory effect of this compound) [18]. As 160 mg/kg and 80 mg/kg had the same effect (data not shown), we choose the effective dose of 80 mg/kg and in addition we included 40 mg/kg and 20 mg/kg doses aiming to obtain a dose-response curve.

2.3 BAL (Bronchoalveolar Lavage)

The trachea was canulated and the lungs were carefully washed with 1.5 mL of phosphate buffered saline, pH 7.4 (PBS) containing 1% of bovine serum albumin (Sigma-Aldrick St. Louis, MA, USA). The total number of leukocytes in the BAL was determined using Trypan blue. Differential cell counts were obtained by using Wright-stained cytospin preparations. Differential counts of at least 100 cells were made in a blind fashion in accordance with standard morphologic protocol. The concentrations of IL-4 and IL-10 in BAL, were quantified by sandwich-ELISA, as recommended by the manufacturer (BD Pharmingen, city, State, USA).

2.4 EPO (Eosinophil Peroxidase) Activity in Lung Cell Lysates

The EPO activity in lung cells was evaluated according to a previously described method [21]. Briefly, cell suspensions were frozen and thawed three times in liquid nitrogen. After centrifugation at 4 ^{o}C for 10 min at 1,000 g, the cell lysates were placed into wells of 96-well plates (75 μL/well), followed by the addition of the chromogen and substrate solution (1.5 mmol/L of o-phenylenediamine and 6.6 mmol/L of H_2O_2 in 0.05 mol/L Tris-HCl, pH 8.0). After 30 min, the reaction was stopped with the addition of 0.2 mol/L citric acid, and the absorbance of the sample determined at 492 nm in an ELISA reader.

2.5 Histopathological Analysis

The histopathological changes were assessed as described previously [22]. Briefly, lung tissue was fixed using 10% (v/v) formaldehyde (Sigma-Aldrich St. Louis, MA, USA). The tissue was dehydrated, embedded in paraffin and cut in 5 μm sections which were stained with hematoxylin and eosin for evaluation of cellular infiltration and with periodic acid Schiff to assess mucus, under light microscopy with 40x magnification.

2.6 Measurement of Anti-Bt IgE, IgG1 and IgG2a Antibody Levels in Serum

Anti-Bt antibody levels in the serum of mice from the different experimental groups were determined by indirect ELISA. 96-well micro titer high-binding plate (Costar, Cambridge, MA, USA) were coated with Bt (100 μg/well) overnight, at 4 ℃, and blocked during 1 hour with PBS-T containing 10% fetal calf serum (FCS, Gibco, Pisley, UK) at RT (room temperature). After this incubation period, the serum samples were added and the plates were incubated overnight at 4 ℃. Biotin-conjugated IgE, IgG1 or IgG2a anti-mouse (BD Pharmingen, San Diego, CA, USA) were added to the wells and incubated during 1 h at RT. A solution of avidin-horseradish peroxidase (BD Pharmingen, San Diego, CA, USA) was then added to each well for 30 min. Finally a solution containing 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (BD Pharmingen, San Diego, CA, USA) was added and incubated during 30 min at RT and the reaction was stopped with 4 M sulfuric acid. The absorbance of the sample was determined at 492 nm in an ELISA reader.

2.7 In vitro Cytokine Production in Spleen Cells of Bt-Sensitized Mice Cultivated in the Presence of Different Concentrations of Eugenol

In vitro estimation of cytokines concentration produced by spleen cells treated *in vitro* with eugenol was performed according to the method described by Bezerra-Santos et al. [23]. In brief, spleen cell suspensions from non-sensitized and Bt-sensitized groups were obtained and washed twice in RPMI medium by centrifugation at 200 g for 10 min. The pellet was resuspended in RPMI medium supplemented with 200 mM l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 5-Mercaptoethanol and 10% FCS (Gibco, Pisley, UK). A total of 5×10^5 viable cells were placed in each well. The cells were incubated with eugenol at different non-cytotoxic concentrations (12.5-200 μm) determined by MTT test (data not shown), and Pokeweed (PWM) (Sigma, St Louis, MA, USA) at 5 μg/mL for 48 h. Supernatants from the cell culture were removed for cytokine measurement by enzymatic immunoassay with their respective antibody pairs following manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

2.8 Effect of Eugenol on Normal and Hyperresponsive Airway Smooth Muscle from AJ Mice

Normal AJ mice were euthanized by overdose of xylazine and ketamine and the tracheae were rapidly removed, cleaned of connective tissue and washed three times with Krebs-bicarbonate solution (composition in mM: NaCl 119, $NaHCO_3$ 25, $CaCl_2 \cdot H_2O$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2 and glucose 11.1). Following, the tracheae were sectioned into rings of 2 mm, containing on average three to four cartilage bands. The rings were suspended on metal rods, attached to a force transducer (FORT10 WPI, Sarasota, USA) and placed in tanks for isolated organ, maintained at 37 ℃ and aerated with a carbogen mixture (95% O_2 and 5% CO_2). The rings were subjected to stabilization for a period of 1 h at 0.5 g. After the stabilization period, the rings were contracted with 10 μM of carbachol (Cch; Sigma, St Louis, MA, USA) to assess the contractile state of the tissue. To evaluate the presence of functional epithelium, the rings were stimulated with bradykinin (Bk; Sigma, St Louis, MA, USA) (10^{-6} M). After stabilization and assessment of the presence of functional epithelium, the rings were washed, again contracted with carbachol (10 μM) and were added cumulatively and increasing concentrations of eugenol. Concentration response curve was constructed and the data analyzed.

To assess the effects of eugenol on the hyper-responsive airway smooth muscle, tracheal rings were previously exposed in culture to IL-13 as previously described [24]. Tissues were placed individually in multiwell plates containing Dulbecco's modified Eagle's medium (containing 25 mM D-glucose, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.2 M L-glutamine, 2.5 μg/mL Fungizone, and 0.1% w/v bovine serum albumin) (Sigma, St Louis, MA, USA). Tracheal segments were incubated at 37 ℃ in a humidified CO_2 gassed incubator in the presence or absence of IL-13 (10 ng/mL, 24 h; BD Pharmingen, San Diego, CA, USA). Cumulative concentration-response curves to CCh after incubation in the absence or presence of IL-13 was built to analyze the hyper reactivity of smooth muscle. After that, concentration-response curve for eugenol was built.

2.9 Statistical Analysis

The ANOVA (one-way analysis of variance) and Tukey's post-test (for data with normal distribution by Kolmogorov-Smirnov test) 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. Concentration response curve for isolated organ experiments and all

the graphs included in this study were performed using GraphPad Prisma 5.

3. Results

3.1 Treatment with Eugenol Reduces the Rosinophilic Cellular Infiltration in BAL of Bt Sensitized Mice

To assess the effects of eugenol on the inflammatory cell infiltration in BAL of the Bt-sensitized and challenged mice, the BAL cellularity was assessed 24 hours after the last challenge. Bt-sensitized mice displayed a significant increase of total cells and eosinophils in relation to the control group ($P < 0.001$; Fig. 1). Oral daily administration of 40 mg/kg or 80 mg/kg of eugenol significantly suppressed the number of total inflammatory cells (Fig. 1a) and eosinophils (Fig. 1b), in relation to the untreated Bt-sensitized mice. No effect was observed on eosinophil count in mice treated with 20 mg/kg of eugenol.

3.2 Treatment with Eugenol Reduces Eosinophil Peroxidase Levels in Lungs of Bt Sensitized Mice

Bt sensitization led to an increase of EPO activity in the lungs ($P < 0.001$) when compared to the control group (Fig. 1c). Treatment with 40 and 80 mg/kg of eugenol decreased EPO activity in lung tissue ($P < 0.001$) of Bt-immunized and challenged mice (Fig. 1c). No effect was observed on eosinophil peroxidase in lungs from mice treated with 20 mg/kg of eugenol.

3.3 Treatment with Eugenol Decreases the Inflammatory Cell Infiltration and Amount of Mucus in Lungs of Bt-immunized Animals

The Fig. 2 shows the typical pathologic features of allergic asthma in lung tissue from Bt-sensitized mice, characterized by the infiltration of numerous inflammatory cells in the peribronchiolar and perivascular regions (Fig. 2b) and airway mucus hypersecretion (Fig. 2f). Treatment with both doses of eugenol (40 mg/kg and 80 mg/kg) markedly reduced the inflammatory cell infiltration around the bronchioles (Figs. 2c and 2d) as well as suppressed mucus secretion in the lung tissue (Fig. 2g and 4h). No effect was observed on leukocyte infiltration and mucus hyper-secretion in lungs from mice treated with 20 mg/kg of eugenol (data not shown).

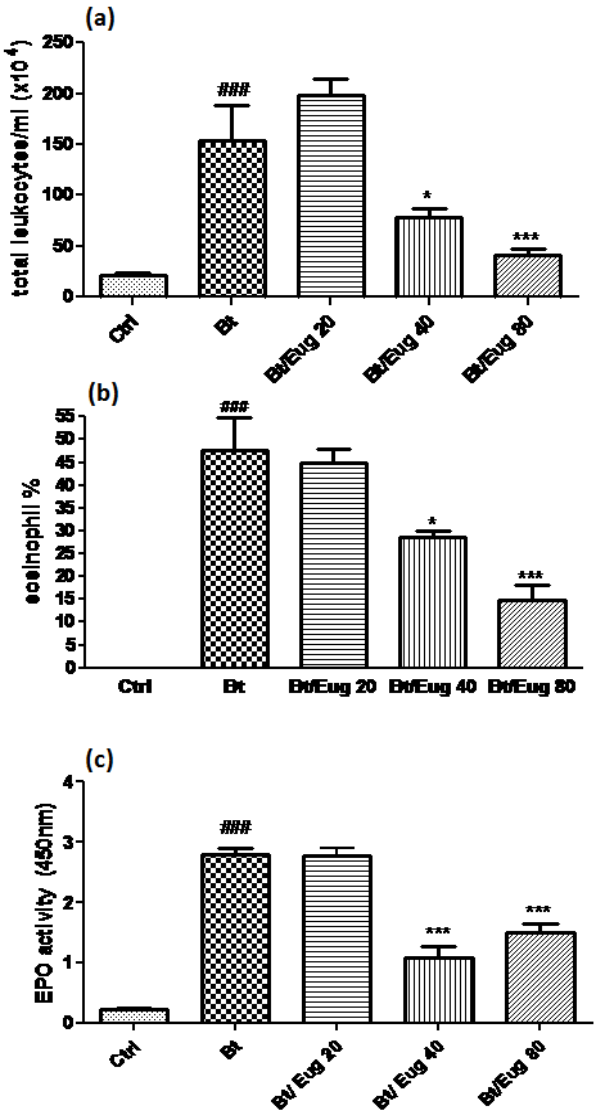

Fig. 1 Effect of Eugenol on leukocytes and EPO activity of Bt sensitized and challenged mouse. (a) Number of total cells; (b) eosinophilia in the BAL and (c) kinetics of EPO (eosinophil peroxidase) activity in lung tissue from Bt-sensitized animals.

Non-sensitized, normal controls mice (Control), Bt-sensitized and challenged animals (Bt), Bt-sensitized and challenged, Eugenol (20 mg/kg, 40 mg/kg or 80 mg/kg) treated mice (Bt/Eug20, Bt/Eug40 or Bt/Eug80). Columns represent the mean values of the results obtained from five animals, and error bars represent the standard error from the means. #$P < 0.05$; ###$P < 0.001$ vs. Control; *$P < 0.05$; ***$P < 0.001$ vs. Bt group.

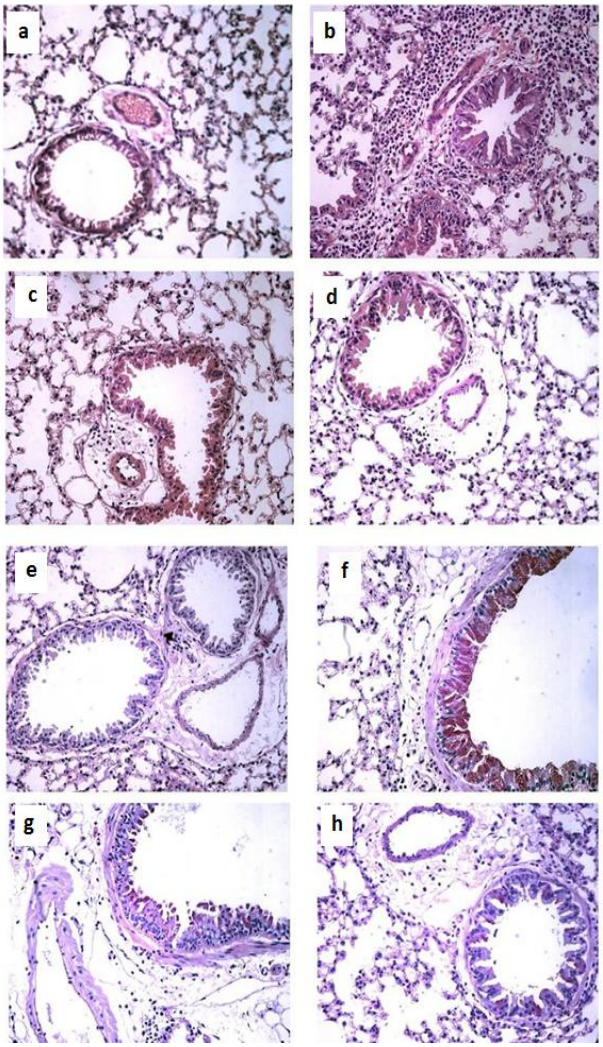

Fig. 2 Effect of Eug (Eugenol) treatment on leukocyte infiltration and mucus production in lung tissues. Sections were stained with hematoxylin-eosin (magnification ×400) (a-d) and sections were stained with periodic acid-Schiff (magnification ×400) (e-h). (a) and (e) Lung section from a control, saline-treated mice; (b) and (f) Lung section from a Bt-immunized and challenged, saline-treated mice; (c) and (g) Lung section from a Bt-immunized and challenged, Eug 40 mg/kg-treated mice; (d) and (h) Lung section from a Bt-immunized and challenged, Eug 80 mg/kg treated mice.

3.4 Treatment with Eugenol Does not Decrease the Levels of Bt-specific IgE, IgG1 and IgG2a Antibodies in the Sera of Bt-sensitized Mice

To assess if eugenol affects anti-*B. tropicalis* specific antibody levels, we evaluated the effect of eugenol on anti-*B. tropicalis* antibody response in Bt-sensitized animals. Bt-sensitized mice produced high levels of specific IgE, IgG1 and IgG2a antibodies than non-sensitized animals ($P < 0.001$). Treatment with eugenol did not reduce significantly serum immunoglobulins levels (Fig. 3).

3.5 Treatement with Eugenol Decreases Levels of Th2-type Cytokines in BAL and Spleen Cells Culture

To determine the possible mechanisms whereby eugenol exerts its modulatory activity in airways from Bt-sensitized animals, levels of the Th (T-helper) type 2 cytokine, typically found during allergic inflammation, were evaluated. Levels of IL-4 in the BAL were higher in Bt-sensitized mice than in the control group ($P < 0.001$) (Fig. 4a). Bt-sensitized animals treated with eugenol had lower levels of this Th2 cytokine in the BAL compared to those untreated mice ($P < 0.01$) (Fig. 4a). However, the oral treatment with eugenol did not affect the levels of regulatory cytokine IL-10 in the BAL (Fig. 4b) of Bt-sensitized mice.

An increased production of Th2 cytokines IL-4, IL-5 and IL-13 was observed in spleen cells stimulated with PWM from Bt-sensitized animals compared with non-stimulated splenocytes. *In vitro* treatment with eugenol (12.5-200 μM) significantly reduced Th2 cytokines (Figs. 5a-5c) in spleen cells culture from Bt-sensitized mice. The concentrations of eugenol herein studied showed 100% cell viability evaluated by MTT assay (data not shown).

3.6 Eugenol Demonstrated a Relaxing Effect o Tracheal Smooth Muscle from Normal Mice

Eugenol (10^{-9}-10^{-3} M), in a concentration-dependent manner, relaxed tracheal smooth muscle isolated from mice without functional epithelium, pre-contracted with the muscarinic agonist carbachol (10 μM). The dataset can be seen in Fig. 6a which shows the concentration-response curve of eugenol where the percentage of maximum relaxation (E_{max}) induced by eugenol was $E_{max} = 112 \pm 3.9$ and $pEC_{50} = 3.7 \pm 0.2$.

In our *in vitro* model of hyper-reactivity using IL-13, tissues that were incubated overnight with IL-13

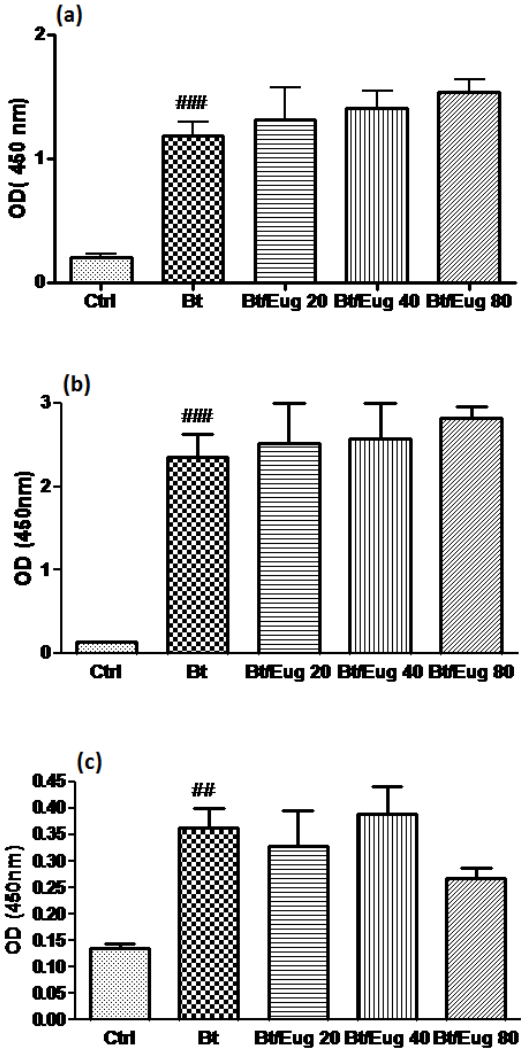

Fig. 3 Effect of Eugenol (Eug) on the levels of (a) IgE, (b) IgG1 and (c) IgG2a anti-*Blomia tropicalis*.

Vehicle-treated and sensitized mice (Control), Bt-sensitized and challenged animals (Bt) and Bt-sensitized and challenged, Eug (20 mg/kg, 40 mg/kg or 80 mg/kg) treated mice (Bt/Eug20, Bt/Eug40 or Bt/Eug80). Antibody levels were measured by indirect ELISA. Columns represent the mean values of the results obtained from five animals, and error bars represent the error deviations from the means. ###$P < 0.001$ vs. Control.

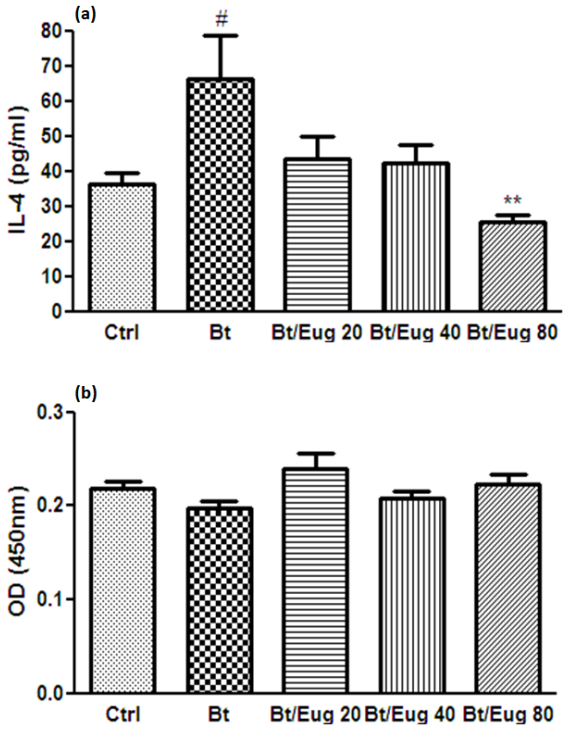

Fig. 4 Effect of Eug (Eugenol) in the cytokines production in BAL. Effect of the treatment with Eug on the levels of (a) IL-4 and (b) IL-10 in the BAL of vehicle-treated and sensitized mice (Control), Bt-sensitized and challenged animals (Bt) and Bt-sensitized and challenged, Eug (20, 40 or 80mg/kg) treated mice (Bt/Eug20, Bt/Eug40 or Bt/Eug80).

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; ** $P <$ 0.01 vs. Bt group and ***$P < 0.001$ vs. Bt group, ANOVA-Tukey.

had a greater constrictor response to CCh (Emax = 0.84 ± 0.09 g), however, without changes in pharmacological potency (pEC_{50} = 6.62 ± 0.14), when compared with non-treated rings (E_{max} = 0.57 ± 0.04 g, pEC_{50} = 6.59 ± 0.08), demonstrating a typical hyper-reactivity in tracheal smooth muscle upon a contracting agent (CCh) (Fig. 6b). The efficacy and potency of eugenol in hyper-reactive tracheal rings sensitized with IL-13 (pEC_{50} = 3.81; E_{max} = 106.70 ± 1.60), were not altered when compared to normal rings, not exposed to IL-13 (pEC_{50} = 3.80; E_{max} = 108.65 ± 1.97) (Fig. 6c).

4. Discussion

The inflammatory response to allergens in the atopic asthmatic lung is a consequence of infiltration on the airway by inflammatory cells, especially eosinophils and is associated with an increased expression of several inflammatory proteins in lung tissue, including cytokines, such as IL-4, IL-5 and IL-13 [25]. The resolution of inflammation is an essential process for the establishment of appropriate host responses and the return to homeostasis [26].

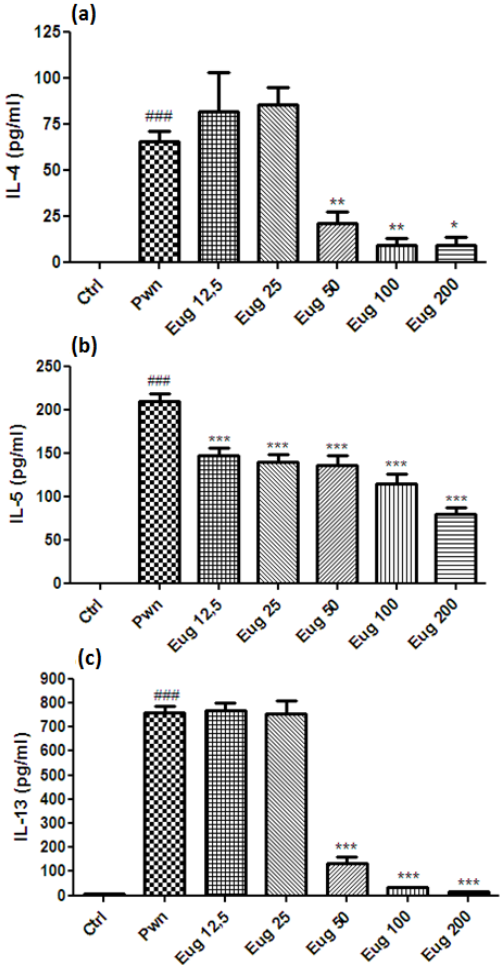

Fig. 5 Effect of Eugenol in the cytokines production in spleen. *In vitro* effect of treatment with eugenol on the levels of: (a) IL-4, (b) IL-5, and (c) IL-13 in spleen cells culture from Bt-sensitized animals. Without stimulation (Control), stimulated with PWM and stimulated with PWM + Eug treatment (12.5-200 µM).

#*P* < 0.05 vs. Control; ###*P* <0.001 vs. Control; **P < 0.01 vs. Bt group and ***P < 0.001 vs. Bt group, ANOVA-Tukey.

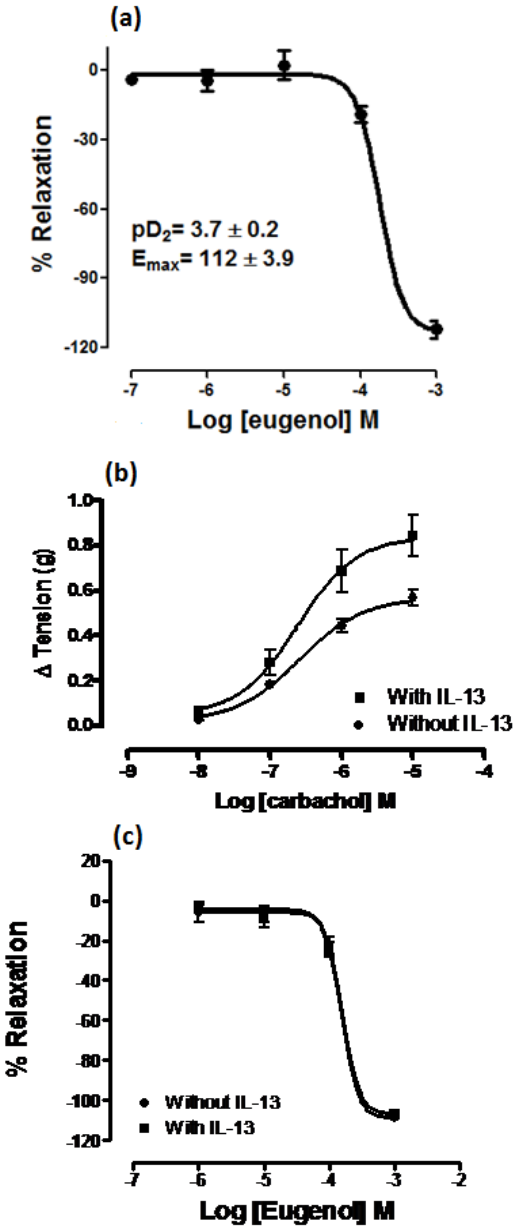

Fig. 6 Effects of Eug (Eugenol) in mice airway smooth muscle. (a) Logarithmic concentration-response curve of relaxant response of eugenol on tracheal rings pre-contracted with 10 µM carbachol, in the absence of functional epithelium (*n* = 6). (b) Concentration response curve showing contracturante effect of the CCh on smooth muscle sensitized or not with IL-13 (*n* = 8) and (c) Concentration response curve showing relaxant effect of the Eug on smooth muscle sensitized or not with IL-13 (*n* = 6). ###*P* < 0,001, ANOVA-Bonferroni.

The use of biologically active natural products is increasing popularity day by day over conventional medicine as an outstanding alternative approach for the treatment of several diseases. However, limited scientific evidence regarding the effectiveness of these natural derivatives, and lack of mechanistic understanding has prevented their incorporation into mainstream medicine and their application in human therapy. Eugenol, an o-methoxyphenol, is of interest for many researchers due to its anti-inflammatory and chemo-preventive effects based on the antioxidant capability of its phenolic group. However, little is known about the effect of eugenol in allergic inflammation mediated by Th2-type cytokines.

The present study was conducted using a murine model of allergic airway disease induced by the sensitization to a common allergen, the *Blomia tropicalis* mite, which was previously characterized by our group as a massive eosinophilic inflammation in the lungs mediated mainly by Th2 cytokines leading to airway luminal narrowing [20]. Using this model, we were able to explore the effect of eugenol on an allergic inflammation induced by a clinically relevant aeroallergen.

As we previously described, sensitization with 100 μg of Bt produced inflammatory cells influx, high levels of EPO and Bt-specific IgE, as well as Th2-type cytokines such as IL-4 in BAL [20]. The treatment with eugenol of Bt-sensitized mice resulted in a great inhibition of airway and lung tissue inflammation, characterized by reduction in: (1) numbers of total inflammatory cells and eosinophils in BAL; (2) inflammatory cell infiltration in the peribronchiolar and perivascular pulmonary region; (3) presence of mucus inside lower airways; (4) levels of EPO in the lung; but did not alter Bt-specific antibodies (IgE, IgG1 and IgG2a) in serum.

Several studies attribute the anti-allergic property of natural products to their ability to reduce the inflammatory cell infiltrate [27-31]. The eosinophilic infiltrate stimulates the production of pro-inflammatory chemokines, cytokines and cytolytic enzymes, including eosinophil cationic protein and major basic protein that degrades integrity of airway epithelium [32]. Accordingly, the increased presence of inflammatory cells and their secreted products in the asthmatic lung often correlates with severity and exacerbation of disease [33]. Reducing eosinophil numbers, when animals are treated with eugenol, may be of relevance to the improvement of inflammation and/or tissue remodeling in allergic asthma. Our results have shown that Eugenol suppresses EPO activity and those results are correlated to the reduction of eosinophilic infiltration observed during the treatment with eugenol.

In order to explore the mechanism whereby eugenol modulates eosinophils infiltration we investigated the effect of this drug on IL-4, IL-5, IL-13 and IL-10 production. Eugenol treatment decreased Th2 cytokines levels of in both *in vitro* (spleen) and *in vivo* (BAL) models. We observed the presence of IL-5 and IL-13 *in vitro* only, probably due to lower levels of these cytokines in BAL in comparison to IL-4. The reduction of IL-4 (*in vitro* and *in vivo*) and IL-5 (*in vitro*) explains, at least in part, modulated inflammation in the lung as well reduced EPO levels in treated animals, since IL-4 is the main cytokine involved in inflammatory Th2-driven response and IL-5 is the principal cytokine involved in the maturation, activation and migration of eosinophils. A study in nasal polyps showed that treatment of the eosinophil-infiltrated tissue with neutralizing anti-IL-5 induced eosinophil apoptosis and decreased tissue eosinophilia [34]. IL-4 is also related to production of IgE, the main immunoglobulin associated with allergic diseases [35], however, no reduction was observed on IgE production. The lack of modulation on IgE despite the decrease in IL-4 and IL-13 could be related to the short-term treatment and the short-term murine model of respiratory disease like ours. IL-13 and IL-4 play an important role in the production of mucus as well. Increased mucus production by goblet cells in the airway epithelium is associated with airway inflammation and asthma. Thus, the reduced production of IL-13 may reflect the decreased production of mucus and improve lung function, as was observed in animals treated with eugenol. The mechanism whereby eugenol inhibits the production of Th2 cytokines is not clear yet. It seems that it was not trough a regulatory mechanism since no alteration was found in IL-10 levels *in vivo* (and *in vitro*, data not shown). Previous studies have shown that eugenol reduces the production of inflammatory cytokines by inhibiting the activation of factor NFkB, an important transcriptional factor that regulates inflammatory response and the expression of inflammatory

cytokines [18, 36]. This may be one of the probable mechanisms of the anti-allergic effect of eugenol.

In addition to anti-inflammatory effect, the experiments with the isolated trachea showed that the eugenol induced a relaxing effect in tracheal smooth muscle pre-contracted with the muscarinic agonist, carbachol, demonstrating a potential as bronchodilator. These results are consistent with studies showing that phenolic compounds are able to relax smooth muscle and improve lung function [19]. Additionally, we evaluated the bronchodilator effect of eugenol in hyperreactive smooth muscle. As a result of inflammation, airway smooth muscle in individuals with asthma becomes hyperresponsive and narrow easily in response to numerous stimuli. This hyperreactivity is non-specific and can occur face to endogenous or exogenous factors [37]. Within the factors involved in hyperreactivity, interleukin-13 (IL-13) has been implicated as a key cytokine [38, 39]. Our study supports these findings. We demonstrated the increased contractility in response to CCh in airway smooth muscle induced by IL-13. The effects of IL-13 on contractility were principally to increase Emax, and no significant changes in EC50 were observed, indicating that IL-13 seemed to increase smooth-muscle contractility rather than induce increased sensitivity to contractile agents (at least CCh), thus demonstrating hyper-reactivity. The effect of eugenol was not altered in hyper-reactive smooth muscle (sensitized with IL-13) when compared to its effect on normal smooth muscle, showing that the bronchodilatory potential of eugenol is not selective for hyperactivity induced by IL-13 pathway. More studies are needed to verify if eugenol may have a selective effect on other mechanisms involved in the hyper-reactivity.

5. Conclusions

The results of the present study, obtained in an experimental model strongly support the potential usefulness of eugenol as antiinflammatory and bronchodilator agent for the treatment of allergic asthma. Additional studies are in progress in our laboratory in order to further elucidate the mechanisms of action whereby eugenol exerts its anti-inflammatory effects.

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