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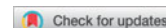
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Essential oil of *Croton Zehntneri* attenuates lung injury in the OVA-induced asthma model

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ABSTRACT

Objective: *Croton zehntneri* Pax et Hoffm. is a Euphorbiaceae species, popularly known as “canela de cunhã,” a native plant of northeastern Brazil, whose essential oil (EOCZ) shows relatively specific myorelaxant action for the smooth muscle of the airways and in the respiratory tract. Based on this information, EOCZ figures as a candidate for testing in the treatment of asthma, and the present study investigated the benefits of using EOCZ in an ovalbumin-induced asthma model. **Methods:** 48 male BALB/c mice were divided into six groups ($n = 8$). In the ST, SO100, and SO300 groups, mice were sensitized and challenged with saline, and then treated with 200 μ L of 0.1% Tween 80, 100 mg/kg EOCZ and 300 mg/kg EOCZ, respectively. In the OT, OO100, and OO300 groups, mice were sensitized and challenged with OVA, and then treated with 200 μ L of 0.1% Tween 80, 100 mg/kg EOCZ and 300 mg/kg EOCZ, respectively. **Results:** Our results demonstrated significant changes in all respiratory mechanics variables analyzed between the OO300 and OT groups demonstrating the effectiveness of EOCZ to attenuate the OVA-induced lung injury. In addition, the use of EOCZ at a dose of 300 mg/kg showed an antioxidant effect and decreased inflammatory cells in the pulmonary parenchyma. In conclusion, our results demonstrated that EOCZ was able to improve the lesion in the respiratory system of mice subjected to OVA-induced asthma. **Conclusions:** The antioxidant action of EOCZ was likely the main mechanism of action in the reversal of this lesion, so more tests should be performed for its confirmation.

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1. Introduction

The use of plants for therapeutic purposes is as old as humans themselves (1). Numerous plants are used for therapeutic purposes in the form of teas, powders, essential oils and roots, where essential oils extracted from aromatic plants are the main object of studies carried out by the scientific community, due to their easy attainment and low cost (2). Many plants rich in essential oils have been used in the treatment of various diseases, including leishmaniasis, malaria, schistosomiasis, and fungal and bacterial infections (3).

Croton zehntneri Pax et Hoffm. is a Euphorbiaceae species, popularly known as “canela de cunhã,” a plant native to northeastern Brazil, whose extracted essential oil has been studied because of its bioactive principles, biological activity, and diversity of its popular use (4). *C. zehntneri* is used in folk medicine mainly as a sedative, appetite stimulant, antispasmodic, antianorexic, analgesic and anxiolytic, and for the treatment of gastrointestinal disorders (5). Analgesic and antiinflammatory effects are important pharmacological properties

attributed to the essential oil of *C. zehntneri* (EOCZ) (6,7). Also important are its larvicidal activity against *Aedes aegypti* (8,9), antifungal and phytotherapeutic potential for dermatophytoses (7), blockade of peripheral nerve excitability (10) and gastroprotective action (11). Because it shows relatively specific myorelaxant action in the smooth muscle of airways (12) and in the respiratory tract, where it totally reverses the contractions maintained by high concentrations of extracellular K^+ (13), EOCZ figures as a major candidate for testing in the treatment of asthma.

Asthma is considered a respiratory disease characterized by an increase in eosinophil infiltration, bronchoconstriction, mucosal hypersecretion, airway inflammation, and hyperresponsiveness (14,15). The prevalence of asthma is increasing rapidly (16); approximately 235–300 million people suffer from asthma worldwide (17). Asthma can be classified as allergic asthma, caused by exposure to an allergen, and nonallergic asthma, caused by intense exercise, illnesses such as a cold or flu, stress, irritants, and pollutants in the air, or by some

medications (18). For many patients with asthma, the addition of a long-acting bronchodilator to inhaled corticosteroid reduces symptoms and decreases the need for short-acting bronchodilators (19), while some treatments for asthma are managed using beta-2 agonists, anticholinergics, methylxanthines, mast cell stabilizers, leukotriene antagonists, and antiimmunoglobulin E (IgE) antibody. Despite recognized benefits, some drugs are associated with adverse effects, such as muscle tremors, restlessness, hypotension, hyperglycemia, tachycardia, convulsions, and adrenal crisis (20).

Due to its importance and complexity, asthma has been the subject of extensive study in recent years. However, numerous questions related to its pathophysiology have not been answered yet, as the development and addition of new therapeutic agents has been limited. Despite the biological activities of EOCZ and its positive results in airway smooth muscle, we are not aware of its use in *in vivo* studies, which led us to evaluate its effect on diseases that affect the respiratory system using the OVA-induced asthma model. Accordingly, we used respiratory system mechanics, micromechanics, biochemical analyses, histopathology, and lung parenchyma morphometry.

2. Materials and methods

2.1. Plant material

The leaves of *Croton zehntneri* were collected near the city of Viçosa in Ceará, Brazil (3°33'48" S; 41°5' 41" W). The identification of the plants was confirmed by Dr. F.J. Abreu Matos (Laboratory of Natural Products, Federal University of Ceará). A voucher specimen (No. 277477) is deposited in the Prisco Viana Herbarium, Federal University of Ceará.

2.2. Extraction and chemical analysis

EOCZ was prepared from freshly chopped leaves by steam distillation and analyzed chemically as previously described (6). Briefly, EOCZ was analyzed as follows: a Hewlett-Packard 6971 GC/MS system; column: dimethylpolysiloxane DB-1 fused silica capillary column (30 m × 0.25 mm; 0.1 µm); carrier gas: helium (1 mL/min); injector temperature: 250 °C; detector temperature: 200 °C; column temperature: 35–180 °C at 4 °C/min and then 180–250 °C at 10 °C/min; mass spectra: electronic impact 70 eV. The composition of EOCZ was 85.7% anethole, 4.8% estragole, 2.95% 1,8-cineole, 2.2% trans-caryophyllene, and 2.23% unidentified.

2.3. Animals

Forty-eight male BALB/c mice (7–8 weeks of age), with body weight of 25 ± 5 g, provided with water and feed *ad libitum*, were used in this study. Mice were housed in plastic cages under controlled environmental conditions. All animal use and care procedures were previously approved by the animal ethics committee of the State University of Ceará (Protocol No. 3114549). Invasive procedures were performed under anesthesia and every effort was made to minimize suffering.

2.4. Induction of murine model of asthma and experimental design

Mice were sensitized and challenged by OVA (Sigma-Aldrich, MO, USA) (21). Briefly, BALB/c mice were subcutaneously sensitized with 100 µg OVA emulsified in 1 mg aluminum hydroxide (Sigma-Aldrich) as adjuvant in 200 µL of phosphate-buffered saline on days 0, 7, and 14. The intranasal challenge was always performed under anesthesia with sevoflurane, for pulmonary aspiration of the OVA solution. The intranasal challenge was performed on days 25, 26, and 27 with OVA (100 µg, diluted in 50 µL of Dulbecco's Phosphate Buffered Saline (DPBS)). The negative control group received the same volume of saline (0.9% NaCl) subcutaneous (days 0, 7, and 14) and intranasal (days 25, 26, and 27).

The experimental design is shown in Figure 1. We used 48 BALB/c mice, randomly divided into six groups ($n = 8$). In the first group (ST), the mice were subjected to the negative asthma control protocol previously mentioned, and 24 h after protocol termination, they received 200 µL of vehicle (0.1% Tween 80) by gavage. In the second group (OT), the mice were sensitized and challenged with OVA as previously mentioned, and 24 h after protocol termination, they received 200 µL of vehicle (0.1% Tween 80). In the third group (SO100), the mice were subjected to the negative asthma control protocol previously mentioned and 24 h after protocol termination, they received 200 µL of EOCZ (100 mg/kg). In the fourth group (OO100), the mice were sensitized and challenged with OVA as previously mentioned and 24 h after protocol termination, they received 200 µL of EOCZ (100 mg/kg). In the fifth group (SO300), the mice were subjected to the negative asthma control protocol previously mentioned, and 24 h after protocol termination, they received 200 µL of EOCZ (300 mg/kg). In the sixth group (OO300), the mice were sensitized and challenged with OVA as previously mentioned, and 24 h after protocol termination, they received 200 µL of EOCZ (300 mg/kg). All analyses were performed 24 h after administration of the vehicle or EOCZ. EOCZ solution was prepared daily by adding

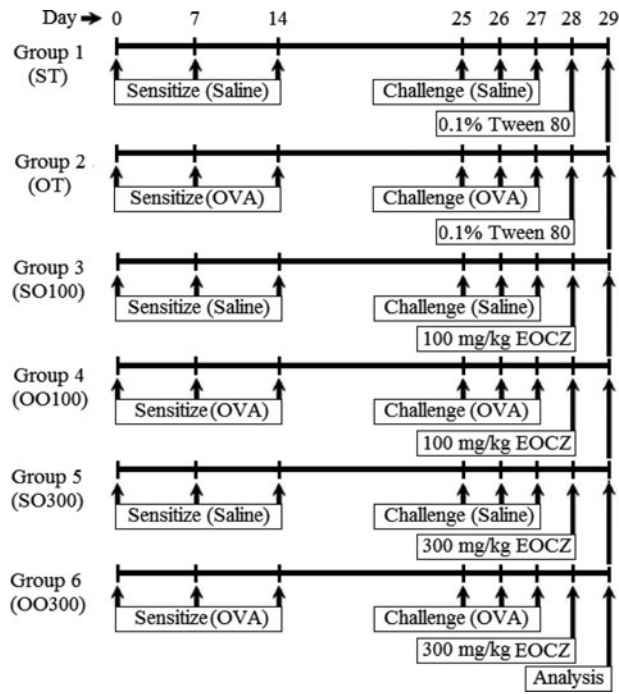


Figure 1. Schematic diagram of the experimental protocol. In group 1 (ST), mice were sensitized with saline on days 0, 7, and 14, challenged with saline on days 25, 26, and 27, and treated with 200 μ L of 0.1% Tween 80. In group 2 (OT), mice were sensitized with 100 μ g OVA emulsified in 1 mg aluminum hydroxide on days 0, 7, and 14, challenged with 100 μ g OVA diluted in 50 μ L of DPBS on days 25, 26, and 27, and treated with 200 μ L of 0.1% Tween 80. In group 3 (SO100), mice were sensitized with saline on days 0, 7, and 14, challenged with saline on days 25, 26, and 27, and treated with 200 μ L of EOCZ (100 mg/kg). In group 4 (OO100), mice were sensitized with 100 μ g OVA emulsified in 1 mg aluminum hydroxide on days 0, 7, and 14, challenged with 100 μ g OVA diluted in 50 μ L of DPBS on days 25, 26, and 27, and treated with 200 μ L of EOCZ (100 mg/kg). In group 5 (SO300), mice were sensitized with saline on days 0, 7, and 14, challenged with saline on days 25, 26, and 27, and treated with 200 μ L of EOCZ (300 mg/kg). In group 6 (OO300), mice were sensitized with 100 μ g OVA emulsified in 1 mg aluminum hydroxide on days 0, 7, and 14, challenged with 100 μ g OVA diluted in 50 μ L of DPBS on days 25, 26, and 27, and treated with 200 μ L of EOCZ (300 mg/kg). All analyses were performed on day 29.

this substance directly in the vehicle (0.1% Tween 80) and then mixed vigorously by hand or vortexed for 3–5 min.

2.5. Respiratory system mechanics

At 24 h after gavage of Tween 80 or EOCZ, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Hypnol[®] 3%, Syntect, Brazil) and tracheotomized. The animals were intubated with a 14-gauge cannula (Eastern Medikit, Delhi, India), which was then connected to a computer-controlled ventilator for small animals (Scirec[®]-flexVent[®], Montreal, QC, Ca). The animals were ventilated at baseline settings: respiratory frequency of 120 breaths/min, tidal volume of 10 mL/kg, limiting

pressure of 30 cmH₂O, and positive end-expiratory pressure (PEEP) of 3 cmH₂O. Mice were then paralyzed with pancuronium bromide (0.5 mL/kg, i.p., Cristália, Brazil).

Initially, we standardized the mechanical history of the respiratory system with two deep inflations (DI, 6-s long, peak pressure: 30 cmH₂O) followed by ventilation for 5 min with baseline settings. Soon after, the impedance of the respiratory system (Z_{rs}) was measured using the forced oscillation technique (22), 12 sequential 30-s sampling intervals, for a total of 6 min (23).

The experimental Z_{rs} was fitted to the constant phase model as previously described (24):

$$Z_{rs} = R_N + I(2\pi f)i + \frac{G - Hi}{(2\pi f)^\alpha} \quad (1)$$

$$\alpha = \frac{2}{\pi} \tan^{-1} \left(\frac{H}{G} \right) \quad (2)$$

where R_N is the Newtonian resistance, which represents the central airways resistance, $i = \sqrt{-1}$, f is the frequency (Hz), I represents airway inertance, and G and H are, respectively, the dissipative and elastic properties of lung tissue (22).

Thereafter, starting at the functional residual capacity defined by the PEEP, the flexiVent delivered seven inspiratory pressure steps for a total pressure of 30 cmH₂O, followed by seven expiratory steps, pausing at each step for 1 s. At each step, plateau pressure (P) was recorded and related to the total volume (V) delivered to produce a quasistatic PV (pressure–volume) curve. Static compliance (C_{ST}) was calculated as the slope of the curve (23). Five quasistatic PV curves were obtained to measure C_{ST} , an estimate of inspiratory capacity (IC), and PV loop area. Another forced oscillation technique ensued to determine respiratory system mechanics.

2.6. Methacholine challenge

Immediately after measurements of respiratory system mechanics, two DIs were done, followed by 5 min of ventilation with baseline settings. Airway smooth muscle hyperresponsiveness was evaluated by inhalation of methacholine (MCh) (Sigma-Aldrich) delivered by aerosol produced by an ultrasonic nebulizer (Inalasonic, NS, São Paulo, Brazil) coupled to the inspiratory line of the ventilator. For such purpose, 4 mL of MCh solution (30 mg/mL) was added to the nebulizer container.

The nebulization was carried out for 30 s under mechanical ventilation (24), and the average amount delivered to the animal was 0.12 mg/kg MCh. After nebulization, the same previous analysis was repeated (forced oscillation, 10-s sequential intervals for 2 min), followed by two DIs and another forced oscillation data gathering.

Data regarding airway hyperresponsiveness (AHR) collected after nebulization of MCh were presented as ΔR_N , where Δ means the parameter after nebulization minus its value before MCh challenge. All Δ values were normalized by the prenebulization values (25).

2.7. Histological study

Slides of left lung sections were stained with hematoxylin and eosin (HE) and examined by light microscopy according to their qualitative and quantitative aspects. An investigator, who was unaware of the origin of the coded material, examined the samples microscopically. Quantitative analysis was performed using an integrated eyepiece with a 100-point and 50-line grid coupled to a conventional light microscope. The area (fraction) of collapsed alveoli (alveolar morphometry), number of polymorphonuclear (PMN) cells, and amount of pulmonary tissue as well were determined by the point-counting technique (26). Cellularity was assessed at 1000 $\times$ magnification across 10–15 random noncoincident microscopic fields in each animal.

The air-space enlargement was quantified by the mean linear intercept length of the distal air spaces (L_m) in 30 randomly chosen fields of tissue sections per group (27). The bronchoconstriction index (BCI) was determined in 10 noncoincident microscopic fields per animal by counting the number of points in the airway lumen (NP) and intercepts through the airway wall (NI) using a reticulum grid and applying the equation: $BCI = NI/\sqrt{NP}$. Only airways in which the long diameter did not exceed the short diameter by more than 20% were accepted for measurement (28). Alveolar morphometric analysis and determination of BCI were done at 400 $\times$ magnification.

2.8. Biochemical analyses

The right lungs of each group were homogenized in 5 mM Tris-HCl buffer (pH 7.4), containing 0.9% NaCl (w/v), 0.5 mM phenylmethylsulfonyl fluoride and 1 μ g/mL aprotinin, followed by centrifugation at 750 $\times g$ for 10 min at 4 $^{\circ}$ C. The supernatants were stored at -80° C for the biochemical analyses (catalase (CAT) activity and levels of malondialdehyde and nitrite). Total protein in the samples was determined by the Bradford method (29).

CAT activity was measured at 240 nm by the rate of decrease in hydrogen peroxide concentration and was expressed as CAT equivalents (U/mg protein). Superoxide dismutase (SOD) activity was assayed by measuring inhibition of adrenaline autooxidation as absorbance at 480 nm and was expressed as SOD equivalents (U/mg protein) (30).

2.9. Statistical analysis

Results are presented as mean $\pm$ SD, where n represents the number of samples. For comparison between groups, we used one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Multiple comparisons were then corrected with a Bonferroni test. A difference was considered significant if $p < 0.05$.

3. Results

Figure 2 (constant phase model) and Figure 3 (quasistatic PV curve) show respiratory system mechanical data of ST ($R_N = 0.125 \pm 0.012$, $G = 3.26 \pm 0.36$, $H = 12.08 \pm 1.35$, $\eta = 0.254 \pm 0.023$, $C_{ST} = 0.109 \pm 0.012$, $CI = 1.12 \pm 0.13$, PV Loop Area = 2.28 ± 0.32), SO100 ($R_N = 0.119 \pm 0.016$, $G = 3.12 \pm 0.50$, $H = 11.74 \pm 1.99$, $\eta = 0.268 \pm 0.039$, $C_{ST} = 0.110 \pm 0.019$, $CI = 1.15 \pm 0.15$, PV Loop Area = 2.12 ± 0.59) and SO300 ($R_N = 0.118 \pm 0.013$, $G = 3.16 \pm 0.51$, $H = 12.10 \pm 1.73$, $\eta = 0.265 \pm 0.052$, $C_{ST} = 0.109 \pm 0.018$, $CI = 1.06 \pm 0.18$, PV Loop Area = 2.11 ± 0.43) groups, where the animals were submitted to the negative asthma control protocol and treated with 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively.

In the OT ($R_N = 0.248 \pm 0.037$, $G = 6.62 \pm 1.19$, $H = 20.98 \pm 3.50$, $\eta = 0.322 \pm 0.062$, $C_{ST} = 0.074 \pm 0.013$, $CI = 0.83 \pm 0.15$, PV Loop Area = 3.98 ± 0.63), OO100 ($R_N = 0.224 \pm 0.032$, $G = 5.73 \pm 1.29$, $H = 19.39 \pm 2.51$, $\eta = 0.296 \pm 0.047$, $C_{ST} = 0.086 \pm 0.012$, $CI = 0.93 \pm 0.13$, PV Loop Area = 3.92 ± 0.53), and OO300 ($R_N = 0.139 \pm 0.034$, $G = 3.40 \pm 0.79$, $H = 13.18 \pm 2.85$, $\eta = 0.268 \pm 0.062$, $C_{ST} = 0.102 \pm 0.018$, $CI = 1.04 \pm 0.19$, PV Loop Area = 2.25 ± 0.52) groups, the animals were sensitized and challenged with ovalbumin, and treated with 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively.

Our results demonstrated significant changes in all respiratory mechanics variables analyzed for the OT group compared to the ST group, confirming the establishment of the OVA-induced lesion. We also observed that mice treated orally with EOCZ at the dose of 300 mg/kg (OO300) showed an improvement in all variables of respiratory mechanics, compared to the untreated group (OT) after challenge with ovalbumin, demonstrating the effectiveness of EOCZ in reversing OVA-induced lung injury.

Figure 4 shows the variation in ΔR_N with time after administration of MCh (30 mg/mL) in all groups. We observed an increase (ΔR_N) at several points of the groups OT and OO100 in comparison to the ST group, evidencing an AHR.

Figure 5 depicts representative lung and airway (inserts) histological images of the ST, OT, SO100,

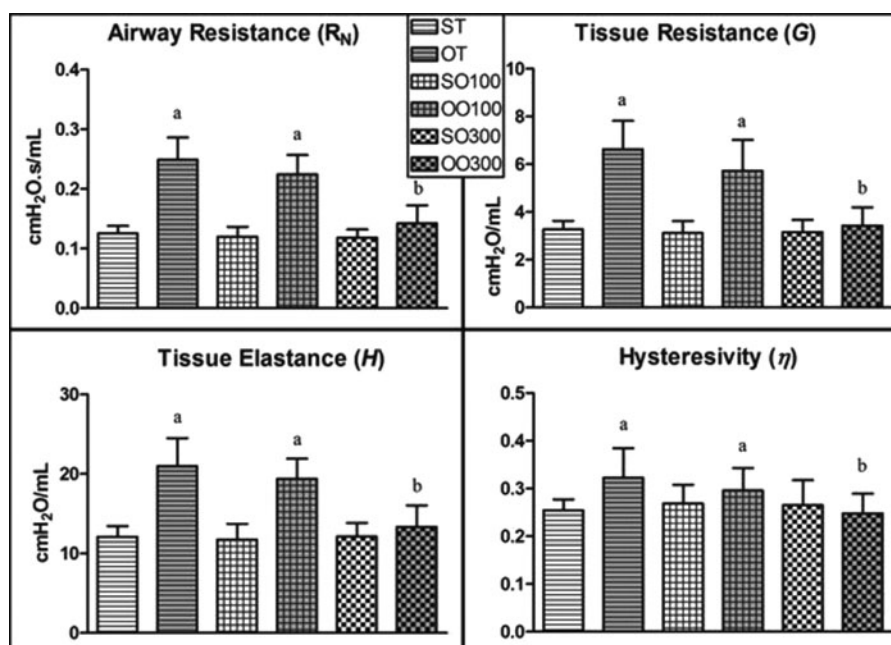


Figure 2. Differences between lung function parameters (forced oscillation technique). Values are mean $\pm$ SD of groups ST, OT, SO100, OO100, SO300, and OO300. One-way ANOVA followed by Student–Newman–Keuls test was performed. ^a Different from ST group ($p < 0.05$). ^b Different from OT group ($p < 0.05$).

OO100, SO300, and OO300 groups. There was the presence of alveolar collapse, thickened septa, cellular infiltration, and constricted airways (inserts) in the photomicrographs of the pulmonary parenchyma of the OT and OO100 groups.

Table 1 displays the alveolar collapse, amount of PMN cells, mean alveolar diameter, and BCI. We observed an increase in alveolar collapse, amount of PMN cells, mean alveolar diameter, and BCI of the OT and OO100 groups when compared to the ST group. Altogether, these findings may suggest pulmonary inflammation and bronchoconstriction.

The results of the biochemical analysis are shown in Table 2. CAT and SOD activities were significantly higher

in the OT and OO100 groups than in the ST group, indicating a redox imbalance in lung homogenates of the animals in those groups. In addition, CAT and SOD activities were significantly higher in the OT group than in the OO300 group, indicating an antioxidant effect in the animals receiving the OECZ oral dose of 300 mg/kg.

4. Discussion

Asthma is an allergic respiratory disease triggered by different spasmogens such as acetylcholine, leukotrienes, and prostaglandins, or by specific exposure to allergens, which reflect the signals of acute bronchoconstriction,

Table 1. Morphometric parameters.

Group	Alveolar collapse (%)	PMN cells ($\times 10^{-3}/\mu\text{m}^2$)	Mean alveolar diameter (μm)	BCI
ST	3.64 $\pm$ 1.02	15.48 $\pm$ 2.61	48.43 $\pm$ 3.31	1.87 $\pm$ 0.11
OT	21.47 $\pm$ 2.52 ^a	34.11 $\pm$ 4.21 ^a	33.49 $\pm$ 3.61 ^a	2.39 $\pm$ 0.19 ^a
SO100	3.09 $\pm$ 0.89	13.83 $\pm$ 2.88	48.08 $\pm$ 5.36	1.84 $\pm$ 0.08
OO100	21.41 $\pm$ 2.38 ^a	30.88 $\pm$ 5.37 ^a	36.32 $\pm$ 4.47 ^a	2.34 $\pm$ 0.16 ^a
SO300	2.69 $\pm$ 0.75	14.09 $\pm$ 3.35	48.98 $\pm$ 4.18	1.88 $\pm$ 0.13
OO300	5.84 $\pm$ 2.01 ^b	15.42 $\pm$ 6.63 ^b	45.77 $\pm$ 4.97 ^b	1.92 $\pm$ 0.15 ^b

Note. Values are mean $\pm$ SD of groups ST, OT, SO100, OO100, SO300, and OO300. The data were collected in ten matched fields per mouse. In ST, SO100, and SO300 groups, mice were sensitized with saline on days 0, 7, and 14, challenged with saline on days 25, 26, and 27, and treated with 200 μL of 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively. In OT, OO100, and OO300 groups, mice were sensitized with 100 μg OVA emulsified in 1 mg aluminum hydroxide on days 0, 7, and 14, challenged with 100 μg OVA diluted in 50 μL of DPBS on days 25, 26, and 27, and treated with 200 μL of 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively. ^aDifferent from ST group ($p < 0.05$). ^bDifferent from OT group ($p < 0.05$).

Table 2. Effect of EOCZ on the activity of some enzymes of lung antioxidant defence system in murine model of asthma.

Group	SOD (U/g)	CAT (U/g)
ST	15.60 $\pm$ 5.85	26.64 $\pm$ 5.08
OT	6.40 $\pm$ 1.64 ^a	18.28 $\pm$ 2.56 ^a
SO100	19.55 $\pm$ 2.73	26.72 $\pm$ 4.15
OO100	9.20 $\pm$ 2.08 ^a	18.05 $\pm$ 3.14 ^a
SO300	17.08 $\pm$ 5.86	34.42 $\pm$ 13.75
OO300	15.44 $\pm$ 3.79 ^b	32.27 $\pm$ 4.23 ^b

Note. Values are mean $\pm$ SD of groups ST, OT, SO100, OO100, SO300, and OO300. The data were collected in ten matched fields per mouse. In ST, SO100, and SO300 groups, mice were sensitized with saline on days 0, 7, and 14, challenged with saline on days 25, 26, and 27, and treated with 200 μL of 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively. In OT, OO100, and OO300 groups, mice were sensitized with 100 μg OVA emulsified in 1 mg aluminum hydroxide on days 0, 7, and 14, challenged with 100 μg OVA diluted in 50 μL of DPBS on days 25, 26, and 27, and treated with 200 μL of 0.1% Tween 80, 100 mg/kg EOCZ, and 300 mg/kg EOCZ, respectively. ^aDifferent from ST group ($p < 0.05$). ^bDifferent from OT group ($p < 0.05$).

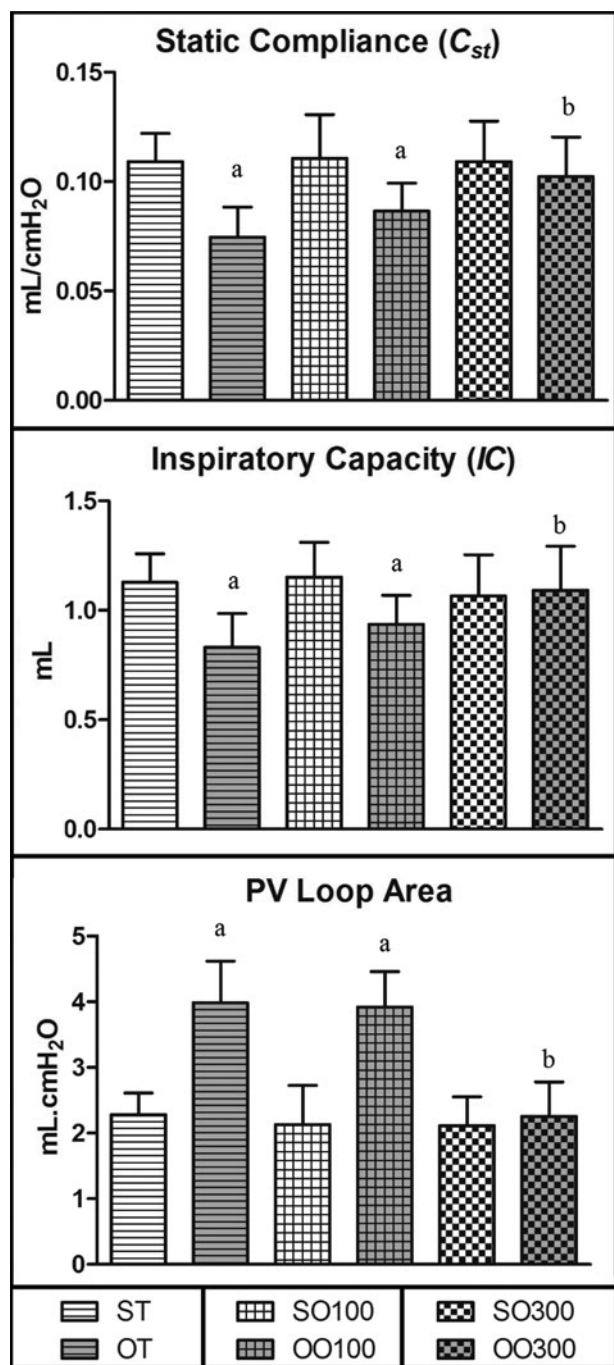


Figure 3. Differences between lung function parameters (quasi-static PV curve). Values are mean $\pm$ SD of groups ST, OT, SO100, OO100, SO300, and OO300. One-way ANOVA followed by Student–Newman–Keuls test was performed. ^a Different from ST group ($p < 0.05$). ^b Different from OT group ($p < 0.05$).

airway inflammation and remodeling (20). More detailed studies identifying and improving our understanding of asthma mechanisms are important, and these would have a greater and faster practical effect if developed in humans. However, for ethical reasons, the types of interventions necessary for the analysis of some

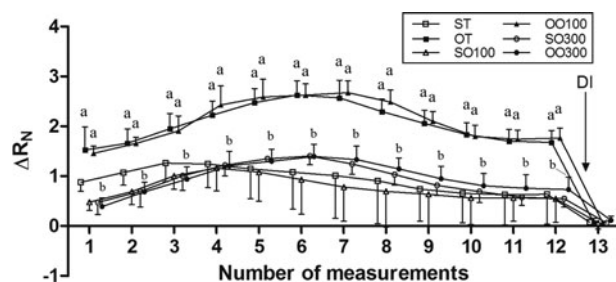


Figure 4. Change in mechanical parameters in ST (open squares), OT (closed squares), SO100 (open triangles), OO100 (closed triangles), SO300 (open circles), and OO300 (closed circles) groups with number of measurements after MCh nebulization (30 mg/mL for 30 s). ΔR_N , lung tissue resistance after nebulization minus its corresponding value before MCh challenge; DI, deep inflation. ^a Different from ST group ($p < 0.05$). ^b Different from OT group ($p < 0.05$).

mechanisms involved at the cellular and molecular levels are not possible in humans.

Considering our knowledge of the complex pathophysiology of asthma and the need to seek new selective active substances of the airway smooth muscle for possible use as a therapeutic agent, this research sought to investigate the possible benefits of EOCZ in influencing the pathophysiology of asthma, as a treatment in an OVA-induced asthma model. The presence of previous studies with EOCZ that demonstrated antiinflammatory (6), myorelaxant (12) effects and reversal of contractions in the respiratory tract (13), raised our curiosity about its beneficial effects in an *in vivo* model, which presents a more complex and representative pathophysiology of a disease of the respiratory system. The OVA-induced asthma model was chosen for investigation in this study.

In relation to our results, we assessed respiratory mechanics by the forced oscillation technique using a constant phase model (Figure 2), and quasistatic PV curve (Figure 3). In the constant phase model, the Newtonian resistance (R_N) represents a good estimate of the total central airway resistance (Bates, 2009), and tissue resistance (G) and tissue elastance (H) are related to the intrinsic properties of the tissue, while hysteresivity (η) correlates with the degree of heterogeneity in lung ventilation (31,32). In the quasistatic PV curve, the static compliance (C_{ST}) indirectly measures the degree of lung tissue distensibility, and estimate of inspiratory capacity (IC) quantifies the volume of air received by the lungs up to a pressure of 30 cmH₂O, while PV loop area provides an estimate of the amount of atelectasis (airspace closure) that existed before the PV loop maneuver (33). To estimate IC and C_{ST} , the pressure and volume data were measured in the upper flatter portion of the expiratory limb of the PV curve.

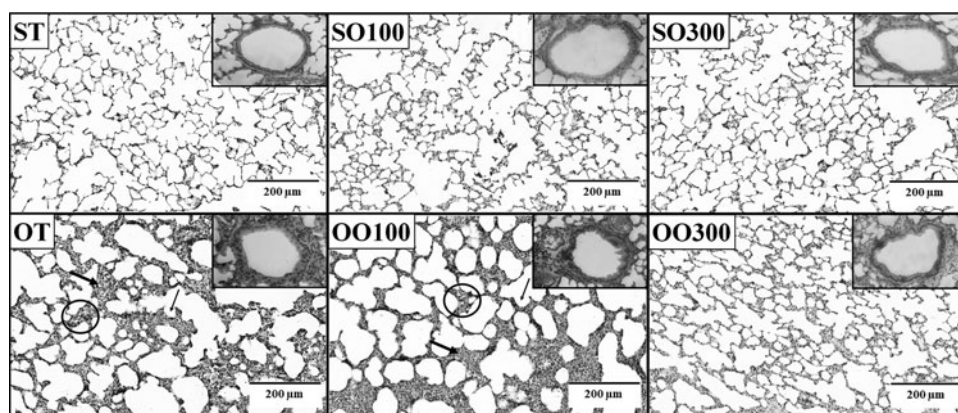


Figure 5. Photomicrographs of lung parenchyma and airway (inserts) stained with HE of ST, OT, SO100, OO100, SO300, and OO300 groups. Thin arrows: thickened septa; thick arrows: cellular infiltrate; and circles: alveolar collapse.

Our results demonstrated significant changes in all respiratory mechanics variables analyzed for the OT and OO100 groups compared to the ST group (Figures 2 and 3). Specifically, the comparison between the OT and ST groups confirmed the establishment of the OVA-induced lesion, since the comparison between OO100 and ST demonstrated that treatment with EOCZ at a dose of 100 mg/kg was not effective in the treatment of the OVA-induced lung lesion.

Therefore, we can assume that the significantly higher values of R_N (Figure 2) might have indicated greater narrowing of the airway lumen or increased stiffness of smooth muscle of the airways. In fact, this hypothesis was supported by histological analysis (Figure 5, inserts) and morphometric data (Table 1, BCI) both confirming narrower airways. The increase in R_N in the OT and OO100 groups was expected, since the acute asthma induction protocol induces an inflammatory process in the airways (34), which can be confirmed by the increase in the number of inflammatory cells in the lung parenchyma of the animals in these groups (Table 1, PMN cells). This inflammatory process in the airways can cause a tissue remodeling characterized by increased goblet cell hyperplasia and the production of matrix metalloproteinases (35), which can cause a decrease in the diameter of the airways, increasing R_N .

Tissue resistance (G) and tissue elastance (H) are related to the intrinsic properties of the tissue. There are several hypotheses to explain their changes. One of them may be the change in tissue rheological properties (32), which could result from thickening of the alveolar septum and presence of atelectatic areas, as observed in our histological analysis (Figure 5), showing an increased amount of collapsed alveoli, reduced alveolar diameter, and increased number of PMN cells (Table 1). Altogether, these findings would suggest lung parenchyma inflammation. Hysteresivity (η) is the ratio of the dissipative to elastic component of overall energy and correlates with

the degree of heterogeneity in lung ventilation (31,32). The increase in R_N (Figure 2), BCI, and areas of alveolar collapse (Table 1), may be associated with the generation of ventilatory heterogeneities and may cause an increase in η .

The reduction in C_{ST} in the OT and OO100 groups compared to ST group was consistent with the distortion of the pulmonary parenchyma with closure of small airways, constituting an effectively smaller lung (36). The higher PV loop area could be attributed to the elastic component of impedance, tissue changes, surfactant-associated mechanism and could be associated with areas of atelectasis (Table 1, Alveolar collapse (%), and Figure 5).

AHR was measured by the pulmonary response to MCh challenge assessed by ΔR_N (Figure 4). After challenge with MCh, we observed significant alterations in the OT and OO100 groups when compared to the ST group, evidencing an AHR of the animals of these groups. This result was expected because AHR is a characteristic present in asthma. AHR is associated with airway inflammation, dominated by eosinophilia, an increase in smooth muscle area, and goblet cell hyperplasia (37). Allergen-induced AHR is also regarded as a manifestation of a Th2-dependent allergic disorder. Th2-associated IL-4, IL-5, IL-9, and IL-13 are believed to be the key cytokines involved in the induction of AHR, lung eosinophil infiltration, and mucus secretion (38). Eosinophils may contribute to remodeling of the airways and to the development of AHR (39).

We did not observe significant alterations in the mechanical values of the respiratory system (Figures 2 and 3) and AHR (Figure 4) between the groups SO100, SO300, and OO300 compared to the ST group. The SO100 $\times$ ST and SO300 $\times$ ST group comparisons might have indicated the nontoxicity of oral EOCZ at doses of 100 and 300 mg/kg, respectively, making its use possible for therapeutic investigation at these doses. The OO300 $\times$

OT group comparison (Figures 2 and 3) demonstrated the effectiveness of EOCZ in reversing OVA-induced lung injury.

In addition, the level of oxidative lung damage through the analysis of oxidative stress markers (SOD and CAT) in lung tissue was demonstrated (Table 2). Our findings showed statistically significant values between the OT and OO100 groups compared to the ST group, corroborating previous studies that demonstrated that OVA-sensitized mice developed an allergic asthma characterized by oxidative stress (40,41). In particular, the comparison between OT and OO300 groups demonstrated the effective antioxidant action of oral EOCZ at a dose of 300 mg/kg in reversing oxidative stress (Table 2). Previous studies have observed that the anethole, the main component of EOCZ, has an antioxidant effect (42,43) and may be responsible for its antioxidant effect on the lung.

Asthma is a chronic inflammatory pathology of the airways, in which changes occur in the levels of various cells, such as eosinophils, mast cells, neutrophils, dendritic cells, and T lymphocytes (44). In the inflammatory focus, these cells, in addition to bronchial epithelial cells, can generate oxidants (45,46). Many of the stimuli that trigger or exacerbate asthma, including viral and pollutant infections, can activate the production of oxidants, inducing inflammation that produces asthma symptoms (47). There is strong evidence that chronic airway inflammation observed in asthma results from the weak or low-functioning antioxidant defenses (48,49), which may cause progressive epithelial damage (50). Epithelial damage to airways contributes remodeling and AHR and suggests that preventing airway epithelial damage and promoting epithelial repair may have therapeutic implications for asthma treatment (51).

The most emphasized treatment in all asthma guidelines is antiinflammatory therapy that uses corticosteroids continuously (52,53). However, the effects of corticosteroids on oxidative stress are controversial. Although some studies report that corticosteroids may increase the number of antioxidant enzymes (54), this is not supported by other studies (55). The antioxidant activity of EOCZ has already been previously demonstrated (56), and our results suggested that this antioxidant action is the main mechanism of therapeutic action of EOCZ in the reversal of OVA-induced lung injury, which makes it a candidate for alternative therapy in conjunction with corticosteroids.

Our study had the following limitations: (1) We used a single-dose acute treatment with EOCZ, performing analyses 24 h after OVA-induced lung injury. Our results demonstrated the benefits of oral EOCZ in the treatment of acute injury, but we could not say if the injury would not return hours or days later. In order to emphasize the

benefits of oral EOCZ use in this animal model, temporal analyzes, performed at different times after EOCZ treatment are necessary. (2) Inflammation biomarkers such as TNF- α , IL-6, and so forth were not determined, so similar studies with the determination of such markers would be important. (3) Future studies with the use of the major compounds of EOCZ (Anethole and Estragole) to determine which compound is the most responsible for the benefits in OVA-induced lung injury are necessary.

5. Conclusion

In conclusion, our results demonstrated that the EOCZ was able to reverse the lesion in the respiratory system of mice submitted to the OVA-induced asthma model. The antioxidant action of EOCZ was likely the main mechanism of action in the reversal of this lesion, so more tests should be performed for its confirmation. The present study serves as a starting point for continuing researches on EOCZ to validate this natural product as alternative asthma therapy. EOCZ use in addition to conventional asthma treatments, such as inhalers and allergy medications, can provide asthma patients with greater comfort. This would also support the use of EOCZ in popular medicine for diseases of the respiratory system.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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