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(RESEARCH ARTICLE)

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Effects of *Rourea coccinea* ethanolic root extract on ovalbumin-induced allergic asthma in rats

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Abstract

Rourea coccinea (syn. Byrsocarpus coccineus) Schum. and Thonn. (Connaraceae) is a medicinal plant that is being used for the treatment of various disorders in Tropical Africa. Previously, we reported anti-inflammatory and antioxidant effects of ethanolic extract of *R. coccinea* root bark (EERc). The present study aimed to test protective potential effects of EERc treatments against ovalbumin-induced airway lung inflammatory and to investigate some of its possible underlying mechanism on allergic asthma. Rats were sensitized and challenged with ovalbumin (OVA), and were orally treated daily with EERc at 200, 400 and 800 mg/kg from day 24 to day 27 post sensitization. The number of total and differential inflammatory cells, leakage of Evans blue dye tracer, histamine and TNF-α level in bronchoalveolar lavage (BAL) fluid were measured. OVA instillation induced airway lung inflammation, which was significantly suppressed by pre-treatment with EERc. Analysis of bronchoalveolar lavage (BAL) revealed that EERc pre-treatment significantly inhibited leukocytosis and reduced Evans blue dye content in the BAL fluid. EERc also significantly decreased the production and release of histamine and TNF-α. Our study shows that EERc possesses significant anti-allergic activity which might be attributed to reduction of histamine, TNF-α in the BAL fluid.

Key word: *Rourea coccinea*; Ovalbumin; Asthma; TNF-A; Inflammatory Cells; Histamine.

1. Introduction

Asthma has been generally recognized as a disease characterized by variable degree of chronic inflammations and structural alterations of the airways [1]. Asthma affects individuals of all ages in countries around the world. Nearly 350 million people are affected in the world and its prevalence has continuously increased [2]. It is a health problem that can be serious and sometimes fatal. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall by leukocytes [3, 4], and is closely associated with the leakage of plasma proteins from the microvasculature into airways [5]. The microvascular leakage facilitates airway oedema, which may consequently produce epithelial cell damage, bronchospasm, and airway obstruction. This phenomenon has been modeled in rats that were sensitized and challenged with ovalbumin [6, 7].

Ovalbumine is known to be an important agent in the development of bronchial-asthma model in rats, by increasing the levels of cytokine in BAL fluid and serum, and by the migration of inflammatory cells (leukocytes such as macrophages, lymphocytes, mast cells, neutrophils, and eosinophils) into areas of inflammation (8, 9).

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These leukocytes, once arrived at the site of inflammation, release pro-inflammatory substances that cause vasodilation, increase vascular permeability and vascular leakage [10, 11]. Mast cells, which are constituent of virtually all organs and tissues are important mediators of inflammatory responses such as allergy [12]. Among inflammatory mediators released from mast cells, histamine remains the best characterized and most potent vasoactive mediator implicated in acute phase of hypersensitivity [12]. Tumor necrosis factor-alpha (TN-α) is also produced by various cell types in response to allergic pulmonary inflammation, including mast cells, macrophages, neutrophils, eosinophils, and epithelial cells [13]. Therefore, suppression of these pro-inflammatory substances may constitute effective therapeutic targets for the treatment of allergic asthma. Due to the various undesirable adverse effects of conventional drugs, the use of natural compounds that have an antioxidant and anti-inflammatory activity might be a useful therapeutic approach beacause several researches proved that oxidative stress is one of the important determinants of asthma [14]. Hence, many plants have been used in traditional medicine because of their potential safety and efficacy.

Rourea coccinea (syn. *Byrsocarpus coccineus*) Schumach. & Thonn, which belongs to the family of Connaraceae, is widely used traditionally to treat inflammation related deseases. Many studies have shown that leaf extract of *B. coccineus* has hepatoprotective [15], antioxidant [15, 16, 17] and anti-proliferative effects [18]. In our previous study, ethanolic extract root bark of *Rourea coccinea* (EERc) was found to possess anti-inflammatory property [19] and antioxidant activity [20] but the underlying mechanism of these properties is unknown. The aim of this study is to uncover the potential anti-inflammatory function and mechanism of *R. coccinea* by using an OVA-induced allergic asthma in rats.

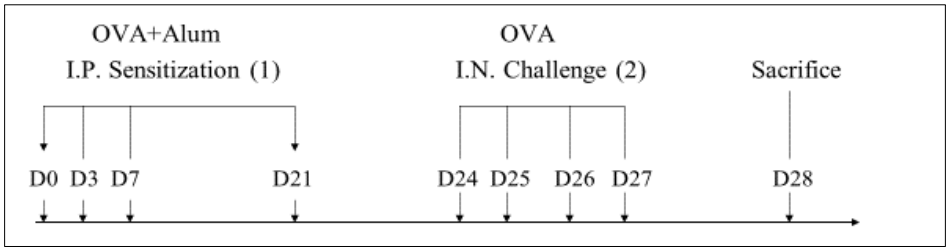
2. Materials and methods

2.1 Preparation of *B. coccineus* root extract

Matured roots of *R. coccinea* were collected around the campus of "Université de Lomé" in February 2021 and authenticated by the botanist at the Department of Botany ("Université de Lomé"). A voucher specimen is deposited in the Herbarium of the department under reference Number TG12604. Root barks were dried at room temperature and powdered. The powder (10 g) was extracted with continuous agitation in ethanol 95° (100 mL) for 72h. The extract was filtered and evaporated at 40°C under reduced pressure. The extraction yield of dried extract was approximately 13.84%.

2.2 Rat sensitization, challenge, and treatment

Animals, except those of non-sensitized or normal control group (NS), were actively sensitized by intraperitoneal injection of 10 mg/kg ovalbumin (OVA) (grade V; Sigma, St. Louis, MO, USA) mixed with 40 mg/kg aluminum hydroxide as adjuvant in normal saline (0.9%) [21]. Non-sensitized (NS) animals were injected with aluminum hydroxide (40 mg/kg) only. Sensitizations were performed 4 times at days 0; 3; 7 and 21. On days 24-27, OVA control (SNT) rats and EERc groups (ST) were challenged with intranasal (I.N.) instillation of 50 µL of a normal saline solution of OVA (20%). Normal control rats (NS) were challenged with 50 µL of normal saline (Figure 1). Thirty minutes before each I.N. and sacrifice on day 28, normal control group and OVA control were orally treated with normal saline (10 mL/kg) and EERc groups were orally treated with different doses of EERc (400 and 800 mg/kg).



OVA control (SNT) rats and EERc groups (ST) were sensitized intraperitoneally on days 0, 3, 7 and 21 (D0, D3, D7 and D21), and challenged intranasally on days 24, 25, 26 and 27 (D24, D25, D26 and D27). Animals were administered vehicle or EERc from days 24 to 28, 30 min before each I.N. Challenge. All animals were sacrificed on day 28. I.N.: intranasal; I.P.: intraperitoneal; EERc: R. coccinea root bark ethanolic extract.

Figure 1 Rats' sensitization and challenge flowchart

2.3 Bronchoalveolar lavage (BAL) fluid

At 24 hours after the last OVA challenge, bronchoalveolar lavage (BAL) was performed by cannulating the trachea and infusing the lung with 5 mL of sterile normal saline solution. BAL fluid was obtained by two aspirations via tracheal

cannulation [21]. Recovery rate of BAL fluid was approximately 78 to 84%. Part of the BAL fluid is used for the white blood cell count. The rest of the BAL is centrifuged (1500 rpm for 5 min) and stored at -80°C for further tests.

2.4 Determination of leukocytes migration to the bronchoalveolar lavage (BAL) fluid

In order to evaluate the potential activity of the plant on leukocytes migration in this model, animals were orally treated 30 min before each OVA intranasal instillation with sterile normal saline solution (10 mL/kg) or different doses of EERc (400 and 800 mg/kg). The non-sensitized groups were considered as the basal and treated with the same volume of sterile saline (vehicle, 10 mL/kg). The total number of leukocytes/mL in BAL fluid was determined using the Malassez technique.

2.5 Histamine assay

For histamine assay, ovalbumin was instilled through days 24 to 28 and the BAL fluid was collected 30 min after the last instillation. Content of histamine in BAL fluid was measured using colorimetric method [22]. In brief, 0.5 mL of sample mixed with both 0.1 mL of 1% sulfanilic acid and 0.1 mL of 5% aqueous sodium nitrite solution was incubated for 10 min. At the end of the incubation period, 1.3 mL of aqueous sodium carbonate (5%) was added to the mixture and two minutes later, 1 mL of ethanol 75° was added. In the interval of 20 min, the absorbance was measured at 530 nm.

2.6 TNFα assay

Concentrations of TNF-α in BAL fluid were quantified using commercially available enzyme-linked immune-sorbent assay (ELISA) kits (RayBio® Rat TNF-alpha, Aachen, Germany). The assay was performed according to the manufacturer's instructions. Cytokine concentrations were expressed in pg. mL^{-1}.

2.7 Vascular permeability assay

Vascular leakage was assessed using Evans Blue dye as a marker for albumin extravasation as described previously [21]. Rats were sensitized and challenged with ovalbumin as described above. Twenty-four hours after the last intranasal instillation, animals were anesthetized with urethane at 1 g/kg. To evaluate the plasma leakage, Evans Blue (30 mg/kg) was administered through the tail vein. Five minutes later, rats were sacrificed by overdose of urethane. Lungs were washed as described previously. The absorbance of the BAL fluid was determined at 620 nm using spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). Evans Blue was dissolved in normal saline and diluted (0-10 µg. mL^{-1} /mL) to generate the standard curve. The amount of Evans Blue permeated into the lung airway was calculated with the standard curve.

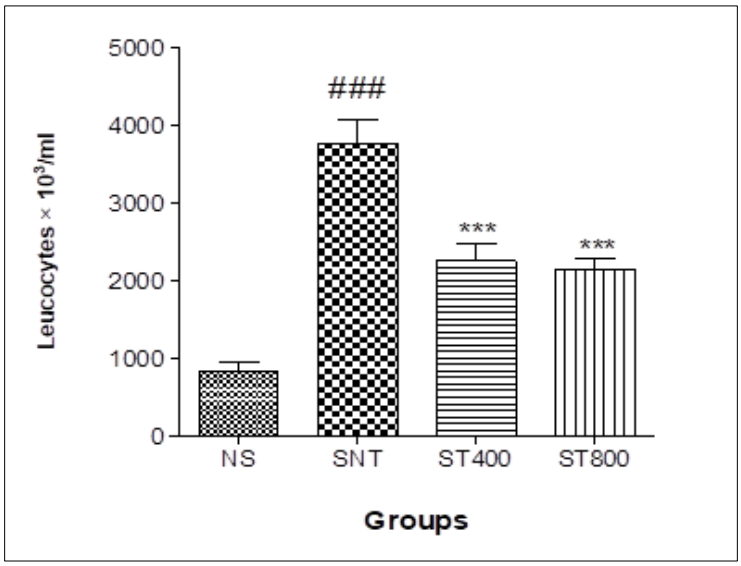
2.8 Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Results were considered to be significant at $P < 0.05$. All statistical analyses were carried out using GraphPad Prism 5.00 (GraphPad Software Inc., CA, USA).

3. Results

3.1 Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid

BAL fluid was collected and the total cells count was determined. The total cell counts in the BAL fluid of OVA control rats were significantly increased compared with the normal control group ($3760\times10^3 \pm 297,99$ vs. $820\times10^3 \pm 130,42$, $P < 0.01$) (Figure 2). As shown in Figure 2, EERc (400 or 800 mg/kg) treatment before OVA inhalation, led to significant lowering of the number of total cells count ($3760 \times10^3 \pm 297,99$ for OVA-sensitive rat vs $2255,5\times10^3 \pm 218,301$ and $2139,65 \times10^3 \pm 135,84$ for 400 and 800 mg/kg of EERc respectively, $P < 0.01$).

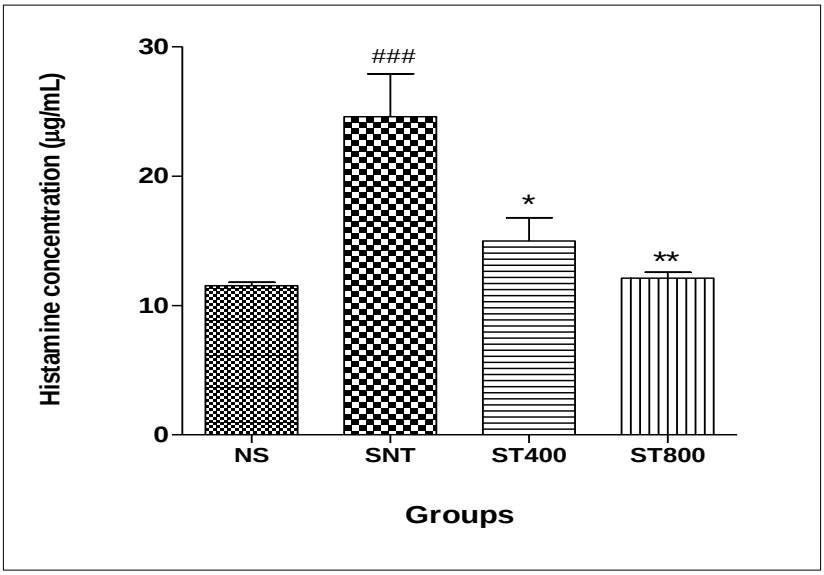


Data are expressed as Mean ± S.E.M, n=5; NS = Non-sensitized group received sterile saline; SNT = Sensitized group treated with saline; ST400, ST800: sensitized and treated groups with the ethanolic extract of the root of R. coccinea 400 and 800 mg/kg, p.o.; ###$P < 0.001$ compared with non-sensitized; ***$P < 0.001$ compared with sensitized.

Figure 2 Effect of EERc on the recruitment of inflammatory cells in BAL fluid obtained from OVA-induced asthma model in rat

3.2 Effect of R. coccinea on histamine release in bronchoalveolar lavage fluid

OVA-challenge of OVA-sensitized rats significantly ($P < 0.001$) increased the histamine content of BAL fluid compared to the normal control (11.535 ± 0.26 to 24.59 ± 3.32 µg/ml). EERc dosed at 400 and 800 mg/kg significantly ($P < 0.05$; $P < 0.01$) decreased the histamine content by 14.99 ± 1.80 µg/ml and 12.15 ± 0.45 µg/ml in BAL fluid, compared to the OVA-challenged control, respectively (Figure 3).



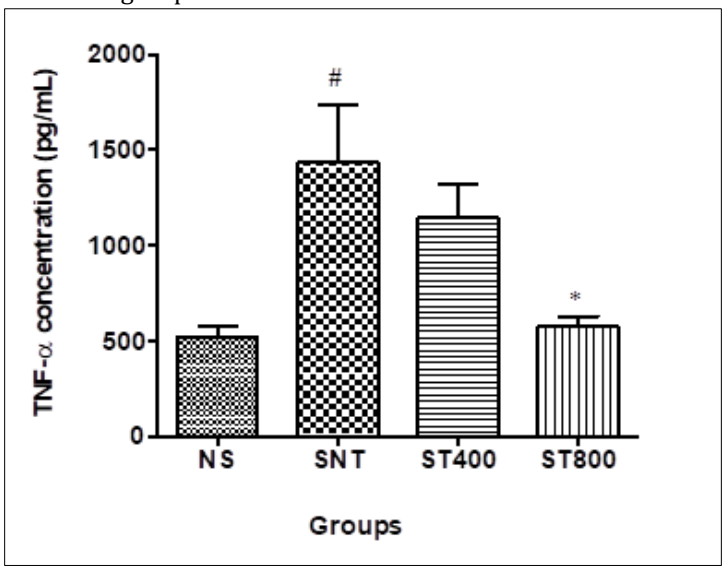
Data are expressed as Mean ± S.E.M, n=5; NS = Non-sensitized group; SNT = Sensitized group treated with saline; ST400, ST800: EERc treated groups with 400, 800 mg/kg, p.o., ###$P < 0.001$ compared with non-sensitize. *$P<0.05$ and **$P<0.01$ compared with sensitized.

Figure 3 Histamine levels in bronchoalveolar lavage in 4 groups of rats

3.3 Effect of R. coccinea root ethanolic extract on the TNF-α concentration in bronchoalveolar fluid

Figure 4 shows that the TNF-α concentration in the BAL fluid of untreated but sensitized rats (SNT) is significantly higher ($P < 0.05$) than that of non-sensitized rats (NS) (1439.00 ± 296.40 pg/mL vs 522.00 ± 54.85 pg/mL). This result indicates that sensitization to ovalbumin followed by intranasal instillation of the same allergen creates an asthmatic

state in Wistar rats. This asthmatic model is therefore a reliable tool for the study of antiasthmatic substances. The groups sensitized and treated with the extract at 400 and 800 mg/kg respectively revealed a decrease in the TNF-α concentration (1146.00 ± 172.80 pg/mL and 78.50 ± 47.68 pg/mL) with significance ($P < 0.05$) at the 800 mg/kg dose compared to the untreated sensitized group.

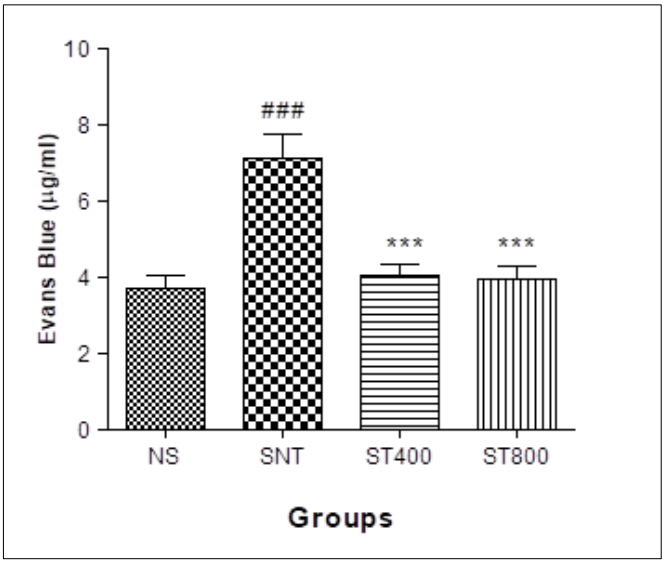


Data represent means ± S.E.M; n = 5. #$P < 0.05$ Tukey's Multiple Comparison Test between non sensitized group (NS: normal control) and sensitized group (SNT: OVA control) ; *$P < 0.05$ Tukey's Multiple Comparison Test between sensitized group (SNT) and extract groups ST400 and ST800.

Figure 4 Effect of EERc on the TNF-α level in BAL fluid

3.4 Effect of *R. coccinea* extract (EERc) on ovalbumin-induced plasma leakage in sensitized rats

Results of EERc showed a dose-dependent inhibitory effect on OVA-induced vascular permeability in rats. Intranasal administration of OVA to OVA-sensitized control rats (SNT) increased vascular permeability by 93.31% ($P < 0.001$) compared to non-sensitized rats (NS). The values of Evans blue concentration in the BAL are 3.69 ± 0.37 µg/mL in the non-sensitized control rats and 7.13 ± 0.59 µg/mL in the sensitized control rats. Pretreatment with the extract at 400 and 800 mg/kg caused significant ($P < 0.001$) (43.24% and 45.01%) inhibition of vascular permeability in the airways compared to the untreated sensitized group. The concentrations of Evans blue in the BAL fluid are respectively 4.05 ± 0.30 µg/mL for the 400 mg/kg dose and 3.92 ± 0.37 µg/mL for the 800 mg/kg dose (Figure 5).



Data are means ± S.E.M; n = 5. ###$P < 0.001$ Tukey's Multiple Comparison Test between non sensitized group (NS: normal control) and sensitized group (OVA control) ; ***$P < 0.001$ Tukey's Multiple Comparison Test between sensitized group (SNT) and extract groups ST400 and ST800.

Figure 5 Effect of *R. coccinea* extract (EERc) on ovalbumin-induced plasma leakage in sensitized rats

4. Discussion

Asthma is a chronic inflammatory airway disease in which multiple complex pathways are involved. Because of its chronic nature, long term medications are required for therapy. Although, there are numerous conventional medicines available, they are unable to prove satisfactory because, they are unable to block all mechanisms that are responsible for causing asthma, low efficacy, various adverse effects, desensitization of receptor and compliance issue.

In this study, we explored the potential protective effects against OVA-induced airway lung inflammatory and investigated the mechanism of action of EERc, an extract which recently demonstrated anti-inflammatory and antioxidant activities [19, 20]. The present study has demonstrated for the first time that EERc had potent anti-inflammatory activity in a murine model of allergic airway inflammation. The suppressive effects of EERC on airway inflammation in rats was showed by a marked decrease in airway recruitment of inflammatory cells, in levels of Evans Blue, histamine and TN-α into BAL fluid.

OVA-induced asthma results from chronic airway inflammation characteristically associated with the infiltration of macrophages, lymphocytes, mast cells, neutrophils and eosinophils into the bronchial lumen [8, 9]. Depletion of these leukocytes in the airways of rats sensitized to ovalbumin inhibits the asthmatic reaction [23]. Results obtained in this study suggest that *R. coccinea* root ethanolic extract might have acted on the passage of leukocytes to the respiratory tract. The total cell number in the BAL fluid were increased after the induction of asthma and significantly decreased by EERc treatment (Fig. 1). Similar results were obtained with *Pluchea ovalis* ethanolic extract in rats [21] and *Juglans regia* ethyl acetate extract in mice. [24]. Previously, *R. coccinea* root bark ethanolic extract has shown inhibitory activity on edema [19]. Data relating to the present study reveal possibilities for the extract mechanisms of action, such as the inhibition of cell migration to the site of inflammation, as observed for reduction of leukocyte migration in OVA-induced asthma model in rat. This could also be the reason why this plant extract promoted reduction of carrageenan-induced paw edema, meaning that its anti-edematogenic action could be due to fewer cell influxes in to the tissue after inflammatory stimuli.

Leukocytes, once arrived at the inflammatory site, release pro-inflammatory substances that cause vasodilation, increased vascular permeability and vascular leakage [10, 11]. Because EERc shows the suppressive effect on leucocytes migration, we examined whether the inhibitory effect of EERc on degree of Mast cells and neutrophils. After migrating into lung tissue, mast cells secrete pro-inflammatory mediators, such as histamine [25]. Histamine is a primary amine released by mast cells after IgE cross-linking by an allergen, with effects such as vasodilatation, mucus hypersecretion, oedema and smooth muscle cell contraction [26, 27, 28]. In this study, a significant increase in histamine levels in OVA-control animals was indicative of the inflammation of lung tissue and the release of mediators. Treatment with EERc significantly decreased histamine levels compared to OVA-control animals.

Various in vivo and in vitro studies have implicated the role of cytokines including TNF-*α*, in asthmatic airway inflammation. In asthma, TNF-*α* blocking activity can be reflected as a possible therapeutic option in patients who are majorly dependent on corticosteroid therapy [29]. TNF-*α* is abundantly found in asthmatic airways and is considered as pro-inflammatory cytokine [30]. We found significantly increased TNF-*α* expression levels in OVA-control group as compared with normal control group. The current study also showed that treatment with EERc significantly suppressed the expression levels of TNF-*α* as compared with positive control or OVA-control. Similar results were obtained with methanol extract and its fractions (n-hexane and ethyl acetate) of *Juglans regia* kernels in mice [31]. The attenuation of allergic airway inflammation by EERc may be, in part, attributed to the suppression of TNF-*α* or may be responsible for the reduction of the number of leucocytes observed.

The leakage of plasma proteins from the microvasculature into airway tissue is an important consequence of asthmatic airway inflammation. The amount of this microvascular leak increases in proportion to the severity of inflammation. The leakage of plasma proteins into the alveolar space depends on the loosening of the vascular endothelium [32, 33]. The leakage of plasma proteins was evaluated by measuring the tissue accumulation of Evans blue dye, which binds to proteins. Consistent with the previous findings, the results of the present investigation also showed that in OVA-induced rats, total protein content in BAL fluid increased compared to the normal control group, whereas EERc administration reduced total protein content in BAL fluid [32]. The present experimental model was undertaken on ovalbumin-sensitized rats that were evoked with intraperitoneal injection of ovalbumin. Our results show that either of ovalbumin can cause microvascular leakage into the airway tissue. The microvascular leakage was observed to be distributed throughout the trachea, bronchi, and intrapulmonary airways. The increase in airway microvascular leakage induced by ovalbumin could be inhibited by pretreatment with EERc ($P < 0.5$).

5. Conclusion

In this study, we show that pretreatment with EERc prevented the development of inflammatory cells lung infiltration in an OVA-induced asthma in rat model. We provided evidence that the anti-inflammatory effect of EERc is associated with, at least in part, the reduction of the infiltration of various inflammatory cells, including eosinophils, mast cells and neutrophils and of the inhibition of pro-inflammatory mediators such us histamine, and TNFα. These activities may be the partial mechanism of the action of EERc. Although R. coccinea has not been prescribed for lung diseases, our study suggests a potential usage of this plant in curing inflammatory lung diseases, which warrants additional studies. Based on our results, we propose that *R. coccinea* can be a new therapeutic option for asthma.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The author declares no conflict of interest.

Statement of ethical approval

The animals were maintained throughout of experiment in accordance with the recommendations of the guide for the care and use of laboratory animals.

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