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The Influence of Essential Oils of Canarium Tree Resin on Expression of TNF-α, Protease Activity, Malondialdehyde (MDA) and Bronchi Histopathology Description in Asthma Rats

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ABSTRACT

Essential oils of canarium tree resin (*Canarium indicum L*) have anti-inflammatory and antioxidant activity. The purpose of this study was to investigate the potential of essential oils of canarium tree resin on the expression of TNF-α, changes in bronchial epithelial cell features, MDA levels and protease activity on asthma rats. This study used four groups of rats, the negative control group, asthma group, therapy group dose of 25 mg/kg body weight, 50 mg/kg body weight and 100 mg/kg body weight. The ashtma rats were prepared by OVA and LPS. TNF-α expression was calculated using Walter calculation, MDA level was measured using Thiobarbituric Acid (TBA), measurement of protease activity was done by spectrophotometric method, and all data analyzed by ANOVA. Histopathologic observation of bronchi was done using Olympus BX51 microscope. The results showed that the therapy of essential oils of canarium tree resin of 25 mg/kg body weight, 50 mg/kg body weight and 100 mg/kg body weight significantly (p<0.05) decreased, MDA up to 54%, protease activity up to 46%, improved bronchial epithelium and TNF-α expression up to 67%. This study proves that essential oils of canarium tree resin can be effectively used as therapy on asthma rats.

Key word: canarium tree resin, asthma, essential oils.

INTRODUCTION

The prevalence of asthma in East Nusa Tenggara is the highest in Indonesia [1]. Patients with asthma in Indonesia increased 4.2% to 5.4% in 2013. Some of the factors that trigger asthma are genetic, pollutants, temperature changes, allergens and bacterial infections. Asthma is characterized by chronic inflammatory airway inflammation that causes shortness of breath, chest tightness, and cough. Airway inflammation and bronchoconstriction occur as a result of the immune response in producing IL-4, IL-5 and IL-13 cytokines by T-helper 2 (Th2) cells and the release of mast cells, eosinophils, and mucus secretion [2]. Allergic asthma occurs due to the provision of OVA [3]. Gram-negative bacteria infection or LPS (*lipopolysaccharide*) as the cause of exacerbations and exacerbate asthma. Mast cells play an important role in asthma pathology through the release of physiological modulators such as histamine and protease. Asthma is associated with increased TNF-α expression in the bronchi biopsy [4]. LPS induces macrophages resulting in the process of phagocytosis in producing ROS. ROS released by macrophages can interact with nitrites (NO_2) and H_2O_2 into reactive nitrogen species (RNS) in the airway causing inflammation and tissue damage in epithelial

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cells [5]. The release of IgE from the expression of cytokines due to mast cell activity can lead to excessive mucus secretion, goblet cell hyperplasia and hypersecretion, smooth muscle hypertrophy, airway obstruction and eosinophil infiltration. Inflammatory immune cells including eosinophils, neutrophils, monocytes and epithelial cells can increase the production of ROS that can damage the lung tissue that causes asthma [6].

Canarium tree resin is used traditionally to cure various diseases: rheumatism, cough, asthma, and epilepsy [7]. The essential oil composition of the canarium tree resin consists of terpenoid compounds, monoterpenoid (93%) and sesquiterpenoid (2.0%). The monoterpene aromatic compounds are γ-terpinene, α-phellandrene, ρ-cymene, α-terpinene, β-pinene, β-phellandrene, terpinolene and α-pinene [8]. Essential oils of canarium tree resin are reported to have antioxidant, anti-inflammatory and antibacterial activity [9]. This study will examine the effect of essential oils of canarium tree resin on MDA levels, protease activity, the severity of asthma based on bronchial histopathology and TNF-α expression.

EXPERIMENT

Chemicals and instrumentation

The materials used in this research are the essential oil of canarium tree resin (*family of Burseraceae*) from steam distillation, ovalbumin, LPS from bacterium porphyromonas gingivalis (*Astarte Biologics*), deionized water, xylol, ethanol, physiological 0,9% NaCl, PBS pH 7.4 (*Phosphate Buffer Saline*), PFA 4% (*Paraformaldehyde Acid*), casein protein block, DAB (Diamono Benzidine), primary antibody (Mouse Monoclonal Anti TNF-α), secondary antibody (*Rabbit Anti-mouse* IgG), polymer anti-rabbit enzyme, $AlOH_3$ (*Aluminium Hydroxide*), H_2O_2 (*Hydrogen Peroxide*), hemotoxylin solution, eosin and entellan solution.

The tool used in this research is Omron CompAir Compressor Nebulizer, vortex, microscope (*Olympus BX51*), UV-VIS spectrophotometry, autoclave, incubator, and centrifugation device (*Denley BR 401*), GC-MS (*Aglient 6980N Network GC System*).

Procedure

Animal Preparations

Wistar rats were used in the study. All experimental animals were placed in cases enclosed with temperatures of 20-25ºC, 50-70% air humidity and lighting. The rats were acclimatized for seven days before being used in the experiment. Animal experiment under a protocol approved by Institutional Animal Care and Use Committee Brawijaya University no. 843-KEP-UB-2018 was followed.

Preparation of Asthma Rats

Injection of ovalbumin OVA I (*SigmaAldrich*) intraperitoneally 10 µg with 1.5 mg $AlOH_3$ in 200 µL PBS (*phosphate buffer saline*) on day 0 and ovalbumin OVA II injection was performed on day 14. Intracurricular lipopolysaccharide LPS injection was performed at 1 µg in the left upper rat molar gingiva mucus on days 10 and 11. The LPS used was LPS1435/1450 from porphyromonas gingivalis (*Astrte Biologics*). Ovalbumin exposure OVA III inhalation was performed on day 21 using a transparent tube connected with Omron CompAir Compressor Nebulizer. Treatment of asthma triggers was performed with OVA nebulose in sterile NaCl with doses of 1 mg / mL for 20 min [10].

Isolation of Essential Oils

Canarium tree resin is distilled of steam distillation for 7 h at 90ºC. Sample added a small amount of anhydrous $MgSO_4$ to remove moisture and flowed nitrogen gas to avoid

oxidation [11]. The essential oils of the obtained canarium tree resin were analyzed using GC-MS [12].

Provision of Resin Essential Oils Therapy of Canarium Tree

The essential oil of canarium tree resin was given to experimental animals of asthma group. Asthma group was treated with essential oil of canarium tree resin 25 mg/kg body weight and 50 mg/kg body weight. Methods of administering oral volume of each rat per 2 mL in each therapy group administered on the 22nd day with essential oil of canarium tree resin for 7 days in a row [13].

Determination of Malondialdehyde (MDA)

The supernatant formed from isolation of protein in lung organ was taken 100 µL inserted into microtube, added 550 µL of distilled water, 100 µL TCA then centrifuged, added 250 µL HCl 1 N and centrifuged again, then it is added with 100 µL Na-Thio 1 % and homogenized. After that, heated in water bath 100 ºC for 30 minutes. After heating, centrifugation was done at 500 rpm for 10 minutes. The supernatant formed was transferred into a new reaction tube [14]. The sample was measured in absorbance by a spectrophotometer (Shimadzu UV-visible spectrophotometer UV-1601) at a maximum wavelength (533 nm) [15].

Protease Activity Assay

Protease activity test was performed on the bronchi isolation results of the optimum condition of pH 6.5 at 37 ºC and incubated for 60 minutes. Protease activity was measured using casein as a substrate and tyrosine as the resultant hydrolysis product. Protease activity measurements included the manufacture of standard tyrosine curves, isolation of protease enzymes, and measurement of protease activity calculated using the Walter method:

$$\text{Enzyme activity} = \frac{(\text{Tyrosin})}{\text{Mr (Tyrosin)}} \times \frac{\text{v}}{\text{p} \times \text{q}} \times \text{df}$$

Information : v = volume of sample (mL)
q = incubation time (minute)
df = dilution factor
p = amount of enzymes (mL)
Mr = relative molecular mass

Histopathological Studies

Organ sample was washed using 0.9% physiological NaCl. Preparation stage includes fixation, dehydration, clearing, embedding, sectioning and pasting in the object glass. Bronchi rats were prepared with hematoxylin and eosin (HE) staining [16]. Bronchi epithelium observed histopathologic feature using Olympus BX51 microscope with 400× magnification.

Immunohistochemistry of TNF-α Expression

Bronchi organ of all groups was incorporated into 4% PFA solution. The histopathology preparations included dehydration, clearing, embedding, sectioning, immunohistochemical staining on glass objects [17]. TNF-α expression in the bronchi was observed and scanned 400× magnification with five field planes for each group.

Statistical Analysis

Data analysis used in this research is quantitative statistical and descriptive qualitative analysis. Quantitative statistical analysis for TNF-α expression and protease activity use calculations according to Walter [18]. Treatment results were analyzed using Microsoft Office Excel and SPSS for Windows with a multiplication analysis (ANOVA) followed by a Tukey test or BNJ (α = 5%) level to determine the difference between treatments. Qualitative descriptive analysis for bronchi histopathological observation using a microscope (Olympus BX51).

RESULT AND DISCUSSION

Effects of Resin Essential Oils of Canarium Tree on MDA Content in Serum

The results of measurements of MDA levels are shown in Table 1. . The rats achieved the highest MDA levels in group B (asthma) with average MDA levels of 8.285 ± 0.029 in comparison to negative control. MDA levels increased to 123%. The Tukey test showed significantly different MDA levels ($p<0.05$) for each treatment group. Therefore, treatment with essential oils of canarium tree resin, rats showed self-recover. This has been proven by decreasing MDA levels from 8.285 ± 0.029 to 7.592 ± 0.208. In the treatment D group of 50 mg/kg body weight, it can be seen that MDA levels are much smaller than MDA levels in the B (asthma) group. A significant effect of treatment with essential oils of canarium tree resin was shown in group E, indicated by decreased MDA levels up to 3.750 ± 0.125. Therapy group of 100 mg/kg body weight decreases MDA levels to 54%. This means treatment with essential oils of canarium tree resin has a significant effect to decrease MDA levels of asthma rat. Decreased MDA levels at doses of 100 mg/kg body weight were caused by monoterpene compounds in the essential oils of canarium tree resin, that suppressed the production of proinflammatory mediators [19].

Table 1. MDA levels in serum rats of asthmatic rats

Treatment Group	MDA level (µg / mL)	MDA level (%)	
		Increasing	Decreasing
Group A (negative control)	3.715 ± 0.141^{a}	–	–
Group B (asthma)	8.285 ± 0.029^{d}	123%	–
Group C (25 mg/kg body weight)	7.592 ± 0.208^{c}	–	8%
Group D (50 mg/kg body weight)	4.115 ± 0.033^{b}	–	50%
Group E (100 mg/kg body weight)	3.750 ± 0.125^{a}	–	54%

Notations a, b, c and d indicate a significant difference between treatments ($p<0.05$)

The identification of GC-MS essential oils of the canarium tree resin of β-phellandrene (10.024%), α-phellandrene (21.684%), γ-terpinene (2.166%) and 2-piperdinone (12.847%) have been reported to inhibit the inflammatory process of 5-LOX (*lipoxygenase*) formation of arachidonic acid, COX-1 (*cyclooxygenase*) and COX-2, TNF-α, IL-6 of the kappa beta transcription factor (NF-kβ) [20]. Decreased PGE (*prostaglandin*) and LTB4 (*leukotriene B4*), LTCA (*Leukotriene C4*) in serum can decrease inflammatory cells in producing ROS. α-phellandrene has been reported to decrease macrophage phagocytosis from blood samples [21]. Decreasing in ROS (*reactive oxygen species*) production may suppress lipid peroxidation that may decrease radical peroxide, lipid hyperoxide and aldehyde formation such as MDA.

Effects of Resin Essential Oils of Canarium Tree on Protease Activity in Bronchi

The measurement of protease activity in the bronchi organs of the asthma model rat given the essential oil of canarium tree resin shown in Table 2. One-way ANOVA statistic test results showed a significant difference ($p<0.05$) in four treatment groups. The highest protease activity in group B (asthma) is 0.0419 ± 0.0013 compared with negative control, protease activity increased to 76%. Treatment with essential oils of canarium tree resin showed a decrease in protease activity from 0.0419 ± 0.0013 to 0.0287 ± 0.0006. A significant reduction of treatment effect with essential oils of canarium tree resin was shown in group D, decreased protease activity until 0.0223 ± 0.0025. This proves that essential oils of canarium tree resin can decrease protease activity in asthmatic mice. The best therapy group was the group with a therapy dose of 50 mg/kg body weight based on a decrease in protease activity of 46%. The increased inflammatory response of allergic asthma due to the high activity of a protease that is mediated by allergens such as the production of reactive oxygen species in the form of neutrophil-induced superoxide anions, hydroxyl radicals, hydrogen peroxide, and nitrite oxide [22]. Increased ROS causes a dangerous pathophysiological disorder in allergic asthma. Excessive ROS can damage carbohydrates, proteins, lipids, and DNA that ultimately lead to increased allergic asthma inflammatory responses.

Table 2. Protease activity in bronchi organs of asthmatic rats

Treatment Group	Protease Activity (µmol/mL.min)	Percentage Protease Activity (%)	
		Increasing	Decreasing
Group A (negative control)	0.0237 ± 0.0011^{b}	–	–
Group B (asthma)	0.0419 ± 0.0013^{d}	76%	–
Group C (25 mg/kg body weight)	0.0287 ± 0.0006^{c}	–	31%
Group D (50 mg/kg body weight)	0.0223 ± 0.0025^{b}	–	46%
Group E (100 mg/kg body weight)	0.0196 ± 0.0007^{a}	–	53%

Notations a, b, c and d indicate a significant difference between treatments ($p<0.05$)

Antioxidant compounds such as terpenoids can prevent ROS as a trap that absorbs energy and electrons, quenching ROS, metal ion bonds to prevent ROS formation, and antioxidants as chain breakers that eliminate and destroy ROS. Essential oils are able to counteract free radicals and anti-inflammatory agents against oxidative explosions [23]. The content of terpene compounds in essential oils of canarium tree resin that is β-phellandrene, α-phellandrene, γ-terpinene and ρ-cymene are reported to provide anti-inflammatory properties by inhibiting migration and decreasing the production of inflammatory mediators, including protease and proinflammatory enzymes such as COX-1 and COX2 [24]. α-phellandrene significantly decreases mast cell degranulation in regulating the inflammatory response. Mast cells are activated to rapidly release various inflammatory mediators such as proteases, histamine, prostaglandins, leukotrienes, and chemokine cytokines including IL-8 contributing to allergic disorders and neutrophil infiltration. α-phellandrene acts as a stabilizer of mast cells and modulates macrophages to decrease phagocytosis caused by ovalbumin and lipopolysaccharide [25]. The essential oils of canarium tree resin inhibit the degranulation of mast cells that can decrease inflammatory cytokines and reduce the infiltration of cell tissue neutrophils resulting in a decrease in the production and activity of protease enzymes.

Effects of Resin Essential Oils of Canarium Tree on Histopathological Examination

OVA-induced allergy and LPS-induced can cause acute airway inflammation in the bronchi. Bronchi conditions in asthma are characterized by subepithelial fibrosis, plain muscle hypertrophy, inflammatory cell infiltration of the bronchi tissue, damage and thickening of the bronchi epithelial cell, smooth muscle hypertrophy, goblet cell hyperplasia. Changes in epithelial cells in the bronchi can be seen through bronchial histopathologic preparations stained with Hematoxylin Eosin (HE) as shown in Figure 1.

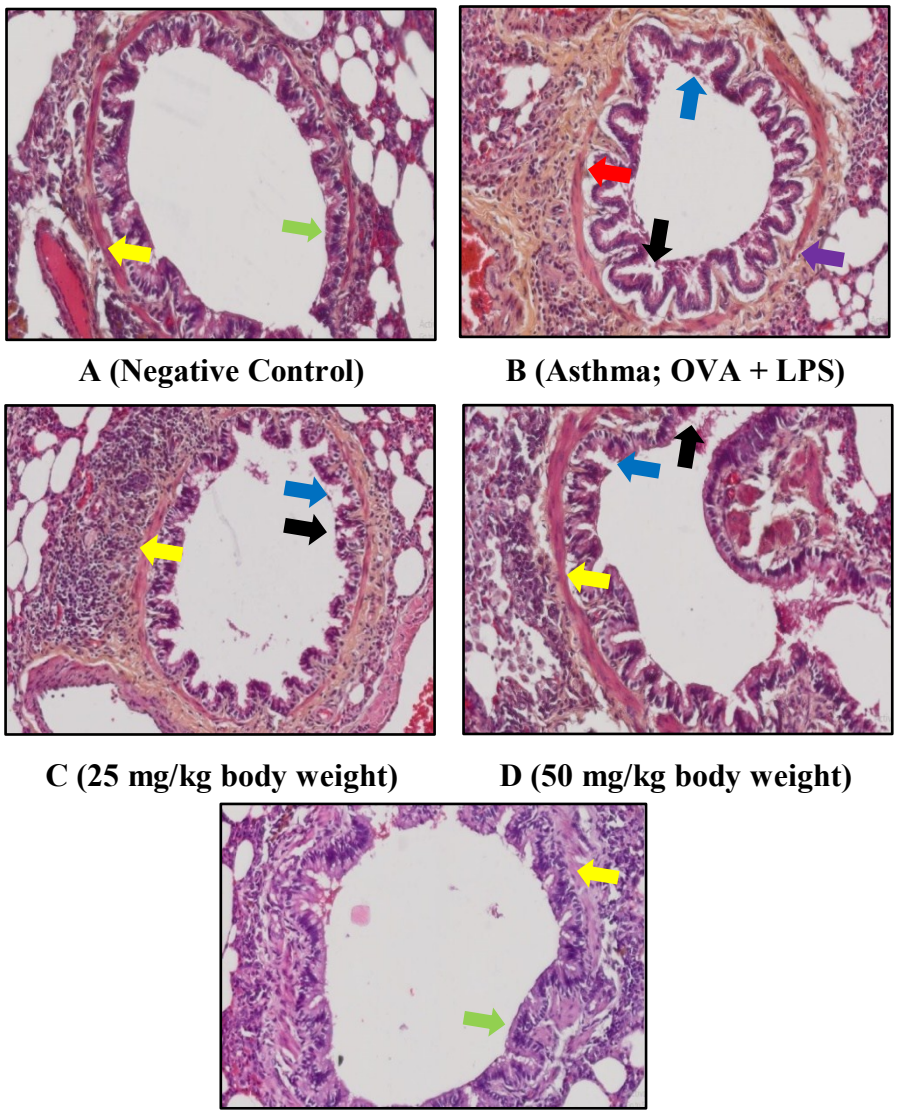

Figure 1. Histopathology Overview Bronchi Muscle of Asthma Rats Using Hemotoxylin-Eosin Staining (HE) (*Magnification 400*×). (A) negative control group; (B) group of asthma, (C) therapy 25 mg/kg body weight, (D) therapy 50 mg/kg body weight and (E) therapy 100 mg/kg body weight. Normal smooth muscle (*yellow arrow*), normal epithelial cell structure (*green arrow*), hypertrophy and smooth muscle hyperplasia (*purple arrow*), detached epithelial cells (*blue arrow*), damaged epithelial cells (*black arrow*), erosion of epithelial cells (*red arrow*).

Bronchi histopathology in group B (Figure 1B) suggests airway inflammation, changes in cell structure resulting in damage from OVA and LPS exposure. OVA increases the thickness of the epithelium, basal membrane, sub-epithelial smooth muscle layer, the number of mast cells and goblet cells [26]. LPS has the potential to induce airway remodeling by releasing TGF-α from epithelial mediated by TNF-α through activation of the TLR4 receptor for delivery to neutrophils and macrophages. ROS produced by neutrophils and macrophages causes epithelial damage and reduces enzyme activity. Epithelial cell destruction is key to the occurrence of respiratory tract remodeling mediated by transformational growth factor-β, epithelial damage or repair in response to inflammation such as IL-13.

Bronchi histopathology in group C (Figure 1C) and group D (Figure 1D) at a dose of 25 mg/kg body weight and 50 mg/kg body weight, showed bronchial smooth muscle repair leading to healing. Damage to epithelial cells in group C and group D has decreased significantly. The best results were shown in group E at 100 mg/kg body weight (Figure 1E) to improve maximal bronchial smooth muscle and regular epithelial cell structure.

Monoterpenes such as β-phellandrene, α-phellandrene, γ-terpinene and p-cymene from essential oils of canarium tree resin are bioactive in aromatic plants that contribute to anti-inflammatory activity in asthma. α-phellandrene plays a role in suppressing cellular populations from immune leukocytes, promoting macrophage phagocytosis, increasing cytotoxic NK cells and inducing cell proliferation of B and T cells after exposure to OVA and LPS. Macrophage modulation produces an inflammatory reaction that can cause bronchial tissue damage [27]. α-phellandrene inhibits the expression of proinflammatory cytokines (*TNF-α, IL-6, and IL-1β*), cyclooxygenase-2 (*COX2*), and NF-kB activation further decreases PGE, LTB4, and LTC4. Decreased inflammation reduce bronchial smooth muscle damage and epithelial cells so that the healing process begins with the repair of epithelial cells. α-phellandrene has been reported to show no cytotoxicity to mammalian cells [28]. Group D, with the dose of 50 mg/kg body weight (Figure 1D) showed significant results because of its ability to repair the bronchial histopathological damage.

Effects of Resin Essential Oils of Canarium Tree on TNF-α Expression in Bronchi

TNF-α is a cytokine as a proinflammatory agent that regulates the function of macrophages released due to OVA and LPS exposure infections as a mediator of tissue inflammation. This study used an immunohistochemical method to observe the expression of TNF-α in the bronchi shown in Figure 2.

The assessment system used for TNF-α is the intensity parameter of staining nucleus and the percentage of cells that gives positive expression [29]. The correlation between TNF-α and the number of size of damage to the infected tissue was statistically tested One-Way ANOVA with BNJ test showed a significant difference ($p<0.05$) between cells and the intensity of the percentage of TNF-α expression shown in Table 3.

The highest of TNF-α expression in group B (*asthma*) average 0.702 ± 0.039, in comparison to negative control, expression of TNF-α increased to 250%. The change in brown color (Figure 2B) on the bronchi nuclear cell tissue organ, indicates an increase in TNF-α positive expression from OVA and LPS exposure. Therapy group of 25 mg/kg body weight decreases TNF-α expression to 35% and therapy 50 mg/kg body weight decreases to 50% of negative control. The significant effect of treatment with essential oils was shown in group E, which has been proven rats showed self-recover indicated by decreased TNF-α expression up to 0.228 ± 0.011 with a percentage decrease of 67%.

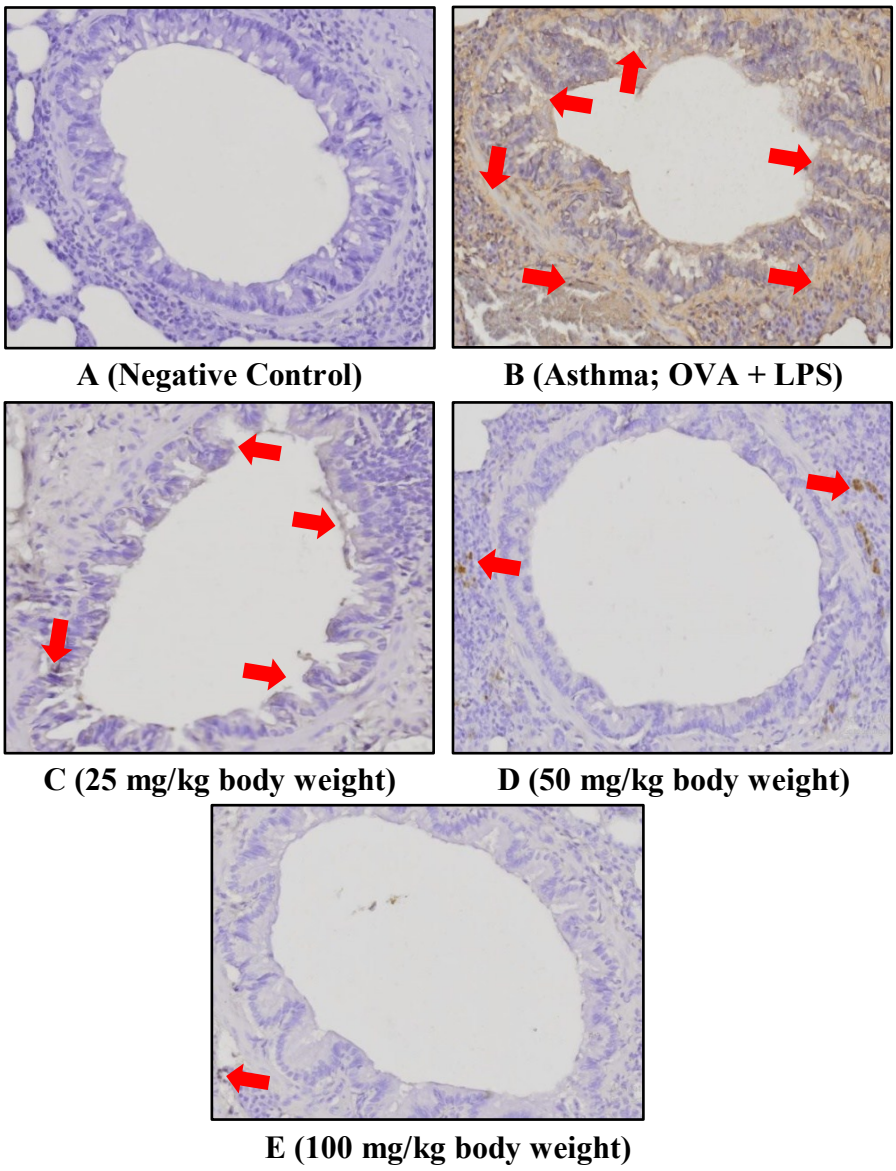

Figure 2. The different levels of expression of TNF-α bronchi of asthma rats exposed by OVA and LPS using immunohistochemical method (400× magnification). (A) negative control group; (B) group of asthma, (C) therapy 25 mg/kg body weight, (D) therapy 50 mg/kg body weight and (E) therapy 100 mg/kg body weight. TNF-α expression (*red arrow*).

Table 3. Expression of TNF-α in bronchi organs of asthmatic rats

Treatment Group	TNF-α Expression	TNF-α Expression (%)	
		Increasing	Decreasing
Group A (negative control)	0.198 ± 0.014^{a}	–	–
Group B (asthma)	0.702 ± 0.039^{d}	250%	–
Group C (25 mg/kg body weight)	0.456 ± 0.023^{c}	–	35%
Group D (50 mg/kg body weight)	0.352 ± 0.024^{b}	–	50%
Group E (100 mg/kg body weight)	0.228 ± 0.011^{a}	–	67%

Notations a, b, c and, d indicate a significant difference between treatments ($p<0.05$)

The cyclic monoterpene compounds have anti-inflammatory properties by inhibition of cell migration and decreased the production of inflammatory mediators, including TNF-α and IL-6 and proinflammatory enzymes such as COX-2. Inflammatory mediators are activated by mast cells such as TNF-α, IL-6, histamine, prostaglandin, leukotriene, and some chemotactic cytokines including IL-8. Inflammatory mediators contribute to allergic disorders, inflammation, and neutrophil infiltration [30]. α-phellandrene is reported to decrease the production of proinflammatory cytokines such as TNF-α and IL-6, besides α-phellandrene can decrease leukocyte migration and degranulation mast cells [31]. α-phellandrene serves to stabilize mast cells so as to suppress the production of proinflammatory cytokines such as TNF-α by inhibiting NF-kB causing damage to bronchial cell tissue and neutrophil infiltration.

CONCLUSION

This study revealed that essential oils of canarium tree resin that contain monoterpenes such as β-phellandrene, α-phellandrene, γ-terpinene and p-cymene are able to suppress inflammation of the respiratory tract by stabilizing mast cells and inhibiting neutrophil infiltration in the asthmatic model rat. This is evidenced by decreased MDA levels of 54%, protease activity 46%, expression of TNF-α to 67 %, and the process of repair and healing epithelial cells in the bronchi.

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