Full Length Research Paper

Protective effect of eucalyptus oil on pulmonary destruction and inflammation in chronic obstructive pulmonary disease (COPD) in rats

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Eucalyptus oil (EO), an essential oil isolated from Eucalyptus leaves, was examined for its effect on lipopolysaccharide (LPS) and *Klebsiella pneumoniae*-induced COPD in rats. The COPD model was induced by instilling intratracheally with LPS and *K. pneumoniae*. The test compound, EO (30, 100 and 300 mg/kg), prednisone acetate (10 mg/kg) or vehicle was instilled intragastrically after three weeks exposure to LPS and *K. pneumoniae*, lasting for 4 weeks. EO significantly reduced amounts of inflammatory cells in bronchoalveolar lavage fluid (BALF) and blood, and decreased bronchiolitis, emphysematous changes and thickness of bronchioles. It also significantly reduced the increased AB-PAS-positive goblet cells in bronchioles. Prednisone acetate attenuated pulmonary inflammation and airway mucus hypersecretion, but no significant difference was found on emphysema. Pretreatment with EO markly reduced the production of proinflammatory cytokines TNF-α and IL-β in lung homogenate, significantly decreased the elevated malondialdehyde (MDA) level and and increased superoxide dismutase (SOD) activity. These findings indicate that EO could exert an protective effect against LPS plus *K. pneumoniae*-induced lung indury via inhibition of proinflammatory cytokines production and improvement of anti-oxidant status. The results provide evidence that EO might have its potential to be a proper candidate drug in the treatment of COPD.

Key words: Eucalyptus globulus, lipopolysaccharide, cytokine, chronic obstructive pulmonary disease.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by chronic inflammation and irreversible airflow obstruction, mainly induced by cigarette smoking and noxious stimuli including infection (Samareh Fekri et al., 2015). Chronic and persistent inflammation results in

emphysema and irreversible airway narrowing that resulted from protease, mucociliary dysfunction, oxidative stress or fibrosis around small airway. There is increasing evidence that bacterial colonization in COPD patients contributed to airway inflammation and excerbated the

progression of decline lung function (Garcha et al., 2012; Korsgren et al., 2012; Cukic, 2013). However, the mechanisms are still not well known. Xu et al. (1999) succeeded to establish a COPD rat model by repeated injecting intranasally of *Klebsiella pneumoniae*, suggesting an important role of bacterial infection in the pathogenesis of COPD.

Eucalyptus essential oil (EO), is commonly used as expectorant for upper respiratory tract infection or inflammation, as well as decongestant and various other inflammatory diseases. It is reported that EO possesses particular anti-inflammatory and anti-oxidative properties (Rantzsch et al., 2009; Juergens, 2014). In murine macrophages, Eucalyptus oil inhibited inducible nitric oxide synthase mRNA expression and NO production induced by lipopolysaccharide and IFN-γ (Vigo et al., 2004). Concomitant therapy of Eucalyptol reduced exacerbations and improves lung function in patients with COPD (Worth et al., 2009). These findings support at least some of the essential oils of Eucalyptus species used in the clinical treatment. However, direct evidence is still lacking to identify its pharmacological proterties in chronic obstructive pulmonary diseases. Therefore, in the present study, the authors established a rat model of COPD through intratracheal adminstration of LPS plus *K. pneumonia*, and determined the effects of Eucalyptus oil on pulmonary destruction and inflammatory responses.

MATERIALS AND METHODS

Male Sprague-Dawley rats, obtained from Shanghai experimental animal center, China, weighing 180 to 230 g, were kept at $23 \pm 2°C$ with a 12 h light/12 h dark cycle. They were allowed free access to food and water. All animals were handled in accordance with the Ethical Principles for Care and Use of Laboratory Animals as previously reported (Zhao et al., 2014). All procedures decribed herein were reviewed and aproved by the local animal ethics committee.

Drugs and reagents

Eucalyptus oil, which was commercially prepared, was used in all of the experiments (Batch No.060711, provided by our laboratory). *K. pneumoniae* (No. 1.1736) was purchased from Agricultural Culture Collection of China (ACCC), LPS (*Escherichia coli* O111: B$_4$) was purchased from Sigma.

Experimental procedure

COPD model was induced by intratracheal LPS plus K.P exposure. Rats were randomly divided into seven groups, and performed as follows: 0.1 mL of *K. pneumoniae* (density, $\geq 6 \times 10^8$ CFU/mL) was instilled intratracheally twice a week and 2 mg/kg of LPS once every two weeks; EO group (30, 100, and 300 mg/kg) was administrated intragastricly after 12 weeks exposure of K.P and LPS and lasted for 4 weeks; Prednisolone acetate (Pred, 10 mg/kg) was administrated as positive control group, aseptic saline was administrated intragastricly as negative control group.

Preparation of bronchoalveolar lavage fluids (BALF) and blood for cell counting

Rats were anesthetized intraperitoneally with sodium pentobarbital, and BALF was harvested 24 h post-last *K. pneumoniae* and LPS exposure. Trachea of each rat was surgically exposed and cannulated. The right lungs were lavaged with 1.5 mL of PBS three times, fluid recovery was routinely ≥90%. A 0.1 mL aliquot was used for total leukocyte number counting. 20 μL of tail vein blood was harvested and added to 0.38 mL of 2% acetic acid solution, and stained with Wright-Giemsa staining. The total inflammatory cell number in the blood film was counted under oil immersion lens.

Histological examination

The lungs were collected and fixed with 10% neutral formalin for one week. After tissues were paraffinized, 5 μm sections were cut and stained with hematoxylin-eosin (H.E.) staining for evaluation of alveolar/interstitial inflammation and emphysema.

Morphological assessment

After the lung tissues were paraffin-embedded and sectioned, 5 μm sections were stained with H.E. To evaluate extent of lung destruction, there was focus on the presence of any of the following: (1) pulmonary mean linear intercept (Lm); (2) mean alveolar number (MAN); (3) ratio of thickness of bronchioles to diameter. Lm, as a measure of interalveolar wall distance, was computed on each slice based on 8 random fields using a cross-line under light microscopy. The total length of the cross-line divided by the numbers of the alveolar wall intersecting the lines was defined as Lm. MAN, an indicator of alveolar density, was computed on each slice based on 8 random fields by counting the numbers of alveoli and dividing the number by the area of the field. Ratio of thickness of bronchioles to diameter was computed on each slice based on 3 to 5 medium bronchi by measuring external diameter and internal diameter and dividing the difference by external diameter (Wang et al., 2014).

Determination of goblet cell hyperplasia

After fixation, 5 μm sections were stained with Alcian blue-Periodic Acid Schiff (AB-PAS). Positive goblet cell number and total cell number of columnar epithelial cells were counted in the main bronchus and 2 to 3 medium bronchi. The ratio of positive goblet cells was calculated by dividing the positive goblet cell number and total cell number of columnar epithelial (Takeyama et al., 1999).

Determination for TNF-α, IL-1β, MDA and SOD activity in lung tissues

Lung tissues stored at -80°C were homogenized in homogenization Tris-buffer on ice using Heidolph Diax 900 Homogenizer (Heidolph, Germany) and then centrifuged at 12000 g for 30 min at 4°C. The supernatants were used to determine the level of TNF-α, IL-1β, SOD and MDA activity. Concentration of the protein in the supernatants was detected using Coomassie brilliant blue G250 method. The concentrations of TNF-α, IL-1β, superoxide dismutase (SOD) and malondialdehyde (MDA) were determined using commercial ELISA kits (BD Bioscience, USA; Jiancheng Bioengineering Institute, Nanjing, China). All procedures were done according to the instructions of the manufactures (Huang et al.,
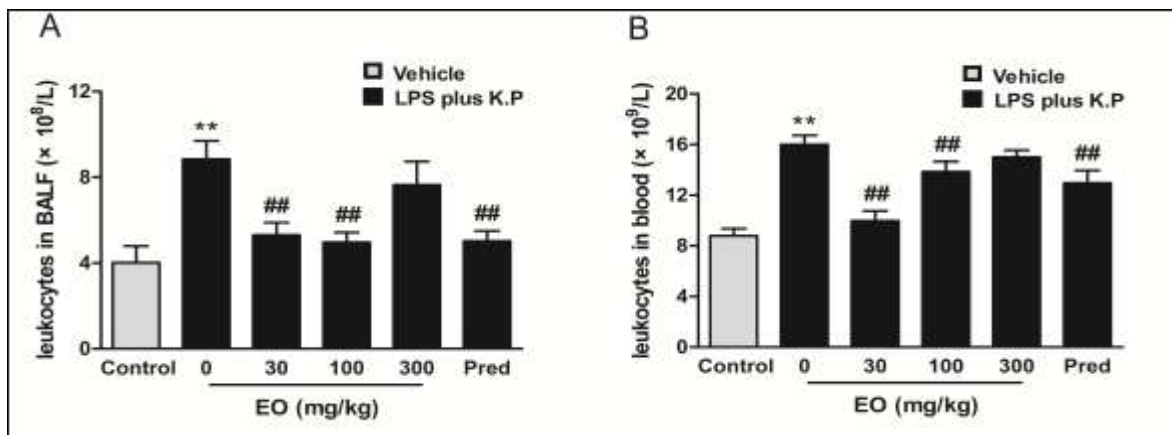
Figure 1. Effect of EO on total cell numbers of inflammatory cells in BALF and blood. 24 h post-last K.P exposure, total cell numbers in BALF (A) and blood (B) were counted and analyzed. Data were expressed as mean ± SD of 8-12 rats /group. $^{**}P < 0.01$ compared with control group, $^{##}P < 0.01$ compared with group.

2013).

Statistical analysis

Data were expressed as mean ± SD and analyzed by a one-way analysis of variance (ANOVA) followed by Dennett's post hoc test with SPSS 15.0 for Windows. For all analyses, significance was calculated with a P value < 0.05 considered statistically significant.

RESULTS

Effect of EO on cell infiltration in BALF and blood

To determine the effect of EO on pulmonary inflammation and peripheral inflammation, the number of leukocytes in the BALF and blood were counted. After LPS plus *K. pneumoniae* exposure for 3 months, the total number of leukocytes in BALF and blood was significantly increased. EO at 30 and 100 mg/kg significantly decreased the infiltrated leukocytes cells in BALF and blood (Figure 1A and B), as compared to the vehicle group. Treatment with EO at 300 mg/kg also obviously decreased the number of leukocytes in BALF and blood, but no significant difference was found. Pred at 10 mg/kg significantly reduced the cell number of leukocytes in BALF and blood.

Effect of EO on emphysematous destruction and bronchiolitis

To observe the effect of EO on emphysema and bronchiolitis, LPS plus *K. pneumoniae* was intratracheally instilled and induced emphysematous destruction, bronchiolitis, small airway remodeling within 4 months. In vehicle group, when compared with the normal alveolar structure, the lung parenchyma was largely destructed

and less alveolus was found after intratracheal instillation of LPS plus *K. pneumoniae*; bronchiolitis was severe since a lot of inflammatory cells gathered around the fine bronchus. Treatment with EO and prednisone obviously reduced emphysematous damage and bronchiolitis around the bronchioles (Figure 2A). Histological analyses showed that Lm and thickness of bronchioles were significantly increased after instillation of LPS plus *K. pneumoniae* (Figure 2B and C), while MAN was significantly decreased (Figure 2D, P < 0.01). Treatment with EO at 100 and 300 mg/kg showed less emphysematous damage, decreased thickness of bronchioles and increased MAN values when compared with vehicle group. No significant difference was found between vehicle and Pred groups.

Effect of Eucalyptus oil on mucus secretion

As shown in Figure3A, there were more AB-PAS-positive goblet cells in bronchioles in the presence of *K. pneumoniae* plus LPS, while less was found in Eucalyptus oil group and Prelon group. The total number of AB-PAS-positive goblet cells was significantly increased in vehicle group as compared to the control group. EO significantly reduced the number of AB-PAS-positive goblet cells in bronchioles at 30, 100 and 300 mg/kg (Figure 3B). Pred (10 mg/kg) also significantly reduced the number of AB-PAS-positive goblet cells in bronchio**les.**

Effect of Eucalyptus oil on TNF-α and IL-1β release

The levels of TNF-α and IL-1β in lung homogenate were significantly increased after challenge of K.P plus LPS for 4 months. EO at dose of 30, 100 and 300 mg/kg
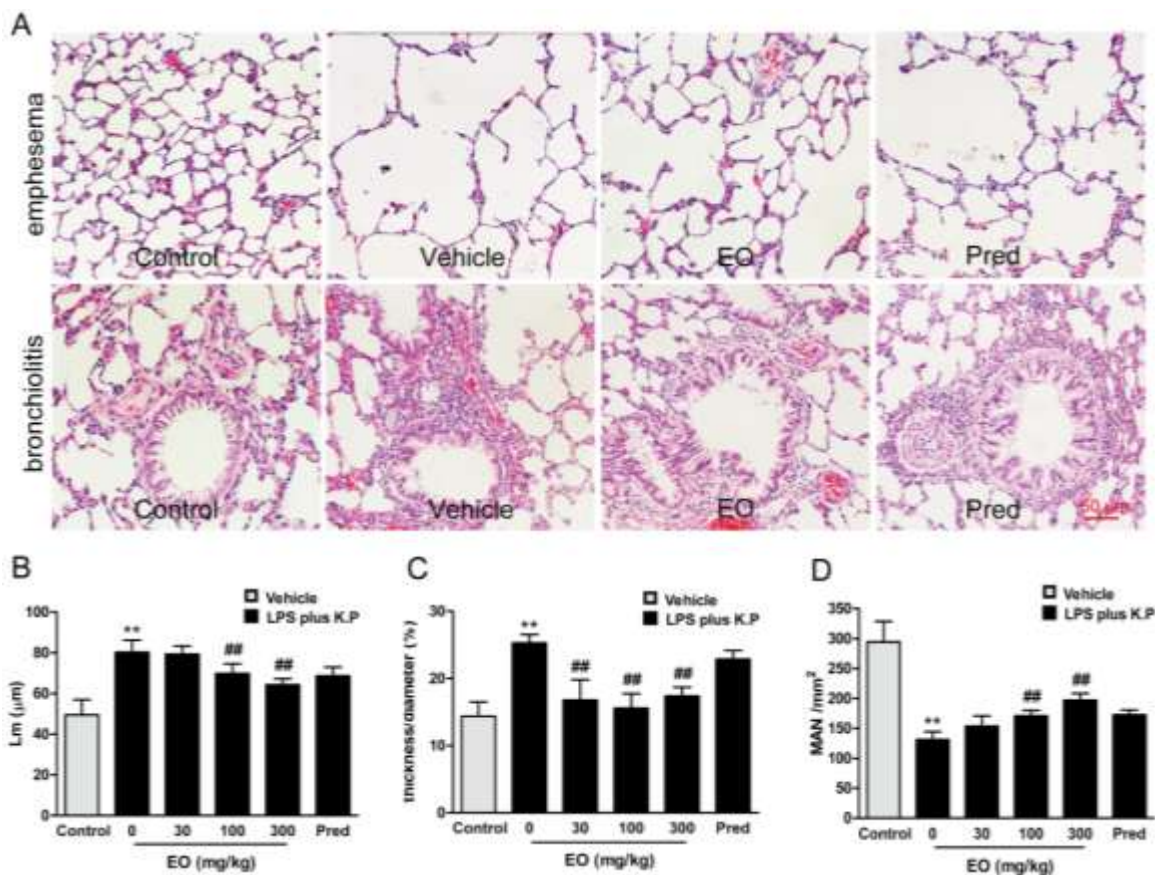
Figure 2. Effect of EO on histochemical changes in rats. Twenty-four hours after the last K.P exposure, the lungs were immersed in 10% neutral formalin for 7 days. After tissues were paraffinized, 5 μm sections were prepared and stained with hematoxylin-eosin for observation of emphysema change and bronchiolitis (A). To estimate the extent of lung destruction in rats, Lm (B), thickness of bronchioles (C) and MAN (D) were evaluated as described in methods. Data were expressed as mean ± SD of 6 rats /group. $^{**}P < 0.01$ compared with control group, $^{##}P < 0.01$ compared with vehicle group.

significantly reduced the production of pro-inflammatory cytokines TNF-α (Figure 4A, P < 0.01) and IL-1β (Figure 4B, P < 0.01). Rats pretreated with Pred at dose of 10 mg/kg also showed decreases in TNF-α and IL-1β productions when compared with the vehicle group.

Effect of EO on MDA production and SOD activity in lung tissues

After exposure with LPS plus *K. pneumoniae*, rats produced a greater amount of MDA in vehicle group when compared with the control group. EO at 30, 100 and 300 mg/kg significantly decreased the LPS plus K.P-induced MDA production in lung homogenate (Figure 4C, P < 0.01). In contrast, SOD activity was elevated obviously in vehicle group when compared with that of control group. While EO at 100 and 300 mg/kg significantly increased the SOD activity in parallel to vehicle group (Figure 4D, P < 0.05). These data indicated

that EO might attenuate the LPS plus *K. pneumoniae*-induced changes via oxidant-antioxidant balance. Among all the tested doses of EO, 100 mg/kg exhibited the best anti-oxidant effect. Pred at dose of 10 mg/kg decreased the MDA production but no significant effect on SOD activity was observed.

DISCUSSION

The present study aimed to evaluate the protective effect of EO using a LPS plus *K. pneumoniae*-induced COPD model and to investigate the underlying mechanisms of the action associated with its anti-COPD activity.

Although, cigarette smoking is the leading risk factor of COPD, only 15-20% of smokers develop the disease. It was postulated that bacterial infections play a major role in the pathogenesis of COPD (Anthonisen, 2004). Recently, increasing evidence supported a clear relationship between bacteria infection and exacerbations
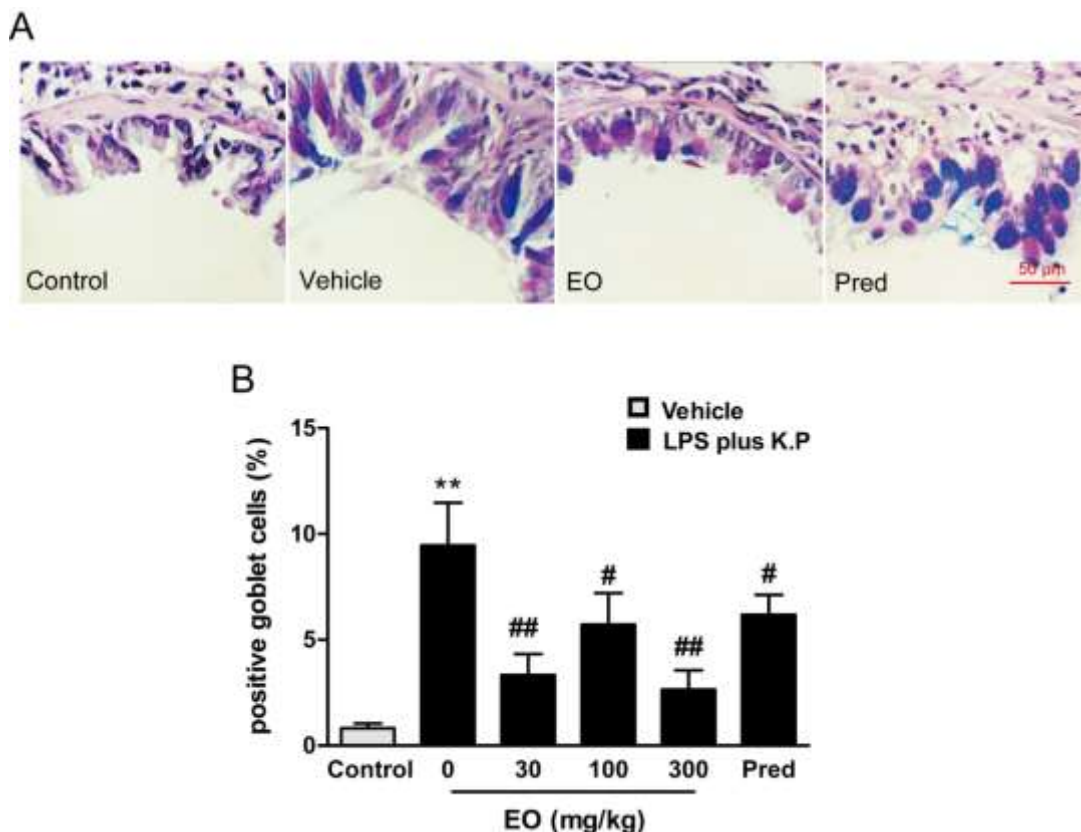
Figure 3. Effect of EO on changes of positive goblet cells in lungs. After challenged with vehicle or K.P plus LPS, lungs were immersed in 10% neutral formalin, paraffinized, and 5 μm sections were prepared and stained with AB-PAS for examination of goblet cell hyperplasia (A). Positive goblet cell number and total cell number of columnar epithelial cells in the main bronchus and 2~3 medium bronchi were counted and analyzed (B). Data were expressed as mean ± SD of 6 rats /group. **$P <$ 0.01 compared with control group, ##$P <$ 0.01 compared with vehicle group.

of COPD, which is strongly associated with mucosal response and neutrophilic inflammatory profile in the sputum (Sethi et al., 2002, 2004, 2008). Here, the authors established a COPD rat model using LPS plus *K. pneumoniae*, characterized by chronic airway inflammation, emphysema and excess mucus. Oral administration of EO effectively reversed chronic bronchiolitis, with significant reduction of mucus hypersecretion. Treatment with EO for one month obviously attenuated the emphysematous changes and thickness of bronchioles while the vehicle group rats showed severe disruption of alveoli and thickened bronchioles. The findings demonstrated that EO had protective effect on LPS plus *K. pneumoniae*-induced pulmonary inflammation and destruction.

It is widely accepted that chronic airway inflammation plays a key role in the pathogenesis of COPD, associated with destruction of airway and lung tissues (Zanini et al., 2015). Previous evidence showed inflammatory cells such as neutrophils, macrophages and lymphocytes was aggregated in blood, sputum and BALF in COPD patients

(Mroz et al., 2015). Release of inflammatory cytokines including TNF-α, IL-1β, IL-6 significantly increased in blood and lung tissues (Shen et al., 2014; Tang et al., 2014). Here the authors detected the elevated extent of TNF-α and IL-1β in lung homogenate, which was obviously reduced by EO treatment in rats. In some trials, anti-inflammatory and antiseptic effect of EO and its extract has been identified (Cermelli et al., 2008; Mulyaningsih et al., 2011; Tsai et al., 2011). Eucalyptol may regulate cytokine production in the airway through TLR$_4$/NF-κB pathway, the mechanisms were further indentified by Zhao *et al.* (2014).

The lungs of patients with COPD are easy to breed micro-organisms including bacteria and virus infections. This results in further lung destruction and host defenses decrease. Bacteria colonization and invasion constitute a lazy immune response, which concomitantly occurs with oxidative stress (O'Rourke et al., 2003). The increased oxidants are very likely to tip the oxidant/antioxidant balance due to the existence of infection and increased immune response (van der Strate et al., 2006). On the
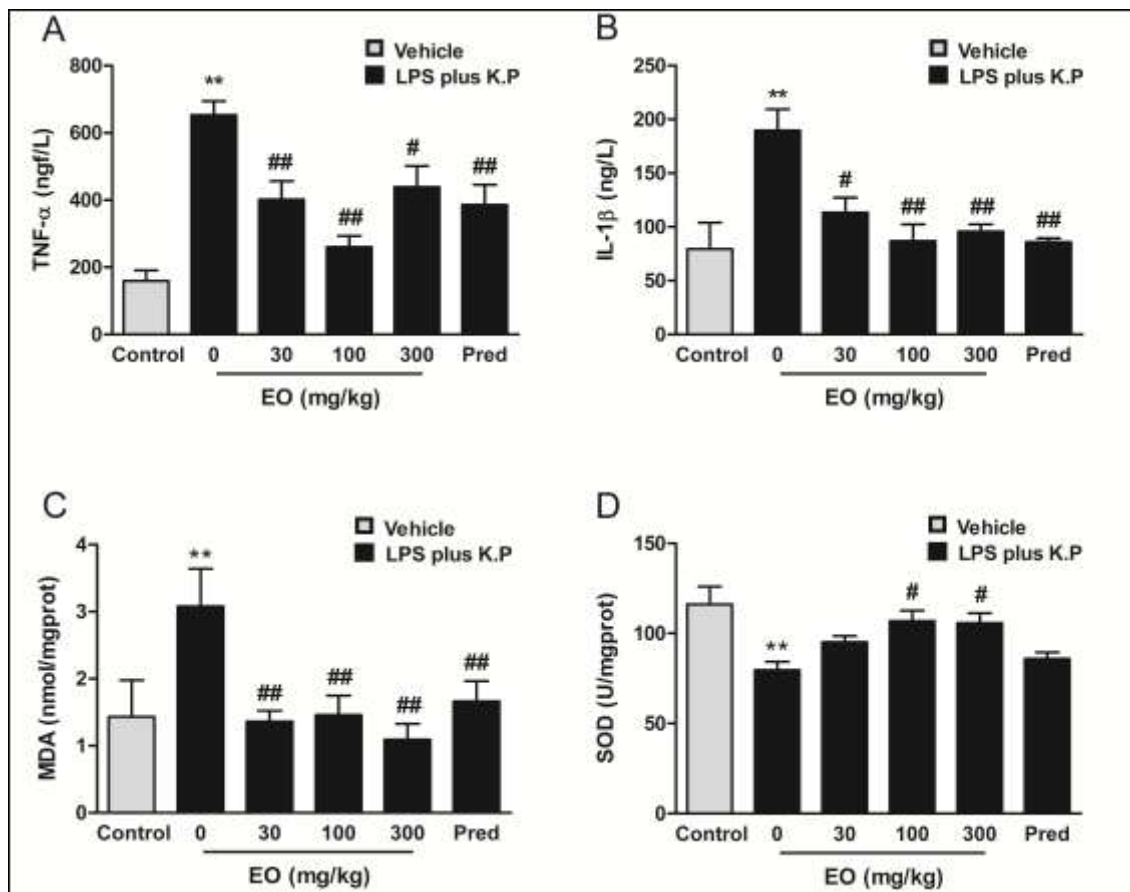
Figure 4. Effect of EO on TNF-α, IL-1β, MDA production and SOD activity in lung homogenate. Lung tissues were harvested 24 h after the last *K. pneumoniae* exposure and used for determining the activity of TNF-α (A), IL-1β (B), MDA (C) and SOD activity (D) among groups. Data were expressed as mean ± SD of 8-12 rats/group. [**]$P < 0.01$ compared with control group, [#]$P < 0.05$, [##]$P < 0.01$ compared with vehicle group.

other hand, organisms have its own enzymatic and non-enzymatic defenses, such as glutathione peroxidase (GSH-Px) and SOD against reactive oxidative stress and lipid peroxidation. To address the role of oxidative stress in the study model, the authors addressed the release of MDA, an index of lipid peroxidation, and SOD activity in lung tissues of rats (Ismail et al., 2015). LPS plus *K. pneumoniae* obviously increased MDA level, accompanied by decreased SOD activity, suggesting a critical role of oxidative stress in COPD model. However, treatment with EO significantly resulted in a significant decrease of MDA formation and increase of SOD activity. The results indicate that EO potentially exerted protective effect against lung destruction via antioxidant mechanism.

Compositions of the major component of EO have been reported differently. Ben Hassine et al. found α-pinene and 1,8-cineol as major components (Ben Hassine et al., 2012), and Bouzabata et al. (2014) found mainly α- and β-pinene. Whatever, EO extract is able to implement the innate cell-mediated immune response besides its anti-septic properties. In LPS-induced chronic bronchitis rats, EO reduced pulmonary inflammation and inhibited hypersecretion of airway mucins (Lu et al., 2004). In accordance with this finding, the data supported the inhibition of mucus hypersecretion in COPD rats. Clinical trials have identified the benefit of concomitant therapy of cineol on improvement of lung function in patients with COPD (Worth et al., 2009). Another placebo-controlled, double-blind trial also showed concomitant therapy using cineole improved lung function and health condition as well as to reduce dyspnea in asthma patients (Worth and Dethlefsen, 2012). The results provided direct evidence that EO attenuated lung destruction and chronic airway inflammation via anti-inflammatory and antioxidant properties, indicating EO as an active controller of lung injury in COPD.

Currently, the clinical use of essential oils has expanded worldwide in the treatment of varieties of inflammatory diseases such as asthma and arthritis (Wang et al., 2007; Shirole et al., 2014). EO has been traditionally used to treat respiratory tract disorders

including bronchitis, pharyngitis and sinusitis. The scientific interest on medical plants in treating chronic pulmonary disease is expanding (Ram et al., 2011). The results provided direct evidence that available Eucalyptus oil might be a proper candidate drug in therapeutic of COPD, at least a good choice to concomitant therapy, apart from the clinical use for sputum clearance in treatment of airway disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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