RESEARCH ARTICLE

The Possible Protective Effect of Safranal on the Lipopolysaccharide-induced Acute Lung Injury in Mice

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ABSTRACT

Experimental evidence suggests that safranal, an organic compound, isolated from saffron (*Crocus sativus*) had anti-inflammatory and antioxidant effects. In the present study, we investigated the protective effect of safranal on the lipopolysaccharide (LPS) induced acute lung injury acute lung injury (ALI) model. Sixty albino BALB/c male (20–30g) mice were divided into five groups: control group (vehicle administration), an induction group received vehicle for 7 consecutive days then LPS 5 mg/kg as a single dose on day 7, two treatment groups received safranal IP (300, 150 mg/kg) respectively for 7 consecutive days then LPS 5mg/kg as a single dose administrated at day seven, and the final group received safranal 300 mg/kg without LPS stimulation. The experiment ended on day eight, bronchoalveolar lavage fluid, blood and lung tissue were collected.total and differential cell count, lung wet/dry ratio, tissue tumor necrosis factor-α (TNF-α), IL-33, and serum IgE levels were significantly ($p < 0.05$) increased after LPS administration and the antioxidant glutathione (GSH) levels were significantly decreased. In contrast, safranal 300 mg/kg could ameliorate ALI by decreasing the levels of (total and differential cell count, wet-dry ratio, and IgE) and the treatment with safranal 150 mg/kg showed a significant reduction in (differential cell count, TNF-α, IL-33, and IgE). And a significant increase in GSH levels. Histopathological scoring levels showed a significant reduction of inflammatory signs in all safranal treated groups when compared to the LPS model group. These results suggest that safranal has a protective effect on LPS induced ALI in mice likely through the anti-inflammatory and antioxidant effect.

Keywords: Acute lung injury, Acute respiratory distress syndrome, Inflammation, Lipopolysaccharide, safranal.

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INTRODUCTION

Acute lung injury (ALI) is an inflammatory disease that is considered a serious condition described by rapid respiratory failure, hypoxemia, and non-cardiogenic pulmonary edema.[1] ALI was first classified in 1994 by the American-European Consensus Conference Committee into two classes according to the severity of the injury: ALI for patients with less severe hypoxemia; acute respiratory distress syndrome (ARDS) for patients with more severe hypoxemia.[2] The main two pathogenic pathways leading to ALI/ARDS are primary (pulmonary; direct) pathway that directly affects the lung (i.e., bacterial, viral, fungal, and parasitic pneumonia and Aspiration of gastric contents) and secondary (extrapulmonary; indirect) that happened indirectly as a result of acute systemic inflammation (i.e., drug overdose and Acute pancreatitis).[2-4] The ALI/ARDS is characterized by the accumulation of protein-rich fluids inside the air space leading to pulmonary edema because of the increase in pulmonary capillary permeability.[5] This can be triggered by many cellular and molecular events, including damage of endothelial and epithelial cells, activation of neutrophils. A network of proinflammatory molecules and cytokines are released, and an imbalance between prooxidants and antioxidants leading to oxidative stress.[6] Recently, ALI characterizes one of the significant pathological changes and the most severe form of the pandemic disease coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

As a consequence, more effective approaches for the resolution of ALI/ARDS are still needed.[7,8] Lipopolysaccharide (LPS) or endotoxin is the glycolipid that forms the main protein of the outer membrane of gram-negative bacteria and it is known to induce severe acute lung injury in pneumonia and sepsis is considered as the most affecting reasons for ARDS.[9] LPS is composed of a hydrophobic domain usually named (lipid A) or (endotoxin), nonrepeating oligosaccharides representing the core, and a distal polysaccharide (O-antigen)[10,11] In animal

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models, inhalation or systemic exposure to LPS was found to cause acute injury in the endothelial and epithelial barriers and cause acute inflammatory reactions in the air space in a short time (not exceeding 48 hours).[11]

Safranal (2, 6, 6-trimethyl-1, 3- cyclohexadiene-1-carboxaldehyde) is an organic compound. It is extracted from saffron (Crocus sativus), an herb found frequently in the Middle East and used as a spice and food colorant.[12,13]

Natural products have been widely used directly or indirectly in the synthesis of pharmacological drugs.Safranal was found to have anti-inflammatory, antioxidant effects,[13] a protective agent against gastric ulcer,[14] a protective effect against pentylenetetrazol-induced seizure,[15] improves hyperglycemia and hyperlipidemia in diabetic disease,16have cardioprotective effect,[17] have protective effect against cataract and preventing the oxidative damage on the lens,[18] and recently found a neuroprotective effect against Alzheimer disease.[19]

Safranal has an important therapeutic effect on lung pathology and tracheal hyperresponsiveness by inhibiting the production of nitric oxide, nitrate, and inflammatory cytokines.[20]

Safranal was found to decrease serum endothelin-1 and total proteins levels in the sensitized guinea pig, which can be considered a prophylactic effect on asthma.[21]In addition, it has an antitussive effect by decreasing the number of coughs on sensitized guinea pigs, whichare attributed to its muscle relaxant activity mainly by its stimulatory effect on B2 adrenoceptors.[22,23] As well, safranal has a suppression effect on lung inflammation found by decreasing the total and differential cell counts in ova albumin sensitized rats.[24] A recent study exhibited an important role of spices including saffron and its derivatives in decreasing the inflammatory response of COVID-19.[25]

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The present study aimed to evaluate the protective effect of safranal on Lipopolysaccharide (LPS) induced acute lung injury (ALI).

MATERIAL AND METHODS

Materials

Safranal and LPS were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Diethyl ether was obtained from ROMAN pure chemistry, UK. Formaldehyde was provided from Sinopharm chemical reagent Co., Ltd, China. IL-33, TNF-α, IgE, and GSH enzyme-linked immunosorbent assay (ELISA) kits were all provided from SHANGHAI YEHUA Biological Technology Co. Ltd, China.

Animals

Sixty Albino BALB/c adult male mice (age 7–8weeks) (weighing between 20–30 gm) were kept in the animal house of the college of the pharmacy/University of Baghdad under specific pathogen-free conditions and provided with water and food ad libitum under a 12 hours light-dark cycle and maintained conventionally during the study with regulated air temperature (15–21°C). The animals were handled under the regulations of the ethical committee at the University of Baghdad as per Helsinki Declaration.

Experimental Protocol for Acute Lung Injury Model

The solution of LPS was prepared by dissolving 10 mg of LPS powder in 10 mL normal saline in a glass tube and mixed by vortex for 30 minutes before each use. For safranal working solution, it was prepared by diluting safranal with liquid paraffin.

All mice were divided into five groups using the stratified randomized grouping method (n = 12) as follow:

- *Group I (Control group):* Twelve mice received liquid paraffin by intraperitoneal injection for seven consecutive days. On day 7, the animals received retro-orbital normal saline. The animal euthanizes on day eight.
- *Group II (Model group):* Twelve mice received liquid paraffin by IP injection for 7 consecutive days. The animal received a retro-orbital LPS (5 mg/kg) dose on day seven and then euthanize 24 hours after LPS administration.
- *Group III (Safranal-treated group):* Twelve mice received safranal (150 mg/kg) by intraperitoneal injection for seven consecutive days. The animal received a retro-orbital LPS (5 mg/kg) dose on day seven and then euthanize 24 hours after LPS administration.
- *Group IV (Safarnal-treated group):* Twelve mice received safranal (300 mg/kg) by IP injection for seven consecutive days. On day seven, the animal received retro-orbital injection of LPS (5 mg/kg) dose and then euthanize 24 hours after LPS administration.
- *Group V:* Twelve mice received safranal (300 mg/kg) by IP injection for seven consecutive days. On day seven retro-orbital normal saline was injected and then euthanize on day 8.

Mice were sacrificed 24 hours after LPS administration. Blood was collected from the retro-orbital route and subsequently centrifuged at 3000g for 20 minutes and 4°C. Thereafter the serum was maintained at -20°C for estimation of IgE. Bronchoalveolar lavage BALF was obtained from six mice of each group, the other six mice were sacrificed by cervical dislocation to obtain the lung tissue.

Collection of Bronchoalveolar Lavage Fluid (BALF)

Six mice of each group were sacrificed by deep anesthesia after blood collection. The lungs were lavaged three times with 1ml of normal saline to collect BALF. A total volume (about

2 mL) was recovered. The BALF samples were centrifuged at 400g for 7 minutes at 4°C. The cell-free supernatants were maintained at −20 °C for pending tests and sediment was kept on ice for total and differential cell count.

White Blood Cells Count and Differentiation

The BALFs were collected and centrifuged (400 rpm/min for 7 minutes) to separate whole cells as pellets. After the supernatant was removed, the pellet of whole cells was isolated on ice. WBC count was measured on the same day and differentiated by Coulter Cellular Analysis System®.

Lung wet/dry Ratio

The left lobe was isolated from the lungs and immediately weighed and then placed in a desiccating oven at 65°C for 48 hours, then the dry weight was achieved. The ratio of wet/dry weight was used to quantify inflammatory lung content.

Preparation of Lung Homogenate

The right inferior and post-raval lobes have been eradicated and washed with chilled phosphate buffer saline (PBS, pH 7.4) at 4°C to remove excess blood and other debris, then the tissue dried with filter paper and weighed. The lung was then minced into small pieces; each 100mg of tissue was put in an Eppendorf tube containing 0.9 mL of chilled PBS. The lung tissue was then homogenized by the homogenizer machine for 1min at speed three after putting the Eppendorf containing the tissue at an ice-containing beaker to keep it cold. The homogenate was centrifuged at cold centrifuge for 20 minutes at 4°C and 3000 rpm. The supernatant was isolated using a micropipette, stored at -20°C, and then utilized for IL-33, TNF-α, and GSH analysis.

Measurement of Inflammatory Cytokines and Glutathione Levels

Lung supernatants were used to measure the levels of IL-33, TNF-α in lung tissue, and serum levels of IgE were measured using commercial ELISA kits according to the manufacturer's instructions. The absorbance values were measured at 450 nm using a microplate spectrophotometer (Human, Germany). The contents of inflammatory cytokines were calculated according to the standard curves.

Statistical Analysis

The data were analyzed using Graphpad Prism 7.0®. Means of groups were compared using one-way ANOVA. All data are expressed as mean ± Standard error of the mean and considered significant at p-value < 0.05.

RESULTS

Effect of Administration of Safranal on White Blood Cell count in BALF for LPS Induced ALI in Mice:

Effect on Total Cell Count

Data presented in Figure 1A reflected that the administration of LPS to mice significantly increased the total cell count in BAL fluid as compared to saline-treated control animals. Pretreatment with safranal at a dose of 300 mg/kg exhibited a significant decline in total cell count post to LPS administration compared to the model group. In contrast, pretreatment with safranal at a dose of 150 mg/kg administration showed a non-significant decline in total cell count post to LPS administration in comparison with LPS model group. Treatment with safranal at a dose of 300 mg/kg without LPS stimulation showed a significant decline in total cell count as compared with the model LPS group.

Effect on Differential Cell Count (Lymphocytes and Neutrophils)

Data presented in Figure 1-B and C reflected that the administration of LPS to mice significantly increased inflammatory cell count (lymphocytes and neutrophils) in BAL fluid as compared to the saline-treated control group. Pretreatment with safranal at a dose of 300 mg/kg exhibited a significant decline in differential cell count post to LPS administration as compared to the LPS model group. Also pretreatment with safranal at a dose of 150 mg/kg showing a significant decline in differential cell count post to LPS administration as compared with the LPS model group. Finally, treatment with safranal at a dose of 300 mg/kg without LPS stimulation and showing a significant decline in differential cell count as compared with the LPS model group.

Effect of Safranal on Lung wet/dry Ratio in LPS Induced Acute Lung Injury in Mice

Data presented in Figure 1D exhibited that the administration of LPS to mice significantly increases the wet/dry ratio as compared to the saline control group. Pretreatment with safranal 300 mg/kg before LPS stimulation shows a significant

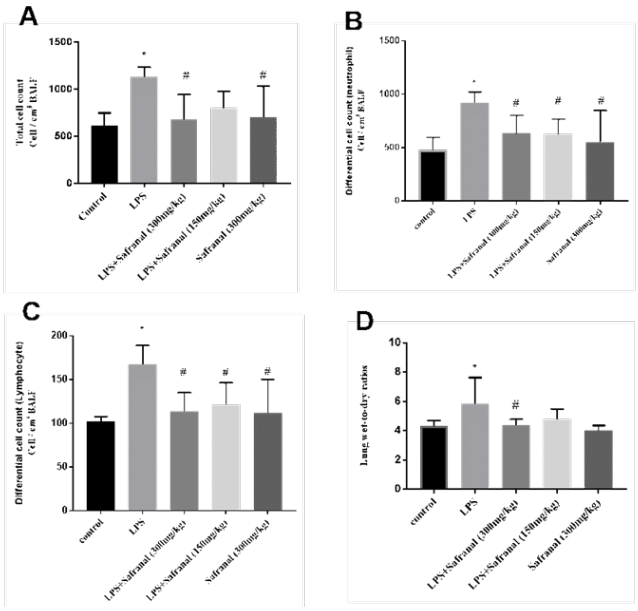

Figure 1: Effect of administration of safranal on total and differential white blood cell count in BALF and wet to dry lung ratio for LPS induced acute lung injury mice.
(Each value represents mean ± SD)
* is significantly different compared with the control group (p < 0.05).
# is significantly different compared with the LPS group (p < 0.05).

decline in wet/dry ratio compared to the LPS model group. While pretreatment with safranal 150 mg/kg before LPS stimulation did not show a significant reduction in lung wet/dry ratio compared to the LPS model group. As well as, the treatment with safranal 300 mg/kg without LPS stimulation had no significant effect on lung wet/dry ratio as compared to the LPS model group.

Effect of Safranal Administration on IL-33 Levels in Lung Tissue Homogenate for LPS Induced Acute Lung Injury in Mice

This study revealed that the levels of IL-33 are significantly elevated in the LPS model group as compared to the saline control group. The pretreatment with safranal at a dose of 300 mg/kg had no significant effect on IL-33 levels as compared to the LPS model group. On the other hand, pretreatment with safranal at a dose of 150 mg/kg exhibited a significant reduction in IL-33 levels as compared to the LPS model group. Finally, treatment with safranal 300 mg/kg without LPS stimulation did not reflect a significant reduction in IL-33 levels as compared to the LPS model group. (Figure 2A).

Effect of Safranal Administration on TNF-α Levels in Lung Tissue Homogenate for LPS Induced Acute Lung Injury in Mice

Data presented in Figure 2B reflected that the levels of TNF-α, a pro-inflammatory cytokine were significantly elevated following LPS administration compared to the saline control group. Pretreatment with safranal at a dose of 300 mg/kg exhibited significant elevation in TNF-α levels posts to LPS administration as compared with the saline control group. Pretreatment with safranal at a dose of 150 mg/kg exhibited a significant decline in TNF-α levels as compared with the LPS model group. On the other hand, treatment with safranal at a dose of 300 mg/kg without LPS stimulation showing a significant decline in TNF-α levels as compared to the LPS model group.

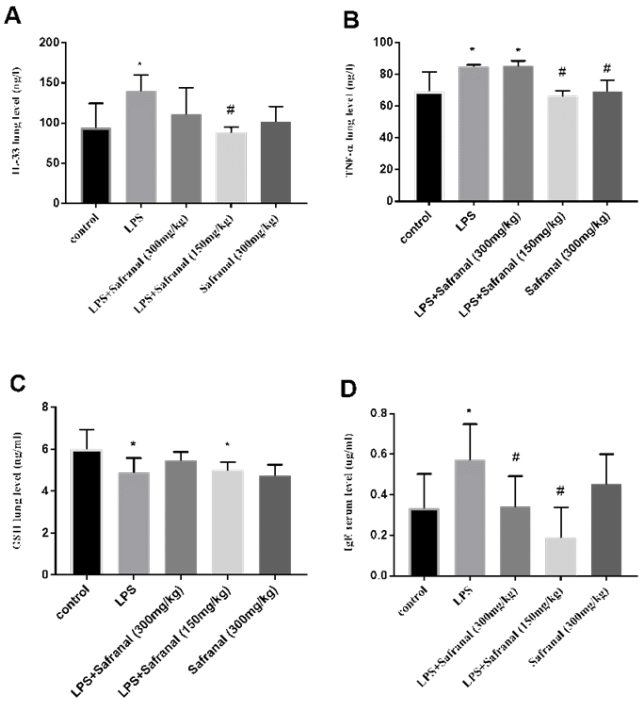

Figure 2: Effects of safranal on the inflammatory contents in serum and lung tissues. The IL-33, TNF-α, GSH, and IgE in serum and lung tissues of LPS-induced ALI were measured using commercial ELISA kits. (Each value represents mean ± SD)
* is significantly different compared with the control group ($p<0.05$).
\# is significantly different compared with the LPS group ($p<0.05$).

Effect of Safranal Administration on GSH Levels in Lung Tissue Homogenate for LPS Induced Acute Lung Injury in Mice

The total glutathione levels (mean ± SD) were measured for determination of the antioxidant status. The data presented in Figure 2C reflect that LPS administration produces a significant reduction in GSH tissue level compared to the saline control group. The pretreatment with a dose of 300 mg/kg did not restore the GSH level as compared to the LPS model group, pretreatment with safranal at a dose of 150 mg/kg reflects a significant elevation as compared to the saline control group. The treatment with safranal at a dose of 300 mg/kg without LPS stimulation did not affect the GSH level.

Effect of Safranal Administration on Serum IgE Levels for LPS Induced Acute Lung Injury in Mice

The Data presented in Figure 2D showed a significant elevation in serum IgE levels after LPS administration compared with the saline control group. The pretreatment with safranal at a dose of 300 mg/kg significantly reduced IgE serum level compared with the LPS model group. Furthermore, pretreatment with safranal 150 mg/kg also produce a significant reduction in serum IgE levels as compared to the LPS model group. Finally, safranal at a dose of 300 mg/kg without LPS stimulation did not reflect significant changes in serum IgE levels as compared to the normal group.

DISCUSSION

LPS is a critical component of the bacterial outer membrane that can induce acute lung injury by overproducing numerous pro-inflammatory cytokines.[26] ALI/ARDS are characterized by an inflammatory response, air–blood barrier dysfunction, alveolar edema, and hypoxemia.[8] Progressive accumulation of neutrophils and lymphocytes in BAL fluid is a major sign of the later phase acute lung inflammation after 24–48 hours LPS treatment.[27,28] The activated immune cells can cause damage to the lung tissues, and the depletion of neutrophils alleviated the disease variety.[29,30] In the present study,our results correspond with the studies in which it found that the LPS model group at a dose of 5 mg/kg caused a significant increase in neutrophils and lymphocytes compared to the control group. While pretreatment with safranal 300 mg/kg showed a significant reduction in total cell count compared to the LPS model group. Furthermore,thesafranal treated groups (300 and 150 mg/kg) significantly reduced neutrophils and lymphocyteinfiltration compared to the LPS model group. Pulmonary edema is an important criterion in LPS-induced ALI.[31] In this study, we

assessed pulmonary edema by detecting lung W/D ratio. The results showed that the lung W/D ratio was significantly increased by the LPS model group compared to the saline control group. However, treatment of safranal 300 mg/kg significantly decreases lung edema in LPS induced ALI. According to previous studies, LPS administrationcan induce an increase in the proinflammatory cytokines (TNF-α, IL-1β, IL-6, G-CSF, IL-8, ENA-78,-1, MIP-1α, and MIP-1, IL-33).[28,32] Our findings regarding TNF-α and IL-33 showed that the level of these two cytokines significantly increased in the LPS model group compared to the control saline group.At the same time,safranalat a dose of 150 mg/kg can cause a significant reduction in these two pro-inflammatory cytokines which may prove the anti-inflammatory activity of safranal against ALI.ALI considered as an oxidative stress-related disease because it results from a redox imbalance between a cascade of a variety of mediators and other outcomes, leading up to oxidative stress,[33] Therefore, ALI/ARDS can be managed by targeting the systems responsible for extreme ROS production, since excessive ROS production by the injured endothelium/ epithelium and the accumulated leukocytes plays a key role in ALI/ARDS exacerbation and lung destruction.[33] LPS induced acute lung injury via the expressions of TLR4 and NF-κB was found to be negatively correlated with the levels of SOD, MPO, and GSH, while positively correlating with levels of MDA and ROS.[35] This study revealed that safranal at a dose of 150 mg/kg significantly attenuated GSH content, a biomarker of oxidative stress. The results indicated that safranal significantly inhibited LPS-induced oxidative stress. The level of IgE in the serum was detected to determine the autoimmune response.[36] IgE levels in serum were significantly upregulated after LPS stimulation. Safranal administration at both doses reversed lgE expression levels significantly.Boskabady et al at 2012 used three different concentrations of safranal to treat lung inflammation on ova albumin sensitized guinea pigs and the results showed that the lower concentration of safranal was more effective than other two higher concentrations on pathological changes, total WBC and eosinophil count and serum histamine level.[20] Another study by feyzi *et al.* in 2016 studied the effect of safranal on Th1/Th2 balance and founds that the medium concentration of safranal shows the maximum effect on IFN-γ/IL-4 ratio which may indicate the non-selective effect of its high concentration. This was attributed to the possibility that a high concentration of safranal may decrease cell viability through mechanisms such as allergy or tolerance.[37] LPS can induce ALI via the activation of both the MYD88 dependent pathway and MYD independent pathway both pathways will end with the activation of many cytokines and pro-inflammatory mediators and transcription factors including IL1β, IL6, IL8, TNF-α, INF-β.[38] Epithelial injury is the first sign of LPS induced ALI since LPS will activate the mitogen-activated protein kinase (MAPK) pathway leading to activation of the NF-kB pathway which in turn will lead to a series of inflammatory responses.[39] Safranal was found to inhibit the Myd88 pathway in addition that saffron found to control the trouble of the "MAPK signaling pathway" which is accompanied by the development of many diseases in humans. Recently, safranal was found to significantly blocked the phosphorylation and expression of MAPK and NF-κB signaling pathway proteins and inhibited the nuclear translocation of AP-1 and NF-κB p6 in LPS stimulated cells *in vitro*.[40,41] These studies could support our theory that safranal may have a protective effect on LPS induced Acute lung injury.

CONCLUSION:

The present study demonstrated that the treatment with safranal exhibited a dose-independent protective effect on LPS-induced ALI in vivo by reducing the pulmonary inflammatory response and tissue damage, reduced neutrophil infiltration, lightened the oxidative stresses through increasing the level of GSH. Safranal apparently can suppress the levels of proinflammatory cytokines (TNFα and IL33). It could be a promising intervention in acute lung injury in the future. Further studies on the high doses of safranal should be performed.

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