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Pulicaria petiolaris effectively attenuates lipopolysaccharide (LPS)-induced acute lung injury in mice

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Abstract: Members of the genus *Pulicaria* have been used in traditional medicine for alleviating several complaints as they have a rich pool of biometabolites. Acute lung injury (ALI) is a serious disease with an elevated mortality rate. The present investigation aimed to evaluate the total phenolic and flavonoid contents and antioxidant capacity of the methanolic extract of *P. petiolaris* Jaub. and Spach. (PP) (Asteraceae). Moreover, the potential protective potential of PP against lipopolysaccharide-(LPS)-induced ALI was assessed. PP is a rich source of phenolics and flavonoids. The total phenolic content (TPC) was 68.05 mg gallic acid equivalent (GAE)/g dried extract, and the total flavonoid content (TFC) was 45.86 mg quercetin equivalent (QE)/g dried extract. Additionally, PP possessed a promising DPPH-scavenging activity, with an IC_{50}=27 μg/mL. Our results showed that PP lessened LPS-induced lung injury. PP effectively reduced pulmonary edema as it lowered total protein and the lung wet/dry weight (W/D) ratio in the bronchoalveolar lavage fluid (BALF). It also significantly ameliorated the level of lactate dehydrogenase (LDH) in the BALF and improved the histopathological lesions in the lung tissue. LPS-induced inflammatory cell infiltration was greatly depressed in PP-treated animals. PP showed antioxidant capacity as it reduced the LPS-induced increase in the lipid peroxidation marker, malondialdehyde (MDA). It also increased the activity of superoxide dismutase (SOD) and the content of reduced glutathione (GSH). This study indicates that PP significantly decreased LPS-induced inflammation in the LPS-mediated ALI murine model, suggesting that it may become a significant preventive strategy for treating nonspecific inflammation of the lungs.

Key words: *Pulicaria petiolaris*; lung injury; lipopolysaccharide; antioxidant; inflammation

INTRODUCTION

Acute lung injury (ALI) has a high mortality and no effective treatment for it is available. ALI is characterized by acute respiratory distress syndrome (ARDS), which is accompanied by an acute inflammatory process in the lung parenchyma and airspaces. The pathogenesis of ALI includes increased vascular permeability, neutrophil accumulation, parenchymal injury and impaired gas exchange. LPS is a component of the Gram-negative bacteria cell wall and it has been used

to produce an experimental model of ALI in mice [1-3]. Administration of LPS leads to the release of many cytokines, such as tumor necrosis factor-α (TNF-α) and interleukins (ILs), which trigger inflammatory responses and alter immune system functioning. LPS induces epithelial cell apoptosis, causes damage to the epithelial cell layers and stimulates the release of reactive oxygen species (ROS) that leads to neutrophilic leukocyte aggregation and eventually to lung tissue injury [4,5]. Mechanical ventilation is the only effective therapy for ARDS [6]. Hence, there is a need to search for new therapeutics that could be used for its treatment.

Natural products display remarkable roles, not only in the synthesis, design, and discovery of new drugs, but also as prominent sources of innovative drugs and bioactive substances. The genus *Pulicaria* (tribe Inuleae and family Asteraceae) comprises around 100 species that are widely distributed in Europe, Asia and Africa [7]. The plants of this genus have been used in traditional medicines for treating various ailments, such as back-pain, inflammation, menstrual cramps, intestinal disorders, dysentery and diarrhea [8,9]. They possess different bioactivities: cytotoxic, antipyretic, antioxidant, antispasmodic, antimicrobial, antihistaminic, analgesic, hepatoprotective, antiinflammatory, cardioprotective and nephroprotective [9-11]. This genus is known to be rich in terpenoids, phenolics, caryophyllene sterols, caryophyllenes and xanthines [9,12]. Moreover, many sesquiterpenes isolated from this genus have been shown to exhibit a wide range of biological activities [9,13,14]. In the course of our biological evaluation of Saudi plants, the protective potential of *P. petiolaris* against LPS-induced ALI was assessed. Furthermore, its flavonoid and phenolic contents as well as antioxidant activity were evaluated.

MATERIALS AND METHODS

Plant material and extraction

The aerial parts of *P. petiolaris* were collected from the Jeddah-Taif road, Saudi Arabia in April 2017. The plant was kindly specified by the staff of the Department of Natural Products and Alternative Medicine, King Abdulaziz University, Saudi Arabia, based on the library database and morphological features [15]. This was confirmed by Dr. Emad Al-Sharif, Biology Department, Faculty of Sciences and Arts, Saudi Arabia. A voucher specimen (PP 2017-1) was stored at the Department of Natural Products and Alternative Medicine herbarium, King Abdulaziz University. The air-dried powdered aerial parts of PP (500 g) were extracted with methanol (2 x 1000 mL) using an IKA Ultra-Turrax T 25 digital instrument (IKA Labortechnik, Staufen, Germany). The solvent was removed under reduced pressure and the dried total methanolic extract (TME) (12.6 g) was kept at 4°C until use in biological tests.

Chemicals and reagents

All chemicals were obtained from commercial sources and were of analytical grade. Gallic acid, quercetin, ascorbic acid, sodium carbonate, sodium hydroxide, 2,2-dyphenyl-1-picrylhydrazyl (DPPH) and sodium nitrate were purchased from Sigma Chemical Co. (Germany). The Folin-Ciocalteu's phenol reagent and aluminum chloride were from Fluka Chemie AG (Buchs, Switzerland). *Escherichia coli* serotype O111:B4 LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA) and freshly dissolved in normal saline.

Preparation of standard solutions

Gallic acid and quercetin (1 g each) were dissolved separately in methanol (100 mL) to obtain standard solutions A and B, respectively.

Determination of the TPC

The Folin-Ciocalteu method was used to assess the TPC of PP [16]. A standard curve for gallic acid was prepared by dilution (0.1, 0.5, 1.0, 2.5 and 5 mg/mL) in methanol from solution A. One hundred µL of each dilution was mixed with 500 µL distilled H_2O and the Folin-Ciocalteu reagent (100 µL) and left for 6 min. Then, 500 µL of distilled H_2O and 1 mL 7% Na_2CO_3 were added to the mixture. The absorbance was measured after 90 min at 765 nm. The same was performed with the PP total methanol extract (TME). All experiments were carried out three times and means were calculated.

Determination of the TFC

The TFC of the TME was estimated by the $AlCl_3$ complex assay [17]. A calibration curve for quercetin (standard) was drawn. Dilutions of 0.1, 0.5, 1.0, 2.5 and 5 mg/mL were prepared in methanol from solution B. From each dilution, 100 µL was mixed with 500 µL of distilled H_2O and 5% $NaNO_3$ (100 µL) and left for 6 min. Then, a 10%-$AlCl_3$ solution (150 µL) was added and kept for 5 min and 200 µL 1M NaOH was added. The absorbance was measured at 430 nm. The same procedure was carried out with PP TME, and the TFC was expressed as the quercetin equivalent (mg QE/g dried extract). All procedures were done in triplicate and the mean values were estimated.

Antioxidant activity

The antioxidant potential was assessed using the DPPH assay as outlined previously [18,19]. One mL of the TME (10, 20, 40, 60, 80, and 100 µg/mL) was mixed with 1 mL of DPPH and kept for 0.5 h. UV absorbance was estimated at 517 nm. Each experiment was carried out in triplicate using ascorbic acid (standard). The percentage of free radical scavenging activity was estimated using the following formula:

$$\text{Antioxidant activity} = 100 \times \left(1 - \frac{\text{absorbance with the sample}}{\text{absorbance of the blank}}\right)$$

The IC_{50} was calculated from the inhibition percentages graph against the concentration of extract plot using a nonlinear regression algorithm.

Animals

BALB/c mice (25-30 g) were supplied by the Animal Facility, College of Pharmacy, Taibah University. The animals were maintained in standard cages and allowed free access to food and water under standard condition of temperature (25°C) and a dark/light cycle. The experimental protocol adhered to the Guidelines of the Ethical Committee of Taibah University, Saudi Arabia which closely adheres to NIH guidelines.

Experimental design

Mice were divided into 4 experimental groups (n=6). The animals were treated according to the following regimen: control group: the mice were given the vehicle once daily for 5 days; LPS group: the mice received a single LPS intraperitoneal (i.p.) injection (10 mg/kg); PP-treated groups: the animals were given PP (50 and 100 mg/kg) by oral gavage (p.o.) for 5 days before LPS injection; 24 h after LPS injection, the blood was obtained from the retro-orbital sinus and the serum was separated and stored at -20°C until further analysis.

The mice were humanely killed with an overdose of anesthesia using diethyl ether. The chest was opened, and the left lung was clamped. The right lung was lavaged using 0.9% saline. BALF was collected and centrifuged at 2000 x g and 4°C for 15 min. The cells were collected and counted. The BALF supernatants were stored at -80°C. A small piece of the left lung was weighed and homogenized in phosphate buffer (pH 7.4, 0.1 mol/L) in an ice bath. The homogenate was centrifuged at 2000 x g and 4°C for 20 min. The supernatants were kept at -80°C for analysis of oxidative stress parameters. The remaining left lung was dissected and washed with ice-cold saline and fixed for 24 h in neutral buffered 10% formalin and submitted for histopathological assessment.

Determination of lung W/D ratio

The lung W/D ratio was estimated to assess the degree of pulmonary edema as described previously [20]. A part of the left upper lung was blotted dry and weighed to determine "wet" weight. Then, it was placed in an oven (80°C) for 24 h to measure the "dry" weight and the lung W/D ratio was determined.

Determination of total and differential cell counts in BALF

Total cell counts were determined using a hemocytometer. Cell pellets were resuspended in 100 µL saline (0.9%), centrifuged onto slides and stained for 8 min with Wright-Giemsa. The differential cell counts were quantified using a light microscope at 40× magnification by counting a total of 200 cells/slide.

Determination of protein

The total protein content in the BALF was estimated colorimetrically using a Pierce BCA Protein Assay Kit

(Cat. NO. 23225, Thermofisher Scientific, MA, USA), according to the manufacturer's protocol.

Determination of LDH activity

LDH activity was measured in BALF using a kit (Human, Wiesbaden, Germany), following the manufacturer's guidelines. Briefly, the samples were mixed with nicotinamide adenine dinucleotide (NADH), sodium pyruvate and TRIS buffer. The change in absorbance was estimated at 340 nm.

Histopathological analysis of lung

Fixed lung samples were embedded in paraffin wax and sectioned (5 μm). Lung specimens were stained with hematoxylin-eosin (H&E) and examined randomly with no knowledge of the group. Histopathological lesions were evaluated on the basis of the degree of inflammation, thickening of the alveolar wall and cellular proliferation. Lesions were semiquantitatively graded as described previously [20].

Determination of oxidative stress

The malondialdehyde (MDA) content is used as an index for lipid peroxidation. SOD activity and the GSH content were assessed and served as indices of the antioxidant capacity of the lung. These parameters were estimated in the supernatants of the lung homogenates. The MDA content was estimated as described previously [21]. In brief, MDA was quantified by reaction with thiobarbituric acid (TBA) and the absorption was measured spectrophotometrically at 532 nm.

SOD activity was assessed by observing the SOD-inhabitable autooxidation of pyrogallol [22]. The change in absorbance was recorded at 420 nm.

The GSH assay relies on the reaction of 5,5-dithiobis-2-nitrobenzoic acid with GSH and the product was measured at 412 nm spectrophotometrically [23].

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's Kramer multiple comparisons test. The data were expressed as means±SE for 6 mice. $P<0.05$ was considered as significant.

RESULTS

Flavonoid and phenolic compound contents and antioxidant activity

P. petiolaris is a rich source of phenolics and flavonoids (TPC: 68.05 mg gallic acid equivalent (GAE)/g dried extract; TFC: 45.86 mg quercetin equivalent (QE)/g dried extract) with a promising DPPH scavenging activity (IC_{50}=27 μg/mL), when compared to ascorbic acid (IC_{50}=32.9 μg/mL). The high phenolic and flavonoid contents of the PP extract, in addition to antioxidant activity, might provide antiinflammatory activity. A positive relation between the flavonoid and phenolic contents and free radical scavenging activity has been reported [24]. It is noteworthy that the plant extracts with high phenolic contents also possess high flavonoid contents, as reported for other *Pulicaria* species [12,25].

Effect on LPS-induced pulmonary edema

The LPS injection caused remarkable increases in the total protein content and the lung W/D ratio as compared to control mice (Fig. 1). Pretreatment with PP resulted in a significant amelioration of these two parameters of pulmonary edema when compared to the LPS group.

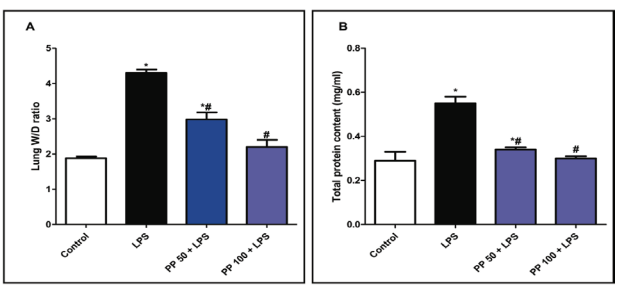

Fig. 1. PP decreased LPS-induced pulmonary edema. **A** – Lung W/D ratio. **B** – Protein content. Mice were treated with two different doses of the methanolic extract of PP (50 and 100 mg/kg, p.o.) once daily for 5 days prior to LPS injection (10 mg/kg, i.p.). Data are the mean±SE n=6. $^{*}P<0.05$ vs control group; $^{\#}P<0.05$ vs LPS group.

Effect on LDH activity in BALF

As demonstrated in Fig. 2, LPS raised the level of LDH in BALF compared to the control group. The PP treatment significantly attenuated the high level of LDH compared to the LPS group.

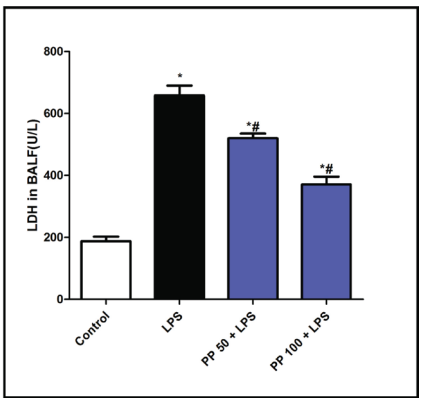

Fig. 2. PP suppressed LDH activity in BALF. Mice were treated with two different doses of the methanolic extract of PP (50 and 100 mg/kg, p.o.) once daily for 5 days prior to LPS injection (10 mg/kg, i.p.). Data are the mean±SE n=6. *P<0.05 vs control group. #P<0.05 vs LPS group.

Effect on lung histopathological examination

Lung sections of normal mice showed normal lung histology, while those of the LPS group showed marked inflammatory cell infiltration alveolar wall thickening. PP-treated animals showed a marked improvement in lung lesions (Fig. 3).

Effect on inflammatory cell counts in BALF

As shown in Fig. 4, LPS administration induced an elevation of inflammatory cell infiltration into lung tissue. Also, there was elevation of the differential and total cell counts, mainly neutrophils, in the BALF in comparison to the control group. The PP pretreatment significantly reduced the differential and total cell counts compared to the LPS group.

Effect on oxidative stress and antioxidant markers

LPS injection increased the oxidative stress parameter and MDA in lung tissue (Fig. 5). LPS attenuated

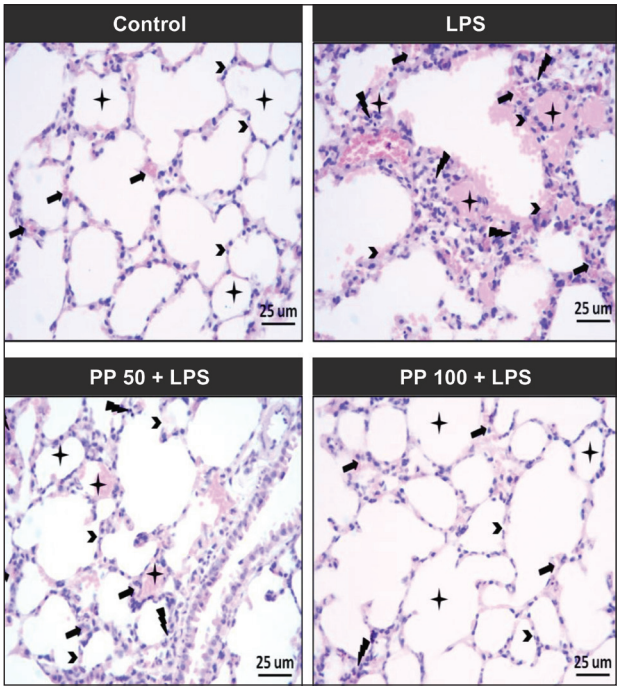

Fig. 3. Lung specimen stained with H&E (×400) showing attenuation of LPS-induced pulmonary damage by PP. **Control** – Sample showing normal alveolar capillaries (arrows), thin alveolar walls (chevrons) and alveolar space (stars). **LPS** – Sample with congested alveolar capillaries (arrows), thickened alveolar walls (chevrons), acute inflammatory cellular infiltrates (lightning bolt), and pink fluid exudates in alveolar spaces (stars). **PP 50 + LPS** – sample displaying moderate alveolar capillary congestion (arrows), moderate thickening of alveolar walls (chevrons) with moderate inflammatory cellular infiltrates (lightning bolt), and less pink exudates in alveolar spaces (stars). **PP 100 + LPS** – Sample exhibiting greater improvement of all LPS-induced inflammatory responses in the lung tissue. Scale bar 25 μm (×400).

the antioxidant activity of the lung, as presented by decreased SOD and GSH levels. The PP pretreatment significantly increased SOD activity and GSH levels, which were accompanied by decreased levels of MDA when compared to the LPS group.

DISCUSSION

ALI is characterized by an extensive inflammatory reaction in the lung tissue along with disruption of the alveolar-capillary barrier, which leads to severe impairment of gas exchange [26]. The current study showed the ability of the PP extract to attenuate LPS-induced ALI, which may be attributed to the antioxidant ability of PP. PP showed a remarkable ability to

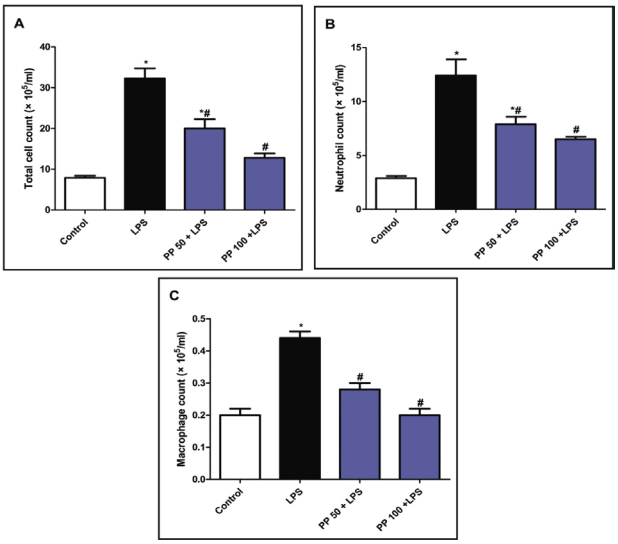

Fig. 4. PP ameliorated inflammatory cell infiltration in lung tissue. **A** – Total cell count. **B** – Neutrophil count. **C** – Macrophage count. Mice were treated with two different doses of the methanolic extract of PP (50 and 100 mg/kg, p.o.) once daily for 5 days prior to LPS injection (10 mg/kg, i.p.). Data are the mean±SE n=6. *P < 0.05 vs control group; # P<0.05 vs LPS group.

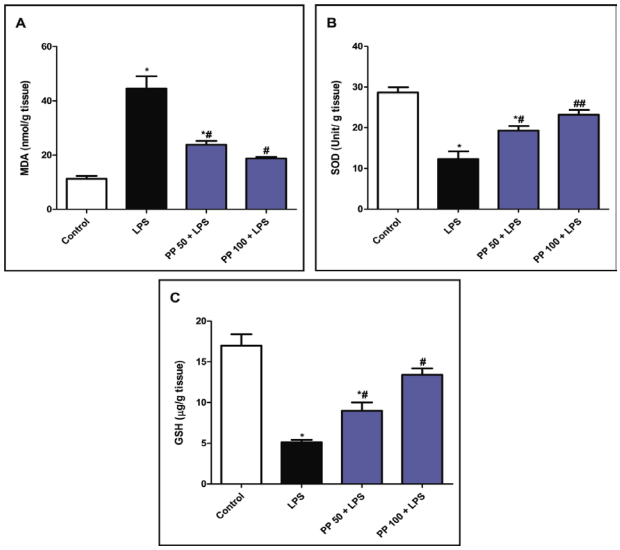

Fig. 5: PP decreased LPS-induced oxidative stress. Mice were treated with two different doses of the methanolic extract of PP (50 and 100 mg/kg, p.o.) once daily for 5 days prior to LPS injection (10 mg/kg, i.p.). Data are the mean±SE n=6. *P<0.05 vs control group; #P<0.05 vs LPS group.

attenuate LPS-induced pulmonary edema and decrease LDH activity, as well as inflammatory cell infiltration. The histopathological lesions in the lungs were greatly improved in PP-treated animals. The results further showed that the protective effect of PP could be linked to its ability to inhibit LPS-induced oxidative stress.

Activation of neutrophil and macrophage infiltration into lung tissue participates in the elevation of permeability of the alveolar/capillary barrier, which results in the development of pulmonary edema. Estimation of the protein content and lung W/D ratio has been used to evaluate the integrity of the alveolar-capillary barrier and the extent of pulmonary edema [27,28]. The results of the present study revealed that LPS injection resulted in the development of pulmonary edema, as there was significant elevation in the total protein content and lung W/D ratio in the BALF. Additionally, LDH activity was significantly elevated in the BALF in the LPS group of experimental animals, which was a reflection of increased cell death. Interestingly, pretreatment with the PP extract reduced these parameters of lung damage.

Inflammatory cell infiltration and accumulation in lung tissue is a pathological hallmark of ALI [29,30]. Normally, the polymorphonuclear leukocytes (PMNs) play essential roles in the clearance of debris and pathogens from the alveolar space. However, excessive and persistent sequestration of PMNs may result in additional damage to the lung tissue through the release of multiple toxic mediators, including proteases, ROS and proinflammatory cytokines, all of which exacerbate ALI [31,32]. During ALI, neutrophils are the first immune cells recruited into the inflammation site. This is usually accompanied by the increase of lung edema and alveolar-capillary barrier permeability. Activated neutrophils extravasate and migrate into the alveolar space where they secrete chemoattractants, such as leukotriene B4 (LTB4) and recruit more leukocytes to expand the inflammation response. Neutrophil influx into the lung is believed to have a key role in the progression of ALI [33]. Clinically, the number of inflammatory cells in the BALF of ARDS patients closely correlates with disease severity [34]. Experimentally, attenuation of inflammatory cell infiltration into lung tissue in LPS-induced ALI results in a reduction of lung damage and improvement of capillary-alveolar function [35]. In line with previous investigations, the present study revealed that LPS administration significantly induced inflamma-

tory cell infiltration, mainly neutrophils, in the lungs. On the other hand, PP pretreatment markedly decreased the LPS-induced influx of inflammatory cells into the lungs, leading to attenuation of lung injury. These findings were supported by the histopathological examination of the lung tissue, which revealed attenuation of LPS-induced inflammatory cell infiltration and improvement of pathological lesions after PP pretreatment. Together, these data suggest that the protective activity of PP against LPS-induced ALI may be mediated through inhibition of inflammatory cell influx, mainly of neutrophils, into the lung.

Recent evidence has suggested that the main pathogenic mechanisms of ALI include oxidative stress, cytokine release, inflammation and apoptosis [36]. During ALI, large amounts of ROS are generated, which cause cell membrane lipid peroxidation and the destruction of lung parenchymal cells. ROS overproduction also damages the capillary basement membrane and other stromal components. LPS-induced oxidative stress is associated with depressed antioxidant activity of the lung, which aggravates lung damage [27,28,37,38]. Several studies have reported on the antiinflammatory capacities of flavonoids and sesquiterpene lactones of the genus *Pulicaria* [9,39,40]. It is noteworthy that Mothana et al. [41] previously demonstrated the selective cytotoxicity of the PP methanol extract against human the urinary bladder carcinoma cell line. However, more studies are needed to elucidate the molecular mechanisms that underlie the biological activity of the methanolic extract of PP.

CONCLUSION

The results of the present study confirmed the elevation of MDA, the parameter of lipid peroxidation, and depression of GSH levels and SOD activity in the lungs of LPS-intoxicated animals. PP pretreatment restored the levels of GSH and SOD to near normal values, which eventually resulted in the suppression of oxidative stress and lipid peroxidation. This study provides evidence of the protective activity of PP against LPS-induced ALI in mice, which may be linked to its antioxidant activity due to the presences of phenolic constituents. Thus, this study encourages further research into the molecular mechanisms of PP activity and isolation of bioactive metabolites.

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Conflict of interest disclosure: None to declare

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