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Anti-inflammatory Effects of Ziziphus Jujube Mill on LPS-induced Acute Lung Injury in Mice

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ABSTRACT

Ziziphus Jujuba Mill (Z.J) is a well-known ethnomedical source of biologically active compounds with anti-inflammatory effects. However, its significance in acute lung injury (ALI) has never been studied. The present study aimed to explore whether Z.J could attenuate lipopolysaccharide (LPS)-induced inflammatory responses in an experimental model of ALI.

Male BALB/c mice received an intratracheal administration of LPS (n=32) or phosphate buffer saline (PBS) (control, n=8). Within 1, 11, and 23 h post-LPS injection, mice were randomly assigned to receive intraperitoneal treatments of saline, dexamethasone (2 mg/kg), and 100 and 200 mg/kg of Z.J extracts, respectively. 24 h after intratracheal administration of LPS, bronchoalveolar lavage fluid and lung tissues were harvested and assessed for inflammatory cell influx, tumor necrosis factor-α (TNF-α) levels, and histological assessments.

Treatment with Z.J extracts (100 and 200 mg/kg) and dexamethasone effectively reduced LPS-induced neutrophil and other inflammatory cell influx into the lung tissue compared to the untreated group. additionally, both doses of Z.J extracts (100 and 200 mg/kg) significantly ameliorated the lung wet-to-dry ratio and histopathological damage. Furthermore, compared to the untreated ALI mice, Z.J extract at the highest dose could significantly reduce the TNF-α level.

The present findings indicated that Z.J could effectively ameliorate LPS-induced ALI inflammatory responses and might be considered a promising alternative therapy for the ALI phenotype.

Keywords: Acute lung injury; Inflammation; Lipopolysaccharides; Ziziphus

INTRODUCTION

Acute lung injury (ALI) is a serious lung inflammatory condition characterized by pulmonary edema and inflammation. This disorder is associated with an increase in vascular permeability, which leads to

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a decrease in lung compliance, damage to the vascular-endothelium, and an excessive infiltration of trans epithelial neutrophils and alveolar macrophages often requiring mechanical ventilation to manage the symptoms [1]. Surfactant inactivation, alveolar collapse, and a rise in the dead space fraction are the most common outcomes of destroying the pulmonary microvascular bed.[2] Mediators like interleukin 8 (IL-8), IL-6, IL-11, IL-1, and tumor necrosis factor-α (TNF-α) lead to tissue damage, respiratory and organ failure, and fluid accumulation in alveolar sacs.[3]

Acute respiratory distress syndrome (ARDS) still carries high mortality and morbidity rates, with over 3 million cases and 75,000 deaths reported globally each year.[4] In recent years, numerous studies have been conducted in search of a medication that could reduce the high mortality rate of ARDS patients. However, the mortality rate in intensive care units remains at around 40%.[5] Trauma, sepsis, smoke inhalation, cardiopulmonary bypass, and toxic ingestions are considered important underlying etiologies of ALI and its most serious type, ARDS.[2,6,7] The innate immune defense is acutely activated in response to exogenous inflammatory factors to rehabilitate affected tissues and minimize systemic inflammation. However pulmonary dysfunction and respiratory failure are plausible when tissue homeostasis is not restored.[8] Infection with endotoxin LPS, the cell wall of gram-negative bacteria, promotes the innate immune system in response to the acute exudative phase with significant damage to the pulmonary tissue.[9,10]

Despite significant progress in the management of ALI and ARDS, approximately 50% of patients are primarily unresponsive to conventional treatments.[11-14] Accordingly, new therapeutic procedures that address the multiple components of this multifactorial disease are urgently needed. *Ziziphus Jujuba* Mill (Z.J) is traditionally used for medicinal purposes due to its antioxidant, anti-inflammatory, anticoagulant, anti-cancer, and immunomodulatory characteristics.[15,16] An expanding body of research suggests that the promising potential of Z.J as a traditional and/or alternative modern medicine can be attributed to the abundance of terpenes and flavonoids present in it.[17-19]

The present study aimed to investigate the potential anti-inflammatory properties of Z.J extract in an experimental model of ALI induced by lipopolysaccharide (LPS). Our objective was to determine if the extract could attenuate LPS-induced inflammatory responses in ALI, by assessing inflammatory cell influx, TNF-α levels, and histological evaluations.

MATERIALS AND METHODS

Preparation Of Ethanolic Extract

The dried fruits of the Z.J mill (voucher number: 327, Birjand University herbarium) were powdered and macerated in 80% ethanol for 48 h at room temperature with continual stirring. The final mixture was filtered through Whatman No. 1 filter papers, and 10 ml of the extract was placed in a Petri dish and dried at 45°C. The Z.J extract was kept at 4°C until it was needed.[20]

Animals

A total of forty male BALB/c mice (6–8 weeks of age) were purchased from the Research Centre of Experimental Medicine (Birjand University of Medical Sciences). Animal studies were approved by the Ethical Committee at Birjand University of Medical Sciences (permission code: IR.BUMS.REC.5149) and followed the Animal Welfare Act. The mice were kept in a temperature-controlled room with light/dark conditions and fed with standard laboratory animal food and tap water ad libitum.

LPS-induced Mouse Model of ALI

The ALI model was developed as previously mentioned.[12,21] In summary, mice were anesthetized using a mixture of ketamine-xylazine (80:10 mg/kg i.p. Alfasan, Netherland), and the model was established through intratracheal instillation of 2 mg/kg of LPS (LPS, 055: B5, Sigma-Aldrich) dissolved in 100 µL PBS. A similar volume of PBS was given to the mice in the control group (100 µL). Systemic analgesia was administered immediately after the ALI operation by subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg/q12).

Forty male Balb/c mice were allocated into five different groups (n=8 per group) of Control, LPS, LPS+ jujube (100 and 200 mg/kg), and LPS+ Dexamethasone at random. It's worth mentioning that the selection of Z.J doses of 100 and 200 are based on our previous unpublished data. LPS instillation (50 µg of LPS dissolved in 100 µL PBS) was performed intratracheally (i.t) in all groups except control animals that received PBS. Mice from the control and LPS groups were treated with vehicle (200 µL saline, i.p.). Animals treated with

jujube received 200 µL of fruit extracts intraperitoneally (i.p) at the doses of 100 mg/kg (LPS+j100) and 200 mg/kg (LPS+j200), respectively. 200 µL of dexamethasone was given to the last group (2 mg/kg, i.p.). PBS or drug administrations were performed at 1, 11, and 23 h post-LPS challenge. Mice were finally sacrificed 24 h after receiving LPS to collect bronchoalveolar lavage fluid (BALF) and lung tissues, which were used to determine inflammatory cell influx into the airways and histopathologic alterations.

Bronchoalveolar Lavage Fluid (BALF) Collection

Mice were euthanized 24 h after LPS injection. As previously mentioned, BALF fluid was obtained by a 20-gauge angiocath.[22] BALF sample was obtained by repeatedly loading and aspirating 0.5 mL of sterile PBS (three times) and centrifuged at 1200 rpm for 10 min at 4°C. Supernatant was kept at −80°C and the cell pellet was resuspended in the cold PBS. Standard hemocytometer was used to count the total number of infiltrating cells. Using staining BALF cell smears with Wright-Giemsa, differential cell counts were estimated based on morphologic parameters on Wright-Giemsa stained cytospin preparations.

Measurement of Lung Wet-to-dry Ratio

To measure pulmonary edema, the wet-to-dry ratio (W/D ratio) was utilized. First, the wet weight of the lower right lungs was measured at the time of scarification. Next, the lungs were incubated in an oven at 60°C for 72 h to determine their dry weight. Finally, W/D ratio was calculated.

Measurement of TNF-α Level in Lung Tissue

Following BALF collection, 100 mg of the right upper lung was taken and gently homogenized with 0.9 mL cold PBS. Homogenate was centrifuged for 15 minutes at 5000 rpm at 4°C. TNF-α levels in lung tissue homogenates were measured using an East-Biopharm rat TNF-α ELISA Kit (Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China) according to the manufacturer's instructions.

Histological Examination

Lung tissues were fixed in 10% formalin, embedded in paraffin, and sectioned into 5 µm thick sections. Hematoxylin and eosin were used to stain the slides after deparaffinization. Under light microscopy, morphologic changes in the lungs were assessed and cellular inflammatory infiltration was scored. Neutrophil infiltration, edema, disorganization of the lung parenchyma, and hemorrhage were utilized to grade the degree of lung injury. More severe lung damage is associated with higher scores in this system: 0 = normal, 1=light, 2 = moderate, 3 = severe, and 4 =very severe.[23]

Statistical Analysis

All data were expressed as mean ± SEM. Statistical differences were evaluated through analysis of variance for repeated measures performed by Bonferroni post hoc test using GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, CA). The same software was used to design the graphs. Substantial differences were defined as *p*-values ($p<0.05$).

RESULTS

Z. J Treatment Ameliorated LPS-induced Cell Influx into the Lungs

Massive infiltration of inflammatory cells into the lung 24 h following intratracheal injection of LPS was found. Data indicated that infiltration of total cells, neutrophils, lymphocytes, and macrophages in BALF considerably increased in the LPS-treated group compared to control animals (Figure 1). Conversely, treatment with dexamethasone (2 mg/kg) and Z.J extracts (100 and 200 mg/kg) reduced total cell count, the number of neutrophils and macrophages profoundly in compared to the LPS-treated animals. In contrast, an increased number of lymphocytes via LPS treatment were not affected by dexamethasone or Z.J administration.

Z. J Reduced LPS-induced Lung Water Content

For measuring the severity of lung edema, the W/D ratio of pulmonary tissue was assessed (Figure 2). 24 h post-LPS injection, the LPS group represented a substantial increase in water content compared to the control mice. Pretreatment with dexamethasone and Z.J extracts (100 and 200 mg/kg) significantly attenuated LPS-induced lung edema.

Z. J Decreased TNF-α Levels in Lung Tissue

The anti-inflammatory effects of Z.J extract were studied additionally by measuring TNF-α production. ELISA assay was applied to detect the expression of pro-inflammatory cytokine TNF-α in pulmonary tissue homogenate. Compared to the control group, LPS administration significantly elevated TNF-α levels

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(p=0.0001, Figure 3). Pretreatment with dexamethasone (2 mg/kg) and Z.J extracts (100 and 200 mg/kg) could apparently reduce the TNF-α levels in LPS-induced ALI mice compared with the control group. The statistical analysis represented that the elevated levels of TNF-α decreased significantly by 21.7% and 39.6%, respectively, in groups that received 100 and 200 mg/kg doses of Z.J compared to the LPS-treated group.

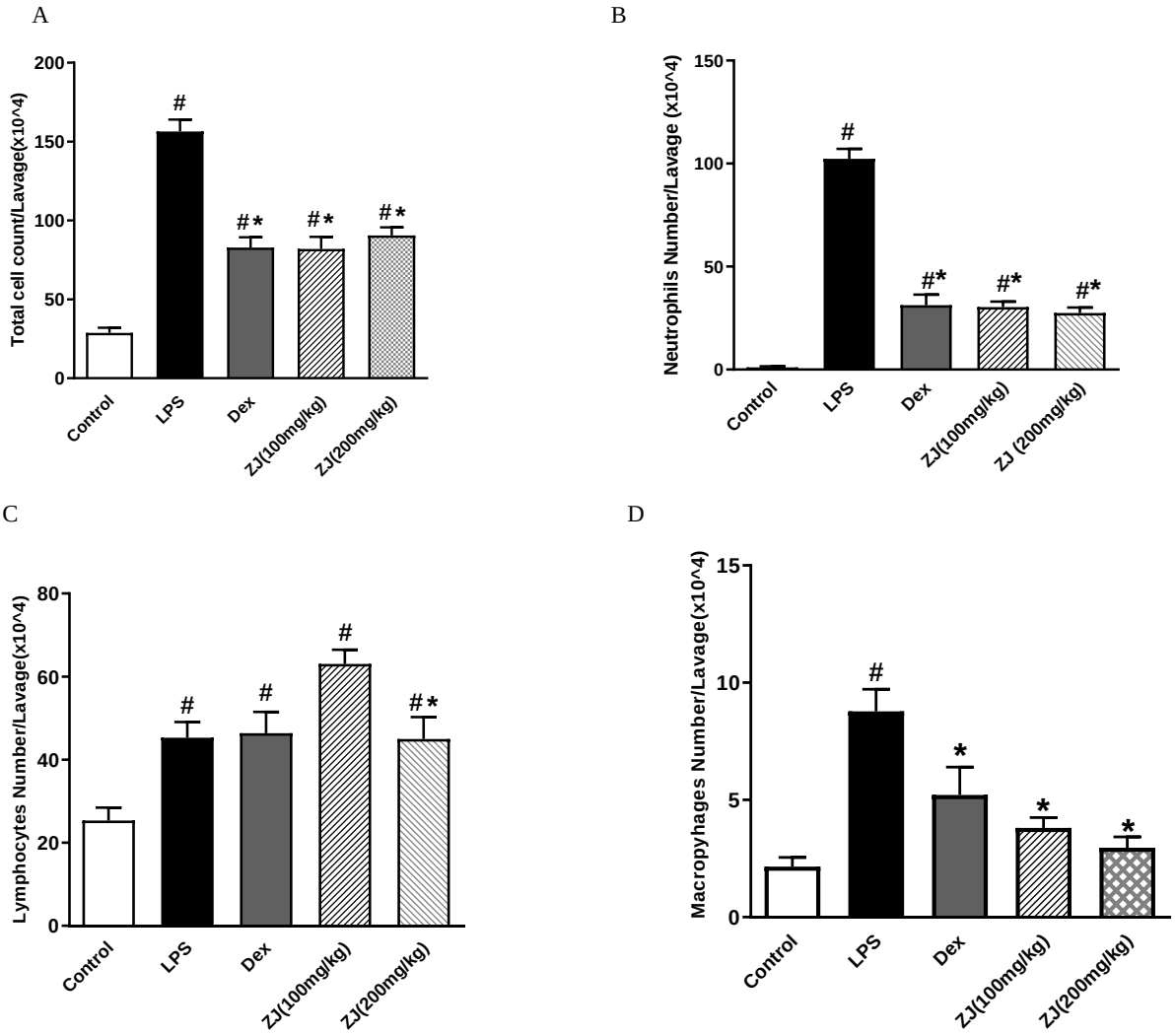

Figure1. Effects of *Ziziphus jujuba* Mill (at doses of 100 and 200 mg/kg, i.p) on bronchoalveolar lavage fluid (BALF) total and differential cell counts in LPS-induced acute lung injury (ALI) mice. Notes: LPS was instilled intratracheally in all groups except the control animals that received PBS. Control and LPS groups only received vehicle (i.p). The other three groups received Z.J (100 and 200 mg/kg, i.p.) or dex (2 mg/kg, i.p.). PBS or drug administrations were performed at 1, 11, and 23 h post-LPS challenge 24 h. later, mice were euthanized and BALF was collected for evaluating the inflammatory cell infiltration. (A) Total inflammatory cells (####*p-value* vs. control < 0.0001, *****p-value* vs. LPS< 0.0001). (B) Neutrophils (####*p-value* vs. control < 0.0001, *****p-value* vs. LPS< 0.0001). (C) Lymphocytes (####*p*-value vs. control < 0.0001, * *p*-value vs. LPS< 0.05). (D) Macrophages (####*p-value* vs. control < 0.0001, ****p-value* vs. LPS= 0.001). For simplicity, only one * and # are shown per column of each diagram. LPS: lipopolysaccharide, Dex: Dexamethasone. The values are shown as means ± standard error of the mean. Data presented with means with SEM. The number of mice/groups: 8.

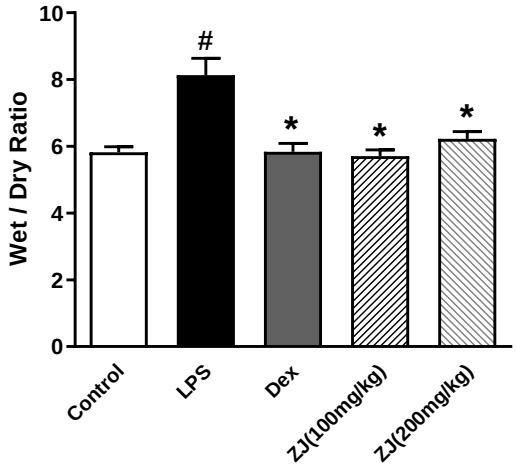

Figure2. Effects of *Ziziphus jujuba* Mill (at doses of 100 and 200 mg/kg, i.p) on the lung W/D ratio in LPS-induced acute lung injury (ALI) mice. Notes: LPS was instilled intratracheally in all groups except the control animals that received PBS. Control and LPS groups only received vehicle (i.p). Other three groups received Z.J (100 and 200 mg/kg, i.p.) or dex (2 mg/kg, i.p.). PBS or drug administrations were performed at 1, 11, and 23 h post-LPS challenge. Twenty-four h later, mice were euthanized and BALF was collected for evaluating the inflammatory cell infiltration. ####*p-value* vs. control < 0.0001, ****p-value* vs. LPS< 0.001. For simplicity, only one * and # are shown per column of each diagram. LPS: lipopolysaccharide, Dex: Dexamethasone. Values are shown as means ± standard error of the mean. Data presented with means with SEM. Number of mice/groups: 8.

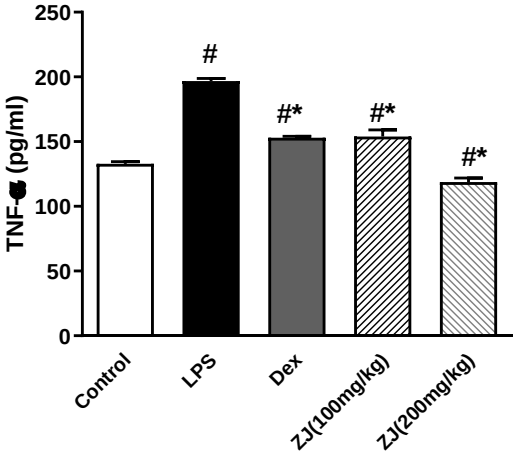

Figure3. Effects of *Ziziphus jujuba* Mill (100 and 200 mg/kg doses, i.p) on tumor necrosis factor-alpha (TNF-α) levels in lung tissue homogenates. Notes: LPS was instilled intratracheally in all groups except the control animals that received PBS. Control and LPS groups only received vehicle (i.p). The other three groups received Z.J (100 and 200 mg/kg, i.p.) or dex (2 mg/kg, i.p.). PBS or drug administrations were performed at 1, 11, and 23 h post-LPS challenge. Twenty-four h later, mice (n=8 in each group) were euthanized, and lung tissue was collected for further evaluation. ###*p-value* vs. control < 0.0001, *****p-value* vs. LPS < 0.0001. For simplicity, only one * and # are shown per column of each diagram. Number of mice/groups: 8. The values are shown as means ± standard error of the mean (SEM). LPS: lipopolysaccharide, Dex: Dexamethasone

Z.J Alleviated LPS-mediated Lung Histopathological Destruction

Histological evaluations revealed LPS-induced pathological structural changes and marked polymorphonuclear leukocyte accumulation in the lung tissue. As shown in Figure 4, pulmonary tissues from the control group represented a normal structure and no inflammatory cell infiltration or hemorrhage under the light microscope (Figure 1a). The LPS group showed airway congestion, alveolar wall thickening, interstitial infiltration of inflammatory cells, edema, and hemorrhage. Both dexamethasone and Z.J extracts (100 and 200 mg/kg) provided significant protective effects compared with LPS-induced mice.

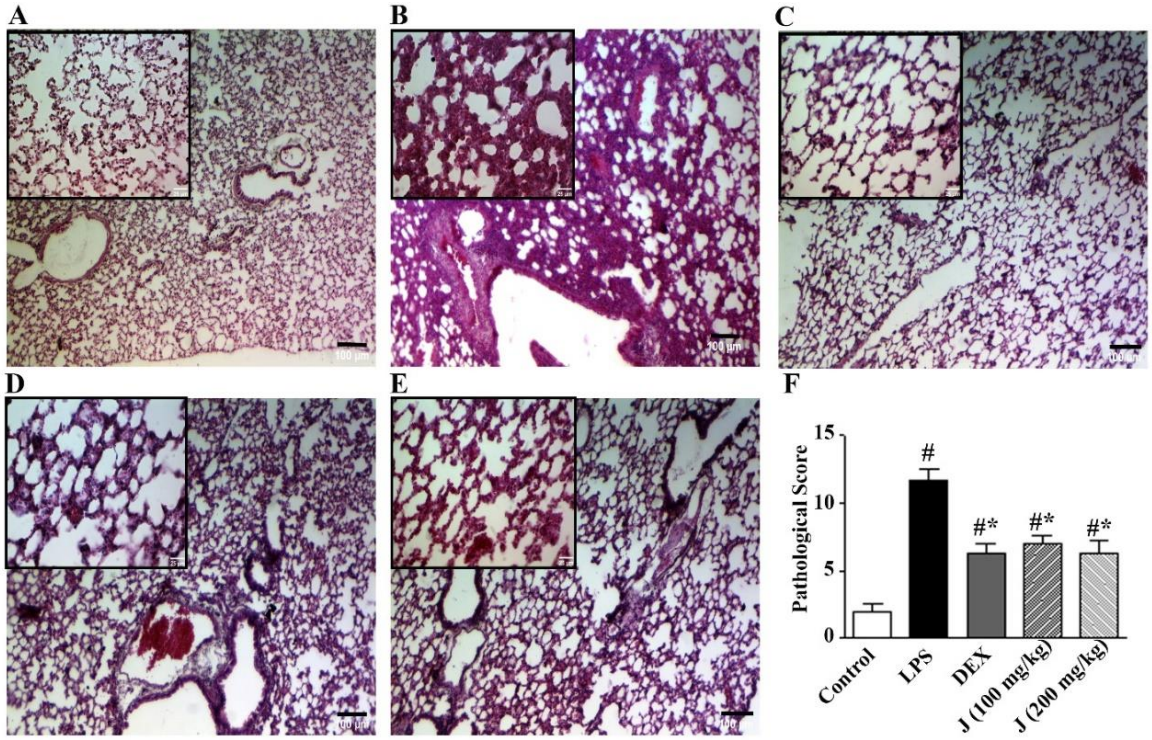

Figure 4. Effects of *Ziziphus jujuba* Mill on lung histopathology in pulmonary tissue homogenates. Notes: LPS was instilled intratracheally in all groups except the control animals that received PBS. Control (A) and LPS (B) groups only received vehicle (i.p). The other three groups received DEX (C) (2 mg/kg, i.p.) or Z.J (100 and 200 mg/kg, i.p.) (D and E, respectively) PBS or drug administrations were performed at 1, 11, and 23 h post-LPS challenge. Systemic analgesia was administered immediately after the ALI operation by subcutaneous injection of buprenorphine hydrochloride. Mice have finally sacrificed 24 h after receiving LPS, and lung tissues were collected for further evaluation. Slides were stained with hematoxylin and eosin reagents (100 X; scale bars=100 µm, 400X; scale bars= 25um). Spongy spaces in any figure are alveolar sacs that surround airways and vessels. In each figure, the magnification of the main part is 100X and the magnification of each of the small squares is 400X. The score of lung injury (F): ####*p-value* vs. control < 0.0001, **p-value* vs. LPS < 0.0001. For simplicity, only one * and # are shown per column of the diagram. Data presented as mean±SEM. Number of mice/groups: 8.

DISCUSSION

We studied the anti-inflammatory properties of ethanolic Z.J extract in an experimental model of ALI in mice. Using LPS endotoxin, we demonstrated that Z.J extracts (100 and 200 mg/kg) significantly attenuated pulmonary inflammation, edema formation, and lung injury. These data are tied with our previous reports wherein the same LPS animal model was recruited to investigate the potency of anti-inflammatory compounds.[12,21-24] Acute inflammation and vascular disruption are essential hallmarks of ALI, a severe disease condition that contributing to significant morbidity and mortality (40–60% mortality rate).[2,25]

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Regardless of the primary cause, pulmonary inflammation is followed by a stereotypic response defined by macrophage activation; neutrophil infiltration, increased lung permeability, interstitial and alveolar edema, and in cases of massive injury, alveolar hemorrhage.[26] Fast initiation of respiratory failure usually needs mechanical ventilation due in part to the quick onset of dyspnea and hypoxemia.[2] Despite the existence of ventilator and non-ventilator-based therapies, no effective treatment has been yet introduced for this devastating disorder.[21] LPS-mediated ALI is a well-established model for the evaluation of new anti-inflammatory compounds partly due to the activation of resident macrophages, the influx of inflammatory neutrophils into the pulmonary tissue, and the over-secretion of cytokines.[25] In the present study, Z.J significantly reduced lung neutrophil and macrophage infiltration after intratracheal injection of LPS. An important feature of ALI (Acute Lung Injury) is the uncontrolled migration of neutrophils into the interstitial and alveolar spaces of the lungs. This has been linked to the severity of the disease and poor outcomes.[27]

While physiological responses typically work to repair and regenerate, the infiltration of neutrophils is instead considered a pathogenic condition. This is due to the fact that when neutrophils accumulate in the lungs, it can result in the production of cytotoxic substances and increased activation of certain agents (including NF-κB, Akt, and p38 kinases), which can exacerbate acute inflammatory conditions.[28,29] On the other hand, neutrophil depletion has confirmed to attenuate lung injury.[30] Jujuboside B (JB), a saponin active ingredient found in jujube ethanolic extract, is a promising compound that has shown to have anti-inflammatory properties. In particular, studies have demonstrated that JB can significantly reduce the number of inflammatory cells in the lungs and decrease the severity of pulmonary inflammation in a mouse model of allergic asthma induced by ovalbumin. JB has also been observed to regulate the elevated levels of T-helper type 2 cytokines found in the serum, bronchoalveolar lavage fluid (BALF), and lung homogenate of the animals studied.[31]

In addition to cell influx, LPS-induced ALI was alleviated by Z.J extracts as evidenced by protective effects on histopathological structural changes and edema formation. The degree of pulmonary edema index (wet-to-dry ratio) is an appropriate way to assess the extent of damage to the alveolar-capillary membrane. When acute pulmonary damage occurs, the tissue itself and elements of the innate immune system initiate a rapid response that leads to acute inflammation and dysfunction.[28] The elevated permeability of the alveolar-capillary barrier and the formation of edema impairs the delivery of arterial oxygen, a characteristic that is similar to the symptoms seen in Coronavirus SARS-CoV-2 (Covid-19).[21,30] In acute lung injury, the increased microvascular permeability leads to a simultaneous rise in lung microvascular hydrostatic pressure and eventually results in pulmonary edema.[2] Impaired epithelial cell barrier function promotes the migration of neutrophils and the inflow of macromolecules, which leads to the accumulation of protein-rich fluid in the alveoli, affecting cell fluid transport and producing edema.[32] In line with the findings of the present study, other experiments also provide strong evidence for the anti-edema capacity of Z.J. The experimental findings from the carrageenan-induced rat paw edema demonstrated that the Z.J ethanolic extract significantly inhibited the paw edema formation.[33] In addition, when extracts from Z.J were used to treat mice models with xylene-induced ear edema and carrageenan-induced paw edema, substantial prevention of edema formation was achieved.

The anti-inflammatory effect of the Z.J extract is probably involved in cyclooxygenase 2 (COX-2) signaling pathways with the consequence of prostaglandin production inhibition.[34] A growing body of evidence confirmed that most of the IL-1β and TNF-α initiating lung inflammatory cascade is generated from the endotoxin stimulation of resident alveolar macrophages. At the same time, adjacent cells release a battery of chemokines and ICAMs that regulate the alveolar recruitment of neutrophils, monocytes, and lymphocytes.[11,27,35] Consistent with previous reports, our study has shown that Z.J extracts significantly attenuated the pulmonary levels of TNF-α.[8] Furthermore, Z.J fruit contains active phenolic compounds of betulinic acid, quercetin, and galangin. TNF-α and the production of nitric oxide (NO) are reduced in LPS-induced mice models treated with Z.J extract, owing to the presence of betulinic acid, a potent anti-inflammatory pentacyclic triterpene molecule found in Z.J, while the anti-inflammatory cytokine IL-10 is increased.[25,33]

The most prominent phenolic components in Z.J extracts are quercetin and galangin which have inhibitory effects on NO production through inducible nitric oxide synthase (iNOS) and IL-6 inhibition. These

chemical ingredients can suppress the nuclear translocation of nuclear factor kappa B (NF-κB) light-chain-enhancer of activated B cells and the activation of extracellular signal-regulated kinase 1/2 (ERK ½) and c-Jun N-terminal kinases (JNK). As previously demonstrated in other investigations, suppressing LPS-induced NF-κB nuclear translocation decreases the protein levels of iNOS and COX-2, which are downstream important enzymes for the generation of nitrite and PGE2.[12,24,36] Although we achieved our main objectives of assessing cell infiltration, pulmonary structure, water content, and TNF-α levels in this study, we lacked detailed knowledge on the molecular mechanisms and other inflammatory mediators affected by Ziziphus jujube mill extract in LPS-induced acute lung injury in mice. Therefore, further evaluations are necessary to clarify its precise efficacy in this model.

Our investigation aimed to assess the effectiveness of Z.J Mill's ethanolic extract on LPS-induced ALI. Our results showed that administering Z.J to ALI mice significantly reduced pulmonary edema, inflammatory cell infiltration, and TNF-α production, leading to amelioration of lung histopathological damage. Detailed inhibitory mechanisms triggered by Z.J should be addressed via further molecular signaling evaluations.

STATEMENT OF ETHICS

Animal studies were accepted through the Ethical Committee at Birjand University of Medical Sciences (approval code: IR.BUMS.REC.5149).

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CONFLICT OF INTEREST

All authors declared that they have NO conflict of interest in the subject matter or materials discussed in this manuscript. They also have NO affiliations with or involvement in any organization or entity with any financial interest. They also have accepted responsibility for the entire content of this submitted manuscript and approved the submission.

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