BJPS

Brazilian Journal of
Pharmaceutical Sciences

DOI: http://dx.doi.org/10.1590/s2175-97902026e24685

Article

Protective effects of bromelain in LPS-induced acute lung injury

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Acute lung injury (ALI) is a severe disease that can cause extensive lung damage and lead to death. Elevated levels of inflammatory cytokines and associated inflammation and oxidative damage irreversibly damage the lung tissue, leading to death. This study aimed to utilize the powerful antioxidant properties of bromelain (BRO) in ALI to determine its beneficial effects. We used an LPS-induced ALI model in rats, and 40 male specimens were divided into five groups: Healthy, Healthy+100BRO, ALI, ALI + 50BRO, and ALI + 100BRO. At the end of the study, histopathological and biochemical analyses of the lung tissue samples were performed. The results showed that tumor necrosis factor, interleukin-1, interleukin-6, and malondialdehyde levels increased in the ALI group and that these levels decreased dose-dependently with BRO treatment. We also demonstrated that superoxide dismutase levels, which decreased in the ALI group, increased dose-dependently in the BRO treatment groups. Histopathologically, significant improvement was detected in the BRO treatment groups. As a result of this research, it is suggested that BRO should be considered as supportive therapy in ALI. However, more detailed experimental and clinical studies on this subject are needed.

Keywords: Acute lung injury. Bromelain. Inflammation. Rat.

INTRODUCTION

Acute lung injury (ALI) is an acute diffuse inflammatory lung injury caused by various pathogenic factors (Tang *et al.,* 2021). A large number of neutrophils infiltrating the lungs after various pathogenic factors increase proinflammatory cytokines and free oxygen radicals (reactive oxygen species), which leads to pulmonary microvascular endothelial and epithelial damage and plays a role in the development of ALI (Wan *et al.*, 2018). ALI and its more severe form, acute respiratory distress syndrome, are important causes of morbidity and mortality. Despite decades of research, the mortality rate ranges from 22% to 40% (Mowery, Terzian, Nelson, 2020). Numerous drugs are used in the treatment of ALI, but a truly radical cure has yet to be found (Zhang, Zhao, Yang, 2022).

Many pulmonary and non-pulmonary conditions can cause ALI, but the most common cause is primary pneumonia due to bacterial, viral, or fungal causes (Matthay, Zemans, 2011). The second most common cause is severe sepsis, which may be associated with a source of infection such as pneumonia or peritonitis (Matthay, Zemans, 2011). Lung infection induced by lipopolysaccharide (LPS), a key component of the cell wall of gram-negative bacteria, is among the most prevalent causes of ALI (Tang *et al.*, 2021). LPS is often used to model experimental ALI (Wan *et al.*, 2018). LPS is preferred in ALI induction over a CLP-induced model because the standard dose of LPS application induces a more standard ALI model than a CLP model with its many variables such as intestine content, ligation errors, and/or puncture differences (Zhou *et al.*, 2023). Proinflammatory cytokines such as the tumor necrosis factor (TNF-α), interleukin-1 (IL-1β), and interleukin-6 (IL-6) mediate the initiation and regulation of inflammatory responses in ALI (Song *et al.*, 2021). Studies have shown that TNF-α

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release occurs rapidly when the lung is exposed to LPS (Jiang *et al.*, 2015). TNF-α induces the inflammatory cascade and is an important parameter in the severity of lung injury (Cinar *et al.*, 2019). TNF-α also leads to an increase in free oxygen radicals (Cadirci *et al.*, 2019). IL-6 exacerbates the body's inflammatory response by enhancing the synthesis and release of acute-phase reactants (Chen *et al.*, 2015a). IL-1β, a crucial mediator in the acute phase of inflammation, plays a role in the repair of alveolar epithelial tissue (Chen *et al.*, 2015b). Due to the interaction of LPS with the TLR4 receptor, free oxygen radicals and proinflammatory cytokines are released. This leads to lipid peroxidation and causes cell and organ damage (Meng *et al.*, 2018). Parameters used as markers of lipid peroxidation in ALI include superoxide dismutase (SOD) and malondialdehyde (MDA) levels. Previous ALI studies indicated that free oxygen radical accumulation, which occurs due to the systemic inflammatory response, plays a vital role in tissue damage (Pendyala *et al.*, 2009). ALI is thought to be prevented by pharmacologic agents that inhibit or scavenge the formation of free oxygen radicals (Cinar *et al.*, 2019). Therefore, using an agent that modulates inflammation and has antioxidant properties in treating ALI may be useful. In prior studies, bromelain (BRO) attracted attention due to its antioxidant and anti-inflammatory role.

BRO is a proteolytic enzyme containing a sulfhydryl group derived from the *Ananas comosus* plant (Hikisz, Bernasinska-Slomczewska, 2021). It possesses anti-inflammatory, cardioprotective, immunomodulatory, anticoagulant, and antioxidant activities (Zhou *et al.*, 2017; El-Demerdash *et al.*, 2022; Hu *et al.*, 2022; Agostinis *et al.*, 2015; Juhasz *et al.*, 2008). Many clinical studies have shown that BRO is safe and effective against various medical conditions such as sinusitis, osteoarthritis, surgical wounds, cardiovascular health, and digestive system diseases (Della Volpe *et al.*, 2022; Gupta *et al.*, 2022; Shoham *et al.*, 2021; Jayachandran, Khobre, 2017; Ley *et al.*, 2016). In vivo studies examining the effects of BRO on lung tissue can also be found. A previous study showed that BRO has protective effects in cadmium-induced lung toxicity (Rafiei-Asl *et al.*, 2021). BRO has also been shown to be therapeutic in allergic airway disease (Secor *et al.*, 2012). In addition, a recent study has demonstrated that BRO may synergistically reduce benzopyrene-induced lung carcinogenesis associated with inflammation and oxidative stress (Majumder *et al.*, 2021).

No studies on the effects of BRO in ALI were found in the literature. Therefore, this study aimed to evaluate the possible impact of BRO on oxidative and inflammatory markers via biochemical and histopathological methods in an LPS-induced ALI model in rats.

MATERIAL AND METHODS

Forty male Wistar albino rats weighing between 240 and 280 g were used in this study. The animals were obtained from the Atatürk University Experimental Research and Application Centre experimental animal laboratory. During the experiment, the rats were given water and pellet feed ad libitum. They were housed in groups in the laboratory at normal room temperature (22 °C) and fed before the experiment.

Experimental protocols on the rats

BRO was administered orally to the experimental group specimens. The manufacturer's instructions were followed to render the powdered substances into a saline solution. The same solvent was similarly administered to the Healthy group.

The rats were randomly divided into the following five groups:

1. Healthy group: the healthy rats received saline solution (0.9% NaCl) as a vehicle (n = 8)
2. Healthy + 100BRO group: the healthy rats were given 100 mg/kg of BRO (n = 8)
3. ALI group: rats with ALI (n = 8)
4. ALI + 50BRO group: the rats with ALI received 50 mg/kg of BRO (n = 8)

5. ALI + 100BRO group: the rats with ALI were administered 100 mg/kg of BRO (n = 8)

BRO was administered to the Healthy + 100BRO, ALI + 50BRO, and ALI + 100BRO groups via oral gavage at the doses (Şehirli *et al.*, 2021) mentioned above. The rats were given these doses 1 h after induction of ALI with LPS (Shaban *et al.*, 2023).

After placing the rats in a supine position, a midline cervical incision was made in the skin. To reduce tissue damage, the fascia, sternocleidomastoid muscle, and parathyroid glands were meticulously excised using cotton swabs, facilitating surgical access to the trachea. A 26G sterile insulin injector was introduced into the intratracheal space via the tracheal cartilage. After retracting the injector's piston to allow air to enter, 5 mg/kg of LPS (Sigma-Aldrich, L2880, LPS from *Escherichia coli* O55:B5), diluted in isotonic NaCl, was gradually administered into the trachea. After the injection, the rats were rotated along three axes to ensure the even distribution of LPS throughout the lungs. The skin was then closed using an Appose ULC 35 W AutoSuture Slim Body Skin Stapler (Covidien, Dublin, Ireland) (Aydin *et al.*, 2022).

All groups were anesthetized via intraperitoneal administration of a ketamine and xylazine combination 12 h after induction of ALI. Cardiac blood and lung tissue samples were collected from the specimens, and they were then euthanized. One half of each lung was perfused with saline and stored at –80 °C for biochemical and molecular analyses; the other half was fixed in a 10% formalin solution for histopathological examination. The blood samples were collected in serum tubes and centrifuged at 4 °C and 4000 rpm for 10 min. The resulting supernatants were collected, frozen at –20 °C, and placed in an ultra-cooler at –80 °C for future biochemical parameter measurement. On the day of the study, the supernatants were removed from the cooler, brought to room temperature, and the experiments were performed.

Biochemical analyses

The collected lung tissues were first homogenized. A TissueLyser II grinder jar set (QIAGEN, Hilden, Germany) was used for this. Centrifugation was then performed (Aydin *et al.*, 2022). The TNF-α, IL-1, and IL-6 contents were analyzed in the lung tissues via enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. The MDA and SOD contents were analyzed via the manual method.

SOD manual method analysis

SOD activity was measured according to the method described by Sun *et al.* (1988). The estimation was based on the production of superoxide anion (O_2^-) generated from the reaction between xanthine and xanthine oxidase, which then reacts with nitro blue tetrazolium to yield formazan dye. SOD activity was subsequently quantified by assessing the extent of inhibition of this reaction at 560 nm.

MDA manual method analysis

MDA levels were measured following the protocol established by Ohkawa, Ohishi, and Yagi (1979). The measurement principle is based on assessing the absorbance at 532 nm of the pink compound formed from the reaction of MDA with thiobarbituric acid, which results from lipid peroxidation.

Histopathological procedure

Histopathological specimens were immediately fixed in a 10% formalin solution for 48 h. After fixation, the tissues were routinely processed and embedded in paraffin. Sections of 3 μm were obtained from the tissue blocks, stained with hematoxylin and eosin (H&E), and photographed for histopathological evaluation using a light microscope equipped with a digital camera. For the histopathological assessment of the edema area, alveolar wall thickness and

inflammatory cell infiltration were evaluated based on the previous literature (Ugan *et al.*, 2018).

Statistical analysis

IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA) was used to compare the data. The results were evaluated via a one-way analysis of variance and compared using Tukey's test; $p < 0.05$ was considered significant.

RESULTS

Biochemical analysis results

IL-6 results

As shown in Figure 1, the IL-6 level in the ALI group was significantly increased compared to the Healthy group in the measurements made via ELISA ($p < 0.001$). The IL-6 levels were significantly lower in the ALI + 50BRO and ALI + 100BRO groups than in the ALI group ($p < 0.001$). The BRO-induced decrease in the IL-6 levels was dose-dependently increased in the ALI groups (Figure 1A).

IL-1β results

As seen in Figure 2, the IL-1β levels in the ALI group were significantly increased compared to the Healthy group in the measurements made via ELISA ($p < 0.001$). In the ALI + 50BRO and ALI + 100BRO groups, the IL-1β results were significantly decreased compared to the ALI group ($p < 0.001$). Among the ALI groups, the decrease in the IL-1β levels was higher in the ALI + 100BRO group ($p < 0.001$) (Figure 1B).

TNF-α results

As shown in Figure 3, ELISA measurements indicated that TNF-α levels in the ALI group were significantly elevated compared to those in the Healthy group ($p < 0.001$). BRO treatment reduced TNF-α levels compared to the ALI group. The decrease in the TNF-α levels in the ALI groups was higher in the ALI + 100BRO group (Figure 1C).

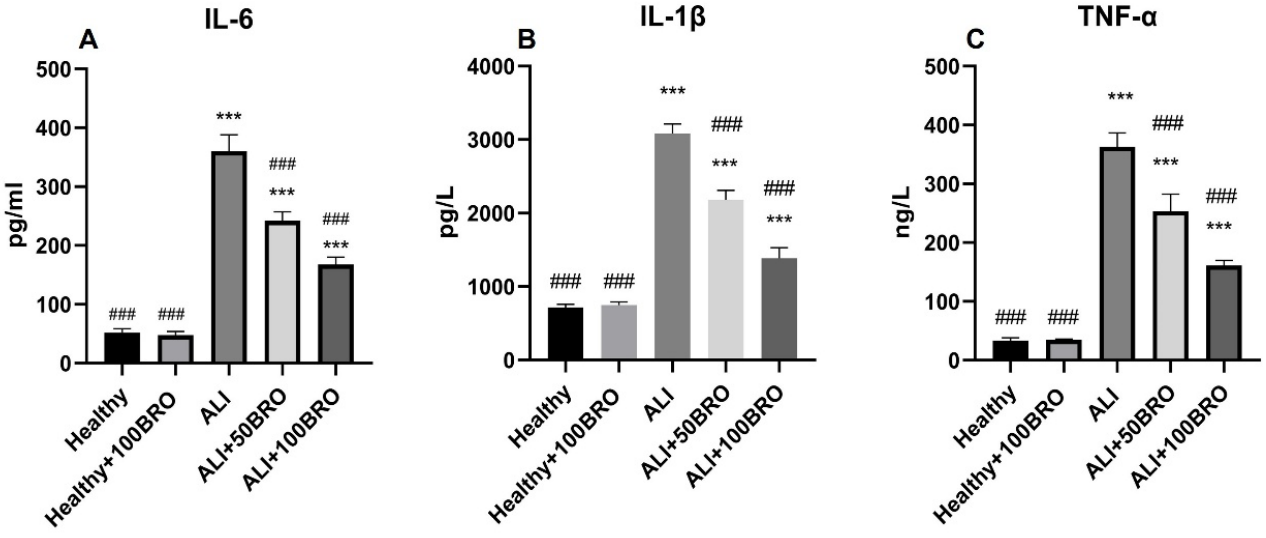

FIGURE 1 - IL-6 (**a**), IL-1β (**b**), and TNF-α (**c**) levels in rat lung tissue. *comparison according to the Healthy group; #comparison according to the ALI group; ***significant difference at $p < 0.001$ compared to the Healthy group; ###significant difference at $p < 0.001$ compared to the ALI group.

SOD results

The SOD enzyme level in the ALI group was significantly decreased compared to the Healthy group. BRO treatment significantly increased SOD levels in both the ALI + 50BRO ($p < 0.01$) and ALI + 100BRO ($p < 0.001$) groups compared to the ALI group. The best SOD activity was realized in the ALI + 100BRO group compared to the ALI group ($p < 0.001$) (Figure 2A).

MDA results

As shown in Figure 4, the measurements revealed that MDA levels in the ALI group were significantly higher than those in the Healthy group ($p < 0.001$). BRO treatment significantly decreased the MDA levels at both doses compared to the ALI group ($p < 0.001$). The ALI + 100BRO group had the highest decrease in MDA levels (Figure 2B).

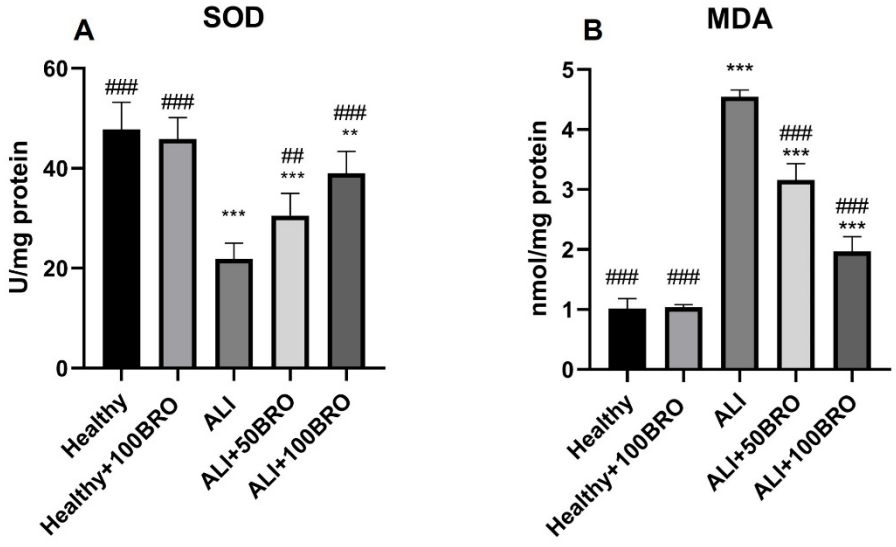

FIGURE 2 - SOD activity and MDA level in rat lung tissue. *comparison according to the Healthy group; #comparison according to the ALI group; ***significant difference at $p < 0.001$ compared to the Healthy group; ###significant difference at $p < 0.001$ compared to the ALI group; **significant difference at $p < 0.01$ compared to the Healthy group; ##significant difference at $p < 0.01$ compared to the ALI group.

Histopathological results

In the histopathological examination of H&E sections in the Healthy and Healthy + 100BRO groups, the bronchi, terminal bronchioles, respiratory bronchioles, alveolar sacs, interalveolar septa, arteries, veins, and capillary vasculature in the lung tissue were examined in detail (Figures 3A and 3B). In the ALI group, advanced edema and inflammation were observed around the vascular structures. The interalveolar septum of the lung parenchyma was enlarged with diffuse parenchymal edema. Areas of interalveolar hemorrhage were also observed (Figures 3C, 3D, and 3E). In the ALI + 50BRO group, edema and inflammation were observed in the H&E sections. However, the findings were more attenuated than those of the ALI group (Figure 3F). Interalveolar septa were observed to be relatively close to normal due to decreased edema in the parenchyma. In the ALI + 100BRO group, edematous areas around the vessels, submucosa, and terminal bronchioles were significantly reduced. Alveolar septum thickening was found to be normal enough to be considered healthy. Inflammation cells were rarely seen in the tissue. In general, a histologic appearance similar to the Healthy group was observed in this group (Figure 3G).

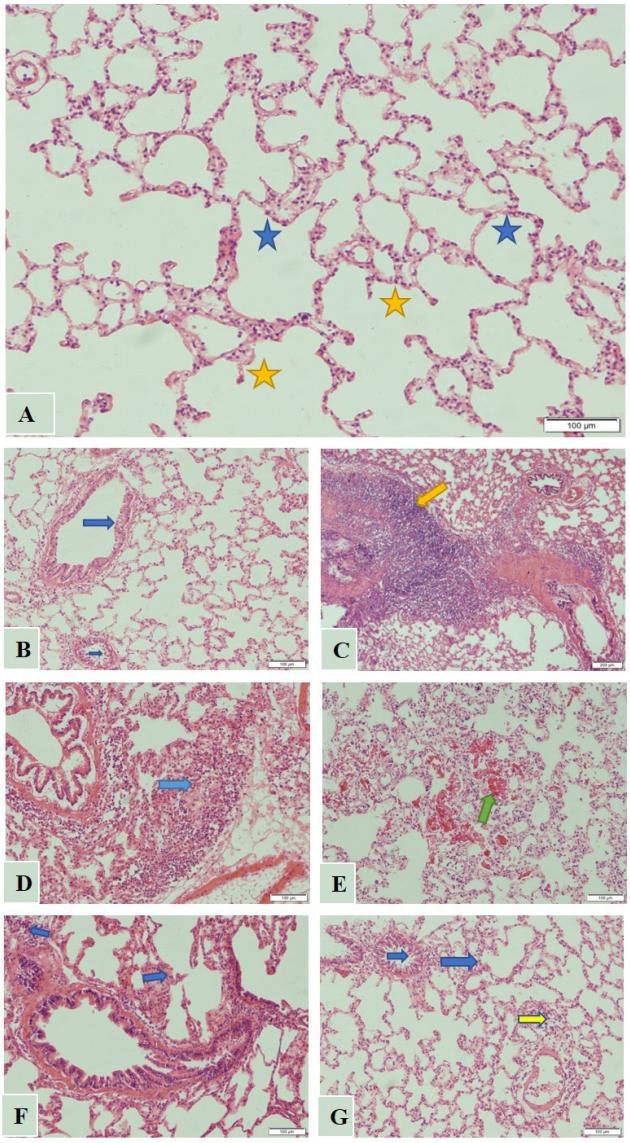

FIGURE 3 - Histopathological examination of the H&E sections of the Healthy group **(A)**, in which no pathological findings were observed histomorphologically (blue star: alveolar space, yellow star: alveolar sac), Healthy + BRO100 group **(B)**, which did not reveal any pathologies in the respiratory and circulatory units of the lung (blue arrow: ordinary bronchial epithelium), ALI group **(C, D, E),** in which areas of interalveolar hemorrhage were also observed **(**yellow arrow: intense inflammation around the vascular structure; blue arrow: parenchymal edema and inflammation; green arrow: intra-alveolar hemorrhage), ALI + 50 group **(F)** (blue arrows: mildly edematous, inflamed lung parenchyma), and the ALI + 100 group **(g)** (blue arrows: intact bronchioles, yellow arrow: mildly edematous parenchymal area).

DISCUSSION

In this study, the effects of BRO—increasingly popular because of its antioxidant and anti-inflammatory properties, on LPS-induced ALI were examined through TNF-α, IL-1β, IL-6, SOD, and MDA levels. In ALI induced by LPS, increased TNF- α, IL-1β, and IL-6 cytokine levels and increased MDA levels, along with oxidative damage, decreased with BRO administration. The levels of SOD, an important part of the antioxidant system, increased in the Healthy + 100BRO, ALI + 50BRO, and ALI + 100BRO groups compared to the ALI group. Histopathological examination showed that BRO administration decreased inflammation and pulmonary edema.

ALI is an acute diffuse inflammatory lung injury in which the endothelial and epithelial barriers of the lung are disrupted. An intense influx of neutrophils into the lungs occurs with increased free oxygen radicals and proinflammatory mediators (Zhang, Zhao, Yang, 2022). The severe inflammatory process occurring in ALI and the resulting high levels of TNF- α, IL-1β, and IL-6 cytokines in the body are positively correlated with the morbidity and mortality of the disease (Mowery, Terzian, Nelson, 2020). Control of excessive inflammatory response and uncontrolled production of free oxygen radicals are critical therapeutic targets in treating ALI (Ge *et al.*, 2020). Numerous mouse models have demonstrated that antioxidant substances can alleviate the severity of ALI (Kellner *et al.*, 2017). Therefore, recent studies have focused on substances with antioxidant or anti-inflammatory properties for treating the disease. When clinical studies were examined, substances with antioxidant properties such as melatonin (Kang *et al.*, 2022), mitoquinone (Zhan *et al.*, 2022), obacunone (Li *et al.*, 2022), and quercetin (Sang *et al.*, 2022) were tested in ALI, and significant results were obtained. BRO, an antioxidant substance, has also come to the forefront in recent studies.

As a proteolytic enzyme obtained from *Ananas comosus*, BRO is classified within the Bromeliaceae

family (Didamoony *et al.*, 2022) and is a protease enzyme containing different thiol groups; these groups protect cells against oxidative stress caused by free radicals due to their sulfhydryl content (Saptarini, Rahayu, Herawati, 2019). In this way, BRO acts as an antioxidant by inhibiting lipid peroxidation and scavenging free oxygen radicals (Khazaeel *et al.*, 2022). It has also been shown to have hepatoprotective (Hu *et al.*, 2020), neuroprotective (Kumar *et al.*, 2022), and pulmonoprotective (Rafiei-Asl *et al.*, 2021) properties. A prior study observed that antioxidant enzymes increased and free oxygen radicals decreased with BRO application in rats with liver damage (El-Demerdash *et al.*, 2022). In another study, the authors found that SOD levels increased and MDA levels decreased after BRO administration to mice given pesticides (Agarwal *et al.*, 2016). In the current study, the SOD level increased, and the MDA level decreased with BRO administration in LPS-induced ALI.

In the pathogenesis of ALI, the accumulation of free oxygen radicals and the increase in proinflammatory cytokines play an essential role. Free oxygen radicals can activate various transcription factors involved in inflammatory pathways. One is NF-κB, an important transcription factor (Sul, Ra, 2021). Activation of NF-κB results in elevated levels of proinflammatory cytokines, contributing to the progression of ALI (Li, Verma, 2002). Research has shown that BRO reduces inflammatory cytokine levels by inhibiting NF-κB translocation, resulting in an anti-inflammatory effect (Hong *et al.*, 2021). BRO has been found to suppress the activity of important cytokines and molecules associated with the inflammatory process in LPS-induced inflammation models in studies involving human (Bhui *et al.*, 2012) and rat (Habashi *et al.*, 2016) cell cultures. Another study found that administering BRO led to reduced levels of TNF-α, IL-1β, IL-6, and MDA alongside elevated SOD levels in rats with ocular toxicity (Okkay *et al.*, 2023).

In the present study, BRO significantly decreased elevated TNF-α, IL-1β, and IL-6 levels in LPS-induced ALI. The histopathological examination revealed perivascular edema, inter alveolar septum thickening, submucosal edema, and severe inflammatory areas in the ALI group. These were improved in the Healthy + 100BRO, ALI + 50BRO, and ALI + 100BRO groups. Considering this information, the biochemical and histopathological analyses demonstrated that BRO elevates antioxidant enzymes and lowers inflammatory cytokine levels, including TNF-α, IL-1β, and IL-6 in ALI. Accordingly, we recommend that BRO should, at a minimum, be considered as supportive therapy in ALI. However, more detailed experimental and clinical studies on this subject are needed.

The study's limitations include the lack of western blot analyses to determine protein levels directly in lung tissue and the fact that we only evaluated BRO's effects on one ALI model.

ACKNOWLEDGMENTS

We would like to thank Prof. Dr. Zekai Halici for his scientific contributions. We also thank Atatürk University's Scientific Research Projects Coordination Unit for supporting this study (grand number TAB-2022-10681).

AUTHOR CONTRIBUTIONS

The study design was conceived by ZB, HH, and TT. Data collection was performed by ATO and CO. Statistical analysis was carried out by SO and TT. Data interpretation was done by ZB, CO, and HH. The manuscript was prepared by ZB and HH, and TT, CO, and ATO conducted the literature search.

ETHICAL APPROVAL

Ethical committee approval was obtained from the Local Animal Care Committee at Atatürk University (Meeting date: 28.02.2022; Number of meetings: 2022/2; Decision No: 29).

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

Data available from the corresponding author upon reasonable request.

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Received for publication on September 20th, 2024
Accepted for publication on November 13th, 2024
Associated Editor: Severino Matias de Alencar