Original Article

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TANAFFOS

Abelmoschus esculentus Seed Ethanol Extract Protects Against Lipopolysaccharide-Induced Lung Injury in Rats through Anti-Inflammatory Properties

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Background: Acute lung injury is respiratory failure due to various causes. Increased inflammatory and oxidative processes are recognized to play an essential role in the etiology of ARDS. *Abelmoschus esculentus* is an herbal product used to treat various diseases due to its anti-inflammatory and antioxidant effects. We aimed to investigate whether *Abelmoschus esculentus* has an effect on acute lung injury.

Materials and Methods: In this experimental study, we used the ethanol extract of *Abelmoschus esculentus* seed. It divided forty male Wistar rats into five equal groups: 1) control, 2) *Abelmoschus esculentus*, 3) lipopolysaccharide, 4) lipopolysaccharide+*Abelmoschus esculentus*, and 5) lipopolysaccharide+ *Abelmoschus esculentus* +dexamethasone groups.

Results: In the lipopolysaccharide group, native thiol, total thiol, IL-10, and IFN-ɣ levels significantly changed. *Abelmoschus esculentus* was effective when used with dexamethasone in increasing native thiol and total thiol values (p=0.008 and p=0.004, respectively). On the other hand, when *Abelmoschus esculentus* was used alone, it significantly increased IL-10 levels and decreased IFN-ɣ levels in the lipopolysaccharide group (p=0.025 and p<0.001, respectively). Additionally, improvements were noted in histological findings of alveolar congestion (p=0.006), intra-alveolar hemorrhage (p=0.006), and intra-alveolar macrophages (p=0.001).

Conclusion: *Abelmoschus esculentus*, with its anti-inflammatory effect, may represent a new potential for treating acute lung injury.

Keywords: *Abelmoschus esculentus*; Acute Lung Injury; IL-10; IFN-ɣ; Thiol

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an acute diffuse lung injury causing hypoxemic respiratory failure. ARDS occurs due to pulmonary or extrapulmonary causes. It is a leading cause of morbidity and mortality in childhood. The cytokine storm is the most significant characteristic of ARDS. Neutrophils have a crucial role in the onset and progression of ARDS. Activated neutrophils and macrophages release numerous pro-inflammatory cytokines. Pro-inflammatory cytokines damage the alveolar epithelium and the vascular endothelium. During these inflammatory processes, reactive oxygen species (ROS) such as hydroxyl, superoxide, and hydrogen peroxide are also increased within the cells. Increased ROSs exacerbate the damage to the alveolo-capillary membrane. This damage increases the permeability of the

capillary membrane. Fluids, proteins, and blood cells can leak from the capillary bed into the pulmonary interstitium and alveoli due to increased capillary permeability, a defining feature of ARDS. ARDS is a significant disease in terms of morbidity and mortality. Studies have shown that ARDS constitutes 5.8% of patients treated in pediatric intensive care units (1). Some of the survivors may have various health problems. Although we have a better understanding of the pathophysiology of the disease and can offer more treatment alternatives, about 1/3 of the cases die (2).

Recently, much research has been conducted to understand the pathophysiology of ARDS and improve prognosis. The imbalance between pro-inflammatory and anti-inflammatory cytokines plays an essential role in the development of the disease. Also, the imbalance between oxidants and antioxidants increases lung damage (3).

Abelmoschus esculentus (AE) is a species of the mallow family (Malvaceae). AE is rich in vitamins, minerals, and fiber. Studies have shown that AE can be used therapeutically in various diseases due to its anti-inflammatory and antioxidant properties (4). AE has significant levels of flavonoids and polyphenols. Polyphenols and flavonoids have been shown to have potent antioxidant properties (5). AE shows its antioxidant activity by changing the secreted cytokines (6). Increased inflammatory and oxidative processes are recognized to play an essential role in the etiology of ARDS.

The purpose of this study was to evaluate whether AE, which has antioxidant and anti-inflammatory properties, is effective in treating ARDS. The study's findings will enable us to provide the herbal supplement AE seed earlier in individuals at risk of developing ARDS; thus the morbidity and mortality of ARDS will be reduced.

MATERIALS AND METHODS

AE seed was extracted by maceration method using 95% ethanol as a solvent (7). Then, 100 g of powdered AE seeds were placed in a closed container. 600 ml of ethanol was added to a container and incubated at 25°C for two days in the dark. The extract mixture was filtered two days later, and the liquid portion (ethanol) was transferred into a round bottom flask. After evaporation of solvent at 40°C, an oily golden yellow AE extract was obtained in the flask and stored in a refrigerator at +4°C until assayed.

The care of the rats and all surgical procedures were planned according to the Universal Declaration of Animal Rights. Rats were cared for under standard laboratory conditions with fed a standard diet and water ad libitum. The temperature was fixed at 19±2°C, humidity at 50%-70%, and the lighting system at 12h light/12h dark cycle.

Male Wistar rats weighing 200-250 grams were obtained from our college's Experimental Animals Application and Research Center. A total of 40 rats were randomly divided into five groups (n=8). The groups were formed as follows:

Control (C) group: a single dose of saline in the same volume was administered intraperitoneally (IP) on the day of the procedure.

AE group: a single dose of 600 mg/kg/day AE extract was administered by gavage for seven days (5).

Lipopolysaccharide (L) group: a single dose of 5 mg/kg L was administered by IP (3). Gram-negative bacteria's outer membrane contains the glycolipid LPS. When LPS circulates throughout the body, it binds to a specific binding protein. This complex stimulates the CD14/TLR-4 receptor complex on monocytes, macrophages, and other cells, causing the production of inflammatory mediators. Based on this, it was decided to use LPS to induce models of bacterial sepsis and acute lung injury (8). Lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA) was used for this procedure.

AE+L group: administration of 600 mg/kg/day AE extract by gavage was started seven days before the procedure. On the day of the procedure, L was administered at a dose of 5 mg/kg by IP, and half an hour later, a single dose of 600 mg/kg/day AE extract was administered by gavage.

Dexamethasone (D)+AE+L (AE+L+D) group: a single dose of 600 mg/kg/day AE extract was administered by

gavage seven days before the procedure. On the day of the procedure, L at a dose of 5 mg/kg was administered by IP, and half an hour later, a single dose of 600 mg/kg/day of AE extract was administered by gavage and dexamethasone at a dose of 1.5 mg/kg was administered by IP (3)(Figure 1).

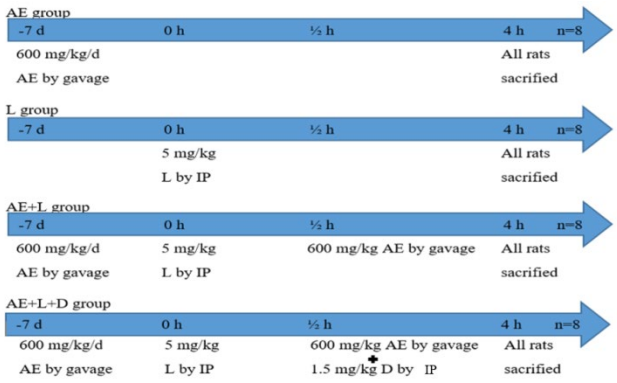

Figure 1. Illustration of the experimental timeline design
(L: Lipopolysaccharide, AE: Abelmoschus esculentus, D: Dexamethasone, IP: Intraperitoneally)

All rats were anesthetized intramuscularly with a 90/10 mg/kg xylazine/ketamine combination and sacrificed 4-hour after the procedure (3). The collected blood was centrifuged at 4000 rpm for 10 minutes and stored in Eppendorf tubes at -80 C until processing. The thorax of the rats was opened, and the right lung was removed, fixed in 10% buffered formaldehyde, and embedded in kerosene blocks for histopathological evaluation. All procedures were performed in a laboratory setting and under sterile conditions.

TNF-α and IFN-γ as inflammatory cytokines, IL-10 as an anti-inflammatory cytokine, and angiopoietin-2 levels as an indicator of endothelial damage were evaluated (9). Rat cytokine ELISA kits were purchased from SinoGeneClon Biotech Co., Ltd., China.

To determine oxidant-antioxidant status from the collected serum, thiol-disulfide levels were measured. For this purpose, the new automated colorimetric measurement method developed by Erel et al. was used(10). This method was used to measure the levels of native thiol, total thiol, and disulfide, as well as the levels of disulfide/native thiol.

For histological examination, 3 µm thick sections were taken from kerosene blocks of the right lung and stained with hematoxylin-eosin dye. The sections were examined by an experienced pathologist using a light microscope (LEICA DM 2000 LED). Damage to lung tissue was assessed semiquantitatively by scoring alveolar congestion, inflammatory infiltration, interalveolar hemorrhage, and interalveolar macrophage; 0 points: no change; 1 point: mild focal changes; 2 points: moderate multifocal changes; 3 points: significant multifocal changes; 4 points: significant diffuse changes(11,12).

Statistical Methods

The SPSS-23 program was used for statistical analysis. Data were expressed as mean ± standard deviation. The SPSS-23 program was used for statistical analysis. Data were expressed as mean+standard deviation. The Kolmogorov-Smirnov test was used to analyze the fit of groups to the normal distribution. The homogeneity of variance was checked. For biochemical data between experimental groups, comparisons were made with a one-way analysis of variance (ANOVA) followed by Tukey tests. Kruskall-Wallis analysis was used to assess differences in histological scores. A Comparison of groups with significant changes was performed using the Mann-Whitney U test. $P<0.05$ was considered significant.

Ethics Statement

The Animal Studies Ethics Committee of Abant İzzet Baysal University approved this study (2020/17). This research was supported by the scientific research fund from Abant Izzet Baysal University (2020.08.23.1466).

RESULTS

There was a significant difference between groups in native thiol levels ($p<0.001$). The L group had lower native thiol levels than the C group ($p<0.001$). Native thiol levels were statistically significantly increased in the L+AE+D group compared to the L group ($p=0.008$). There was no

significant difference in native thiol levels between the other groups (p>0.05) (Table 1).

There was a significant difference in total thiol levels between groups (p<0.001). The L group had lower total thiol levels than the C group (p<0.001). The increase in total thiol levels in the L+AE+D group compared to the L group was statistically significant (p=0.004). There was no significant difference in total thiol levels between the other groups (p>0.05) (Table 1).

There was a significant difference in IFN-ɣ levels between groups (p<0.001). Higher IFN-ɣ levels were detected in the L group than in the C group (p<0.001). IFN-ɣ levels in the group receiving AE for prophylactic purposes were lower than in the L group (p=0.033). IFN-ɣ levels in the L+AE and L+AE+D groups were statistically significantly lower than in the L group (p<0.001 and p < 0.001, respectively) (Table 1).

There was a significant difference in IL-10 values between groups (p<0.001). The L group had lower IL-10 values than the C group (p=0.041). There was a statistically significant increase in IL-10 values in the L+AE and L+AE+D groups compared to the L group (p=0.025 and p<0.001). There was a significant difference between the L+AE and L+AE+D combinations, leading to increased IL-10 values in rats exposed to L (p=0.03) (Table 1). There was no significant difference in disulfide, ANGPT2, TNF-α levels, and disulfide-native thiol ratios between groups (p>0.05) (Table 1).

There was a significant difference between groups in alveolar congestion values (p<0.001). Higher congestion values were found in the L group than in the C group (p<0.001) (Figure 2a, 2b, 2c). Congestion levels in the group receiving AE for prophylactic purposes were significantly lower than in the L group (p=0.005) (Figure 2e, 2f). Compared to the L group, congestion levels were statistically significantly reduced in the L+AE and L+AE+D groups (p=0.006 and p<0.001, respectively) (Table-2) (Figure 2g, 2h) (Figure 2j, 2k).

There was a significant difference between groups in intraalveolar hemorrhage values (p=0.006). Higher bleeding values were observed in the L group than in the C group (p=0.001). The bleeding values in the group receiving AE for prophylactic purposes were significantly lower than in the L group (p=0.037). Compared to the L group, bleeding levels were significantly reduced in the L+AE and L+AE+D groups (p=0.006 and p=0.011, respectively) (Table 2).

A significant difference between the groups was observed in the values for the increased alveolar wall thickness (p<0.001). Higher values for the increase in alveolar wall thickness were found in the L group than in the C group (p<0.001) (Figure 2a, 2b, 2c, 2d). The values for the increase in alveolar wall thickness in the group receiving AE for prophylactic purposes were statistically different from those in the L group (p=0.006). Compared to the L group, the value for the increase in alveolar wall thickness was significantly reduced only in the L+AE+D group (p<0.001) (Table 2).

Table 1. Comparison of the biochemical data between the groups

	C[1]	L[2]	AE[3]	AE+L[4]	AE+L+D[5]	P
NThiol (µmol/l)	206.1±14.9	145.7±21.2	175.7±24.9	159.4±26.5	187.3±25.6	**<0.001**
TThiol (mmol/l)	262.9±10.3	199±20	232.6±27.4	211.8±28.6	245.8±28.6	**<0.001**
IFN-ɣ (pg/ml)	264.2±17.8	325±5.4	291.6±20.6	254±23.1	250.6±14.7	**<0.001**
IL-10 (pg/ml)	95.7±6.3	55.9±15.3	73.2±9.4	100.9±20.3	143.5±41.6	**<0.001**
Dis (pg/ml)	28.4±3.4	29.2±3.5	28.4±7.6	26.2±2.4	26.6±5.5	0.68
Dis/NThiol	13.9±2.5	15.7±2.4	16.4±5.1	16.7±2.8	18.8±5.4	0.17
ANGPT2 (ng/ml)	11.7±3.7	11.7±1.5	11.2±0.5	9.4±1.1	10.5±1.1	0.18
TNF-α (pg/ml)	171±47.3	172.4±52	149±15.6	154.9±19.9	153.3±11.7	0.56

(C: Control, L: Lipopolysaccharide, AE: *Abelmoschus esculentus*, D: Dexamethasone, NThiol: Native thiol, TThiol: Total thiol, Dis: Disulfide, ANGPT2: Angiopoietin-2)

Table 2. Comparison of the histological scores between the groups

	C[1]	L[2]	AE[3]	AE+L[4]	AE+L+D[5]	P
Congestion	0.8±0.3	3.2±0.7	1.7±0.7	2±0.5	1.1±0.3	**<0.001**
Intraveolar hemorrhage	0.6±0.5	2.1±0.6	1.1±0.8	0.8±0.6	1.1±0.6	**0.006**
Thickening of the alveolar wall	1.2±0.4	2.8±0.3	2±0.5	2.4±0.5	1.2±0.4	**<0.001**
Intraalveolar macrophage	0.2±0.4	2.3±0.5	1.5±1.1	0.4±0.5	0.1±0.3	**<0.001**
Inflammation	1±0.5	3.1±0.8	2.2±0.7	2.4±0.5	1.2±0.4	**<0.001**
Abscess	0±0	1.2±1.7	0.1±0.3	0±0	0±0	0.058

(C: Control, L: Lipopolysaccharide, AE: *Abelmoschus esculentus*, D: Dexamethasone)

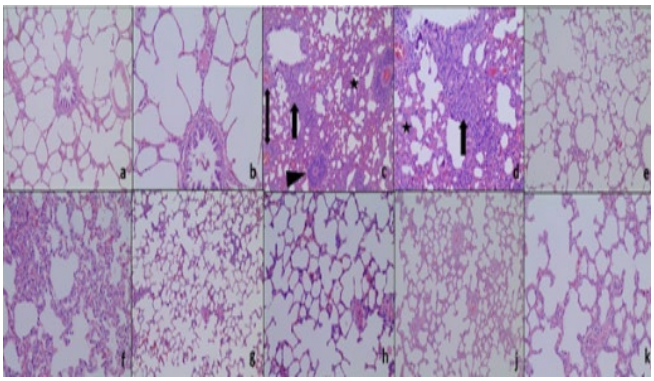

Figure 2. Histopathological changes of AE in L-induced acute lung injury.
a) Control group (HEX100), b) Control group (HEX200), c) L group: Active inflammation accompanied by neutrophils (arrow), inflammation in the perivascular space (arrowhead), alveolar congestion (bidirectional arrow), thickening of the alveolar wall (asterisk) (HEX100), d) L group: inflammation (arrow), thickening of the alveolar wall (asterisk) (HEX200), e) AE group (HEX100), f) AE group (HEX200), g) L+AE group (HEX100), h) L+AE group (HEX200), j) L+D+AE group (HEX100), k) L+D+AE group (HEX200)
(L:Lipopolisaccaride, AE:Abelmoschus Esculentus, D:Dexamethasone, HEX: Hematoxylin-eosin stain)

There was a significant difference in intraalveolar macrophage scores between groups (p<0.001). Intraalveolar macrophage scores were higher in the L group than in the C group (p<0.001). Compared to the L group, interalveolar macrophage scores were significantly reduced in the L+AE and L+AE+D groups (p=0.001 and p<0.001, respectively) (Table 2).

There was a significant difference in inflammation scores between groups (p<0.001). Inflammation levels were higher in the L group than in the C group (p=0.001) (Figure 2a, 2b, 2c, 2d). Compared to the L group, inflammation scores were significantly reduced only in the L+AE+D group (p=0.001). There was no significant difference in abscess scores between groups (p=0.058) (Table 2).

DISCUSSION

One of the most common conditions treated in the ICU is ARDS. Early diagnosis and treatment are the most critical factors for the prognosis of ARDS. The longer the diagnosis is delayed, the more invasive procedures are required, which increases morbidity and mortality. Maintenance of normal lung function is possible if the balance between oxidants and antioxidants in cells is maintained. In inflammatory processes, ROSs such as hydroxyl, superoxide, and hydrogen peroxide increase in the cell. The amount of antioxidants decreases as antioxidants are used to detoxify ROSs. It has been demonstrated that the so-called oxidative stress plays an essential role in the development of ARDS. In ARDS, various cytokines are released by neutrophils, macrophages, and lymphocytes that proliferate in the alveoli. In addition to these inflammatory cells, capillary endothelial cells, alveolar cells, and epithelial cells also produce ROSs (13). ROSs increase to protect against the harmful agent. In cases where the pathogen cannot be eliminated, the increasing ROSs cause cell death by destroying proteins, carbohydrates, lipids, and DNA in the cells (14,15). Disrupting the balance between inflammation and anti-inflammation and between oxidants and antioxidants damages the capillary endothelium and alveolar epithelium. The damage to the alveolar epithelium leads to alveolar edema. Eventually, the surfactant can no longer be produced, and alveolar collapse occurs (16).

Lymphocytes play an essential role in the development of ARDS. Tryptophan is necessary for the healthy functioning of rapidly proliferating cells such as

lymphocytes. On the other hand, IFN-ɣ, a pro-inflammatory cytokine, increases in response to inflammation, regulating the functions of monocytes, macrophages, and T lymphocytes, but increased IFN-ɣ causes the degradation of tryptophan (17). Studies have shown that AE is rich in tryptophan and lysine amino acids (18,19). This property may be one reason why AE is effective in ARDS. Lysine is known to be an auxiliary amino acid in reducing the inflammatory response (20).

Studies have shown that AE is also rich in antioxidants such as ascorbic acid and tocopherol (21, 22). AE shows its antioxidant activity by increasing the production of many endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (5). During oxidative stress, the thiol content decreases as it is used to neutralize ROSs. Our study showed that treatment of AE and dexamethasone significantly increased the thiol group content, suggesting that AE may contribute to the inhibition of oxidative stress.

It is well known that IFN-ɣ, a pro-inflammatory cytokine, plays an essential role in ARDS. This cytokine has been shown to increase capillary permeability and neutrophil migration (23,24). It has been suggested that the efficacy of ARDS treatments can be measured by decreasing serum IFN-ɣ levels (25). AE shows its anti-inflammatory effect by altering the secretion of TNF-α, IFN-ɣ, IL-10, and IL-12 (6). Studies have shown that AE increases IFN-ɣ secretion (26). On the other hand, antagonizing the action of IFN-ɣ in ARDS is thought to have a curative effect (24). Mock et al. (27) found that IFN-ɣ is increased in ARDS, suggesting that suppression of this increase plays a crucial role in controlling inflammation. In our study, we found that IFN-ɣ is increased in ARDS. Our study showed that the increase of IFN-ɣ in ARDS can be reduced with both AE+D and AE alone. On the other hand, it has been shown that administration of AE may have a prophylactic effect by keeping IFN-ɣ levels of lipopolysaccharide low. Consequently, AE may have both therapeutic and prophylactic effects in ARDS by reducing IFN-ɣ levels.

It is known that IL-10, an anti-inflammatory cytokine, plays an essential role in ARDS. The balance between inflammation and inflammatory response, which is disturbed in ARDS, increases lung injury. Increasing IL-10 inhibits the inflammatory response and restores this balance. The reason for the decrease in IL-10 level in ARDS is the increased IFN-ɣ (28). Effective ARDS treatments increase IL-10 levels (25). While there are studies stating that AE reduces IL-10 secretion (26), there are also publications stating that it significantly increases IL-10 secretion (6). Our study showed that the decreased IL-10 secretion in ARDS could be increased with both AE+D and AE alone. On the other hand, it has been shown that the administration of AE may have a prophylactic effect by keeping IL-10 levels of L high. Consequently, AE may show therapeutic and prophylactic effects in ARDS by increasing IL-10.

Local measurements of cytokine expression at the tissue location are thought to be the most accurate (29). This may be one reason why other cytokines did not show a statistically significant difference in our study, and another reason could be the shortness of the duration.

Studies have shown that AE reduces the inflammatory response levels of rats' gastric mucosa (30). Our study showed that increasing congestion, hemorrhage, and intraalveolar macrophages in alveoli in ARDS could be reduced by both AE+D and AE alone. AE reduced congestion and alveolar wall thickness, alleviated inflammation, and decreased intraalveolar macrophages that destroy alveolar septa, thus regressing histological damage. This indicates that the deteriorated alveolar structure may be corrected with AE, which has an anti-inflammatory effect. In orchestrating inflammation and ARDS recovery, alveolar macrophages are essential players. The damage in the lung is increased once alveolar macrophages are triggered because they attract neutrophils and circulating macrophages to the pulmonary sites of injury, in this way, the damage in the lung is exacerbated, and the decrease in the number of macrophages will allow this damage to be reduced (31). On the other hand,

administration of AE has been shown to have a prophylactic effect in ARDS by reducing congestion/hemorrhage in the alveoli and the alveolar wall thickness. These results suggest that AE may histologically show therapeutic and prophylactic effects in ARDS.

Our study's limitations are not using quercetin, the main active component of *Abelmoschus esculentus*, and not performing immunohistochemical analysis.

CONCLUSION

It is thought that AE may be a new potential treatment to eliminate inflammation in acute lung injury. AE may also show a prophylactic effect with the same effect.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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