

Research Article

Anti-Inflammatory Effects of *Clematis terniflora* Leaf on Lipopolysaccharide-Induced Acute Lung Injury

Ji-Yeong Park,¹ Min-Jong Kim,¹ Young-Ae Choi,¹ Yeon-Yong Kim,² Soyoung Lee,² Jae-Min Chung,³ Sang-Yong Kim ⁰,⁴ Gil-Saeng Jeong ⁰,⁵ and Sang-Hyun Kim ⁰

¹Cell and Matrix Research Institute, Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 41944, Republic of Korea

²Functional Biomaterial Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongeup 56212, Republic of Korea

³Department of Gardens and Education, Korea National Arboretum, Pocheon 11186, Republic of Korea

⁴DMZ Botanic Garden, Korea National Arboretum, Yanggu 24564, Republic of Korea

⁵College of Pharmacy, Chungnam National University, Daejeon 34134, Republic of Korea

Correspondence should be addressed to Sang-Yong Kim; rosaksy@korea.kr, Gil-Saeng Jeong; gsjeong@cnu.ac.kr, and Sang-Hyun Kim; shkim72@knu.ac.kr

Received 9 June 2023; Revised 3 October 2023; Accepted 22 December 2023; Published 9 January 2024

Academic Editor: Rômulo Dias Novaes

Copyright © 2024 Ji-Yeong Park et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

For centuries, natural products are regarded as vital medicines for human survival. *Clematis terniflora* var. *mandshurica* (Rupr.) Ohwi is an ingredient of the herbal medicine, Wei Ling Xian, which has been used in Chinese medicine to alleviate pain, fever, and inflammation. In particular, *C. terniflora* leaves have been used to cure various inflammatory diseases, including tonsillitis, cholelithiasis, and conjunctivitis. Based on these properties, this study aimed to scientifically investigate the anti-inflammatory effect of an ethanol extract of leaves of *C. terniflora* (EELCT) using activated macrophages that play central roles in inflammatory response. In this study, EELCT inhibited the essential inflammatory mediators, such as nitric oxide, cyclooxygenase-2, tumor necrosis factor- α , interleukin- (IL-) 6, IL-1 β , and inducible nitric oxide synthase, by suppressing the nuclear factor- κ B and mitogen-activated protein kinase activation in macrophages. Acute lung injury (ALI) is a fatal respiratory disease accompanied by serious inflammation. With high mortality rate, the disease has no effective treatments. Therefore, new therapeutic agents must be developed for ALI. We expected that EELCT can be a promising therapeutic agent for ALI by reducing inflammatory responses and evaluated its action in a lipopolysaccharide- (LPS-) induced ALI model. EELCT alleviated histological changes, immune cell infiltration, inflammatory mediator production, and protein-rich pulmonary edema during ALI. Collectively, our results may explain the traditional usage of *C. terniflora* in inflammatory diseases and suggest the promising potential of EELCT as therapeutic candidate for ALI.

1. Introduction

Plants are essential elements for treating various diseases in oriental medicine. *Clematis* is a herb genus containing approximately 300 species in the family Ranunculaceae [1]. Wei Ling Xian, which comes from the roots and rhizomes of several *Clematis* species, is prescribed as an analgesic, antipyretic, anticancer, and anti-inflammatory agent in the ancient books of East Asia, including Chinese Pharmacopeia and

Donguibogam (Korean traditional ancient book completed in 1613 by Jun Heo) [2, 3]. One particular plant, *Clematis terniflora* var. *mandshurica* (Rupr.) Ohwi, is mainly distributed in China, Japan, and Korea. It is known as "Earri" or "Wi Ryeong Seon" in Korea and also has been used to address pain and fever issues in oriental medicine [4, 5]. In particular, the leaf of *C. terniflora* is described as treatment for inflammationrelated diseases, such as tonsillitis, cholelithiasis, and conjunctivitis in Traditional Chinese Medicine Dictionary [6].

Inflammation is the first defense response of the innate immune system to protecting the body against infections and injuries. However, excessive inflammation causes enormous tissue damage and multiple disorders [7]. Macrophages play important roles in inflammatory processes by secreting diverse inflammatory mediators, including nitric oxide (NO), cyclooxygenase (COX)-2, tumor necrosis factor- (TNF-) α, interleukin- (IL-) 6, IL- 1β , and inducible nitric oxide synthase (iNOS) [8]. The expression of these mediators is associated with lifethreatening inflammatory disorders, e.g., sepsis-related multiple organ dysfunction/multiple organ failure, neurodegenerative disorders, microbial infections, and acute lung injury (ALI) [9]. Inflammatory mediators are regulated by intracellular signaling pathways, in particular, nuclear factor- (NF-) kB, which is a pleiotropic transcription factor active during innate immunity and inflammation responses [10, 11]. Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases, which phosphorylate substrates, to activate or deactivate target proteins, specifically in macrophages [12]. Consecutive phosphorylation triggers MAPK cascades, which have important roles in the pathogenesis and development of inflammation [13].

ALI is a pulmonary disease characterized by excessive inflammatory responses and dysfunction of the respiratory tract. ALI can be caused by both direct and indirect lung damage, including sepsis, aspiration pneumonia, inhalation injury, pancreatitis, and blood transfusions [14]. Whatever etiology causes, the ALI pathogenesis is attributed to out-of-control inflammatory response. As irreversible inflammation and pulmonary damage can lead to death, it is recognized as a serious public health problem [15]. ALI treatments can be divided into supportive care and pharmacological interventions (corticosteroids, antiinflammatory agents, and antioxidants); however, these therapies cannot effectively cure ALI and improve patients' quality of life [16, 17]. Therefore, it is urgent to develop new drugs for ALI. As Gram-negative bacterial infection is one of the most common causes of ALI, the intratracheal injection of lipopolysaccharide (LPS) into animals is typically considered as a well-established experimental model, which imitates human ALI [18, 19]. In this model, LPS induces several inflammatory symptoms, including tissue damage, protein-rich edema, immune cell infiltration, and increased inflammatory mediators in the lung [20].

The study of traditional medicinal plants is considered an attractive means for developing promising complementary and alternative medicine in current health problems. Accordingly, we examined the mechanism action of ethanol extract of leaves of *C. terniflora* (EELCT) in inflammatory responses using LPS-stimulated macrophages. Moreover, we expected that EELCT has a therapeutic possibility against inflammatory diseases and identified the anti-inflammatory activity in an LPS-induced ALI model.

2. Materials and Methods

2.1. Reagents. Reagents were obtained from the following suppliers: Dulbecco's modified Eagle's medium (DMEM) (12800-017) and fetal bovine serum (FBS) (16000-044) from Gibco, Grand Island, NY. Antibiotic-antimycotic solution (SV30079.01) from HyClone Laboratories, Logan, UT. LPS from *Escherichia coli* 055:B5 (L2637), dexamethasone (Dex) (D4902), sodium nitrite (S2252), sulfanilamide (S9251), and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (N9125) from Sigma-Aldrich, St. Louis, MO.

2.2. EELCT Preparation and Identification. Clematis terniflora var. mandshurica (Rupr.) Ohwi was obtained from the Korea National Arboretum in Pocheon, Republic of Korea (voucher specimen no. JMC15119). Material (30 kg) was ground and extracted in ethanol at 70°C for 5 h twice in water bath. Then, the extract was filtered, lyophilized, and then stored at 4°C. The dried extract yield was approximately 5.3% from starting crude materials. To identify individual components in EELCT, we used high-performance liquid chromatography (HPLC) as previously reported [21]. Analyses were performed using Agilent HP 1260 Infinity Series liquid chromatograph (Agilent Technologies, Santa Clara, CA) with a Capcell Pak C18 column $(5 \mu m \times 4.6 mm \times 250 mm;$ Shiseido, Japan) and Agilent 6120 single quadrupole. Chromatography was performed at room temperature at a flow rate of 1 mL/min, and a $10 \mu \text{L}$ sample was analyzed over 120 min. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in a ratio specified by the following binary gradient with linear interpolation: 0 min 20% B, 60 min 30% B, 70 min 60% B, 100 min 70% B, and 120 min 20% B. UV spectra were collected using a diode array detector system (DAD) (Agilent Technologies) every 0.4 s from 260 nm to 345 nm with a resolution of 2 nm. Mass spectrometry was carried out using Agilent 6120 single quadrupole (Agilent Technologies) and analyzed using Mass Hunter Qualitative Analysis Software Version B.06.00 (Agilent Technologies). The analyses were performed under the following conditions: capillary voltage, 3.2 kV; sample cone voltage, 27 V; source temperature, 100°C; gas flow, 600 L/h; and gas temperature, 40°C. The mass scan range was 50–1000 m/z. Raw data were processed based on retention time and characteristic behavior of MS, including the exact mass, guasi-molecular ions, and in-source fragmentation. These data were compared with those of known compounds from an in-house plant database and existing literature. These data are shown in Figure 1.

2.3. Cell Culture and Measurement of NO Production. RAW 264.7 cells were grown in DMEM supplemented with heat-inactivated 10% FBS and antibiotic-antimycotic solution in an atmosphere of 5% CO₂ at 37°C. In this study, Dex (3.925 μ g/mL; 10 μ M), a typical corticosteroid medicine for inflammation, was used as a positive control [22]. To



FIGURE 1: HPLC profiling of EELCT. (a) HPLC-DAD analysis was performed on Capcell Pak C18 column ($5 \mu m \times 4.6 \text{ mm} \times 250 \text{ mm}$). The main peaks were detected from the chromatogram. (b) The peaks were identified as kaempferol-3-O-glucoside-7-O-rhamnoside and kaempferol-3-rutinoside by mass spectrum.

measure NO production, RAW 264.7 cells (1×10^5 cells/well in a 96-well plate) were pretreated with EELCT (1, 10, or 100 µg/mL) or Dex (10 µM) for 1 h and stimulated with LPS (100 ng/mL). After 24 h, supernatants were collected and centrifuged at 400 g for 5 min at 4°C. NO concentration was measured using Griess reagent consisting of 1% sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride, and 2.5% phosphoric acid. In a 96-well plate, equal volumes of culture media and Griess reagent were incubated for 5 min at room temperature. The absorbance was measured at 540 nm using a spectrophotometer (Molecular Devices). A standard curve using serial dilutions of sodium nitrite was constructed, and the amount of NO was calculated.

RNA *Extraction and qPCR.* RAW 2.4.264.7 cells $(5 \times 10^5$ cells/well in a 12-well plate) were pretreated with EELCT (1, 10, or $100 \,\mu\text{g/mL}$) or Dex ($10 \,\mu\text{M}$) for 1 h and stimulated with LPS (100 ng/mL) for 6 h. Before qPCR, total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA). cDNA was then synthesized from $1 \mu g$ of total RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. qPCR was conducted using an Applied Biosystems StepOnePlus[™] Real-time PCR System (Life Technologies Co., Kallang Avenue, Singapore) according to the manufacturer's protocol. In brief, 1 μ L of cDNA, 1 μ L of each sense and antisense primer solution $(0.5 \,\mu\text{M})$, $10 \,\mu\text{L}$ of QGreenBlue Master Mix High ROX (QBHR-05 2X, Thermo Fisher Scientific), and $7 \,\mu\text{L}$ of nuclease-free water were mixed in reaction tubes. Relative quantification of target gene was calculated using the $2-^{\Delta\Delta Cq}$ method in StepOne-Plus PCR system software (Thermo Fisher Scientific). Primer sequences used in this study are shown in Supplementary Table S1.

2.5. *ELISA*. Inflammatory cytokine levels in BALF and culture media were measured by ELISA. RAW 264.7 cells $(5 \times 10^5 \text{ cells/well} \text{ in a } 12 \text{ well plate})$ were pretreated with EELCT (1, 10, and 100 µg/mL) and Dex (10 µM) for 1 h and stimulated with LPS (100 ng/mL) for 12 h or 24 h. ELISA was performed on a 96-well immune plate (Nunc, Rochester, NY) using specific kits according to the manufacturer's instructions. The following kits were used: TNF- α and IL-6 (BD Biosciences, San Diego, CA); IL-1 β (Invitrogen, Carlsbad, CA); and myeloperoxidase (MPO) (R&D Systems, Minneapolis, MN). The absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices).

2.6. Western Blot. The protein levels in lung tissues and macrophages were determined using Western blot. RAW 264.7 cells $(1.5 \times 10^6 \text{ cells/well in a 6-well plate})$ were pretreated with EELCT (1, 10, and $100 \,\mu\text{g/mL}$) and Dex ($10 \,\mu\text{M}$) for 1 h and stimulated with LPS (100 ng/mL) for 15 min or 12 h. To obtain total protein extract, cells and tissues were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Nadeoxycholate, 0.1 M DTT, and 1 mM Na₃VO₄) plus a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged at $15,000 \ q$ for 20 min at 4°C. Equal amounts of total protein quantities were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8.5% gel and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin in Tris-buffered saline containing 0.5% Tween 20, membranes were incubated overnight with target primary antibodies and then with appropriate anti-IgG horseradish peroxidase-conjugated secondary antibodies. All antibodies used for Western blot are listed in Supplementary Table S2.

2.7. LPS-Induced ALI Model. To investigate the antiinflammatory effect of EELCT *in vivo*, we used an LPSinduced ALI model characterized by serious inflammatory response [23]. BALB/c mice (male, 6 weeks old, and body weight: 19–21 g) were purchased from the Dae-Han Experimental Animal Center in Daejeon, Republic of Korea. Animals were housed at a temperature of $22^{\circ}C \pm 1^{\circ}C$, humidity of $55\% \pm 5\%$, light/dark cycle of 12 h, and air exchange rate of 15 min/h. Feed and water were supplied *ad libitum*. Animal experiments were conducted in accordance with Public Health Service Policy on the Humane Care and Use of Laboratory Animals Guidelines and approved by the Institutional Animal Care and Use Committee of Kyungpook National University (IRB #2022-0389). First, 30 mice were randomly divided into six groups: control; ALI; ALI and 0.1, 1, and 10 mg/kg EELCT; and 2 mg/kg Dex. The dosages were selected based on our preliminary experiments. Mice were intraperitoneally injected with a phosphate-buffered saline (PBS):ketamine: Rompun (7:2:1) mixture anesthetic and intratracheally injected with 50 μ L of LPS (5 mg/kg). An equal volume of PBS was injected into control group. EELCT and Dex were orally administrated 1 h before ALI induction. After 24 h, mice were humanely sacrificed by anesthetic overdose and lung tissue and bronchoalveolar lavage fluid (BALF) were collected for analysis.

2.8. Histopathological Evaluations. The left lung lobe was excised and fixed in 4% paraformaldehyde. After 72 h, tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) [24]. Pathological changes were observed under light microscopy (PerkinElmer, Waltham, MA) at ×200 magnification.

2.9. BALF Analysis. To obtain BALF, an 18-gauge catheter was inserted into the trachea and perfused twice with $800 \,\mu\text{L}$ of autoclaved PBS through the tracheal cannula as described previously [14]. Collected BALF was then centrifuged at 400 g for 10 min at 4°C, supernatant was transferred to fresh tubes, and pellets were resuspended in PBS. Total protein content in BALF was determined using a Bradford assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. In brief, 10 µL of BALF and $200\,\mu\text{L}$ of Bradford agent were added to a 96-well plate and incubated for 5 min at room temperature. The absorbance was measured at 595 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). To count differential cells, pellets were resuspended in PBS, fixed onto slides, and stained using a Diff-Quik staining kit (Sysmex Co., Kobe, Japan). Total cell numbers in BALF were determined using a hemacytometer.

2.10. Statistical Analysis. Statistical analyses were performed in GraphPad 7 software (GraphPad Software Inc., San Diego, CA) using one-way analysis of variance followed by Dunnett's multiple comparisons test. p values <0.05 were considered statistically significant. The results were expressed as mean \pm standard deviation (SD) for *in vitro* experiments and mean \pm standard error of the mean (SEM) for *in vivo* experiments.

3. Results

3.1. Effects of EELCT on Inflammatory Mediators in Macrophages. To investigate the mechanism action of traditional usage of *C. terniflora* on inflammation, *in vitro* studies were conducted using LPS-stimulated macrophages. First, to rule out the cytotoxicity of EELCT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assays were performed. Cell viability was not affected up to 1,000 μ g/mL; therefore, we used below

100 μ g/mL EELCT in our study (Supplementary Figure S1). The various mediators from activated macrophages are closely involved in development of inflammation. From our data, EELCT significantly inhibited LPS-induced NO production in macrophages (Figure 2(a)). In this regard, expression of iNOS was also investigated. Our data showed that EELCT suppressed the gene and protein expression of COX-2, iNOS, TNF- α , IL-6, and IL-1 β in a concentration-dependent manner (Figures 2(b)-2(d) and Figure 3).

3.2. Effects of EELCT on NF- κ B and MAPK Activation in Macrophages. To identify signaling mechanisms of the EELCT action, NF- κ B and MAPK activation was assessed by determining phosphorylation of subfamilies. We observed that LPS-induced phosphorylation of p65-NF- κ B, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 was reduced by EELCT (Figure 4). Collectively, EELCT appeared to inhibit the expression of major inflammatory mediators, such as NO, COX-2, TNF- α , IL-6, IL-1 β , and iNOS, through suppression of NF- κ B and MAPK activation in macrophages.

3.3. Effects of EELCT on Histology and Immune Cell Infiltration in ALI. To investigate the pharmacological effects of EELCT in the ALI model, mice were administrated EELCT or Dex and intratracheally injected with LPS (5 mg/ kg). After 24 h, lung tissue and BALF were collected. Lung tissue was stained with H&E for histological analysis. In the ALI group, lung destruction, including immune cell infiltration, alveolar collapse, hemorrhage in the stroma, and airspace edema were observed, but these were alleviated in the EELCT-administrated group (Figure 5(a)). The immune cell infiltration was investigated by counting cell numbers in BALF. In our data, increased total cell numbers during ALI were reduced by oral administration of EELCT (Figure 5(b)). In addition, the infiltration of macrophages and neutrophils, which are important effector cells in ALI pathogenesis, was determined using Diff-Quik staining. As a result, both macrophage and neutrophil numbers were increased by LPS and decreased by EELCT in a dose-dependent manner (Figures 5(c) and 5(d)).

3.4. Effects of EELCT on Inflammatory Mediators and Pulmonary Edema in ALI. To investigate the anti-inflammatory effect of EELCT during ALI, we evaluated TNF- α , IL-6, IL-1 β , and MPO levels in BALF. Oral administration of EELCT dose-dependently suppressed the LPS-induced TNF- α , IL-6, IL-1 β , and MPO production (Figures 6(a)–6(c) and Supplementary Figure S2). In addition, we confirmed the expression of important proteins in lung tissues. As a result, LPS increased the expression of NF- κ B, COX-2, and iNOS that reduced by the oral administration of EELCT. In addition, EELCT inhibited the phosphorylation of NF- κ B and MAPKs (ERK) induced by LPS (Figure 6(d)). Protein-rich pulmonary edema occurs from alveolar barrier dysfunction 5

and is a major symptom of ALI [25]. To determine the extent of protein accumulation, total protein concentration in BALF was measured. The mouse body and lung were also weighed using precision balance (Ohaus, Parsippany, NJ) to investigate the effects of EELCT in edema. As a result, total protein levels and lung/body weight ratios were decreased by EELCT in the LPS-induced ALI model (Figures 6(e) and 6(f)). The body weight and spleen weight changes are important resulting factors of ALI. Our data showed that the body weight was reduced by approximately 30% in LPStreated group compared with normal state, and it was alleviated by oral administration of EELCT (Figure 6(g)). EELCT also inhibited the increased spleen/body weight ratio during ALI (Figure 6(h)). These results indicated that oral administration of EELCT can attenuate the inflammatory symptoms and systemic damage during ALI.

4. Discussion

As mentioned above, C. terniflora leaf is a traditional herbal medicine to treat diverse inflammatory diseases. This study aimed to provide the scientific evidence for antiinflammatory effect of C. terniflora leaf using activated macrophages. Macrophages are closely involved in inflammatory response by releasing various mediators, such as NO, COX-2, and inflammatory cytokines. Excessive NO production induces the overexpression of inflammatory cytokines that can conversely promote the NO production, forming a positive feedback cycle that causes inflammation. In addition, COX-2 has been implicated in several inflammatory diseases, such as asthma, arthritis, and cancer. In in vitro study, EELCT markedly inhibited LPS-induced high levels of NO, COX-2, TNF- α , IL-6, IL-1 β , and iNOS in macrophages. LPS stimulates macrophages via toll-like receptor 4 leading to activation of NF- κ B, MAPKs, and other intracellular signaling pathways [26]. Upon macrophage stimulation, NF-kB is phosphorylated and translocated to the nucleus following degradation of $I\kappa B$. In the nucleus, NF- κ B binds (as a transcription factor) to promoter region in target genes [27-29]. MAPKs are well-characterized signaling pathways related to inflammatory reaction in activated macrophages [30]. Indeed, it is well known that expression of inflammatory mediators mentioned above is regulated by NF- κ B and MAPKs. We confirmed that EELCT inhibited p65-NF-κB, ERK, JNK, and p38 phosphorylation in LPS-stimulated macrophages. Taken together, these findings indicate that EELCT exerted anti-inflammatory effects by suppressing the expression of NO, COX-2, TNF- α , IL-6, IL-1 β , and iNOS through regulation of NF- κ B and MAPK activation in activated macrophages.

ALI and its more severe form (ARDS) are lifethreatening inflammatory diseases with high fatality rates [31]. However, there is no effective pharmacotherapy, and the ALI management remains in supportive care [32]. In recent years, considerable research has been conducted to develop new ALI treatment from natural products [16]. On the basis of the anti-inflammatory effect of EELCT, we investigated the therapeutic activity of EELCT against ALI. Multiple immune cells regulate the immune responses and



FIGURE 2: Effects of EELCT on NO production and COX-2 and iNOS expression in RAW 264.7 cells. (a) Cells were pretreated with/without drugs (EELCT or Dex) for 1 h and stimulated with LPS (100 ng/mL). After 24 h, NO production in culture media was determined using Griess reagent. (b, c) After 6 h, relative gene expression was verified by qPCR. (d) After 12 h, protein expression was determined by Western blot. Graph data represent mean \pm SD. * p < 0.05 compared with the LPS-stimulated group. β -Actin was the loading control.

the microenvironment in the lungs by releasing different inflammatory mediators [33, 34]. Notably, resident or recruited macrophages and neutrophils mainly contribute to pathogenesis of ALI. Alveolar macrophages have essential roles in initiating and maintaining the inflammatory responses. Additionally, activated neutrophils release several toxic mediators, which enhance tissue damage and increase vascular permeability and alveolar edema [35, 36]. These immune cells mediate inflammatory responses by releasing various inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β [37, 38]. These cytokines can promote other signaling cascade and directly lead to lung injury. TNF- α and IL-6 are major cytokines, which stimulate other cytokine and chemokine secretion and contribute to severity of ALI [39, 40]. Furthermore, IL-1 β is an important early mediator in ALI and inflammatory conditions [41]. MPO is one of the main toxic mediators from neutrophils and leads to tissue disruption during pulmonary inflammation [42]. As mentioned earlier, NF- κ B is an important signaling molecule that regulates various inflammatory factors, ultimately participating in lung damage [43]. Accordingly, inhibition of this system can be a critical strategy for therapeutic capabilities in inflammation during ALI. In this study, oral administration of EELCT reduced the macrophage and neutrophil numbers in BALF in addition to the expression of inflammatory mediators during ALI. Therefore, our results suggest that EELCT suppressed inflammatory responses during ALI.

Infiltrated immune cells induce alveolar barrier dysfunction and microvascular permeability increase, which causes protein-rich pulmonary edema [25]. Once pulmonary edema fluid accumulates, it causes increased breathing and impairs gas exchange leading to hypoxemia and ultimately acute respiratory failure [44]. Our data showed that the accumulated protein amounts in lung and mouse lung/ body weight ratios were increased in ALI group and decreased in EELCT-administrated group. Therefore, oral administration of EELCT reduced protein-rich pulmonary edema, suggesting that EELCT alleviated alveolar barrier dysfunction during ALI. In addition, ALI induces a reduction of body weight, which is known to be involved in ARDS clinical outcomes [45]. The spleen is the largest peripheral lymphoid organ and performs a wide range of immunological functions as the first line of defense against bacterial endotoxins [46]. In this regard, the increased spleen/body weight ratio is regarded as a resulting factor of ALI [47, 48]. In our results, the oral administration of



FIGURE 3: Effects of EELCT on inflammatory cytokine expression in RAW 264.7 cells. Cells were pretreated with/without drugs (EELCT or Dex) for 1 h and stimulated with LPS (100 ng/mL). (a–c) After 6 h, relative gene expression was quantified by qPCR. (d–f) After 12 h, inflammatory cytokine secretion was determined by ELISA. Graph data represent mean \pm SD. * p < 0.05 compared with the LPS-stimulated group.



FIGURE 4: Effects of EELCT on NF- κ B and MAPK activation in RAW 264.7 cells. Cells were pretreated with/without drugs (EELCT or Dex) for 1 h and stimulated with LPS (100 ng/mL). After 15 min, total proteins were extracted and p65-NF- κ B, ERK, JNK, and p38 phosphorylation was assayed by Western blot. β -Actin was the loading control.



FIGURE 5: Effects of EELCT on histopathology and immune cell infiltration in ALL. Mice were intratracheally injected with LPS (5 mg/kg) before oral administration of EELCT or Dex. After 24 h, lung tissues and BALF were collected. (a) Representative images of H&E-stained lung sections (×200 magnification). (b–d) Diff-Quik-stained immune cell numbers in BALF. Scale bar = 100μ m. Graph data represent mean ± SEM. * *p* < 0.05 compared with the ALI group.





FIGURE 6: Effects of EELCT on inflammatory mediator production and pulmonary edema in ALI. Mice were intratracheally injected with LPS (5 mg/kg) before oral administration of EELCT or Dex. After 24 h, lung tissue and BALF were collected. (a–c) TNF- α , IL-6, and IL-1 β levels in BALF were measured by ELISA. (d) NF- κ B, ERK, COX-2, and iNOS expressions in lung tissues were investigated using Western blot. (e) Total protein concentrations in BALF were determined using the Bradford assay kit. (f) Graph shows lung/body weight ratios. (g) Mice were weighed before EELCT or Dex administration and before sacrifice. The graph reveals the body weight change. (h) The graph represents spleen/body weight ratios. Graph data represent mean ± SEM. * p < 0.05 compared with the ALI group. β -Actin was the loading control.

EELCT attenuated the reduction of body weight and increased spleen/body weight ratio during ALI. Collectively, EELCT suppressed typical ALI symptoms, such as tissue damage, immune cell infiltration, production of inflammatory mediators, and pulmonary edema. Thus, this study suggested that EELCT has therapeutic effect against ALI.

A previous study reported that Clematis species contained many bioactive compounds, including triterpenoid saponins, flavonoids, coumarins, and alkaloids [2]. Our HPLC profile of EELCT implied that kaempferol-3-O- β -rutinoside and kaempferol-3-O-glucoside-7-O-rhamnoside were the major EELCT components (Figure 1). Kaempferol is a dietary flavonoid commonly existing in fruits, vegetables, and Chinese herbs, with distinct antiinflammatory properties [49]. In addition, kaempferol has been proposed to prevent and treat inflammatory diseases including ALI [50-53]. A study identified that kaempferol-3-O- β -rutinoside, the most abundant compound in EELCT, suppressed the expression of inflammation-related mediators, such as iNOS, COX-2, TNF- α , and IL-6, through regulation of NF-*k*B and MAPK pathways in LPS-stimulated RAW 264.7 cells [54]. Thus, the anti-inflammatory effects of EELCT confirmed in this study may be due to a combined effect of active compounds, including kaempferol, resulting in a final anti-inflammatory action.

5. Conclusion

In summary, EELCT inhibited several critical inflammatory mediators, such as NO, COX-2, TNF- α , IL-6, IL-1 β , and iNOS, by suppressing the NF- κ B and MAPK activation in LPS-stimulated macrophages. Additionally, EELCT suppressed inflammatory response, including tissue damage, immune cell infiltration, production of inflammatory mediators, and protein-rich edema in an LPS-induced ALI model. Therefore, we propose that EELCT may function as a therapeutic candidate for ALI. However, more comprehensive research is needed for the practical use of EELCT as a pharmacotherapy.

Data Availability

The data used and analyzed in this study are included within the article and the supplementary information files.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PJY performed all experiments and wrote the draft manuscript. KMJ, CYA, and KYY assisted in experiment and performed the statistical analysis. LS and CJM performed additional experiments. KSY, JGS, and KSH participated in study design and coordination and helped to draft the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2021R1A5A2021614, 2022M3A9G8018189, and 2020M3A9D3038894) as well as the Korea National Arboretum (grant no. KNA 1-2-34, 17-9).

Supplementary Materials

Supplementary material consists of the additional data and the detailed information of primer sequences and antibodies. Supplementary Figure S1: effects of EELCT on cell viability. Supplementary Figure S2: effect of EELCT on MPO production in LPS-induced ALI model. Supplementary Table S1: primer sequences used for qPCR. Supplementary Table S2: antibody information used in Western blot. (*Supplementary Materials*)

References

- R. Chawla, S. Kumar, and A. Sharma, "The genus *Clematis* (Ranunculaceae): chemical and pharmacological perspectives," *Journal of Ethnopharmacology*, vol. 143, no. 1, pp. 116–150, 2012.
- [2] D. C. Hao, X. J. Gu, P. G. Xiao, and Y. Peng, "Chemical and biological research of *Clematis* medicinal resources," *Chinese Science Bulletin*, vol. 58, no. 10, pp. 1120–1129, 2013.
- [3] L. Kyung-Lock, "The practical use of the *Clematis* root during the Koryo to the early days of the Chosŏn — A case of the East Asian botany," *Daedong Munhwa Yeongu*, no. 77, pp. 189– 222, 2012.
- [4] C. W. Lee, S. M. Park, Y. S. Kim et al., "Biomolecular evidence of anti-inflammatory effects by *Clematis mandshurica* Ruprecht root extract in rodent cells," *Journal of Ethnopharmacology*, vol. 155, no. 2, pp. 1141–1155, 2014.
- [5] E. K. Park, M. H. Ryu, Y. H. Kim et al., "Anti-inflammatory effects of an ethanolic extract from *Clematis mandshurica* Rupr," *Journal of Ethnopharmacology*, vol. 108, no. 1, pp. 142–147, 2006.
- [6] F. Zheng, Y. Jia, and L. Xie, "Han Ying Ying Han Zhong yi da ci dian = Traditional Chinese medicine dictionary in Chinese-Enghich and English-Chinese. Di 1 ban," *Tianjin: Tianjin da xue chu ban she: Xin hua shu dian Tianjin fa xing suo fa xing*, vol. 10, p. 1416, 1994.
- [7] T. S. Xiao, "Innate immunity and inflammation," *Cellular and Molecular Immunology*, vol. 14, no. 1, pp. 1–3, 2017.
- [8] T. Venkatesan, Y. W. Choi, J. Lee, and Y. K. Kim, "Pinus densiflora needle supercritical fluid extract suppresses the expression of pro-inflammatory mediators iNOS, IL-6 and IL-1β, and activation of inflammatory STAT1 and STAT3 signaling proteins in bacterial lipopolysaccharide-challenged murine macrophages," *Daru Journal of Pharmaceutical Sciences*, vol. 25, no. 1, p. 18, 2017.

- [9] I. M. Jou, C. F. Lin, K. J. Tsai, and S. J. Wei, "Macrophagemediated inflammatory disorders," *Mediators of Inflammation*, vol. 2013, Article ID 316482, 9 pages, 2013.
- [10] M. G. Dorrington and I. D. C. Fraser, "NF-κB signaling in macrophages: dynamics, crosstalk, and signal integration," *Frontiers in Immunology*, vol. 10, p. 705, 2019.
- [11] T. Liu, L. Zhang, D. Joo, and S. C. Sun, "NF-κB signaling in inflammation," *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, Article ID 17023, 2017.
- [12] G. L. Johnson and R. Lapadat, "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases," *Science*, vol. 298, no. 5600, pp. 1911-1912, 2002.
- [13] J. Lloberas, L. Valverde-Estrella, J. Tur, T. Vico, and A. Celada, "Mitogen-activated protein kinases and mitogen kinase phosphatase 1: a critical interplay in macrophage biology," *Frontiers in Molecular Biosciences*, vol. 3, p. 28, 2016.
- [14] Y. Y. Kim, S. Lee, M. J. Kim et al., "Tyrosol attenuates lipopolysaccharide-induced acute lung injury by inhibiting the inflammatory response and maintaining the alveolar capillary barrier," *Food and Chemical Toxicology*, vol. 109, no. 1, pp. 526–533, 2017.
- [15] V. Kumar, "Pulmonary innate immune response determines the outcome of inflammation during pneumonia and sepsisassociated acute lung injury," *Frontiers in Immunology*, vol. 11, p. 1722, 2020.
- [16] Y. Q. He, C. C. Zhou, L. Y. Yu et al., "Natural product derived phytochemicals in managing acute lung injury by multiple mechanisms," *Pharmacological Research*, vol. 163, Article ID 105224, 2021.
- [17] D. Mokra, P. Mikolka, P. Kosutova, and J. Mokry, "Corticosteroids in acute lung injury: the dilemma continues," *International Journal of Molecular Sciences*, vol. 20, no. 19, p. 4765, 2019.
- [18] R. Kapur and J. W. Semple, "Alleviation of gram-negative bacterial lung inflammation by targeting HECTD2," *Annals of Translational Medicine*, vol. 4, no. 24, p. 488, 2016.
- [19] H. Chen, C. Bai, and X. Wang, "The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine," *Expert Review of Respiratory Medicine*, vol. 4, no. 6, pp. 773–783, 2010.
- [20] N. Nguyen, S. Xu, T. Y. W. Lam, W. Liao, W. S. F. Wong, and R. Ge, "ISM1 suppresses LPS-induced acute lung injury and post-injury lung fibrosis in mice," *Molecular Medicine*, vol. 28, no. 1, p. 72, 2022.
- [21] M. J. Kim, Y. A. Choi, S. Lee et al., "Prunus serrulata var. spontanea inhibits mast cell activation and mast cell-mediated anaphylaxis," Journal of Ethnopharmacology, vol. 250, Article ID 112484, 2020.
- [22] L. H. Feng, X. D. Li, X. Y. Zhang, P. J. Cheng, and Z. Y. Feng, "Dexamethasone for the treatment of acute respiratory distress syndrome: a systematic review and meta-analysis," *Medicine (Baltimore)*, vol. 101, no. 39, Article ID e30195, 2022.
- [23] E. R. Johnson and M. A. Matthay, "Acute lung injury: epidemiology, pathogenesis, and treatment," *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, vol. 23, no. 4, pp. 243–252, 2010.
- [24] S. Wang, H. Zhou, L. Zheng et al., "Global trends in research of macrophages associated with acute lung injury over past 10 years: a bibliometric analysis," *Frontiers in Immunology*, vol. 12, Article ID 669539, 2021.
- [25] R. Herrero, G. Sanchez, and J. A. Lorente, "New insights into the mechanisms of pulmonary edema in acute lung injury," *Annals of Translational Medicine*, vol. 6, no. 2, p. 32, 2018.

- [26] J. B. Soares, P. Pimentel-Nunes, R. Roncon-Albuquerque, and A. Leite-Moreira, "The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases," *Hepatology International*, vol. 4, no. 4, pp. 659–672, 2010.
- [27] J. Wan, Y. Shan, Y. Fan et al., "NF-κB inhibition attenuates LPS-induced TLR4 activation in monocyte cells," *Molecular Medicine Reports*, vol. 14, no. 5, pp. 4505–4510, 2016.
- [28] D. Bhatt and S. Ghosh, "Regulation of the NF-κB-mediated transcription of inflammatory genes," *Frontiers in Immunology*, vol. 5, p. 71, 2014.
- [29] O. Ernst, S. J. Vayttaden, and I. D. C. Fraser, "Measurement of NF-κB activation in TLR-activated macrophages," *Methods in Molecular Biology*, vol. 1714, pp. 67–78, 2018.
- [30] F. Qian, J. Deng, G. Wang, R. D Ye, and J. W Christman, "Pivotal role of mitogen-activated protein kinase-activated protein kinase 2 in inflammatory pulmonary diseases," *Current Protein & Peptide Science*, vol. 17, no. 4, pp. 332–342, 2016.
- [31] M. Diamond, H. L. Peniston, D. Sanghavi, and S. Mahapatra, "Acute respiratory distress syndrome," in *StatPearls*, Treasure Island (FL), 2023.
- [32] D. Mokra, "Acute lung injury- from pathophysiology to treatment," *Physiological Research*, vol. 69, no. 3, pp. S353– S366, 2020.
- [33] D. Ling, X. Zhang, J. Wu, Q. Xu, Z. He, and J. Zhang, "Identification of immune infiltration and effective immune biomarkers in acute lung injury by bioinformatics analysis," *Cell Transplantation*, vol. 31, Article ID 096368972211244, 2022.
- [34] R. B. Goodman, J. Pugin, J. S. Lee, and M. A. Matthay, "Cytokine-mediated inflammation in acute lung injury," *Cytokine and Growth Factor Reviews*, vol. 14, no. 6, pp. 523–535, 2003.
- [35] J. Grommes and O. Soehnlein, "Contribution of neutrophils to acute lung injury," *Molecular Medicine*, vol. 17, no. 3-4, pp. 293–307, 2011.
- [36] X. Huang, H. Xiu, S. Zhang, and G. Zhang, "The role of macrophages in the pathogenesis of ALI/ARDS," *Mediators of Inflammation*, vol. 2018, Article ID 1264913, 2018.
- [37] S. Han and R. K. Mallampalli, "The acute respiratory distress syndrome: from mechanism to translation," *The Journal of Immunology*, vol. 194, no. 3, pp. 855–860, 2015.
- [38] G. Matute-Bello, C. W. Frevert, and T. R. Martin, "Animal models of acute lung injury," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 295, no. 3, pp. L379–L399, 2008.
- [39] S. Mukhopadhyay, J. R. Hoidal, and T. K. Mukherjee, "Role of TNF-α in pulmonary pathophysiology," *Respiratory Research*, vol. 7, no. 1, p. 125, 2006.
- [40] K. H. Park, E. Y. Chung, Y. N. Choi, H. Y. Jang, J. S. Kim, and G. B. Kim, "Oral administration of *Ulmus davidiana* extract suppresses interleukin-1β expression in LPS-induced immune responses and lung injury," *Genes and Genomics*, vol. 42, no. 1, pp. 87–95, 2020.
- [41] B. Lin, Z. Jin, X. Chen et al., "Necrostatin1 protects mice from acute lung injury by suppressing necroptosis and reactive oxygen species," *Molecular Medicine Reports*, vol. 21, no. 5, pp. 2171–2181, 2020.
- [42] J. Blazquez-Prieto, I. Lopez-Alonso, C. Huidobro, and G. M. Albaiceta, "The emerging role of neutrophils in repair after acute lung injury," *American Journal of Respiratory Cell* and Molecular Biology, vol. 59, no. 3, pp. 289–294, 2018.
- [43] T. Y. Liu, L. L. Zhao, S. B. Chen et al., "Polygonatum sibiricum polysaccharides prevent LPS-induced acute lung injury by

inhibiting inflammation via the TLR4/Myd88/NF- κ B pathway," *Experimental and Therapeutic Medicine*, vol. 20, no. 4, pp. 3733–3739, 2020.

- [44] L. A. Huppert, M. A. Matthay, and L. B. Ware, "Pathogenesis of acute respiratory distress syndrome," *Seminars in Respiratory and Critical Care Medicine*, vol. 40, no. 01, pp. 031–039, 2019.
- [45] Y. Zhang, X. Li, J. J. Grailer et al., "Melatonin alleviates acute lung injury through inhibiting the NLRP3 inflammasome," *Journal of Pineal Research*, vol. 60, no. 4, pp. 405–414, 2016.
- [46] S. M. Lewis, A. Williams, and S. C. Eisenbarth, "Structure and function of the immune system in the spleen," *Science Immunology*, vol. 4, no. 33, Article ID eaau6085, 2019.
- [47] T. Zuo, Y. Yue, X. Wang, H. Li, and S. Yan, "Luteolin relieved DSS-induced colitis in mice *via* HMGB1-TLR-NF-κB signaling pathway," *Inflammation*, vol. 44, no. 2, pp. 570–579, 2021.
- [48] A. Goraca, H. Huk-Kolega, P. Kleniewska, A. Piechota-Polanczyk, and B. Skibska, "Effects of lipoic acid on spleen oxidative stress after LPS administration," *Pharmacological Reports*, vol. 65, no. 1, pp. 179–186, 2013.
- [49] J. Ren, Y. F. Lu, Y. H. Qian, B. Z. Chen, T. Wu, and G. Ji, "Recent progress regarding kaempferol for the treatment of various diseases," *Experimental and Therapeutic Medicine*, vol. 18, no. 4, pp. 2759–2776, 2019.
- [50] W. Alam, H. Khan, M. A. Shah, O. Cauli, and L. Saso, "Kaempferol as a dietary anti-inflammatory agent: current therapeutic standing," *Molecules*, vol. 25, no. 18, p. 4073, 2020.
- [51] O. Kadioglu, J. Nass, M. E. Saeed, B. Schuler, and T. Efferth, "Kaempferol is an anti-inflammatory compound with activity towards NF-κB pathway proteins," *Anticancer Research*, vol. 35, no. 5, pp. 2645–2650, 2015.
- [52] R. Zhang, X. Ai, Y. Duan et al., "Kaempferol ameliorates H9N2 swine influenza virus-induced acute lung injury by inactivation of TLR4/MyD88-mediated NF-κB and MAPK signaling pathways," *Biomedicine & Pharmacotherapy*, vol. 89, pp. 660–672, 2017.
- [53] X. Chen, X. Yang, T. Liu et al., "Kaempferol regulates MAPKs and NF-κB signaling pathways to attenuate LPS-induced acute lung injury in mice," *International Immunopharmacology*, vol. 14, no. 2, pp. 209–216, 2012.
- [54] D. Hwang, M. J. Kang, C. W. Kang, and G. D. Kim, "Kaempferol-3-O-β-rutinoside suppresses the inflammatory responses in lipopolysaccharide-stimulated RAW264.7 cells via the NF-κB and MAPK pathways," *International Journal of Molecular Medicine*, vol. 44, no. 6, pp. 2321–2328, 2019.