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Botanical formulation, TADIOS, alleviates lipopolysaccharide (LPS)-Induced acute lung injury in mice via modulation of the Nrf2-HO-1 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: TADIOS is an herbal formulation prepared from a mixture of Taraxacum officinale (L.) Weber ex F.H.Wigg, Dioscorea batatas Decaisne and Schizonepeta tenuifolia (Benth.) Briquet. These plants have traditionally been used in Asia to treat a variety of respiratory diseases. A bulk of literature on traditional Korean medicine describe their activities and functions for respiratory problems. Therefore, we hypothesized that the combination of these plants might be effective in alleviating respiratory symptoms.

Aim of the study: In this study, we investigated whether TADIOS ameliorates LPS-induced acute lung injury via regulation of the Nrf2-HO-1 signaling pathway.

Materials and methods: The LPS-induced acute lung injury mouse model was used to determine the anti-inflammatory and anti-oxidative stress effects of TADIOS. The amount of marker compounds contained in TADIOS was quantified using high-performance liquid chromatography (HPLC) analysis. The protein level of pro-inflammatory cytokines in culture supernatant was measured by ELISA. Changes in the RNA level of pro-inflammatory cytokines in mice lungs and RAW264.7 cells were measured by quantitative RT-PCR. The relative amounts of reactive oxygen species (ROS) were measured by DCF-DA assay. Western blot analysis was used to evaluate expression of cellular proteins. Effects of TADIOS on antioxidant responsive elements (AREs) were determined by luciferase assay. The severity of acute lung injury was evaluated by Hematoxylin & Eosin (H&E) staining. To test the effects of TADIOS on LPS-induced oxidative stress, myeloperoxidase (MPO) activity and the total antioxidant capacity were measured.

Results: TADIOS was prepared by extraction of a blend of these three plants by ethanol, and quality control was performed through quantification of marker compounds by HPLC and measurement of bioactivities using cell-based bioassays. In the murine macrophage cell line RAW264.7, TADIOS effectively suppressed the production of pro-inflammatory cytokines such as IL-6 and IL-1β, and also ROS induced by LPS. When RAW264.7 cells were transfected with a luciferase reporter plasmid containing nucleotide sequences for AREs, TADIOS treatment increased the level of relative luciferase units in a dose-dependent manner. In the LPS-induced acute lung injury mouse model, orally administered TADIOS alleviated lung damage and neutrophil infiltration induced by LPS. Consistent with the in vitro data, treatment with TADIOS inhibited the LPS-mediated expression of pro-inflammatory cytokines and oxidative stress, and activated the Nrf2-HO-1 axis.

Conclusion: Our data suggest the potential for TADIOS to be developed as a safe and effective therapeutics for the treatment of acute respiratory distress syndrome.

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1. Introduction

Acute respiratory distress syndrome (ARDS) is the most common cause of severe respiratory failure triggered by damage to the alveoli and capillary barrier (Gonzales et al., 2015). ARDS occurs in the United States at a rate of 0.065−0.08%, with 75% of cases categorized as moderate or severe (Diamond et al., 2020). The case fatality rate from mild to severe ARDS stands at approximately 27−45%, which is significantly higher than for other acute/chronic diseases such as myocardial infarction, pneumonia, and asthma (Diamond et al., 2020). The causative infectious agent SARS-CoV-2 (Fan et al., 2020; Gibson et al., 2020; Li and Ma, 2020) at the heart of the COVID-19 pandemic has escalated the threat of ARDS from a major healthcare concern to a global crisis. Excessive inflammation of the airway induced by inhalation of virulent substances into the bronchi is thought to be important in the pathogenesis of ARDS (Han and Mallampalli, 2015). Only symptomatic relief therapies are currently available, such as inhaled nitric oxide (iNO) or therapies for ARDS since they contain multiple compounds with health benefits. Many such therapeutic extracts have been extensively documented for their safety and efficacy, and contain a variety of chemical compounds that may regulate the cellular or biochemical entities involved in the pathogenesis of ARDS (Chen et al., 2018; Dai et al., 2014; Han et al., 2013; Huang et al., 2018).

2. Materials and methods

2.1. Preparation of TADIOS extracts from plants

 Dioscorea batatas Decaisne, Taraxacum officinale (L.) Weber ex F.H.Wigg and Schizonepeta tenuifolia (Benth.) Briquet. In Korea, they are all classified as dietary ingredients. Dioscorea batatas Decaisne is known to have the lung-nourishing and cough relieving properties (Kang B, 2000; Ma et al., 2018; Min-Hye Yang, 2009). It has been shown to contain anti-oxidant and anti-inflammatory activities, and inhibit PM$_{2.5}$-induced lung injury, whose pathological phenotypes are similar to ARDS (Lee, W. et al., 2019a). Taraxacum officinale (L.) Weber ex F.H.Wigg has traditionally been used to treat respiratory problems, including sore throat and lung abscess (Hee-Jung Ha, 2011; Kang B, 2000). A water-soluble extract from Taraxacum officinale (L.) Weber ex F.H.Wigg has been reported to inhibit LPS-induced ALI via regulation of oxidative stress- and inflammation-related responses (Ji et al., 2010). Schizonepeta tenuifolia (Benth.) Briquet is widely prescribed in Korea for a variety of respiratory infections such as the common cold, fever, and sore throat (Fujita and Fujita, 1973; Fung and Lau, 2002; Kang B, 2000). It was reported that Schizonepeta tenuifolia (Benth.) Briquet suppresses pulmonary inflammation by regulating the TLR4 signaling pathway (Byun, 2014). Since the aforementioned plants are typically taken in an ethanol extracted form, this study combined the three plants followed by ethanol extraction, to generate TADIOS (also called HX1108) (Lee et al., 2020). The LPS-induced ALI mouse model was then used to evaluate the therapeutic and preventative effects of TADIOS. TADIOS was much more effective in suppressing the inflammatory responses induced by LPS than the individual plant extracts. When the murine macrophage cell line RAW264.7 was employed to understand the underlying mechanisms for these responses, TADIOS was found to activate the axis of the Nrf2-HO-1 signaling pathway. Data obtained from TADIOS-treated mouse experiments were highly comparable to above in vitro results. These results indicated that TADIOS might be developed as an effective and safe therapeutics to alleviate clinical condition associated with ARDS.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| LPS          | lipopolysaccharide |
| Nrf2         | nuclear factor erythroid 2-related factor 2 |
| HO-1         | heme oxygenase-1 |
| HPLC         | high-performance liquid chromatography |
| ELISA        | enzyme-linked immunosorbent assay |
| DCF-DA       | dichlorodihydrofluorescein diacetate |
| TNF-α        | tumor necrosis factor alpha |
| IL-6         | interleukin-6 |
| IL-1β        | interleukin-1β |
| WST-1        | water-soluble tetrazolium salt-1 |
| ROS          | reactive oxygen species |
| AREs         | antioxidant responsive elements |
| ARDS         | acute respiratory distress syndrome |
| iNO          | inhaled nitric oxide |
| ALI          | acute lung injury |
| NC           | normal control |
| H&E          | Hematoxylin & Eosin |
| BALF         | bronchoalveolar lavage fluid |
| TAC          | total antioxidant capacity |
| PVDF         | polyvinylidene difluoride |
| MPO          | myeloperoxidase |
| MAPK         | mitogen-activated protein kinase |
| NF-kB        | nuclear factor kappa-light-chain-enhancer of activated B cells |
of 1:1:1, then the combination of plants was extracted with 25% ethanol. After filtering through 10-μm cartridge paper, the extracts were concentrated through vacuum evaporation system (Eyela, Tokyo, Japan). The voucher specimens used in this study were stored at the herbarium of Helixmith Co., Ltd. (Seoul, Korea). Voucher Specimen No: S14161213 for Dioscorea batatas Decaisne; F02170525 for Taraxacum officinale (L.) Weber ex F.H.Wigg; H2719121 for Schizonepeta tenuifolia (Benth.) Briquet.

2.2. High-performance liquid chromatography (HPLC) analysis

Batch-to-batch consistency was monitored by quantifying marker compounds via HPLC analysis. Representative marker compounds for each plant (allantoin (Sigma-Aldrich, St. Louis, MO, USA) for Dioscorea batatas Decaisne, chicoric acid (Sigma-Aldrich, St. Louis, MO, USA) for Taraxacum officinale (L.) Weber ex F.H.Wigg and luteolin-3′-glucuronide (Chengdu biopurify, Chengdu, Sichuan, China) for Schizonepeta tenuifolia (Benth.) Briquet) were selected based on previously published information (Cai et al., 2020; Kajla et al., 2017; Wang et al., 2017). Standard solutions of representative markers were prepared by dissolving their reference compounds in 50% methanol and filtered with a 0.45 μm PVDF filter before use. Sample solutions of the extracts were also prepared in the same manner. HPLC analysis was performed using Waters Alliance 2695 HPLC system coupled with UV detector (Waters, Millford, MA, USA). Details on the conditions of HPLC analysis can be found in Table S1.

2.3. Experimental animals

All experimental procedures were conducted in compliance with the guidelines set by the Institutional Animal Care and Use Committee of Helixmith Co., Ltd. Male C57BL/6 mice at seven weeks of age were purchased from Raonbio Inc. (Yongin, Korea). All mice were housed for 1 week to adapt to their surroundings before surgery in an air-conditioned facility under a fixed 12-h light/dark cycle. All animal experiments were carried out in accordance with the Guide for Animal Experimentation of Helixmith Co., Ltd. The protocol was approved by the Institutional Animal Care and Use Committee of Helixmith Co., Ltd (Approval Number: VIC-20-11001).

2.4. LPS-induced acute Lung injury mice model

Male C57BL/6 (8-week-old) mice were divided into 7 groups as follows (n = 8 per group): i) normal control (NC) group; ii) LPS group; iii) Dioscorea batatas Decaisne (500 mg/kg) group; iv) Taraxacum officinale (L.) Weber ex F.H.Wigg (500 mg/kg) group; v) Schizonepeta tenuifolia (Benth.) Briquet (500 mg/kg) group; vi) TADIOS (500 mg/kg) group; and vii) TADIOS (1000 mg/kg) group. Extracts of individual plants were dissolved with distilled water and administered orally 1 h before LPS treatment to each group.

2.5. H&E staining

Mouse lungs were fixed in 10% normalized buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) and embedded in a paraffin block. Then, 6-μm paraffin sections of the lungs were stained with H&E to visualize tissue areas.

2.6. Measurement of total cells and neutrophils in bronchoalveolar lavage fluid (BALF)

Lungs were lavaged with 0.7 mL PBS through the cannulated trachea. The BALF obtained was centrifuged at 1500 rpm for 5 min at 4℃ to separate the supernatant from the cells. Cell pellets were counted in a hemocytometer and H&E-stained to identify the differential profiles after cytosin preparation. Total cells and neutrophils were counted via light microscope (Olympus, Tokyo, Japan) according to morphological criteria and staining.

2.7. Measurement of oxidative-stress in Lung tissue

The antioxidant enzymatic activities were measured using Total antioxidant capacity (TAC) assay kit (DoGen, Seoul, Korea) and myeloperoxidase (MPO) assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers’ protocol. Quantification of total proteins was performed using a Bradford protein assay reagent (Invitrogen, Carlsbad, CA, USA).

2.8. Western blot analysis

The lung tissue was homogenized with T-per tissue extraction reagent (ThermoFisher, Waltham, MA, USA) and mixed with PhosSTOP™ and eComplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) for 20 min on ice. The cell extracts were prepared using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitors. The tissue and cell homogenates were then centrifuged at 12,000 g for 15 min and the supernatant was collected. The total protein in the lung tissue was quantified by the Bradford protein assay reagent (Invitrogen, Carlsbad, CA, USA). After reconstituting in sample buffer, protein samples were subjected to SDS-PAGE on Bolt™ 10% Bis-Tris Plus Gels (Invitrogen, Carlsbad, CA, USA). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA) using Power Blotter Station system (Invitrogen, Carlsbad, CA, USA) and the membrane was incubated in 5% skim milk in 0.1% TBST at room temperature for 1 h to block nonspecific binding. The membrane was then incubated with antibodies specific to HO-1 (#70081), Nrf2 (#12721; Cell signaling, Beverly, MA, USA) and β-actin (A5441, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4℃ followed by incubation with horse-radish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG (1:100,000; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h. The blot was developed by Immobilon ECL HRP substrate (Millipore, MA, USA) and visualized by ChemiDoc MP imaging system (BIO-RAD, CA, USA).

2.9. Cell culture and reagents

The RAW264.7 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. LPS and OB-24 were purchased from Sigma-Aldrich (St. Louis, MO, USA). siNrf2 was purchased from Invitrogen (Carlsbad, CA, USA).

2.10. Enzyme-linked immunosorbent assay (ELISA)

IL-6 and IL-1β in cell culture supernatants were measured using commercially available ELISA kits (R&D systems, Minneapolis, MN).

2.11. RNA isolation and qRT-PCR analysis

Both mouse lung and cell line RNA were obtained using TriZol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using oligo dT primers (QIAGEN, Valencia, USA) and Reverse Transcriptase XL (avian myeloblastosis virus (AMV)) (Takara, Shiga, Japan) from 1 μg of RNA. Real-time quantitative RT-PCR was performed with SYBR Premix (Takara, Shiga, Japan) and Thermal Cycler Dice Real Time System TP800 (Takara, Shiga, Japan). PCR conditions were denaturation at 95℃ for 5 s, followed by annealing and extension at 60℃ for 30 s. The
sequences of synthesized PCR primer (Bioneer Co. Ltd., Seoul, Korea) are presented in Table 1.

2.12. Nitric oxide (NO) and reactive oxygen species (ROS) measurement

A Griess Reagent Kit (Invitrogen, Carlsbad, CA, USA) was used to measure NO. Briefly, the level of nitrite was measured by adding 100 μL of Griess reagent to 50 μL of culture supernatant. The optical density at 550 nm was measured with a microplate reader and then determined from a sodium nitrite standard curve. ROS production was quantified using a DCF-DA ROS detection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

2.13. WST-1 assay

A WST-1 assay kit (DoGen, Seoul, Korea) was used to measure cell viability. Briefly, RAW264.7 cells were cotreated with LPS and different concentrations of TADIOS for 24 h, followed by the addition of WST-1 reagent. After 1 h, the cell viability was determined by measuring the OD at 450 nm using a microplate reader (ThermoFisher, Waltham, MA, USA).

2.14. Luciferase reporter plasmid assay

An inducible AREs-responsive luciferase reporter assay kit was purchased from QIAGEN (Valencia, USA), and the assay was performed as described before (Lee, W. et al., 2019b; Lee et al., 2018a; Lee et al., 2018b). RAW264.7 cells were briefly transfected with either ARE-reporter plasmid or negative control plasmid using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, the cells were treated first with various concentrations of TADIOS for 30 min, then with LPS (100 ng/mL) for 18 h. Cell lysates were prepared and a luciferase activity assay was performed using the dual luciferase reporter assay system (Promega, Madison, WI, USA) and Varioskan LUX (ThermoFisher, Waltham, MA, USA) according to the manufacturer’s protocol.

2.15. siRNA transfection

The siRNA specific to Nrf2 and scrambled siRNA (ThermoFisher, Waltham, MA, USA) were transfected into RAW264.7 cells using RNAiMAX (ThermoFisher, Waltham, MA, USA). Twenty-four hours after the siRNA-induced knockdown of Nrf2, cells were treated with LPS and TADIOS and then subjected to further experiments. Knockdown efficiency was tested using an antibody against Nrf2 (1:1000, Cell signaling, Beverly, MA, USA).

2.16. Statistical analysis

All values are represented as mean ± S.E.M. of three independent experiments. Statistical significance was determined using unpaired Student’s t-test or one-way ANOVA with Tukey correction, provided by GraphPad Prism software version 7 (GraphPad, USA). The F statistic values of the ANOVA tests were presented in Table S2. Data were considered statistically significant if the p-value was < 0.05.

3. Results

3.1. The combined use of T. officinale, S. tenuifolia and D. batatas provides synergistic effects on LPS-Induced acute lung injury

TADIOS, the botanical extract composed of 3 plants, was tested for its effects on LPS-induced inflammatory responses in this model. First, TADIOS was compared to the effects of individual plant extracts from Dioscorea batatas Decaisne, Taraxacum officinale (L.) Weber ex F.H.Wigg and Schizonepeta tenuifolia (Benth.) Briquet. As Fig. 1 shows, the expression of pro-inflammatory cytokines increased dramatically in the LPS-treated group, while extracts from individual plants did not have significant effects. However, TADIOS treatment lowered the RNA level of TNF-α, IL-6 and IL-1β in a statistically significant manner.

3.2. Quality of TADIOS is measured by HPLC and cell-based bioassay

The content of each marker compound contained in TADIOS was quantified using HPLC analysis in order to verify the batch-to-batch consistency (Fig. 2A and B). The measured values from the three batches used in this study are shown in Fig. 2C. The quantity of respective marker compounds has been highly consistent from batch to batch. In addition, the quality of TADIOS was also biologically controlled using cell-based bioassays. The effect of TADIOS on nitric oxide production was measured to determine the half maximal inhibitory concentration (IC50) value (Fig. 2D). Only TADIOS batches showing IC50 values between 0.3 and 0.6 mg/mL were used in the experiments.

3.3. TADIOS inhibits LPS-Induced expression of pro-inflammatory mediators in RAW264.7 cells

We investigated the anti-inflammatory effects of TADIOS in RAW264.7 cells. As shown in Fig. 3A, the nitric oxide production induced by LPS was significantly reduced by TADIOS treatment in a dose-dependent manner.

We then tested the capacity of TADIOS to inhibit the LPS-induced production of pro-inflammatory cytokines. Cells treated exclusively with LPS showed highly increased production levels of IL-6 and IL-1β, but TADIOS cotreatment inhibited such LPS effects in a dose-dependent manner (Fig. 3B and C). Interestingly, the LPS-induced production of TNF-α was not affected, likely due to the specific characteristics of this particular murine macrophage cell line as previously reported by Choi et al. (2012).

To test the effect of TADIOS on the RNA level of these pro-inflammatory cytokines, quantitative RT-PCR was performed. Consistent with protein results, TADIOS treatment significantly reduced the RNA levels of IL-6 and IL-1β, but not of TNF-α (Fig. 3D and E). To ascertain that this TADIOS-mediated inhibition of pro-inflammatory gene expression was not related with the cytotoxic effects of the agents used in the study, the effects of LPS and TADIOS on cell viability were determined using WST-1 assay. Under our experimental conditions, no significant cytotoxic effects were observed (Fig. 3F).

3.4. TADIOS exerts anti-oxidative stress effects through up-regulation of HO-1

We investigated the effects of TADIOS on the ROS production. As shown in Fig. 4A, the LPS-induced production of ROS was inhibited by TADIOS in a dose-dependent manner.

To test the effects of TADIOS on the expression of HO-1, Western blot and quantitative RT-PCR analyses were performed. In these experiments, TADIOS treatment increased the HO-1 protein levels in a dose-
dependent manner (Fig. 4B). RNA levels of HO-1 were consistently enhanced by TADIOS in a dose-dependent manner (Fig. 4C).

To test whether TADIOS can suppress the ROS production through the regulation of HO-1, HO-1 inhibitor OB-24 was used. OB-24 treatment effectively blocked TADIOS-mediated inhibition of ROS production (Fig. 4D).

3.5. Nrf2 plays a key role in TADIOS-Induced HO-1 gene expression

Since Nrf2 is well-known to regulate the expression of HO-1, we tested whether TADIOS could control the Nrf2 signaling pathway using a luciferase reporter plasmid. As shown in Fig. 5A, TADIOS treatment increased the relative luciferase units in a dose-dependent manner.

To further study the role of Nrf2 in the TADIOS-mediated induction of HO-1, changes in the protein levels of Nrf2 were analyzed. Levels of the Nrf2 protein were very low under normal and LPS-treated conditions, but increased with TADIOS treatment in a dose-dependent manner (Fig. 5B).

To validate the causative relationship, Nrf2-specific siRNA was used. The protein levels of Nrf2 were very low when cells were transfected with siRNA specific to Nrf2 (Fig. 5C). TADIOS treatment greatly raised the expression levels of HO-1 in cells transfected with control siRNA, whereas this effect was much lower in cells transfected with Nrf2 siRNA (Fig. 5D).

3.6. TADIOS ameliorates LPS-Induced acute Lung injury

Based on the above in vitro data, we investigated the possibility that TADIOS might regulate pulmonary inflammation in LPS-induced ALI mouse model. In mice treated with LPS alone, significant changes in histopathology were observed due to this endotoxin-mediated inflammation in the lungs, including a manifest inflammatory cell infiltration and thickening of the alveolar wall, compared with those of control mice (Fig. 6A and B). However, TADIOS treatment effectively ameliorated the LPS-induced pathological changes in a dose-dependent manner (Fig. 6C and D).

Next, the effects of TADIOS were tested on inflammatory cell infiltration. As shown in Fig. 6E, LPS treatment significantly increased the number of total cells and neutrophils in the BALF, but it was decreased in a dose-dependent manner when mice were administered with TADIOS (Fig. 6F and G).

3.7. TADIOS reduces LPS-Induced gene expression of pro-inflammatory cytokines in mice Lung

To unravel the molecular mechanisms of TADIOS, the effects on a variety of inflammation-related genes in control group animals were compared to TADIOS-treated mice. Twenty-four hours after exposure to vehicle or LPS, the RNA level of pro-inflammatory cytokines was highly increased by LPS treatment, while this effect was markedly inhibited when mice were administered with TADIOS (Fig. 7A).

To test the effects of TADIOS on LPS-induced oxidative stress, MPO activity and the total antioxidant capacity in mice lungs were measured 24 h after the LPS challenge. When mice were treated with LPS alone, the levels of MPO activity and of antioxidants were changed as expected, but returned to normal levels upon treatment with TADIOS (Fig. 7B).

Based on in vitro data, effect of TADIOS on the Nrf2-HO-1 signaling pathway was tested in this mouse model. As presented in Fig. 7C, LPS-mediated reduction of the HO-1 and Nrf2 proteins was suppressed by TADIOS treatment in a dose-dependent manner.

4. Discussion

In this study, we developed the plant formulation, TADIOS, an ethanol extract from a blend of three herbs (Taraxacum officinale (L.) Weber ex F.H.Wigg, Dioscorea batatas Decaisne and Schizonepeta tenuifolia (Benth.) Briquet). We demonstrated that TADIOS was highly effective in regulating inflammation and oxidative stress both in vivo and in vitro experiments, and alleviating the pathologic features of acute lung injury caused by the intratracheal injection of LPS. It was shown that the combined use of the three plants more effectively inhibited the expression of proinflammatory cytokines, TNF-α, IL-6 and IL-1β than single-source extracts from individual plants. In RAW264.7 cells, TADIOS was shown to suppress the LPS-induced ROS production through activation of Nrf2-HO-1 signaling pathway, all in a dose-dependent manner. In the LPS-induced lung injury mouse model, orally administered TADIOS mitigated lung injury and neutrophil infiltration. Similar results were obtained in lung tissues to those in RAW264.7 cells, including reduced proinflammatory cytokine production and MPO activity, increased total antioxidant capacity, and upregulated protein levels of HO-1 and Nrf2. The high level of consistency between in vitro and in vivo data suggest that TADIOS may indeed be capable of acting as an effective therapeutics for ARDS.

The indications that TADIOS regulates the Nrf2-HO-1 signaling axis, a key pathway against oxidative stress, has significant implications for ARDS. The common denominators of almost all ARDS cases are uncontrolled oxidative stress and inflammation that lead to infiltration of inflammatory cells into the airways, overproduction of pro-inflammatory cytokines, and in extreme cases, a cytokine storm, all of which eventually cause substantial damage to the lungs (Cheng and Matthay, 2003; Diamond et al., 2020; Gibson et al., 2020; Huppert et al., 2019; Su et al., 2012). Our data clearly show that TADIOS increased the HO-1 expression by regulating Nrf2, and effectively dealt with excess production of inflammatory mediators and ROS.

MAPK/NF-κB signaling pathways, important in the progress of inflammation, may also be regulated by TADIOS. Dioscorea batatas
Fig. 2. Description of the quality control of TADIOS.
Decaisne has been shown to inhibit NF-κB and ERK1/2 in RAW264.7 cells (Jin et al., 2010), while Taraxacum officinale (L.) Weber ex F.H. Wigg has been reported to reduce oxidative damage via the Nrf2-MAPK–HO–1 signaling pathway (Yoon and Park, 2019). On the other hand, treatment with Schizonepeta tenuifolia (Benth.) Briquet extract suppressed collagen stimulated platelet function by inhibiting MAPK phosphorylation (Jeon et al., 2019). Data from our own experiments involving RAW264.7 cells also suggested that the LPS-induced activation of MAPK and NF-κB signaling pathways might effectively be inhibited by TADIOS treatment (Fig. S1). Taken together, TADIOS is likely to exert effects on inflammation through multiple factors and several different pathways, including Nrf-1, HO-1, MAPK and NF-κB.

The active compounds responsible for the observed effects of TADIOS described in this study have not yet been identified. It is likely that the effects of TADIOS are the result of the combined actions of several compounds rather than of one specific molecule. Marker compounds used in this study have already been shown to control the expression of pro-inflammatory cytokines (for allantoin and luteolin-3′-glucuronide) and HO-1 (for chicoric acid) (Florentino et al., 2016; Kitakaze et al., 2020; Liu et al., 2017). In addition, the three plants that make up TADIOS also contain a variety of flavonoid and steroidal saponins, which have been reported to exhibit anti-inflammatory and antioxidiant properties (Chen et al., 2016; Erden Inal et al., 2001; Kim et al., 2010; Qin et al., 2014; Wu et al., 2020; Yang et al., 2017). Given the potential for the highly effective therapeutic effects and relevant biological activities of TADIOS observed in this study, further experimentation is warranted to identify active compounds, or to obtain a fraction containing concentrated bioactivity from this botanical extract.

It is remarkable that TADIOS works effectively in such a rapidly progressing LPS-induced lung injury model. In this model, 4–6 h after LPS injection, the degree of inflammatory cell infiltration, as measured in the BALF, is greatly increased, and hemorrhagic lung injury is aggravated over time, with its damage level reaching a plateau after 24 h (Asti et al., 2000). In our experiments, mice were orally administered to 1 h before LPS treatment, and effects in the lungs were analyzed 24 h after LPS introduction. This suggests that the effects of TADIOS observed in this study were the result of TADIOS’s activity during that 24-h period.

When TADIOS is considered for human use, this could have significant consequences. Many of the infection cases of SARS-CoV-2 are discovered in the relatively early asymptomatic stage, owing to the intensive surveillance systems adopted by most developed nations (Gao et al., 2020). With TADIOS’s ability to do effective work in such a rapidly progressing lung injury model, it is a logical next step to consider the feasibility of this botanical product being administered immediately upon positive diagnosis of the virus to test whether it can inhibit further disease progression. We are currently exploring the feasibility of conducting such a human study using TADIOS for SARS-CoV-2 infection.

5. Conclusion

Our findings clearly indicate that TADIOS effectively alleviates LPS-induced ALI by exerting potent anti-inflammatory and anti-oxidative stress activities via regulation of the Nrf2-HO-1 signaling pathways. The safety and effectiveness of the three plants used for the preparation of TADIOS have been well established throughout their long history of use in Asian countries, particularly to improve respiratory health. Taken together, TADIOS may have the potential to be developed as a safe and
effective therapeutic agent for different types of inflammatory lung diseases including ARDS, emphysema, and pneumonia.

**Author contributions**

W. Lee and S. Kim designed the project. W. Lee, C. H. Lee, J. Lee, Y. Jeong, J. H. Park, I. J. Nam, J. Song and S. Choi conducted the experiments. D. S. Lee, H. Lee, J. H. Lee and N. Yun were involved in the HPLC
W. Lee and S. Kim analyzed the data and made interpretations. W. Lee, C. H. Lee and S. Kim drafted and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

W. Lee, C. H. Lee, J. Lee, Y. Jeong, J. H. Park, I. J. Nam, D. S. Lee, H. Lee, J. H. Lee, N. Yun, J. Song, S. Choi and S. Kim are the employees of Helimith Co., Ltd. S. Kim own stocks of this company.

Appendix A. Supplementary data

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References

Aust, C., Ruggieri, V., Pozzio, S., Chiusaroli, R., Melillo, G., Caselli, G.F., 2000. Lipopolysaccharide-induced lung injury in mice. I. Concomitant evaluation of inflammatory cells and haemorrhagic lung damage. Palm. Pharmacol. Therapeut. 13 (2), 61–69.

Bosmann, M., Grailler, J.J., Russkamp, N.F., Ruenmiller, R., Zetoune, F.S., Sarma, J.V., Ward, P.A., 2013. CD11c+ alveolar macrophages are a source of IL-23 during lipopolysaccharide-induced acute lung injury. Shock 39 (5), 447–452.

Byun, M.W., 2014. Schizonepeta tenuifolia ethanol extract exerts anti-inflammatory activity through the inhibition of TLR4 signaling in lipopolysaccharide-stimulated macrophage cells. J. Med. Food 17 (3), 350–356.

Cai, Z., Liao, H., Wang, C., Chen, J., Tan, M., Mei, Y., Wei, L., Chen, H., Yang, R., Liu, X., 2020. A comprehensive study of the aerial parts of Lonicera japonica Thunb. based on metabolite profiling coupled with PLS-DA. Phytochem. Anal. 31 (6), 786–800.

Chen, L., Li, W., Qi, D., Wang, D., 2018. Lycium barbarum polysaccharide protects against LPS-induced ARDS by inhibiting apoptosis, oxidative stress, and inflammatory effects of Memora nodosa and allantoin in mice. J. Ethnopharmacol. 196, 298–304.

Delgado-Roche, L., Mesta, F., 2020. Oxidative stress as key player in severe acute respiratory syndrome coronavirus (SARS-CoV) infection. Arch. Med. Res. 51 (5), 384–387.

Diamond, M., Penistion Feliciano, H.L., Sanghavi, D., Mahapatra, S., 2020. Acute Respiratory Distress Syndrome (ARDS). StatPearls, Treasure Island (FL).

Dong, Z., Yuan, Y., 2018. Accelerated inflammation and oxidative stress induced by LPS in acute lung injury: iotainhibition by ST1926. Int. J. Mol. Med. 41 (6), 3405–3421.

Erdem Inal, M., Kahraman, A., Koken, T., 2001. Beneficial effects of quercetin on oxidative stress induced by ultraviolet A. Clin. Exp. Dermatol. 26 (6), 536–539.

Fan, E., Brandt, J.R., Brochard, L., Calfee, C.S., Ferguson, N.D., Slutsky, A.S., Brodie, D., 2020. COVID-19-associated acute respiratory distress syndrome: is a different approach to management warranted? Lancet Respir Med 8 (8), 816–821.

Florentino, F.L., Silva, D.P.B., Galdino, P.M., Lino, R.C., Martins, J.L.R., Silva, D.M., de Paula, J.R., Tresvenzol, L.M.F., Costa, E.A., 2016. Antinociceptive and anti-inflammatory effects of Memora nucha and allantoin in mice. J. Ethnopharmacol. 186, 298–304.

Fujita, S., Fujita, Y., 1973. [Miscellaneous contributions to the essential oils of the plants from various territories. XXXII. On the components of the essential oils of Schizonepeta tenuifolia Briq (author’s transl)]. Yakugaku Zasshi 93 (12), 1622–1626.

Fung, D., Lau, C.B., 2002. Schizonepeta tenuifolia: chemistry, pharmacology, and clinical applications. J. Clin. Pharmacol. 42 (1), 30–36.

Gao, Z., Xu, Y., Sun, C., Wang, X., Guo, Y., Qiu, S., Ma, K., 2020. A systematic review of asymptomatic infections with COVID-19. J. Microbiol. Immunol. Infect. In press.

Gebistorf, F., Karam, O., Wetterwey, J., Afschari, A., 2016. Inhaled nitric oxide for acute respiratory distress syndrome (ARDS) in children and adults. Cochrane Database Syst. Rev. 6, CD002787.

Gilson, P.G., Qiu, L., Puh, S.H., 2020. COVID-19 acute respiratory distress syndrome (ARDS): clinical features and differences from typical pre-COVID-19 ARDS. Med. J. Aust. 213 (2), 54–56 e51.

Gonzales, J.N., Lucas, R., Verin, A.D., 2015. The acute respiratory distress syndrome: mechanisms and perspective therapeutic approaches. Austin J Vasc Med 2 (1),
