Water extract of *Helminthostachys zeylanica* attenuates LPS-induced acute lung injury in mice by modulating NF-κB and MAPK pathways

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**ABSTRACT**

*Ethnopharmacological relevance:* Previous studies showed that *Helminthostachys zeylanica* (L.) Hook. could reduce inflammatory responses in macrophage and brain astrocytes.  

**Aim of the study:** In the present study, we evaluated whether an ethyl acetate extract (HZE) or a water extract (HZW) of *H. zeylanica* could reduce inflammatory responses in lung epithelial cells and ameliorate lipopolysaccharide (LPS)-induced acute lung injury in mice.  

**Methods:** Human lung epithelial A549 cells were pre-treated with HZE or HZW (1–10 μg/mL), then stimulated with LPS. RALB/c mice received oral HZW for 7 consecutive days, then an intratracheal instillation of LPS to induce lung injury.  

**Results:** HZW reduced chemokine and proinflammatory cytokine production in LPS-activated A549 cells. HZW also suppressed ICAM-1 expression and reduced the adherence of acute monocytic leukemia cells to inflammatory A549 cells. HZE had less efficacy than HZW in suppressing inflammatory responses in A549 cells. In vivo, HZW significantly suppressed neutrophil infiltration and reduced the TNF-α and IL-6 levels in bronchoalveolar lavage fluid and serum from LPS-treated mice. HZW also modulated superoxide dismutase activity, glutathione, and myeloperoxidase activity in lung tissues from LPS-treated mice. HZW decreased the phosphorylation of mitogen-activated protein kinase and nuclear factor kappa B, and promoted heme oxygenase-1 expression in inflamed lung tissue from LPS-treated mice.  

**Conclusion:** Our findings suggested that HZW reduced lung injury in mice by reducing oxidative stress and inflammatory responses. HZW also reduced inflammatory responses in human lung epithelial cells.

1. Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome are characterized by neutrophil accumulations in the lung. This accumulation leads to severe pulmonary inflammation, reduced lung capacity, and increased pulmonary edema (Butt et al., 2016). ALI also causes fluid accumulation in alveoli, which leads to difficulty breathing, rapid breathing, shortness of breath, and asphyxia, which increases the risk of mortality (Ghidoni et al., 2015). ALI can also develop into a respiratory disease with devastating complications, such as chronic obstructive pulmonary disease, ischemia/reperfusion injury, cystic fibrosis, asthma, and severe sepsis (Mokra and Kosutova, 2015).

Bacterial lipopolysaccharide (LPS), an important cell wall component of pathogenic gram-negative bacteria, can activate macrophages...
and neutrophils to defend against bacterial invasion. The airways of patients with ALI are infected with gram-negative bacteria, and their lung tissues secrete proinflammatory cytokines and chemokines, which aggravate immune inflammatory responses and cause pulmonary oxidative damage (Gong et al., 2014). In experimental animal models of ALI (ALI mice), delivery of LPS with an intratracheal or tail vein injection induced pulmonary inflammation and fever (Butt et al., 2016; Niu et al., 2015). Previous studies demonstrated that LPS could bind to toll-like receptor-4 expressed on immune cell membranes. This binding activated inflammatory signaling pathways (Rossol et al., 2011), including nuclear factor kappa B (NF-kB), an important transcription factor that induces the expression of inflammatory cytokines and mediators (Peri et al., 2010). LPS also contributed to the production of reactive oxygen species (ROS), which worsened cellular and lung tissue injuries in ALI mice (Mokra and Kosutova, 2015; Wang, 2014).

Furthermore, mitogen-activated protein kinase (MAPK) pathways were induced ALI mice (Chen et al., 2012). Therefore, we reasoned that tissue injuries in ALI mice (Mokra and Kosutova, 2015; Wang, 2014).

of reactive oxygen species (ROS), which worsened cellular and lung tissue injuries in ALI mice (Mokra and Kosutova, 2015; Wang, 2014).

Furthermore, mitogen-activated protein kinase (MAPK) pathways were activated, which also stimulated inflammatory gene expression in LPS-induced ALI mice (Chen et al., 2012). Therefore, we reasoned that suppressing both the NF-kB and MAPK pathways might ameliorate inflammatory responses and oxidative damage in ALI.

*Helminthostachys zeylanica* (L.) Hook. (Ophioglossaceae) is a common herb used to relieve fever symptoms and inflammatory diseases in Taiwan (Su et al., 2016). Previous studies found that the flavonoid, uoginin K, isolated from *H. zeylanica*, could reduce proinflammatory cytokine production in LPS-induced macrophages (Lee et al., 2011). An extract from *H. zeylanica* also reduced inflammatory responses and MMP-9 expression in bradykinin-activated brain astrocytes (Hsieh et al., 2015). In the present study, we evaluated whether a water extract (HZW) or an ethyl acetate extract (HZE) of *H. zeylanica* could protect ALI mice from LPS-induced lung injury. We evaluated the inflammatory response and oxidative stress in this animal model.

2. Materials and methods

2.1. *H. zeylanica* collection and extraction

The root of *H. zeylanica* was purchased in Wanhua, Taiwan. The herb was identified by Mr. Jun-Chih Ou. The roots of *H. zeylanica* (1.0 kg) were extracted with ethanol at 50 °C for 4 h. Then, the concentrated ethanol extract (38.0 g) was partitioned between the ethyl acetate extract (HZE, 14.2 g, yield 1.42%) and a water extract (HZW, 23.8 g, yield 2.38%).

2.2. HPLC analysis of *H. zeylanica*

The HPLC fingerprint of *H. zeylanica* was examined with the Hitachi HPLC system (L-2000 series, Tokyo, Japan). We injected 20 μL of extract (4 mg/mL) into a reverse-phase column (Cosmosil 5C18-AR-II, 5mm, 25 cm × 4.6 mm I.D.), and the UV detection wavelength was set at 260 nm.

2.3. HZW and HZE treatment of A549 cells

A549, a human lung epithelial cell line, was obtained from the Bioresource Collection and Research Center in Taiwan. Cells were cultured in F-12 nutrient mixture medium (Life Technologies, Carlsbad, CA, USA), which contained 10% fetal bovine serum (Biological Industries, Haemek, Israel), penicillin (100 units/mL), and streptomycin (100 μg/mL). HZE was dissolved in DMSO, and HZW was dissolved in PBS, to obtain stock solutions of 10 mg/mL. In all cell experiments, the DMSO concentration in the culture medium was ≤0.1%. A549 cells were pretreated with (1–10 μg/mL) HZE or HZW for 1 h, then stimulated with 1 μg/mL LPS for 24 h. The supernatants were collected and assayed with ELISA kits for the specific detection of selected chemokines or cytokines. Moreover, A549 cell viability was tested with the MTT reagent; results were assayed at 570 nm on a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

2.4. Cell-cell adhesion assay

A549 cells were treated with various doses of HZW or HZE and stimulated with LPS for 24 h, as previously described (Liou et al., 2016). Next, a calcine-AM solution (Sigma) was used to stain acute monocytic leukemia cells (THP-1 cells). THP-1 cells were co-cultured with the A549 cells to observe THP-1 adhesion. Adherent cells were detected (green fluorescence) with fluorescence microscopy (Olympus, Tokyo, Japan).

2.5. Animals

Female BALB/c mice (20–25 g, 6–8 weeks old) were purchased from the National Laboratory Animal Center in Taiwan. Mice were given a standard chow diet and clear water, and they were maintained in central air-conditioned animal housing. All experimental procedures were approved by the Laboratory Animal Care Committee of the Chang Gung University of Science and Technology (IACUC approval number: 2013-001).

2.6. HZW treatment and LPS-induced acute lung injury in mice

Mice were randomly assigned to five groups, with 8 mice in each group. The N group comprised normal control mice; the LPS group comprised mice challenged with LPS administered intratracheally; and three HZW groups comprised LPS-treated mice that were pretreated with 1 mg/kg HZW (HZW1), 5 mg/kg HZW (HZW5), or 10 mg/kg HZW (HZW10). On days 1–7, experimental mice received oral administrations of normal saline or the designated dose of HZW. On day 8, mice were anesthetized and treated with 50 μL LPS (1 μg/mL) or normal saline, administered intratracheally. Then, 4 h later, mice sacrificed, and the bronchoalveolar lavage fluid (BALF), lung tissue, and serum were harvested for the experiments.

2.7. BALF collection and cell count

BALF was collected as described previously (Huang et al., 2016). Briefly, mice were sacrificed, trachea were intubated, and the lungs were flushed with normal saline. Isolated BALF cells were stained with Liu stain (Polysciences, Inc., Taipei, Taiwan). Neutrophils were counted with an optical microscope (Olympus, Tokyo, Japan). BALF supernatant was collected to evaluate secreted cytokines and chemokines.

2.8. Histological analyses of lung tissue

Lung tissues were fixed in formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin (HE). Neutrophils were observed with an optical microscope (Olympus).

2.9. Wet-to-dry (W/D) weight ratio of lung tissue

Mice were sacrificed, and the right lung was excised to obtain its wet weight (W). Next, the lung was dried in an oven at 80 °C for 48 h, then weighed to obtain the dry weight (D). The W/D ratio was calculated to assess lung edema.

2.10. Myeloperoxidase and malondialdehyde activity

Lung tissues were homogenized in cool normal saline. Myeloperoxidase (MPO) activity was measured with a MPO fluorometric activity assay kit (Sigma). We used a lipid peroxidation assay kit (Sigma) to evaluate malondialdehyde (MDA) activity in the lungs,
according to the manufacturer's instructions. MPO and MDA activities were measured and calculated on a multi-mode microplate reader (BioTek Synergy HT, Bedfordshire, United Kingdom).

2.11. Glutathione and superoxide dismutase assay

Lung tissue was homogenized and glutathione (GSH) levels were evaluated with a glutathione assay kit (Sigma). Superoxide dismutase (SOD) activity was assayed with a SOD determination kit (Sigma), according to the manufacturer’s instructions. Results were measured on a microplate reader (Multiskan FC, Thermo).

2.12. Western immunoblot analyses

Lung tissues were homogenized, and total proteins and nuclear proteins were extracted as described previously. Proteins were separated on a 10% SDS-PAGE, then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were incubated with specific primary antibodies against ERK1/2, JNK, p38 (Millipore), phosphorylated-1xB-α, phosphorylated-ERK 1/2, phosphorylated-JNK, phosphorylated-p38 (Cell Signaling Technology, Boston, MA, USA), p65, phosphorylated-p65, 1xB-α, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase (HO)-1, lamin B1 (Santa Cruz, CA, USA), and β-actin (Sigma). Then, membranes were washed and incubated with HRP-conjugated secondary antibody for 1 h. Luminol/Enhancer Solution (Millipore) was added, and protein signals were detected with the BioSpectrum 600 system (UVP, Upland, CA, USA).

2.13. Serum collection

Mice were anesthetized with isoflurane, and blood was collected from the orbital vascular plexus. Samples were centrifuged at 6000 rpm for 5 min, as previously described (Huang et al., 2014). Serum was stored at –80 °C, before assaying cytokines.

2.14. Cytokine assay

We analyzed serum, BALF, and cell culture medium samples with specific ELISA kits (R & D Systems, Minneapolis, MN, USA) to quantify IL-6 IL-8, MCP-1, CCL5, ICAM-1, and TNF-α expression, the cDNA was used as template in a real-time PCR kit (Bio-Rad Laboratories, Hercules, CA, USA). Next, cDNA was synthesized with a cDNA synthesis kit (Life Technologies). To detect specific gene expression, the cDNA was used as template in a real-time PCR kit (Bio-Rad Laboratories, Hercules, CA, USA) and amplified on a spectrofluorometric thermal cycler (iCycler; Bio-Rad). In this experiment, we used the specific primers shown in Table 1.

2.15. Real-time PCR

RNA was extracted from lung tissue with the TRizol reagent (Life Technologies, Carlsbad, CA, USA). Next, cDNA was synthesized with a cDNA synthesis kit (Life Technologies). To detect specific gene expression, the cDNA was used as template in a real-time PCR kit (Bio-Rad Laboratories, Hercules, CA, USA) and amplified on a spectrofluorometric thermal cycler (iCycler; Bio-Rad). In this experiment, we used the specific primers shown in Table 1.

2.16. Statistical analyses

Experimental data were examined with a one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparisons. Results were expressed as the mean ± SEM, and a P-value < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of HZW and HZE effects on activated A549 cells

HZW and HZE were analyzed with HPLC. We found that the HZE comprised mainly flavonoids (ugonin J and ugonin K) (Fig. 1). Next, we found that doses ≤10 µg/mL of HZW or HZE did not significantly affect the viability of A549 cells (data not shown). Therefore, we used 1–10 µg/mL HZW and HZE to evaluate their anti-inflammatory effects. First, we assessed whether HZW or HZE influenced the secretion of chemokines and cytokines in LPS-stimulated A549 cells (Fig. 2). Our results demonstrated that HZW pretreatment significantly reduced IL-6 and IL-8 levels compared to LPS-stimulation alone (IL-6; HZW 1 µg/mL: 154.9 ± 4.9 pg/mL, p < 0.01; HZW 5 µg/mL: 101.8 ± 5.8 pg/mL, p < 0.01; HZW 10 µg/mL: 59.3 ± 4.7 pg/mL, p < 0.01; vs. LPS-treated control: 207.1 ± 6.7 pg/mL) (IL-8; HZW 1 µg/mL: 4.25 ± 0.21 ng/mL, p < 0.05; HZW 5 µg/mL: 3.59 ± 0.13 ng/mL, p < 0.01; HZW 10 µg/mL: 1.63 ± 0.11 ng/mL, p < 0.01; vs. LPS-treated control: 5.31 ± 0.23 ng/mL). HZW also significantly, dose-dependently reduced the levels of MCP-1 and CCL5 in LPS-stimulated A549 cells. Surprisingly, HZE did not significantly reduce the levels of IL-6, IL-8, MCP-1, or CCL5. Only the highest dose (10 µg/mL), of HZE could significantly reduce the levels of IL-6 and IL-8. This showed that HZW could reduce the inflammatory response more effectively than HZE, in LPS-stimulated A549 cells.

3.2. Effects of HZW and HZE on LPS-induced ICAM-1 expression

A549 cells were pretreated with HZW or HZE, then stimulated with LPS for 24 h. The results showed that HZW significantly reduced ICAM-1 expression compared to LPS-stimulation alone in A549 cells (ICAM-1; HZW 1 µg/mL: 184.9 ± 7.3 pg/mL, p < 0.01; HZW 5 µg/mL: 147.8 ± 4.2 pg/mL, p < 0.01; HZW 10 µg/mL: 92.7 ± 8.7 pg/mL, p < 0.01; vs. LPS-treated control: 322.3 ± 8.5 pg/mL; Fig. 3A). We also found that HZW inhibited adherence of THP-1 cells to LPS-stimulated A549 cells (Fig. 3B). However, compared to HZW, HZE did not significantly reduce ICAM-1 levels or THP-1 cell adherence. Therefore, only HZW was investigated further to determine its effects on inflammation and oxidation in ALI mice.

3.3. Effects of HZW on neutrophil infiltration in LPS-induced lung injury

Mice treated with LPS alone showed more neutrophil infiltration in lung biopsies compared to untreated mice (N group). The HZW groups showed reduced neutrophil infiltration (Fig. 4). HZW also significantly inhibited the neutrophil count compared to the LPS-treated group (neutrophil counts: HZW1: 5.2×105 ± 1.7×104/mL, p=0.20; HZW5: 3.5×105 ± 1.5×104/mL, p≤0.05; HZW10: 2.4×105 ± 1.1×104/mL, p ≤ 0.01 vs. LPS: 6.4×105 ± 2.2×105/mL; Fig. 5A).

3.4. Effects of HZW on cytokine and chemokine levels in BALF and lung tissue

Among ALI mice, HZW treatment significantly suppressed IL-6 and TNF-α levels in BALF compared to LPS alone (IL-6: HZW1, 333.2 ±

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<td><strong>Primers used in real-time PCR analyses of the expression of proinflammatory mRNAs.</strong></td>
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14.5 pg/mL, \(p = 0.052\); HZW5, 226.6 ± 19.6 pg/mL, \(p < 0.05\); HZW10, 72.1 ± 15.2 pg/mL, \(p < 0.01\) vs. LPS: 367.1 ± 36.0 pg/mL) (TNF-\(\alpha\): HZW1, 439.4 ± 58.4 pg/mL, \(p = 0.31\); HZW5, 305.1 ± 52.5 pg/mL, \(p < 0.05\); HZW10, 113.0 ± 21.9 pg/mL, \(p < 0.01\) vs. LPS: 528.6 ± 57.3 pg/mL; Fig. 5B–C).

Gene expression assays in lung tissues showed that HZW significantly reduced iNOS, COX-2, IL-1\(\beta\), IL-6, TNF-\(\alpha\), CCL5, MCP-1, and ICAM-1 expression compared to expression levels observed with LPS-treatment alone (Fig. 6). However, HZW did not significantly inhibit CCL11 gene expression. Furthermore, we found that HZW also significantly reduced protein levels in BALF compared to LPS alone (Fig. 7A).

3.5. Effects of HZW on lung W/D ratios in LPS-treated mice

The W/D ratios reflect the levels of lung edema. We found that LPS significantly increased the W/D ratio compared to no treatment (N group). Interestingly, HZW significantly reduced the W/D ratio compared to LPS alone (Fig. 7B).

3.6. The effects of HZW on MOP, MOD, GSH, and SOD activities in lung

We found that LPS significantly promoted MOP and MDA activities, and repressed GSH and SOD activities, compared to no treatment (N group; Fig. 7C–F). Conversely, HZW significantly decreased LPS-induced MOP and MDA activity, and increased GSH and SOD production.

3.7. Effects of HZW on serum cytokine levels

Serum analyses demonstrated that HZW treatment suppressed IL-6 and TNF-\(\alpha\) production, compared to LPS treatment alone (Fig. 8).

3.8. Effects of HZW on the phosphorylation of NF-\(\kappa\)B and MAPK in lung tissue

HZW treatment reduced the phosphorylation of NF-\(\kappa\)B subunits, I\(\kappa\)B-\(\alpha\) and p65, compared to LPS alone (Fig. 9A). HZW also decreased the phosphorylation of MAPK pathway proteins, ERK1/2, p38, and JNK, in LPS-stimulated lung tissues (Fig. 9B).

3.9. Effects of HZW on HO-1 and Nrf2 expression in lung tissue

LPS treatment induced HO-1 expression in lung tissue compared to no treatment (N group). HZW further increased HO-1 expression and increased Nrf2 expression compared to LPS treatment alone (Fig. 9C).
4. Discussion

Previous studies confirmed that *H. zeylanica* extract had anti-inflammatory effects on LPS-stimulated macrophages (Su et al., 2016). It was also shown that neougonin A, a flavonoid isolated from *H. zeylanica*, could suppress inflammatory responses in RAW 264.7 macrophages by blocking the NF-κB pathway (Cao et al., 2016). Another study showed that *H. zeylanica* extract could improve liver damage and modulate serum enzymes in Wistar rats (Suja et al., 2004). Moreover, ugonin U was shown to activate phospholipase C, which improved the inflammatory, oxidative, and immune effects of neutrophils (Chen et al., 2014). Ethyl acetate could isolate lipophilic alkaloids and flavonoids; while, water extract could isolate hydrophilic flavonoids, polysaccharides, or alkaloids. In the present study, we investigated HZE and HZW to determine whether they could suppress inflammation in lung epithelial cells, and ameliorate the lung inflammation and oxidative stress associated with ALI in mice. Our results showed that HZW significantly reduced the production of IL-6, IL-8,
CCL5, and MCP-1 in LPS-stimulated A549 cells. HZW also repressed ICAM-1 expression and reduced THP-1 cell adherence to LPS-stimulated A549 cells. Furthermore, we found that HZW could effectively inhibit neutrophil infiltration in the lungs and BALF, which led to diminished inflammatory and oxidative responses.

The HPLC profiles showed that HZE contained two major known compounds (ugonin J and ugonin K). However, HZE could not reduce the inflammatory response or decrease ROS levels as well as HZW, in LPS-stimulated A549 cells. HZW fraction contained the main compound that could reduce the oxidative stress and inflammatory response in ALI mice. Therefore, in the future, we plan to identify, isolate, and purify the bioactive compounds from HZW that ameliorated the damage induced by LPS in ALI mice.

ALI is a severe clinical illness characterized by neutrophil infiltration into pulmonary parenchyma. This infiltration accelerates disease progression. It induces alveolar barrier disruption and edema, increases microvascular permeability, decreases tidal volume, induces hypoxemia, and can even lead to pulmonary function disorder and death (Butt et al., 2016). When gram-negative bacteria infect the airway, they release LPS, which causes lung inflammation and edema (Rossol et al., 2011). Neutrophils and macrophages are recruited to defend against infective microbes; they release inflammatory mediator enzymes to kill the bacteria (Konrad and Reutershan, 2012; Mokra and Kosutova, 2015). Unfortunately, neutrophils secrete mediators that can cause more serious inflammation and oxidative damage to infected lung tissue. Our findings showed that HZW effectively suppressed neutrophil infiltration in BALF and lung tissues, which might reduce the edema caused by alveolar rupture and fluid accumulation.

In the current study, mice were orally administered HZW for 7 days. Then, mice were given intratracheal LPS to induce lung injury. Four hours later, the mice were sacrificed. Hence, we did not induce death from ALI in this experimental mouse model. However, we did observe significant increases in the levels of proinflammatory cytokines and chemokines in lung tissue. Oral administration of HZW decreased
the levels of IL-6 and TNF-α in the serum and BALF of ALI mice. We also found that HZW significantly decreased the expression of proinflammatory cytokines, mediators, and chemokines in lung tissues compared to LPS alone.

Interestingly, HZW did not decrease the levels of CCL11, a chemokine that induces eosinophilic infiltration. Therefore, HZW might operate primarily by preventing neutrophil infiltration, and this might be sufficient to block LPS-induced lung disease. Moreover, we found that HZW diminished ICAM-1 expression and decreased THP-1 adherence to LPS-activated A549 cells in vitro. HZW also reduced ICAM-1 expression in lungs, which blocked neutrophil infiltration into the lungs, which reduced oxidative damage and the inflammatory response.

Other studies have shown that LPS could activate proinflammatory signaling pathways to increase the expression of genes that encode proinflammatory cytokines and mediators (Guha and Mackman, 2001). Triptolide, an NF-κB inhibitor, had anti-inflammatory effects in LPS-induced ALI mice (Wei and Huang, 2014). Additionally, a MAPK inhibitor attenuated inflammatory and oxidative damage in the lungs of ALI mice (Qiu et al., 2016). In the present study, we demonstrated that HZW significantly reduced the phosphorylation of MAPK and NF-κB pathway proteins in lung tissues of ALI mice. That result suggested that HZW had the potential to diminish ALI by modulating both NF-κB and MAPK pathways in ALI mice.

Inflammatory tissue is often accompanied by oxidative stress, which exacerbates tissue damage (Antus, 2016). Oxidative metabolism releases free radicals and superoxide anions, which cause DNA breaks and cellular damage (Suzuki et al., 2015). Furthermore, neutrophils release oxidant enzymes to defend against invasive bacteria in the lungs of ALI mice (Konrad and Reutershan, 2012; Porto and Stein, 2016). In particular, myeloperoxidase (MPO) is produced in the granulocytes of neutrophils. When MPO is released, it produces hypohalous acids to kill pulmonary bacteria (Colgan, 2015; Ferrari and Andrade, 2015). However, MPO activity can also cause oxidative damage to the host tissue, during its attack against the bacterial invasion. Here, we found that HZW significantly reduced neutrophil infiltration and suppressed MPO activity, which ameliorated the lung injury in ALI mice. Other antioxidant flavonoids were previously shown to protect from oxidative damage and attenuate inflammatory responses in the lungs of ALI mice (Ferrari and Andrade, 2015). Our results demonstrated that HZW also increased SOD and GSH production to defend against oxidative damage in lung tissue. In addition, HZW effectively reduced ROS levels in inflammatory tracheal epithelial cells, and it reduced the expression of MDA, a lipid peroxidation marker, in lung tissues of ALI mice. Furthermore, HZW increased Nrf2 and HO-1 expression, which defend against oxidative damage. Hence, our results suggested that HZW could effectively reduce lung injury by decreasing oxidative stress in LPS-induced ALI mice.

Fig. 6. Effects of HZW on gene expression of cytokines, chemokines, and inflammatory mediators in the lung. Fold-changes in gene expression are shown for (A) IL-1β, (B) IL-6, (C) TNF-α, (D) INOS, (E) COX-2, (F) ICAM-1, (G) CCL5, (H) CCL11, and (I) MCP-1. Expression was measured with real-time PCR analysis of RNA extracted from lung tissues. Fold-changes in expression were evaluated relative to β-actin expression (internal control). Data are presented as the mean ± SEM. *p < 0.05 compared to LPS-treated mice. **p < 0.01 compared to LPS-treated mice.
Fig. 7. HZW effects in the lung tissues of LPS-induced ALI mice. Graphs show effects on (A) the levels of protein, (B) W/D ratio, (C) MPO activity, (D) MDA activity, (E) GSH activity, (F) and SOD activity. Data are presented as the mean ± SEM. *p < 0.05 compared to LPS-treated mice. **p < 0.01 compared to LPS-treated mice.

Fig. 8. HZW decreased serum levels of proinflammatory cytokines. ELISA results show changes in serum (A) IL-6 and (B) TNF-α levels. All data are presented as the mean ± SEM. *p < 0.05 compared to LPS-treated mice. **p < 0.01 compared to LPS-treated mice.

Fig. 9. HZW inhibits phosphorylation and expression of proinflammatory proteins. HZW inhibited LPS-induced phosphorylation of (A) the IκB-α and p65 subunits, in the NF-κB pathway; and (B) proteins in the MAPK pathway. (C) HZW increased HO-1 and Nrf2 protein expression in lung tissues from mice treated with LPS.
5. Conclusions

Our results showed that HZW, but not HZE, could reduce neutrophil infiltration and ameliorate inflammatory responses in ALI mice by blocking NF-κB and MAPK pathways and reducing oxidative stress. Thus, HZW showed potential as an anti-inflammatory and anti-oxidative therapeutic for maintaining lung function in ALI.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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References


