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柑橘來源黃酮類化合物 Diosmetin 減輕脂多醣誘發之急性肺損傷：抗氧化防禦與黏附分子相關發炎反應之調控

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Diosmetin, a Citrus-Derived Flavonoid, Attenuates Lipopolysaccharide-Induced Acute Lung Injury by Preserving Antioxidant Defense and Reducing Adhesion Molecule-Associated Inflammatory Responses

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Abstract Acute lung injury (ALI) involves inflammatory cell recruitment, disruption of the alveolar–capillary barrier, and oxidative injury. Diosmetin is a citrus-derived flavonoid with reported anti-inflammatory and antioxidant activities, but its relationship with adhesion molecule-associated inflammatory responses in ALI remains incompletely characterized. This study investigated the protective effects of diosmetin in lipopolysaccharide (LPS)-induced ALI using network pharmacology and an experimental mouse model. Potential diosmetin targets and ALI-associated genes were analyzed to identify shared targets and enriched pathways. BALB/c mice received diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg) before intratracheal LPS challenge (5 mg/kg). Lung histopathology, bronchoalveolar lavage fluid (BALF) protein concentration, leukocyte and neutrophil recruitment, myeloperoxidase (MPO) activity, lipid peroxidation, inflammatory cytokines, ICAM-1 and VCAM-1 levels, and pulmonary antioxidant status were evaluated. Network analysis identified 74 shared targets and showed enrichment of several inflammation-related pathways, including PI3K-Akt, NF-κB, MAPK, TNF, and Toll-like receptor signaling. LPS caused marked lung injury, increased BALF protein, inflammatory cell accumulation, MPO activity, lipid peroxidation, TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1, together with reductions in SOD and catalase activities and GSH content. Diosmetin at 25 mg/kg produced little change, whereas significant protection was observed at 50 mg/kg and was generally more pronounced at 100 mg/kg. Overall, diosmetin reduced

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inflammatory cell recruitment and adhesion molecule-associated responses while limiting oxidative injury and preserving pulmonary antioxidant capacity. These results support further investigation of diosmetin as a citrus-derived bioactive compound for inflammation-associated ALI.

Key words: Diosmetin; Acute Lung Injury; Citrus flavonoid; Adhesion molecules; Oxidative stress; Network pharmacology

INTRODUCTION

Acute lung injury (ALI) is characterized by acute pulmonary inflammation and disruption of the alveolar–capillary barrier, leading to increased vascular permeability, pulmonary edema, and impaired gas exchange [1,2,3]. Both alveolar epithelial cells and the pulmonary vascular endothelium are affected during the development of lung injury. Lipopolysaccharide (LPS) challenge reproduces several major features of inflammatory ALI, including cytokine release, leukocyte recruitment, oxidative injury, and increased pulmonary permeability [1,2,4]. It is therefore widely used to investigate the pathophysiology of inflammatory lung injury and potential protective interventions [1,2,4].

The pulmonary endothelium contributes actively to the inflammatory response rather than serving only as a structural component of the alveolar–capillary barrier. Following inflammatory stimulation, endothelial cells increase adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), thereby facilitating leukocyte attachment and migration into injured tissue [3]. Neutrophils are particularly important during the early phase of ALI. Once recruited, they release reactive oxygen species (ROS), proteases, and myeloperoxidase (MPO)-derived oxidants that further aggravate pulmonary injury [4]. At the same time, excessive ROS production promotes lipid peroxidation and impairs endogenous antioxidant defenses [5]. Endothelial activation, inflammatory cell recruitment, and oxidative injury are therefore closely related events in LPS-induced pulmonary inflammation [3, 4, 5].

Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) is a naturally occurring flavone found in plant-derived foods, particularly Citrus species. Diosmetin and related glycosides, including diosmin and neodiosmin, have been identified in citrus fruits and juices and represent part of the flavonoid composition of these foods [6]. This dietary origin has attracted interest because citrus flavonoids have been investigated for their anti-inflammatory and antioxidant properties.

Experimental studies have shown that diosmetin can modulate oxidative and inflammatory responses [7,8]. In endothelial cells exposed to oxidative stress, diosmetin reduced ROS generation and lipid peroxidation while improving superoxide dismutase, catalase, and glutathione peroxidase activities [8]. Protective effects have also been reported in experimental ALI. Diosmetin attenuated LPS-induced histopathological injury, inflammatory cell infiltration, cytokine production, MPO activity, and oxidative damage in association with activation of Nrf2 and suppression of the NLRP3 inflammasome [9]. Another study showed that diosmetin improved pulmonary barrier dysfunction and altered ZO-1 and occludin in association with RhoA/ROCK signaling [10].

Because flavonoids can act on several molecular targets rather than a single pathway, network pharmacology may help identify biological processes potentially involved in their effects [11,12]. These studies support a protective role for diosmetin in inflammatory lung injury, although its relationship with adhesion molecule-associated inflammatory responses has received less attention. In particular, changes in ICAM-1 and VCAM-1 have not been examined together with leukocyte recruitment, oxidative injury, barrier disruption, and pulmonary antioxidant status within the same experimental setting. To further characterize the pulmonary actions of diosmetin, network pharma-

cology was integrated with an LPS-induced mouse model of ALI. Lung histopathology, alveolar barrier injury, inflammatory cell recruitment, MPO activity, lipid peroxidation, inflammatory cytokines, ICAM-1 and VCAM-1, and antioxidant status were examined. Particular attention was given to the relationship between adhesion molecule-associated responses, inflammatory cell recruitment, and oxidative injury.

MATERIALS AND METHODS

In Silico Toxicity Prediction

The potential toxicity of diosmetin was evaluated using ProTox 3.0 [13]. The chemical structure of diosmetin was submitted to the platform to predict its median lethal dose (LD_{50}), acute toxicity class, and toxicity-related endpoints. The analysis included general toxicity, organ toxicity, nuclear receptor-associated activity, stress-response pathways, and other molecular toxicity targets. Predicted probabilities were summarized to provide a preliminary in silico toxicity profile of diosmetin.

Network Pharmacology Analysis

Potential diosmetin targets and genes associated with ALI were obtained from publicly available target-prediction and disease-related databases. Target names were standardized to human gene symbols, and duplicate entries were removed. Diosmetin-related targets were intersected with ALI-associated genes to identify shared targets potentially relevant to the effects of diosmetin [14]. These targets were submitted to the STRING database [15] to construct a protein–protein interaction (PPI) network. The network was subsequently imported into Cytoscape [16] for visualization and topological analysis. Hub targets were identified using the CytoHubba plugin [16] and ranked according to degree values. Functional enrichment analysis of the common targets was then performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses [17, 18]. GO analysis included biological process, cellular component, and molecular function categories. KEGG analysis was used to identify signaling pathways enriched among the shared target.

Animals and experimental protocol

Male BALB/c mice, approximately 9-12 weeks of age and weighing 25±2 g, were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Animals were housed at 21±2℃ and 60±5% relative humidity under a 12-h light/dark cycle, with free access to standard laboratory chow and water. Mice were allowed to acclimatize for one week before experimentation. Housing and experimental procedures were based on protocols previously established in our laboratory for LPS-induced ALI [19,20,21]. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC approval no. 1720). Mice were randomly assigned to six groups (n = 6 mice per group) : control, LPS, LPS plus diosmetin (25 mg/kg), LPS plus diosmetin (50 mg/kg), LPS plus diosmetin (100 mg/kg), and LPS plus dexamethasone (1 mg/kg). LPS and dexamethasone were obtained from Sigma-Aldrich (MO, USA), and diosmetin was purchased from ChemFace (Hubei, China). Diosmetin or dexamethasone was administered intraperitoneally 30 min before LPS challenge. ALI was induced by intratracheal administration of LPS (5 mg/kg; *Escherichia coli* O111:B4; Sigma-Aldrich), as described in our previous studies [19, 22]. The selection of diosmetin doses was based on previous findings demonstrating its protective effects against LPS-induced acute lung injury at 5 and 25 mg/kg following intraperitoneal administration [9]. In the present study, 25 mg/kg was selected as the lowest dose, while 50 and 100 mg/kg were included to further investigate the protective effects of diosmetin at higher doses under the current experimental conditions. Control animals received the corresponding vehicle. Six hours after LPS administration, mice were euthanized and bronchoalveolar lavage fluid (BALF) and lung tissues were collected.

Lung specimens for histological examination were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at approximately 5 μm, and stained with hematoxylin and eosin. Histopathological lung injury was evaluated based on pulmonary edema, interstitial and alveolar wall thickening, inflammatory cell infiltration, and hyaline membrane formation, as previously described [20]. Histological changes were graded using a semiquantitative scoring system ranging from 0 (normal lung histology) to 4 (severe lung injury).

Collection and Analysis of Bronchoalveolar Lavage Fluid

BALF was collected according to procedures previously established in our laboratory [19,20]. After euthanasia, the trachea was cannulated and the lungs were gently lavaged three times with 1 mL of precooled sterile phosphate-buffered saline (PBS ; Sigma-Aldrich). Recovered BALF was centrifuged at 1000×g for 10 min at 4℃ . The supernatant was retained for protein and soluble mediator measurements, while the cell pellet was used for flow cytometric analysis. BALF protein concentration was measured by the Bradford method using bovine serum albumin as the standard and was used as an index of alveolar-capillary barrier disruption. For leukocyte analysis, BALF cell pellets were resuspended in PBS containing 1% bovine serum albumin and incubated with fluorochrome-conjugated antibodies against CD45 and Ly6G (BioLegend, San Diego, CA, USA). Total leukocytes were identified as $CD45^+$ cells and neutrophils as $CD45^+Ly6G^+$ cells. Unstained and isotype-matched controls were included to establish background fluorescence and gating. Samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) [19,22].

Pulmonary myeloperoxidase activity

Pulmonary MPO activity was determined as an indicator of neutrophil-associated inflammatory activity [21,23]. Lung tissue was homogenized in PBS containing cetyltrimethylammonium bromide (Sigma-Aldrich) and guaiacol (Sigma-Aldrich). Following centrifugation, the supernatant was reacted with hydrogen peroxide, and guaiacol oxidation was measured spectrophotometrically. MPO activity was normalized to tissue protein content.

Determination of lipid peroxidation

Pulmonary lipid peroxidation was evaluated using a TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions [20,23]. Lung tissues were homogenized on ice in lysis buffer containing protease inhibitors and centrifuged at 1,600×g for 10 min at 4℃ . The resulting supernatants were reacted with SDS and Color Reagent and incubated at 95℃ for 1 h. After cooling on ice and centrifugation, absorbance was measured at 540 nm. TBARS values were expressed as malondialdehyde (MDA) equivalents and normalized to tissue protein content.

Measurement of Inflammatory Cytokines and Adhesion Molecules

BALF concentrations of TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1 were measured using mouse-specific ELISA kits (BioLegend, San Diego, CA, USA), following procedures established in our previous studies [19,20]. Concentrations were calculated from individual standard curves and expressed as pg/mL.

Assessment of pulmonary antioxidant status

Lung tissue was homogenized under ice-cold conditions and centrifuged to obtain the soluble fraction. Superoxide dismutase (SOD) activity, catalase activity, and reduced glutathione (GSH) content were measured using the corresponding commercial assay kits (Cayman Chemical, Ann Arbor, MI, USA). Measurements followed procedures adapted from our previous studies of oxidative injury in LPS-exposed lungs [20,21,23].

Statistical analysis

Results are presented as mean±standard devia-

tion (SD). The assumptions of approximate normality and homogeneity of variances were considered when applying parametric statistical analysis. Differences among groups were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test. The same statistical approach was applied to all quantitative experimental endpoints. A value of $P < 0.05$ was considered statistically significant.

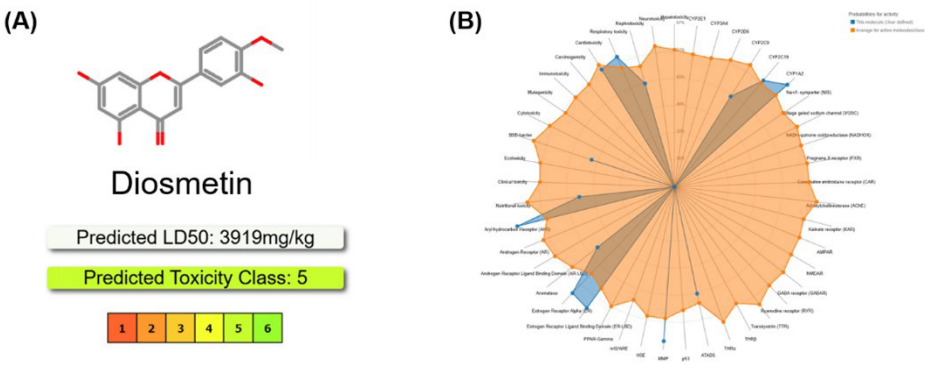
RESULTS

In Silico Toxicity Prediction of Diosmetin

ProTox analysis predicted an oral LD_{50} of 3919 mg/kg for diosmetin, corresponding to Toxicity Class V (Figure 1A). The toxicity profile did not show a prominent predicted cytotoxicity signal across the evaluated endpoints (Figure 1B). These computational findings indicated a relatively low predicted acute oral toxicity and were used as preliminary background information for subsequent experiments. However, this prediction reflects acute oral toxicity and should not be interpreted as direct evidence of safety following the intraperitoneal administration used in the present study.

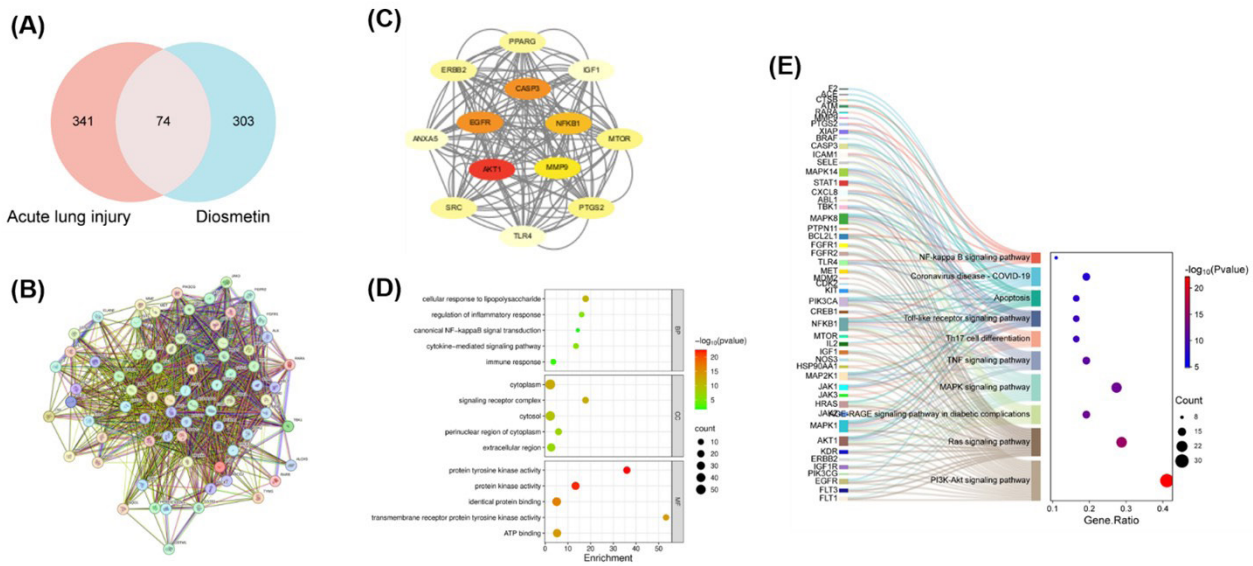
Network Pharmacology Identifies Potential Diosmetin Targets Related to ALI

Comparison of diosmetin-related and ALI-associated targets identified 74 overlapping genes (Figure 2A). These shared targets were used to construct a PPI network (Figure 2B). Degree-based ranking identified *AKT1, EGFR, CASP3, NFKB1, MMP9, MTOR, TLR4, PTGS2, SRC, PPARG, ERBB2, IGF1*, and *ANXA5* among the more highly connected nodes (Figure 2C). GO biological process analysis showed enrichment in terms related to cellular responses to LPS, inflammatory regulation, canonical NF-κB signaling, cytokine-mediated signaling, and immune responses. Enriched cellular component terms included the cytoplasm, signaling receptor complex, cytosol, perinuclear region, and extracellular region, while molecular function analysis highlighted protein kinase activity, protein tyrosine kinase activity, protein binding, receptor tyrosine kinase activity, and ATP binding (Figure 2D). KEGG analysis identified several inflammation- and injury-associated pathways, including PI3K-Akt, Ras, AGE-RAGE, MAPK, TNF, Toll-like receptor, NF-κB, apoptosis, and Th17 cell differentiation pathways (Figure 2E).



圖一　Diosmetin 的電腦模擬毒性評估。

Figure 1. In silico toxicity assessment of diosmetin. (A) Predicted acute oral toxicity of diosmetin, with an estimated LD_{50} of 3919 mg/kg and classification as Toxicity Class V. (B) Predicted toxicity profile across toxicological endpoints and toxicity-associated signaling pathways.

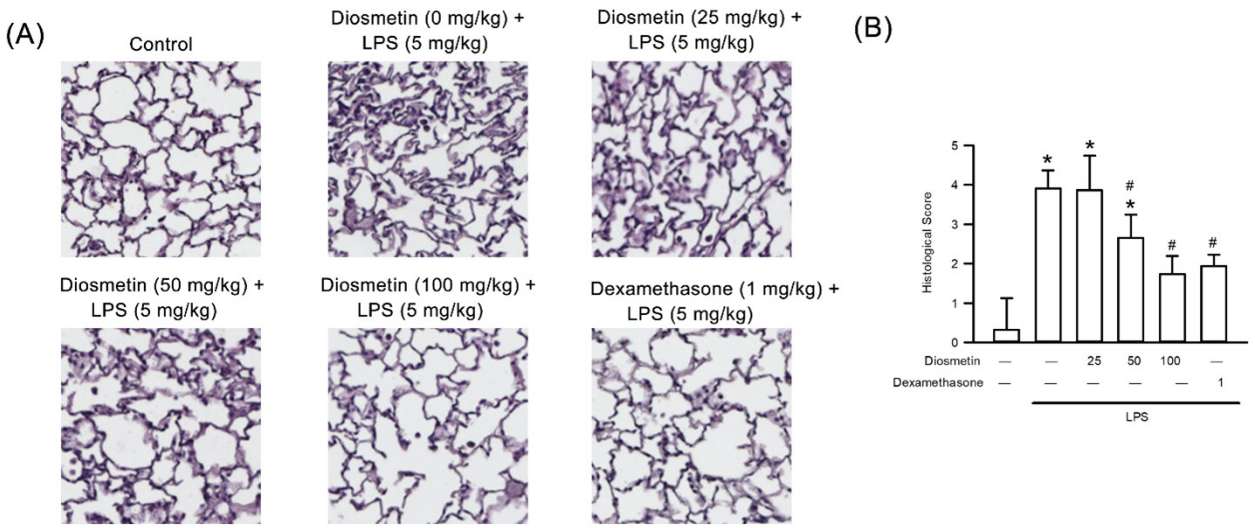


圖二　Diosmetin 對抗急性肺損傷之網絡藥理學分析。

Figure 2. Network pharmacology analysis of diosmetin against ALI. (A) Venn diagram showing 74 shared targets between diosmetin and ALI. (B) Protein–protein interaction network. (C) Hub targets ranked according to degree. (D) Gene Ontology enrichment analysis, including biological process, cellular component, and molecular function. (E) KEGG pathway enrichment analysis.

Diosmetin Attenuates LPS-Induced Histopathological Lung Injury

Control lungs showed preserved alveolar architecture and thin alveolar septa (Figure 3A). In contrast, LPS caused marked alveolar wall thickening, narrowing of alveolar spaces, and inflammatory cell accumulation. Little morphological improvement was apparent with 25 mg/kg diosmetin. At 50 mg/kg, alveolar architecture was better preserved and inflammatory cell accumulation was reduced. The improvement was more evident with 100 mg/kg diosmetin, whereas dexamethasone also attenuated the histopathological alterations induced by LPS. Semiquantitative histopathological analysis further showed that LPS significantly increased lung injury scores compared with the control group (Figure 3B). Diosmetin at 25



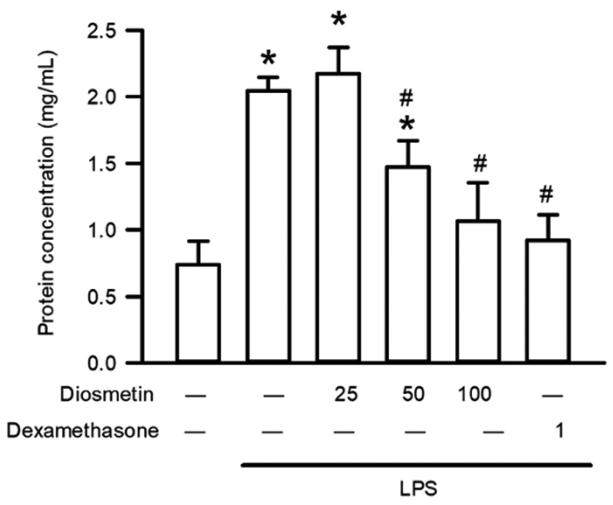
圖三　Diosmetin 對 LPS 誘導小鼠肺組織病理損傷之影響。

Figure 3. Effects of diosmetin on LPS-induced histopathological lung injury in mice. (A) Representative H&E-stained lung sections from control mice and mice challenged with LPS (5 mg/kg) in the absence or presence of diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). (B) Semiquantitative histopathological lung injury scores. Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

mg/kg did not significantly reduce lung injury scores, whereas 50 and 100 mg/kg significantly decreased the scores compared with the LPS group. Dexamethasone treatment also significantly reduced histopathological scores.

Diosmetin Reduces LPS-Induced Alveolar Barrier Disruption

The protein concentration in BALF was measured as an indicator of LPS-induced disruption of the alveolar–capillary barrier (Figure 4). LPS challenge markedly increased BALF protein levels compared with the control group, indicating increased pulmonary permeability. Treatment with 25 mg/kg diosmetin did not significantly alter the elevated BALF protein concentration. At 50 mg/kg, diosmetin significantly reduced BALF protein levels compared with the LPS group, although the value remained higher than that of the control group. A further reduction was observed at 100 mg/kg, at which BALF protein concentration was no longer significantly different from the control level. Dexamethasone treatment also significantly reduced BALF protein accumulation. These findings indicate that diosmetin attenuates LPS-induced alveolar barrier disruption, with a clearer protective effect observed at 50 and 100 mg/kg.



圖四　Diosmetin 對 LPS 誘導肺泡屏障破壞之影響。

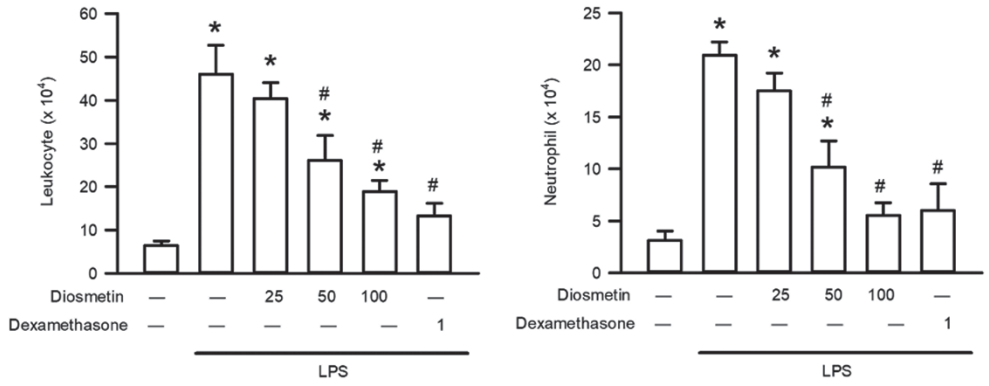
Figure 4. Effects of diosmetin on LPS-induced alveolar barrier disruption. BALF protein concentration was used as an index of pulmonary barrier permeability. Mice challenged with LPS (5 mg/kg) were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

Diosmetin Reduces Pulmonary Leukocyte and Neutrophil Recruitment

LPS challenge markedly increased the numbers of leukocytes and neutrophils in BALF compared with the control group (Figure 5). Treatment with 25 mg/kg diosmetin produced only a modest reduction in both cell populations, with no significant difference from the LPS group. In contrast, 50 mg/kg diosmetin significantly decreased both leukocyte and neutrophil counts, although the values remained higher than those observed in the control group. At 100 mg/kg, diosmetin further reduced inflammatory cell accumulation. Total leukocyte counts remained significantly above the control level, whereas neutrophil counts were no longer significantly different from those of the control group. Dexamethasone treatment also significantly reduced LPS-induced leukocyte and neutrophil accumulation. These results indicate that diosmetin limits LPS-induced pulmonary inflammatory cell recruitment, with greater effects observed at 50 and 100 mg/kg.

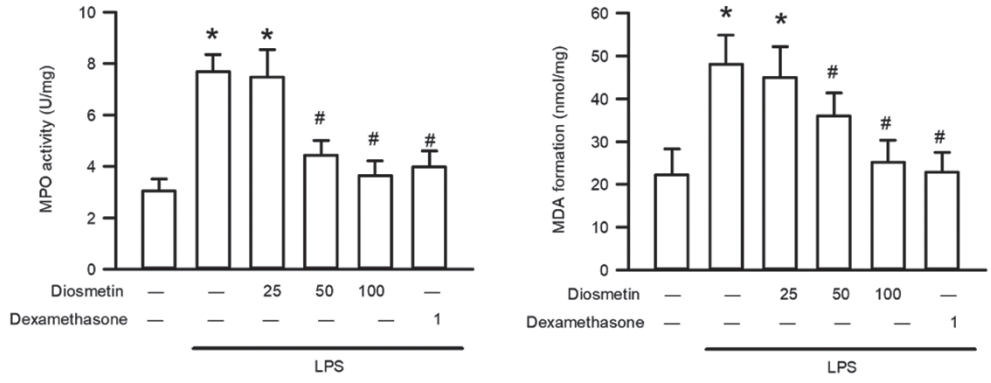
Diosmetin Suppresses LPS-Induced Pulmonary MPO Activity and lipid peroxidation

Pulmonary MPO activity and MDA formation were examined to assess neutrophil-associated inflammatory activity and lipid peroxidation, respectively (Figure 6). LPS challenge markedly increased both MPO activity and MDA levels compared with the control group. Treatment with 25 mg/kg diosmetin did not significantly alter either parameter, with values remaining comparable to those observed in the LPS group. In contrast, 50 mg/kg diosmetin significantly reduced MPO activity and MDA formation, and neither value was significantly different from the control



圖五　Diosmetin 對 LPS 誘導肺部白血球浸潤之影響。

Figure 5. Effects of diosmetin on LPS-induced pulmonary leukocyte infiltration. Total leukocyte and neutrophil numbers were determined in BALF following LPS challenge. Mice were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.



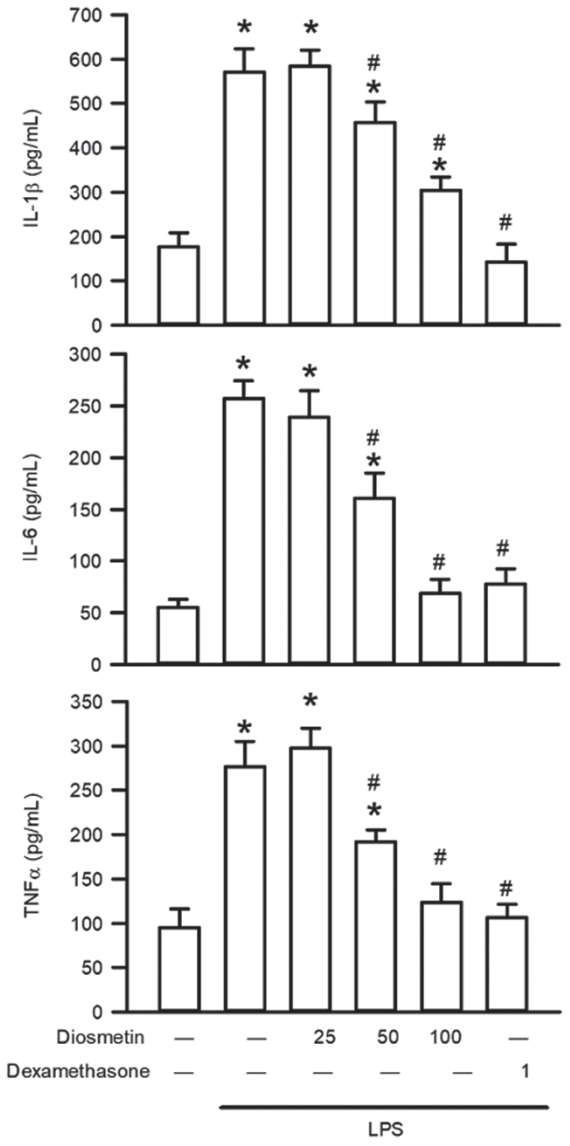
圖六　Diosmetin 對 LPS 誘導急性肺損傷中肺部髓過氧化酶活性及脂質過氧化之影響。

Figure 6. Effects of diosmetin on pulmonary myeloperoxidase activity and lipid peroxidation in LPS-induced ALI. MPO activity and MDA formation were measured in lung tissue as indices of neutrophil-associated inflammatory activity and lipid peroxidation, respectively. Mice challenged with LPS (5 mg/kg) were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

level. Treatment with 100 mg/kg produced a further numerical reduction, with both parameters remaining close to those of the control group. Dexamethasone suppressed the LPS-induced increases in MPO activity and MDA formation. These results show that diosmetin, particularly at 50 and 100 mg/kg, reduces neutrophil-associated inflammatory activity and lipid peroxidation in LPS-challenged lungs.

Diosmetin Reduces BALF Pro-Inflammatory Cytokine Concentrations

LPS challenge markedly increased pulmonary levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 compared with the control group (Figure 7). Treatment with 25 mg/kg diosmetin did not significantly reduce any of the three cytokines, with levels remaining comparable to those observed in the LPS group. In contrast, 50 mg/kg diosmetin significantly decreased TNF-α, IL-1β, and IL-6, although their levels remained higher than those in the control group. At 100 mg/kg, a further reduction was observed. TNF-α and IL-6 levels were no longer significantly different from the control values, whereas IL-1β remained elevated despite a significant decrease relative to the LPS group. Dexamethasone suppressed



圖七　Diosmetin 對 LPS 誘導急性肺損傷中促發炎細胞激素生成之影響。

Figure 7. Effects of diosmetin on pro-inflammatory cytokine production in LPS-induced ALI. Pulmonary levels of TNF-α, IL-6, and IL-1β were measured following LPS challenge. Mice were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

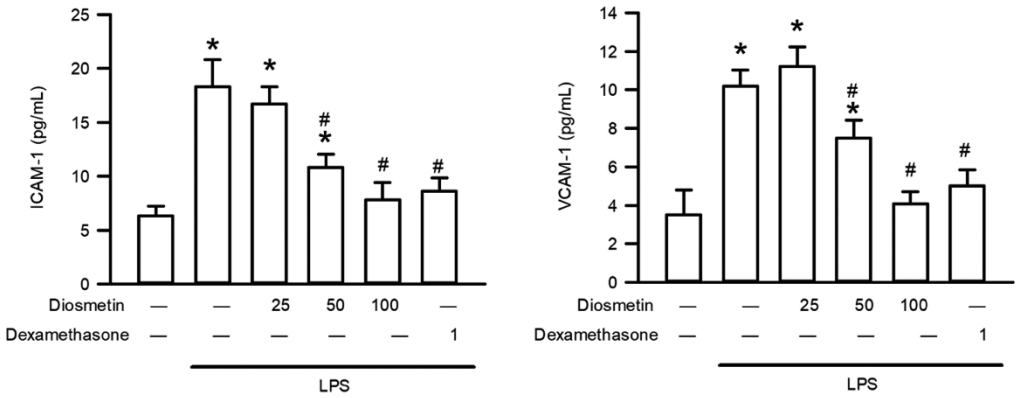
all three cytokines to levels comparable to those of the control group. These findings indicate that diosmetin attenuates the LPS-induced pulmonary inflammatory response, with more pronounced effects at 50 and 100 mg/kg.

Diosmetin Reduces Soluble ICAM-1 and VCAM-1 Concentrations in BALF Following LPS Challenge

LPS challenge markedly increased pulmonary ICAM-1 and VCAM-1 levels compared with the control group (Figure 8). Treatment with 25 mg/kg diosmetin did not significantly reduce either adhesion molecule, and both remained elevated relative to the control group. In contrast, 50 mg/kg diosmetin significantly decreased ICAM-1 and VCAM-1 levels compared with the LPS group, although both remained significantly higher than the corresponding control values. At 100 mg/kg, diosmetin further reduced ICAM-1 and VCAM-1, with neither level differing significantly from that of the control group. Dexamethasone suppressed both adhesion molecules to levels comparable to those of the control group. These findings indicate that diosmetin suppresses LPS-induced increases in pulmonary ICAM-1 and VCAM-1, with clear effects observed at 50 and 100 mg/kg.

Diosmetin Preserves Pulmonary Antioxidant Enzyme Activities in LPS-Induced ALI

LPS challenge markedly impaired pulmonary antioxidant status, as reflected by significant reductions in SOD activity, catalase activity, and GSH content compared with the control group (Figure 9). Treatment with 25 mg/kg diosmetin produced only minor numerical increases in these parameters, with no significant differences from the LPS group. At 50 mg/kg, diosmetin significantly increased SOD and catalase activities as well as GSH content. Although SOD activity remained significantly lower than that of the control group, catalase activity and GSH content were no longer significantly different from control values. Treatment with 100 mg/kg resulted in a further recovery of all three antioxidant indices, with SOD activity, catalase activity, and GSH content reaching levels comparable to those of the control group. Dexamethasone preserved pulmonary antioxidant status. These findings indicate that diosmetin counteracts the



圖八　Diosmetin 對 LPS 誘導急性肺損傷中肺泡沖出液中 ICAM-1 與 VCAM-1 表現量之影響。

Figure 8. Effects of diosmetin on soluble ICAM-1 and VCAM-1 concentrations in BALF following LPS challenge. The concentrations of ICAM-1 and VCAM-1 in BALF were measured following LPS challenge. Mice were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n=6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

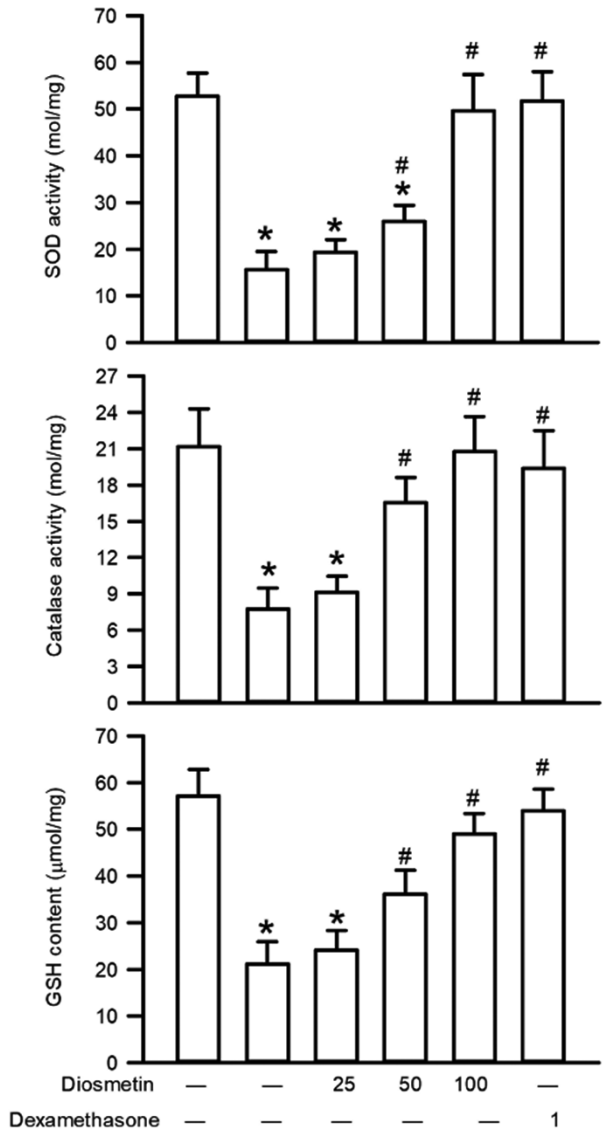
LPS-induced loss of pulmonary antioxidant capacity, with clear protective effects at 50 and 100 mg/kg.

DISCUSSION

The present study studied the protective effects of diosmetin in LPS-induced ALI, with particular attention to leukocytes and neutrophils recruitment, adhesion molecule-associated responses, and pulmonary redox status. LPS challenge produced typical features of ALI, including disruption of alveolar structure, increased BALF protein concentration, accumulation of leukocytes and neutrophils, enhanced MPO activity and lipid peroxidation, and increased concentrations of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. These changes were accompanied by higher ICAM-1 and VCAM-1 levels and reductions in SOD and catalase activities and GSH content. Diosmetin at 25 mg/kg had little influence on most of these responses, whereas significant protection became evident at 50 mg/kg and was generally more pronounced at 100 mg/kg. The overall findings indicate that diosmetin affects several interacting components of LPS-induced ALI rather than the single inflammatory endpoint.

The in silico toxicity analysis provided preliminary information on the predicted safety profile of diosmetin. ProTox 3.0 estimated an oral LD_{50} of 3919 mg/kg and classified diosmetin as Toxicity Class V [13]. This prediction is consistent with a relatively low potential for acute oral toxicity. However, it should not be considered direct evidence of safety under the present study. Diosmetin was administered intraperitoneally at 25-100 mg/kg, whereas the ProTox estimate refers to predicted oral toxicity. Route-dependent differences in absorption, metabolism, systemic exposure, and organ distribution may substantially influence toxicity. Thus, the in silico analysis is useful as supportive background information, but dedicated toxicological and pharmacokinetic studies are still required before the safety of diosmetin can be defined more precisely. The ProTox result in the present manuscript is therefore best interpreted as a preliminary toxicity prediction rather than experimental confirmation of safety.

Network pharmacology was used to explore possible biological processes associated with the protective effects of diosmetin. Seventy-four targets were shared between predicted diosmetin targets and ALI-associated genes. Among the more highly connected targets were *AKT1, EGFR, CASP3, NFKB1, MMP9, MTOR, TLR4, PTGS2, SRC, PPARG, ERBB2, IGF1*, and *ANXA5*. Functional enrichment further highlighted the PI3K-Akt, NF-κB, MAPK, TNF, Toll-like receptor, Ras, and apoptosis-related pathways. These pathways are relevant to inflammatory



圖九　Diosmetin 對 LPS 誘導急性肺損傷中肺部抗氧化能力之影響。

Figure 9. Effects of diosmetin on pulmonary antioxidant capacity in LPS-induced ALI. SOD activity, catalase activity, and GSH content were measured in lung tissue to evaluate pulmonary antioxidant status. Mice challenged with LPS (5 mg/kg) were treated with diosmetin (25, 50, or 100 mg/kg) or dexamethasone (1 mg/kg). Data are presented as mean ± SD (n = 6 mice per group). *P < 0.05 versus the control group; #P < 0.05 versus the LPS group.

signaling, cell survival, and tissue injury and are therefore broadly consistent with the inflammatory and oxidative changes observed in the animal experiments. The network results also support the possibility that diosmetin acts through several interconnected targets rather than a single molecular pathway, a pattern often encountered with natural products [11,12].

Disruption of the alveolar–capillary barrier is a central feature of ALI and promotes leakage of plasma proteins and fluid into the alveolar compartment [1,2,3]. In the present model, LPS caused marked alterations in alveolar morphology and substantially increased BALF protein concentration. These changes were attenuated by diosmetin, particularly at 50 and 100 mg/kg. At 100 mg/kg, BALF protein concentration was no longer significantly different from the control level. Previous studies have similarly shown that diosmetin reduces pulmonary edema, inflammatory cell accumulation, BALF protein leakage, and histopathological injury in LPS-induced ALI [9]. Protection of pulmonary barrier integrity and preservation of junctional proteins, including ZO-1 and occludin, have also been associated with modulation of RhoA/ROCK signaling [10]. The current findings are consistent with these earlier observations and further show that improvement in barrier-related injury occurs together with changes in inflammatory cell recruitment and adhesion molecule-associated responses.

Recruitment of neutrophils is particularly important during the early phase of LPS-induced ALI. Activated neutrophils release ROS, proteases, and MPO-derived oxidants, all of which can amplify pulmonary inflammation and tissue injury [4]. In the present study, increases in BALF leukocytes and neutrophils were accompanied by elevated pulmonary MPO activity. Diosmetin at 50 and 100 mg/kg reduced both inflammatory cell accumulation and MPO activity, whereas 25 mg/kg produced little measurable effect. The agreement between BALF cellular findings and tissue MPO activity strengthens the interpretation that diosmetin attenuates neutrophil-associated inflammatory activity rather than merely altering a single inflammatory marker.

Changes in pro-inflammatory cytokines showed a similar treatment pattern. LPS markedly increased TNF-α, IL-1β, and IL-6, while 50 and 100 mg/kg diosmetin significantly reduced all three mediators. The

response, however, was not identical for each cytokine. At 100 mg/kg, TNF-α and IL-6 were reduced to values that were not significantly different from those of the control group, whereas IL-1β remained elevated despite a clear reduction relative to the LPS group. This difference suggests that the anti-inflammatory action of diosmetin is not uniform across all inflammatory mediators. It is therefore more appropriate to describe the effect as an attenuation of the overall inflammatory response rather than complete suppression of LPS-induced cytokine signaling. Previous experimental evidence showing reduced cytokine production and inflammatory cell infiltration after diosmetin treatment is consistent with this interpretation [9].

An important observation in this study was the reduction in ICAM-1 and VCAM-1. During inflammatory lung injury, activation of the vascular endothelium is accompanied by increased production and expression of adhesion molecules that facilitate leukocyte attachment and migration across the vascular wall [3]. LPS markedly increased ICAM-1 and VCAM-1 levels, whereas diosmetin significantly reduced both markers at 50 and 100 mg/kg. At 100 mg/kg, their levels were no longer significantly different from those of the control group. These changes occurred in parallel with lower BALF leukocyte and neutrophil numbers. The correspondence between the adhesion molecule measurements and cellular infiltration therefore supports an association between attenuation of adhesion molecule-associated inflammatory responses and reduced leukocyte recruitment. This finding provides an additional perspective on the pulmonary effects of diosmetin. Previous ALI studies have focused mainly on Nrf2/NLRP3-associated inflammatory and antioxidant signaling [9] or preservation of endothelial barrier integrity through pathways associated with RhoA/ROCK [10]. The present study further links changes in ICAM-1 and VCAM-1 with leukocyte recruitment, MPO activity, inflammatory cytokines, and barrier injury within the same experimental setting. However, ICAM-1 and VCAM-1 were measured as soluble proteins in BALF rather than as endothelial cell-surface proteins. Because endothelial localization and leukocyte transmigration were not directly assessed, these findings support an association with reduced inflammatory cell recruitment rather than demonstrating a direct effect of diosmetin on endothelial adhesion molecule expression or function.

Oxidative imbalance was another prominent feature of the response to LPS. Pulmonary MDA formation increased substantially, whereas SOD and catalase activities and GSH content declined. This combination is consistent with increased lipid peroxidation together with impairment of endogenous antioxidant defenses, both of which can contribute to pulmonary endothelial and epithelial dysfunction [5]. Diosmetin significantly improved these indices at 50 and 100 mg/kg. At 100 mg/kg, SOD and catalase activities and GSH content were comparable to control values, while MDA remained markedly reduced. Previous endothelial-cell studies have shown that diosmetin can decrease oxidative injury and improve cellular antioxidant capacity [8]. Experimental ALI studies have also associated diosmetin treatment with increased Nrf2/HO-1 signaling and reduced oxidative damage [9]. The current findings therefore agree with the antioxidant profile previously reported for diosmetin. Although Nrf2-related signaling was not examined directly in the present study, network pharmacology identified several potentially relevant pathways, including PI3K-Akt, NF-κB, MAPK, TNF, and Toll-like receptor signaling. Their mechanistic involvement, however, requires direct experimental validation.

Inflammation and oxidative stress are closely interconnected during ALI, as activated leukocytes generate ROS and MPO-derived oxidants that can further aggravate pulmonary injury [3-5]. In the present study, the concurrent reductions in inflammatory cell recruitment, MPO activity, cytokines, adhesion molecules, and lipid peroxidation, together with recovery of antioxidant defenses, suggest that diosmetin attenuates both inflammatory and oxidative injury in LPS-exposed lungs. A relatively consistent treatment

pattern was observed across the three doses. Diosmetin at 25 mg/kg had little effect on most endpoints, whereas significant protection became evident at 50 mg/kg and was generally more pronounced at 100 mg/kg. However, because plasma and lung concentrations of diosmetin were not determined, the present findings should not be interpreted as a formal pharmacological dose–response relationship.

The occurrence of diosmetin and related glycosides such as diosmin and neodiosmin in Citrus species provides a nutritional context for the present findings [6]. However, the experimental conditions used here differ substantially from normal dietary exposure. Diosmetin was administered intraperitoneally before LPS challenge, thereby bypassing intestinal absorption and first-pass metabolism. Following oral intake, bioavailability, metabolic conversion, and circulating metabolites may substantially influence systemic exposure. Therefore, the present findings support the biological activity of a citrus-derived flavonoid but should not be interpreted as evidence that ordinary citrus consumption provides comparable protection against ALI. Further studies using oral administration and pharmacokinetic analysis are needed to clarify its nutritional relevance.

Several limitations should be acknowledged. Diosmetin was administered before LPS challenge and evaluated only at an acute time point, so the present study mainly reflects preventive effects rather than treatment of established ALI. ICAM-1 and VCAM-1 were not localized specifically to pulmonary endothelial cells, and endothelial functional assays were not performed. In addition, the signaling mechanisms underlying the antioxidant effects and the targets predicted by network pharmacology were not experimentally validated. Finally, the in silico toxicity analysis provides only preliminary information and cannot replace dedicated toxicological or pharmacokinetic studies. Further studies using post-injury treatment, oral administration, endothelial-specific analyses, and molecular validation are needed to strengthen the mechanistic and translational relevance of these findings.

In conclusion, diosmetin attenuated LPS-induced ALI, with the clearest protective effects observed at 50 and 100 mg/kg. These effects were accompanied by preservation of pulmonary morphology and alveolar–capillary barrier integrity, reduced inflammatory cell recruitment, lower MPO activity and cytokine levels, decreased ICAM-1 and VCAM-1, reduced lipid peroxidation, and improved pulmonary antioxidant capacity. The combined experimental and network pharmacology findings suggest that diosmetin acts through multiple inflammatory and redox-related processes rather than a single dominant mechanism. The parallel reductions in adhesion molecule-associated responses and inflammatory cell recruitment further support its potential role in limiting pulmonary inflammation. These findings warrant further investigation of diosmetin as a citrus-derived bioactive compound in inflammation-associated lung injury.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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柑橘來源黃酮類化合物 Diosmetin 減輕脂多醣誘發之急性肺損傷：抗氧化防禦與黏附分子相關發炎反應之調控

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摘要 急性肺損傷（acute lung injury, ALI）的病理特徵包括發炎細胞募集、肺泡 – 微血管屏障破壞及氧化性損傷。Diosmetin 為存在於柑橘類食物中的黃酮類化合物，具有抗發炎與抗氧化活性，但其在 ALI 中與黏附分子相關發炎反應之關係仍未完全釐清。本研究結合網路藥理學與脂多醣（lipopolysaccharide, LPS）誘發之小鼠 ALI 模型，探討 diosmetin 的保護作用。首先分析 diosmetin 潛在作用標的與 ALI 相關基因，以篩選共同標的及其富集路徑。BALB/c 小鼠於氣管內給予 LPS（5 mg/kg）前，分別接受 diosmetin（25、50 或 100 mg/kg）或 dexamethasone（1 mg/kg）處理。實驗評估肺組織病理變化、支氣管肺泡灌洗液（bronchoalveolar lavage fluid, BALF）蛋白濃度、白血球與嗜中性球募集、髓過氧化酶（myeloperoxidase, MPO）活性、脂質過氧化、發炎細胞激素、ICAM-1 與 VCAM-1，以及肺部抗氧化狀態。網路分析共鑑定出 74 個 diosmetin 與 ALI 的共同標的，並顯示多條發炎相關路徑富集，包括 PI3K-Akt、NF-κB、MAPK、TNF 及 Toll-like receptor 訊息傳遞路徑。LPS 刺激造成明顯肺組織損傷，並增加 BALF 蛋白濃度、發炎細胞累積、MPO 活性、脂質過氧化，以及 TNF-α、IL-1β、IL-6、ICAM-1 與 VCAM-1 水平，同時降低 SOD 與 catalase 活性及 GSH 含量。Diosmetin 25 mg/kg 對多數指標的影響有限，而自 50 mg/kg 起即出現顯著保護作用，且於 100 mg/kg 時整體效果更為明顯。整體而言，diosmetin 可降低發炎細胞募集及黏附分子相關反應，同時減輕氧化性損傷並維持肺部抗氧化能力。本研究結果支持進一步探討 diosmetin 作為柑橘來源生物活性成分應用於發炎相關肺損傷之可能性。

關鍵詞：Diosmetin、**急性肺損傷、柑橘類黃酮、黏附分子、氧化壓力、網路藥理學**

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