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Exploring the Effect and Mechanism of DaYuan Yin Against Acute Lung Injury by Network Pharmacology, Molecular Docking, and Experimental Validation

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Background: DaYuan Yin (DYY), a traditional Chinese medicine for lung diseases, requires further study to understand how it improves acute lung injury (ALI). This study seeks to elucidate the material basis and molecular mechanisms underlying the treatment of ALI with DYY through network pharmacology, molecular docking, and experimental validation.

Methods: DYY's active components and targets were identified using TCMSP and UHPLC-MS/MS, and a herb-component-target network was created with Cytoscape 3.7.2. ALI target genes were sourced from GeneCards, DisGeNET, and DrugBank. A PPI network was built, with core targets analyzed through GO and KEGG enrichment via Metscape. The therapeutic effects and mechanisms of DYY on LPS-induced ALI in rats were explored, and molecular docking evaluated the interactions between Nrf2, HO-1, TLR4, and the components.

Results: The study identified 95 active compounds, 234 therapeutic targets, and 2529 ALI-related genes, with 111 shared targets between DYY and ALI. KEGG analysis indicates that the PI3K-AKT, MAPK, and oxidative stress pathways are associated with DYY's anti-ALI effects. Network pharmacology and UHPLC-MS/MS analysis revealed active ingredients like quercetin, Magnolol, and Wogonin. Compared with the model group, DYY reduced the lung dry–wet ratio (W/D) of ALI rats from (5.31 ± 0.51) to (4.47 ± 0.73)($P < 0.05$). Meanwhile, the contents of IL-6 and TNF-α in bronchoalveolar lavage fluid (BALF) and MDA, NO and ROS in lung tissue were also significantly decreased. Notably, DYY enhances UCP2 mRNA expression, boosts Nrf2 and HO-1 expression, and inhibits TLR4-mediated pro-inflammatory mediators. Molecular docking analysis showed that the main components of DYY had strong binding ability with HO-1.

Conclusion: DYY can alleviate inflammation, oxidative stress, and ALI-related changes by targeting the Nrf2/HO-1 mediated TLR4 pathway, providing insights for developing effective ALI treatments.

Keywords: DaYuan Yin, Acute lung injury, Oxidative stress, Anti-inflammatory, Network pharmacology

Introduction

ALI is a life-threatening disease with a US incidence rate of 78.9 per 100,000 people annually for those over 15, and a high fatality rate of 30–40%, this highlights the critical need for thorough research on ALI.[1,2] Current treatments, such as supportive mechanical ventilation and drugs like glucocorticoids or antibiotics, have limited effectiveness, and even lead to permanent damage.[3,4] Therefore, the overall synergy of TCM may bring a better choice for the treatment of ALI.

In ALI, severe lung inflammation disrupts endothelial barrier integrity, resulting in pulmonary edema. Additionally, infection-induced inflammatory infiltration and pro-inflammatory cytokine production cause endothelial cell death, further compromising the lung endothelial barrier.[5] It has been confirmed that the complex pathogenesis of ALI involves uncontrolled pulmonary inflammation,[6,7] driven by cytokines like TNF-α, IL-1β, and IL-6.[8] Excessive inflammation leads to neutrophil infiltration in lung tissue, lung cell injury, alveolar-capillary permeability increase, and impaired gas exchange.[9] Furthermore, ferroptosis may play a role in the onset and progression of ALI. This mode of non-apoptotic cell death is primarily attributed to the accumulation of lipid reactive oxygen species (ROS) induced by iron, which results in

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cellular peroxidation and significantly impacts various physiological processes, particularly inflammation.[10,11] Phagocytes generate ROS to combat microorganisms and inflammation, but regulation is crucial to avoid tissue damage.[12] Excessive ROS can increase endothelial permeability, promoting inflammatory cell migration and resulting in oxidative stress and acute inflammation.[13,14] It can be seen that blocking oxidative stress and inflammation of the lung may be a potential strategy for the treatment of ALI.

DYY is a classic prescription used by Wu Youke, a famous Chinese doctor in the early Qing Dynasty, to treat infectious diseases.[15] It can effectively reduce the viral load and inflammatory factors such as IL-6, IL-1β and TNF-α in the lung tissue during the occurrence of viral pneumonia, and play a significant antiviral and immunomodulatory role.[16] The whole prescription is composed of seven traditional Chinese medicines: Radix Paeoniae Alba, betel nut, licorice, *Scutellaria baicalensis*, *Magnolia officinalis* and *Anemarrhena asphodeloides* (Table 1).[17] Recent pharmacological research has demonstrated that DYY can regulate respiratory tract infections,[18] decrease inflammatory factors, and protect lung tissue.[19] Magnolol and honokiol,[20,21] and licorice flavonoids,[22] the main active ingredients in DYY, also reduce inflammation and enhance antioxidation. However, the effectiveness and mechanism of DYY for ALI require further investigation.

Network pharmacology helps to understand how traditional Chinese medicine compounds work in treating diseases by analyzing their effects and mechanisms.[23] By studying the interaction network of multiple genes, targets, and pathways, we can predict the potential targets and mechanisms of traditional Chinese medicine in treating different diseases.[24] In this study, in order to clarify the potential anti-inflammatory and antioxidant mechanism of DYY in the treatment of ALI, we adopted the method of network pharmacology combined with in vivo experiments and molecular docking.

Materials and Methods

Study on Network Pharmacology of DYY Anti-ALI

DYY Herbal Compounds and Their Targets

The active components and target proteins of DYY are derived from traditional Chinese medicine systems pharmacology (TCMSP, https://old.tcmsp-e.com/tcmsp.php), with the conditions of oral bioavailability (OB) ≥30%, caco-2 permeability (Caco-2) ≥ −0.4, blood–brain barrier (BBB) ≥−0.3, drug half-life (HL) ≥4 h and drug likeness (DL) ≥0.18,[25,26] the active compounds and their protein targets were obtained. The target proteins were limited to humans, and unified in UniProt protein database (https://www.uniprot.org/) standardize the specification.[27] Cytoscape 3.7.2 was used to create the "herb-active ingredients-targets" network.[28]

Search of ALI-Related Genes

With "acute lung injury" as the keyword, the duplicates were removed from GeneCards (https://www.genecards.org/), (value ≥ median), DisGeNET (https://www.disgenet.org) and OMIM (https://www.omim.org/)databases, and "Homo sapiens" was selected as the species.

Table 1 Detailed Information of Herbs in DYY

Pharmaceutical name	Botanical plant name	Chinese name	Weight (g)	Part(s) used
Arecae Semen	*Areca catechu* L.	Binglang	7.46	Seed
Magnolia Officinalis	*Magnolia officinalis Rehd.et* Wils.	Houpo	3.73	Barks
Amomum tsao-ko	*Amomum tsao-ko Crevost et* Lemaire.	Caoguo	1.87	Fruit
Anemarrhena asphodeloides	*Anemarrhena asphodeloides* Bunge	Zhimu	3.73	Rhizome
Radix paeoniae Alba	*Paeonia lactiflora* Pall.	Baishao	3.73	Roots
Scutellaria Baicalensis	*Scutellaria baicalensis* Georgi	Huangqin	3.73	Roots
Glycyrrhiza glabra	*Glycyrrhiza uralensis* Fisch.	Gancao	1.87	Roots and rhizomes

Construction of Protein–Protein Interaction (PPI) Network and Selection of Key Targets

Venn diagram (https://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify common target genes for DYY and ALI, which were then analyzed in STRING12.0 (https://cn.string-db.org) with a minimum network interaction score confidence of 0.7,[29] which indicates that the PPI network is composed of proteins interacting with each other to participate in various aspects of life processes such as biological signal transmission, gene expression regulation, energy and material metabolism, and cell cycle regulation. After removing free nodes for visualization, the target's network topology parameters were analyzed using CytoNCA, including degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC). The central target of DYY was selected according to the degree value greater than the respective median (DC > 13, BC > 0.003, CC > 0.45).[30] Filter the PPI network using the MCODE plug-in in Cytoscape using various cutoff values: degree = 2, k-core = 2, node score −0.2, maximum depth = 100.[31]

Cytoscape Gene Ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) Enrichment Analysis

Enrichment analysis extends beyond single-gene annotation to analyze groups of genes, as biological processes often involve multiple genes. Initially, various methods identify numerous relevant genes, such as differentially expressed or co-expressed gene sets. Then, significant enrichment analysis helps interpret the biological roles of gene sets and reveal what role they play inside or outside the cell. Using Metascape, GO and KEGG pathway enrichment analyses were conducted on DYY and ALI targets (https://metascape.org/).[32] Terms with a P-value under 0.05, including the top 20 for biological process (BP), cellular component (CC), and molecular function (MF), and the top 30 KEGG terms, were visualized on a bioinformatics platform to identify key molecular biology processes, significantly enriched GO nodes or KEGG pathways within these gene sets are identified, facilitating more detailed experimental studies.

Molecular Docking Verification

We identified active ingredients associated with ALI using "herb-active ingredients-targets" network and UHPLC-MS/MS results. The structures of these ingredients were downloaded from the TCMSP database in 2D and SDF formats. Using chem 3D software, we converted the structures to 3D structure in mol2 format. The 3D structures of nuclear factor erythrocyte 2-associated factor 2 (Nrf2), Heme Oxygenase-1 (HO-1), and Toll-like receptor 4 (TLR4) were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/), and the crystallographic structures of targets were prepared for dehydration and hydrotreatment before using Autodock 1.5.7 (http://autodock.scripps.edu/) for molecular docking.[33] Autodock Vina 1.1.2 (http://vina.scripps.edu/) was selected to calculate binding energy between active ingredients and proteins. A binding energy ≤−5.0 kJ/mol is considered a standard for good binding efficiency.[34] Results were visualized using Pymol and LigPlot.[35]

Preparation and Quality Control of DYY

Materials and Reagents

Arecae Semen (Binglang), Magnolia Officinalis (Houpo), Amomum tsao-ko (Caoguo), Anemarrhena asphodeloides (Zhimu), Radix paeoniae Alba (Baishao), Scutellaria Baicalensis (Huangqin), Glycyrrhiza glabra (Gancao) were purchased from Suzhou Tianling traditional Chinese Medicine Co., Ltd. and identified by Wu Xuerong, a pharmaceutical expert in Kunshan Hospital of Traditional Chinese Medicine. The criteria for the quality of the herbs were in accordance with the 2020 Chinese pharmacopoeia.[36] Methanol and acetonitrile were purchased from EMD Millipore Corporation (Germany), and formic acid from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China), and all chemicals and solvents were analytical reagent or chromatographic grade. Ultra-pure water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Extraction for DYY Testing Sample

According to the proportion of DYY in Table 1, all the herbs were soaked in water with 10 times the amount of raw medicine for 30 minutes, and then extracted twice with electric heating sleeve for 1 hour each time.[37] The extract was mixed and concentrated to about 1g/kg.

UHPLC-MS Analysis

Instruments

Vanquish Ultra performance liquid chromatograph and QE Ultra Resolution Mass Spectrometer (Thermo Fisher Scientific, USA), ACQUITY UHPLC HSST3 (100 mm × 2.1 mm, 1.8 μm) column (Waters, USA), 5430R table-top high-speed refrigerated centrifuge (Shanghai Eppendorf, China), KQ-2200E ultrasonic cleaning machine (Kunshan Ultrasonic instrument Co., Ltd., China).

Sample Treatment

Took 1mL sample, added 2 times the volume of methanol-acetonitrile solution (1:1, v/v), vortex for 60s, sonicated for 30 min, and centrifugation for 20 min (12,000 rpm, 4°C), the supernatant was filtered using a 0.22 μm organic filter film, and transferred to insert-equipped vials for LC-MS analysis.

Liquid Chromatography-Mass Spectrometry Conditions

The sample extracts were analyzed using an UHPLC-Orbitrap-MS system (UHPLC, Vanquish; MS, HFX). The analytical conditions were as follows: UHPLC: column, Waters HSS T3(100 × 2.1 mm,1.8μm); column temperature, 40 C; flow rate, 0.3 mL/min; injection volume, 2μL; solvent system, water (0.1% Acetic acid): acetonitrile (0.1% Acetic acid); gradient program,100: 0 V/V at 0–1 min, 5: 95 V/V at 9.0 min, 5: 95 V/V at 9.0–13.0 min, 100: 0 V/V at 13.1–17 min.

HRMS data were recorded on a Q Exactive HFX Hybrid Quadrupole Orbitrap mass spectrometer equipped with a heated ESI source utilizing the Full-msddMS2 MS acquisition methods. The ESI source parameters were set as follows: spray voltage, -2.8 kV/3.0 kV; sheath gas pressure, 40 arb; aux gas pressure, 10 arb; sweep gas pressure, 0 arb; capillary temperature, 320°C; and aux gas heater temperature, 350°C.

The original data were processed by metabolomics software Progenesis QI (Waters, Milford, USA) for baseline filtering, peak identification, integration, retention time correction and peak alignment to obtain a data matrix of retention time, mass-to-charge ratio and peak intensity. The main parameters include: (1) only retain variables with non-zero values of more than 80% in any group of samples; (2) total peak normalization, and then delete variables with relative standard deviation (RSD) ≥30% of QC samples. Finally, Human Metabolome Database (http://www.hmdb.ca/)[38] and METLIN (https://metlin.scripps.edu/)[39] were used for qualitative analysis.

Animal Experiments

Reagents and Instruments

Lipopolysaccharides (LPS), RIPA Cracking Buffer and malondialdehyde (MDA) kit were obtained from Solarbio Science & Technology Co.,Ltd. (Beijing, China). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were supplied by Enzyme-linked Biotechnology Co.,Ltd. (Shanghai, China). Nitric oxide (NO) kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China.) 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) kit was purchased from Biyuntian Biotechnology Co. (Shanghai, China). Trizol kit was obtained from Yeasen Biotechnology Co.,Ltd. (Shanghai, China). BCA kit was provided by Fisher Scientific Inc (Shanghai, China). Uncoupling protein 2 (UCP2) was obtained General biology Co., Ltd (Anhui, China). All antibodies Nrf2, HO-1, and TLR4 were supplied by Proteintech Group, Inc (Wuhan, China).

Animals

SPF male Sprague-Dawley rats (6–8 weeks old) from Pengyue Experimental Animal breeding Co.,Ltd. (Jinan, China) were fed under ventilated and temperature-controlled conditions (humidity of 25.55%, light/dark cycle for 12 hours. All animal procedures were approved by the Animal Experimental Ethics Committee (SWS20240130) and followed the guiding principles of the GB/T35892-2018 Guidelines for Ethical Review of the Welfare of Experimental Animals.[40]

Establishment of ALI Model and Treatment of DYY

After 1 week of domestication, according to complete randomization method, 18 rats were randomly divided into 3 groups (n = 6): Con, LPS, and DYY groups. ALI was induced by a single intraperitoneal injection of 10mg/kg LPS.

DYY (4.7g/kg/d) was given by gavage once a day for 1 week. Con and LPS groups received saline. Rats were euthanized with excessive pentobarbital 12 hours after the last treatment, BALF and lung tissue were collected.

Histopathological Analysis

Left lung tissues of rats were taken, fixed with 10% formalin, then embedded in paraffin wax and made into 5μm thick sections, washed with PBS buffer, stained with hematoxylin and eosin, sealed with neutral gum, and images were captured by microscope (NikonTi, Japan).

Pulmonary Edema Assessment

Extract lung tissue, rinsed with normal saline and remove residual tissue. After absorbing the surface liquid, the wet mass of the lung was obtained, and then dried in an oven at 70°C for 48 hours to obtain the dry weight. The ratio of W/D was calculated according to the weight to evaluate the degree of pulmonary edema.[41,42]

Determination of Proinflammatory Factors in BALF

The BALF liquid was centrifuged at 3000 rpm for 10 min at 4°C, then according to the manufacturer's instructions, the supernatants were used for detection of cytokines levels, such as TNF-α and IL-6.[43,44]

Determination of MDA, NO and ROS in Lung Tissue

ROS, MDA and NO are usually used to express local or systemic oxidative stress.[45] The lung tissue was broken into small pieces and homogenized in lysozyme at 37°C for 1 hour. Then, filter the homogenate, centrifuge 3000g 20 min at 4°C, and collect the supernatant. Then, quantify the MDA according to the manufacturer's recommended scheme[46] and use a reagent based on the Gliese reaction to determine the nitrite level to indirectly evaluate the NO content.[47] The level of ROS in lung tissue was detected by DCFH-DA kit. In short, after harvesting the single cell suspension, 5×10^5 cells were resuscitated in 1mL PBS with 1 μL DCFH-DA and analyzed for ROS response with the displayed fluorescence value using a microwell plate reader.[48]

Real-Time Quantitative PCR Analysis

According to the manufacturer's instructions, the Trizol kit was used to extract total RNA from lung tissue and obtain cDNA. Finally, a two-step PCR amplification reaction was used to obtain threshold cycle (Ct) and average value from triplicate samples. Using GAPDH as the internal reference, 2-ΔΔCt was used to calculate the relative expression level of mRNA of the target gene.[49] The primers were synthesized by General Biotechnology Co., Ltd. (Anhui, China). Primer sequences are listed in Table 2.

Western Blot Analysis

The lung tissue was homogenized using RIPA lysis buffer, and proteins were extracted. Total protein was quantified using a BCA kit. The protein was then separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with antibodies against Nrf2, HO-1, TLR4, and GAPDH overnight at 4 °C, followed by a 2-hour incubation with a secondary antibody coupled with HRP. After washing with phosphate-buffered saline, antibodies were developed and exposed to enhanced chemiluminescence (ECL) to observe protein bands.[50] The strips were analyzed using Image J software (Bio-Rad, California, USA) with GAPDH as the loading control.

Table 2 Primer Sequence

Gene	Primer	Sequence
UCP2	F	ATGTGGTAAAGGTCCGCTTCC
	R	ACAGTTGACAATGGCATTTCG
GAPDH	F	GGTCATCAACGGGAAACCCATCA
	R	CGCCAGTAGACTCCACGACATAC

Statistical Analysis

All experimental data were expressed as mean ±SD and analyzed by SPSS26.0 software (IBM Inc., USA). One-way analysis of variance was used for comparison among multiple groups, student *t* test was used for comparison between two groups, and modified by Bonferroni method. $P < 0.05$ was considered statistically significant.

Results

Network Pharmacology Predicted the Potential Mechanisms of DYY for Treating ALI

Collection of DYY Targets and ALI Targets

A total of 95 active components were identified in the DYY prescription based on set screening conditions (Supplementary Material 1). Three compounds, Magnolol, honokiol, and quercetin, were considered active despite not meeting ADME parameters due to their known anti-inflammatory or antioxidant effects.[20,21,51] A total of 234 therapeutic target proteins for these compounds in DYY were identified and their gene names were adjusted using the UniProt database (Supplementary Material 2). Then the herbal-ingredient-target gene network was created using Cytoscape 3.7.2 (Figure 1A). It includes different herbs and active ingredients (the surrounding circle), shared ingredients (the upper hexagon), and targets (the middle prism). There were 15 medicinal ingredients with a degree of ≥ 15 (Supplementary Material 3). 2529 ALI-related targets were gathered from GeneCards, DisGeNET, and OMIM databases (Figure 1B). 111 overlapping genes between DYY target and ALI target were identified by Venn map (Figure 1C and Supplementary Material 4).

PPI Network Analysis and Core Target Screening

We imported 111 genes into the STRING database, creating a PPI network with 108 nodes and 1716 edges due to 3 proteins did not participate (Figure 2A). Core targets were identified to construct a network based on their DC, BC, and CC values (Figure 2B), with the top 15 targets sorted by degree shown in Figure 2C and detailed in Table 3. Subnetworks identified by MCODE were divided into four groups for further analysis (Figure 2D).

GO and KEGG Enrichment Analysis

To better understand how DYY works against ALI, we analyzed 111 overlapping targets using GO and KEGG in Metscape. Figure 3A–C displays the each top 20 enrichment items for MF, CC, and BP. MF includes kinase binding, transcription factor binding, protein kinase activity, protein homodimerization activity, cytokine receptor binding, and more. CC includes membrane raft, membrane microdomain, transcriptional regulatory complex, mitochondrial membrane, and Bcl-2 family protein complex. BP involves response to hormones, response of cells to nitrogen compounds, response to peptides, regulation of apoptosis signal pathway, response to lipopolysaccharide, response of cells to cytokine stimulation, etc. DYY therapy can regulate the immune system, mitochondrial stress, apoptosis, and signal transduction to alleviate ALI symptoms. KEGG analysis revealed the top 30 signaling pathways, such as cancer pathway, AGE-RAGE signaling pathway in diabetic complications, fluid shear stress, and atherosclerosis, PI3K-Akt, MAPK and p53 signaling pathways, may be involved in the treatment of ALI by DYY (Figure 3D and E and Supplementary Material 5), we observed that 28 and 23 core targets were involved in the upstream and downstream regulation of PI3K-AKT and MAPK signaling pathways, respectively, and excess ROS could activate Nrf2 through these two signaling pathways, and then promote the transcriptional expression of HO-1, the downstream gene of Nrf2, to cope with the damage caused by oxidative stress.[52] In addition, Nrf2/HO-1 signal transduction can regulate TLR4-driven inflammatory response during stress.[53] These results suggest that DYY may treat ALI by regulating the pathways related to inflammation and oxidative stress.

Identification and Prediction of Active Ingredients in DYY

The effective components of DYY prescription were identified by UHPLC-MS/MS. The representative LC-MS total ion current chromatography (TIC) obtained in positive (ESI+) and negative (ESI-) modes is shown (Figure 4A and B). Table 4 identified and labeled the representative compounds of DYY Chinese herbal medicine, in which Anhydroicaritin,

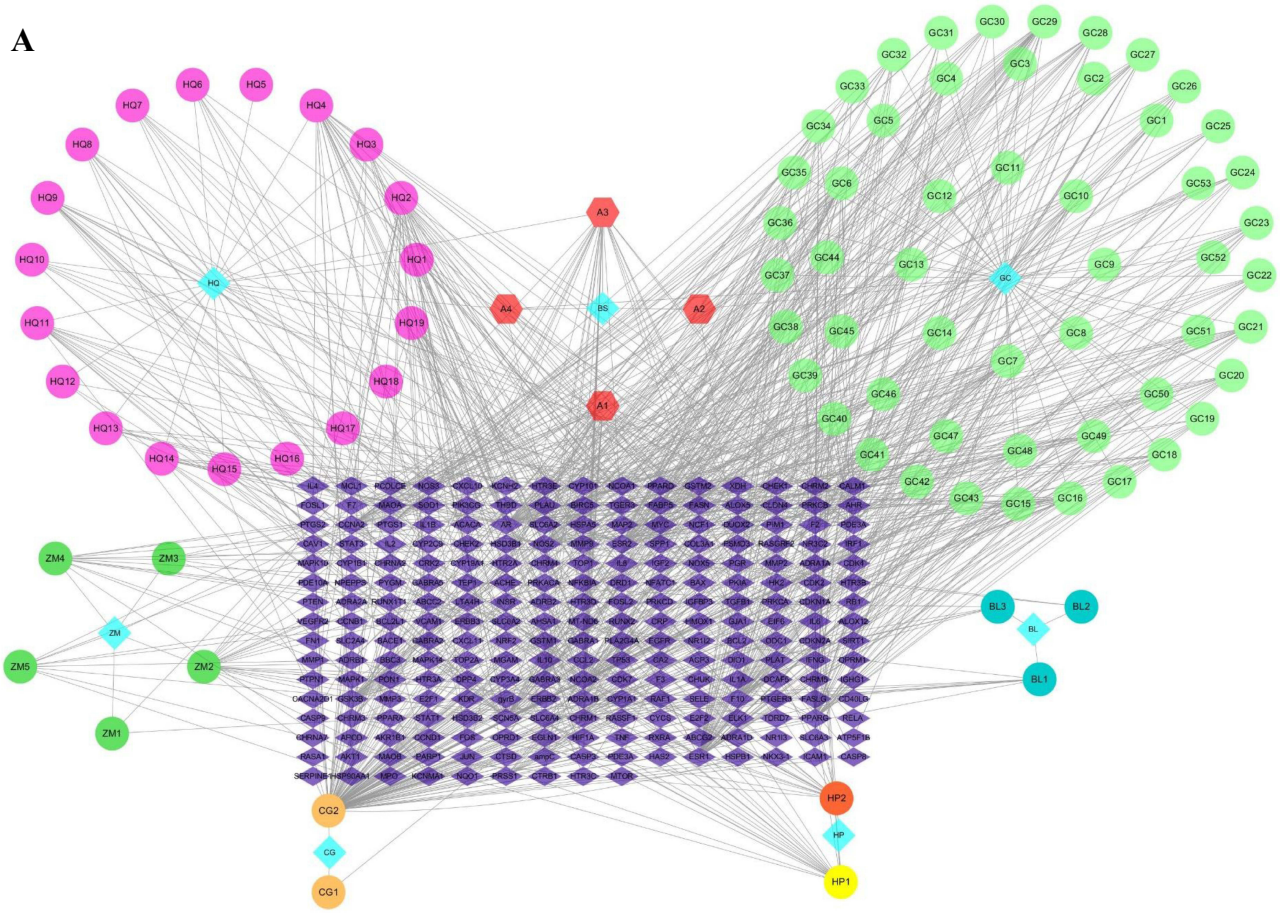

Figure 1 Targets related to ALI and active ingredient-targets of DYY. (**A**) Herb-ingredient-targets gene network. (**B**) The Venn diagram of ALI therapeutic targets. (**C**) Venn diagram of ALI targets and DYY targets.

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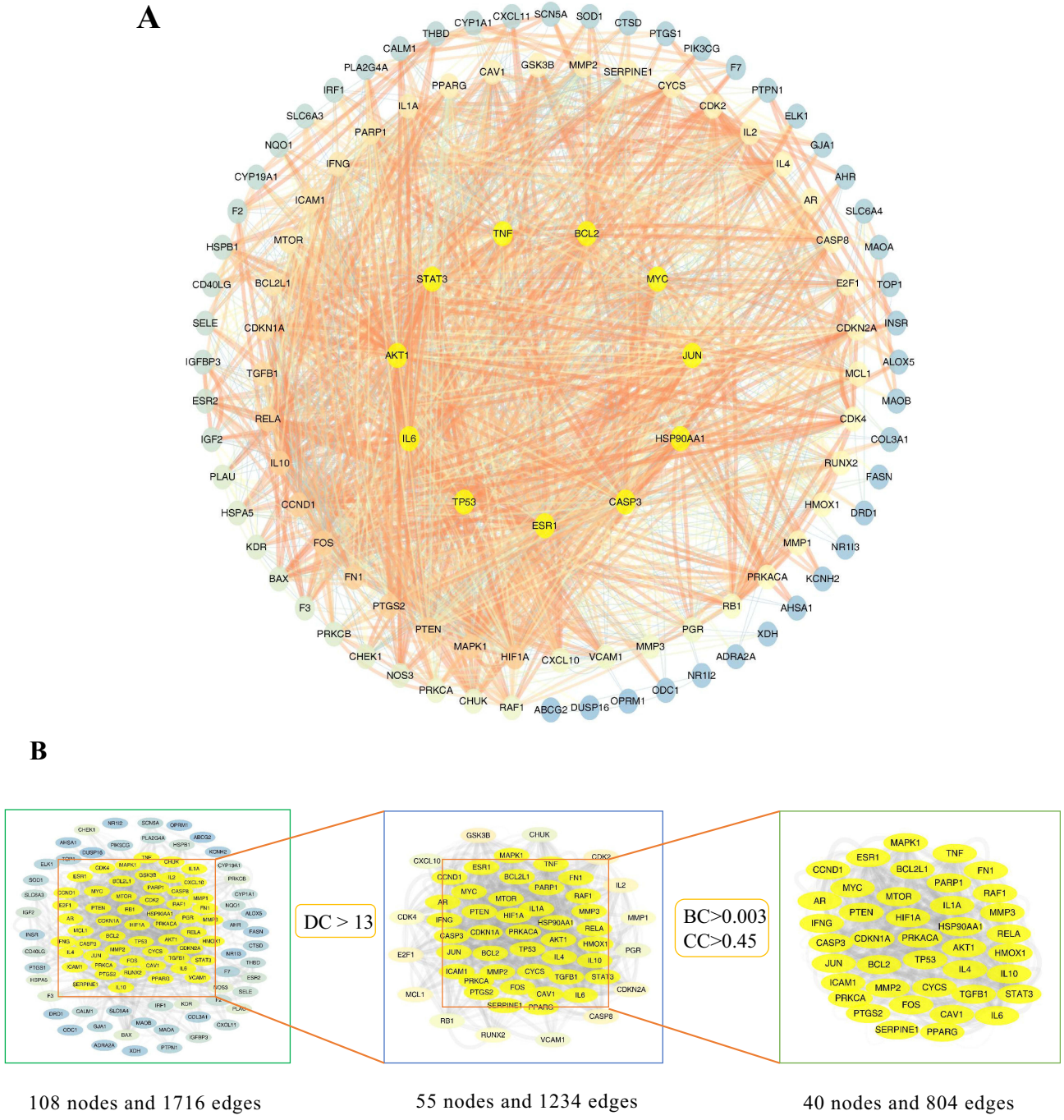
quercetin, licochalconea, Wogonin and Honokiol were the main components confirmed by network pharmacological analysis.

DYY Improves LPS-Induced ALI in Rats

To study DYY's role in ALI, we induced ALI in rats using LPS. Pulmonary edema, a common ALI change, can be measured by W/D ratio. Compared to the Con group, rats treated with LPS had significantly higher W/D ratios, which was reduced by DYY (Figure 5A). HE staining of lung tissue showed severe cell inflammation, thickening of alveolar



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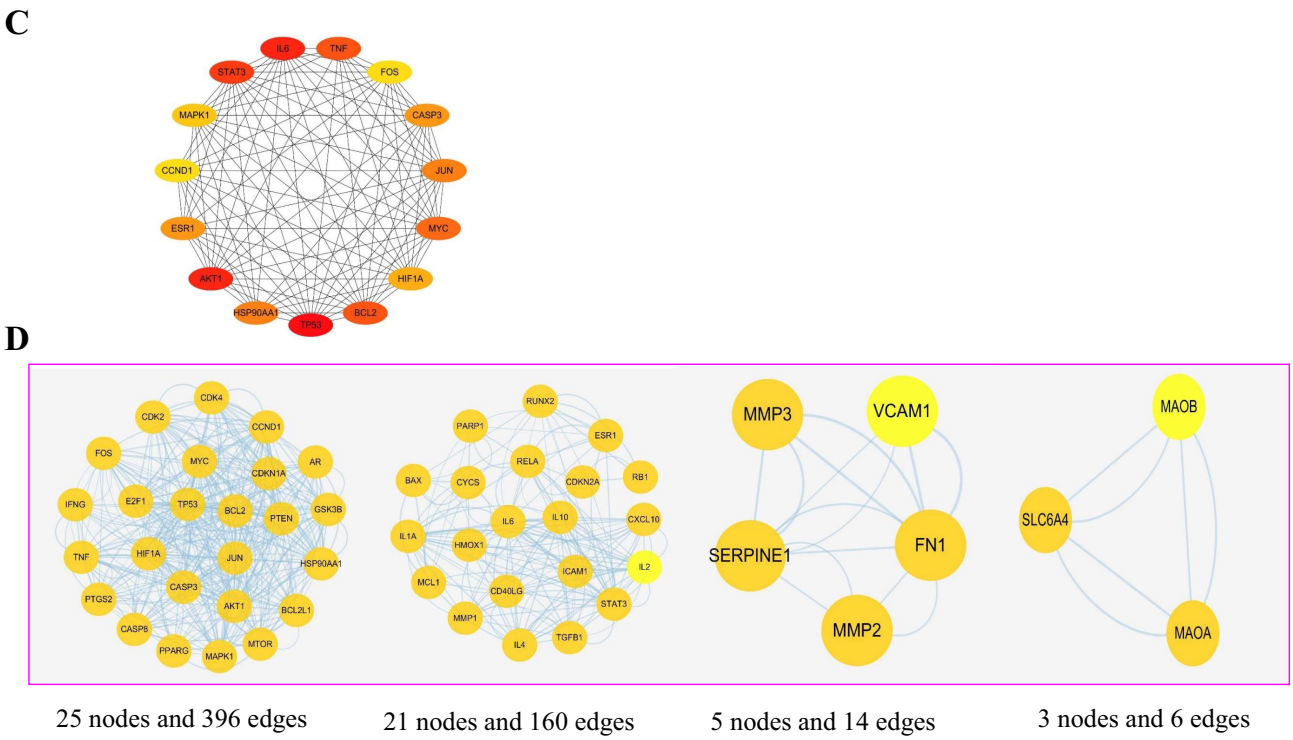

Figure 2 The PPI network of DYY's targets for the treatment of ALI. (**A**) PPI network of potential targets. (**B**) Topology screening process for PPI networks. (**C**) Top 15 core targets. (**D**) PPI network based on cluster analysis.

septum and partial destruction of alveolar structure in LPS group, which was improved by DYY (Figure 5B). In summary, the results confirmed the protective effect of DYY prescription on ALI rats.

DYY Inhibits Lung Inflammation and Oxidative Stress

As previously described, uncontrolled inflammation and oxidative stress can worsen ALI.[54] To study DYY's effects on LPS-induced inflammation and oxidative stress, we measured levels of IL-6, TNF-α, ROS, MDA and NO in rats. Results showed DYY reduced levels of inflammatory factors in BALF (Figure 6A and B), meanwhile DYY significantly reduced

Table 3 Information of 15 Core Targets

No.	UniProt ID	Gene symbol	Protein name	Degree
1	P04637	TP53	Cellular tumor antigen p53	52
2	P31749	AKT1	RAC-alpha serine/threonine-protein kinase	49
3	P05231	IL6	Interleukin-6	49
4	P40763	STAT3	Signal transducer and activator of transcription 3	47
5	P10415	BCL2	Apoptosis regulator Bcl-2	42
6	P01375	TNF	Tumor necrosis factor	42
7	P01106	MYC	Myc proto-oncogene protein	41
8	P07900	HSP90AA1	Heat shock protein HSP 90-alpha	40
9	P05412	JUN	Transcription factor Jun	40
10	P03372	ESR1	Estrogen receptor	38
11	P42574	CASP3	Caspase-3	38
12	Q16665	HIF1A	Hypoxia-inducible factor 1-alpha	33
13	P28482	MAPK1	Mitogen-activated protein kinase 1	32
14	P24385	CCND1	**G1/S-specific cyclin-D1**	31
15	P01100	FOS	**Protein c-Fos**	31

levels of ROS, MDA, and NO in lung tissue (Figure 6C–E), which is consistent with the results of related studies,[55] showing anti-inflammatory effects and reducing oxidative stress to improve lung injury.

DYY Up-Regulates UCP2 mRNA Expression in Lung Tissue

UCP2 is involved in the maintenance of mitochondrial function, the regulation of immune response and oxidative stress under physiological or pathological conditions.[49] As shown in Figure 6F, LPS decreased the expression of UCP2 mRNA in lung tissue ($P<0.01$). However, DYY administration could up-regulate UCP2mRNA expression ($P < 0.05$). This suggests that UCP2 plays an active role in improving lung injury caused by LPS.

DYY Regulates the Expression of Nrf2/HO-1-Mediated TLR4 Pathway Protein

Environmental and pathological stress activates Nrf2, leading to the regulation of downstream antioxidant factors like HO-1. This helps protect against oxidative stress and inflammation, and the associated TLR4 pathway plays an anti-inflammatory role.[56,57] KEGG analysis shows that PI3K-AKT and MAPK pathways are enriched, but its upstream and downstream gene Nrf2/HO-1-mediated TLR4 pathways are crucial for eliminating oxidative stress and inflammation, which was an important mechanism of ALI therapy. To study the effects of DYY on Nrf2, HO-1, and TLR4 proteins, we found through WB that DYY treatment reduced TLR4 expression and increased Nrf2 and HO-1 expression in response to LPS (Figure 7A–D), indicating DYY's anti-inflammatory and oxidative stress-regulating effects through the Nrf2/HO-1-mediated TLR4 pathway.

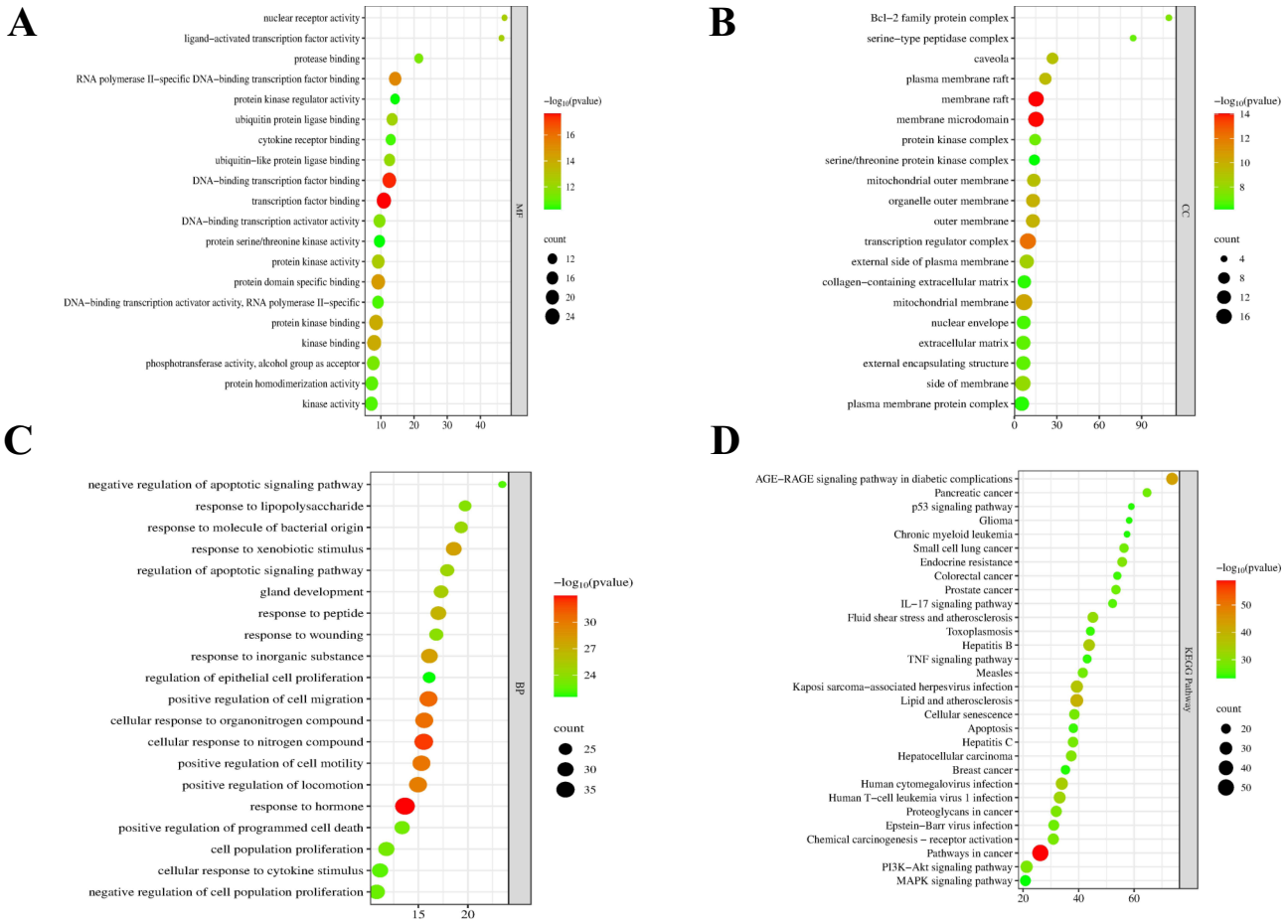

Figure 3 Continued.

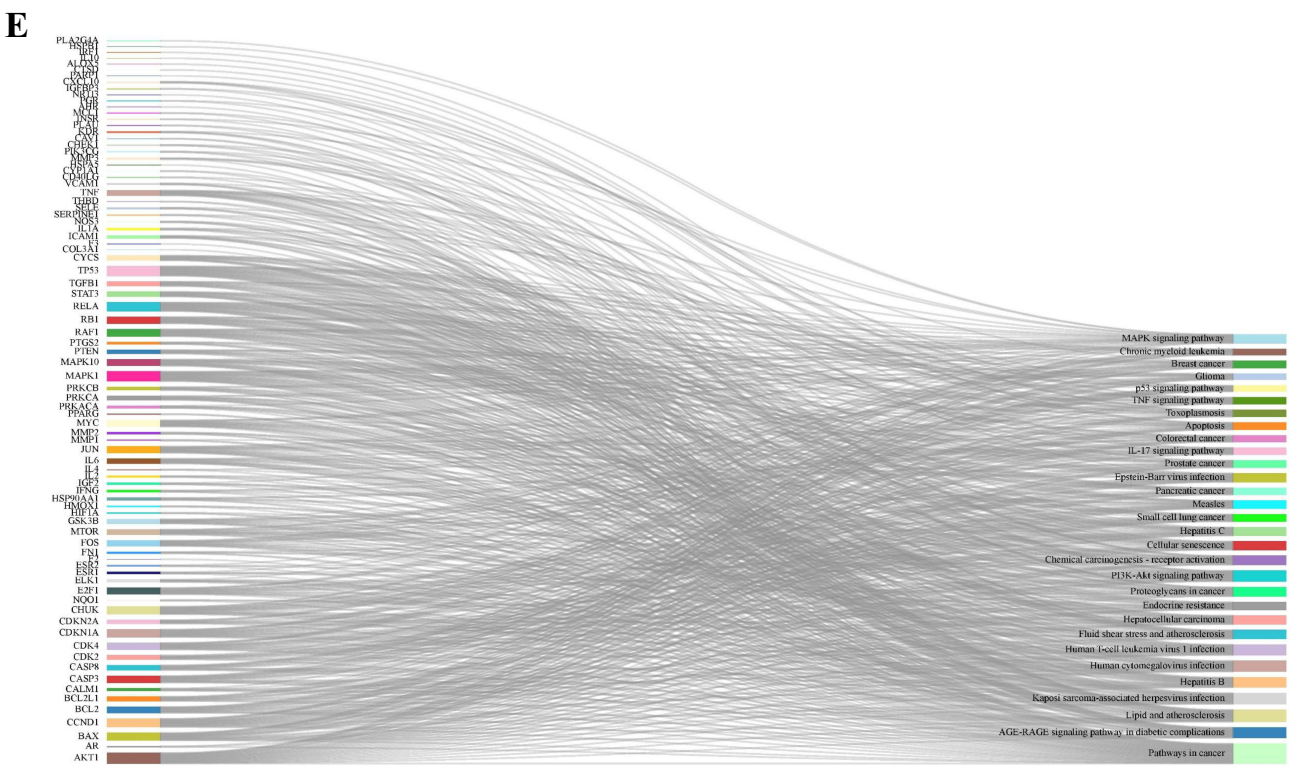

Figure 3 GO and KEGG enrichment analysis of 111 targets. (**A**) Molecular function category. (**B**) Cellular component category. (**C**) Biological process category. (**D**) KEGG pathway analysis. (**E**) Sankey diagram for KEGG signaling pathway analysis. The lines join the targets on the left and pathways on the right.

Predicting Active Compounds of DYY Through Molecular Docking

We selected Anhydroicaritin, Wogonin, Licochalcone A, quercetin, and Honokiol for molecular docking with Nrf2, HO-1, and TLR4 proteins. Lower binding energy indicates stronger interaction.[58] Anhydroicaritin demonstrated the highest docking score (−8.1 kcal/mol) by primarily forming hydrogen bonds with GLN38 on HO-1. Honokiol, with the lowest score (−4.9 kcal/mol), still showed stability, binding mainly to GLY139 on Nrf2 through conventional hydrogen bonding. Other compounds also effectively bind to Nrf2, HO-1, and TLR4 through similar interactions, indicating potential significance in ALI treatment. The typical docking results are shown in Table 5 and Figure 8A–E.

Discussion

ALI is a syndrome with various causes, where oxidative stress and inflammation are key factors affecting lung function.[59,60] Despite extensive research in determining the influencing factors and repair mechanisms[61], current treatments have not significantly reduced the high mortality rate of ALI.[62]

Research has demonstrated that TCM is effective in treating coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2.[63] TCM targets inflammation, oxidative stress, and organ injuries associated with the virus, providing unique clinical advantages.[64] TCM is now a crucial component in preventing and treating ALI, classified as “lung heat syndrome“ and ”asthma syndrome” in TCM. The pathogenesis incloud evil toxin trapped in the lung causes heat to consume body fluid, leading to lung dysfunction and accumulation of phlegm and heat.[65] This can progress to ALI and potentially acute respiratory distress syndrome (ARDS). DYY has been used in China for centuries for epidemic diseases. It has the effect of eliminating turbid, clearing heat and detoxification. It is usually used to treat influenza, cold, fever and other upper respiratory diseases, showing a good effect.

ALI is characterized by uncontrolled inflammation and redox imbalance,[66] with a focus on oxidative stress, inflammatory response and apoptosis in treatment research.[67] Network pharmacology is commonly used to uncover the complex pharmacological mechanisms of traditional Chinese medicine for treating complex diseases. This study used

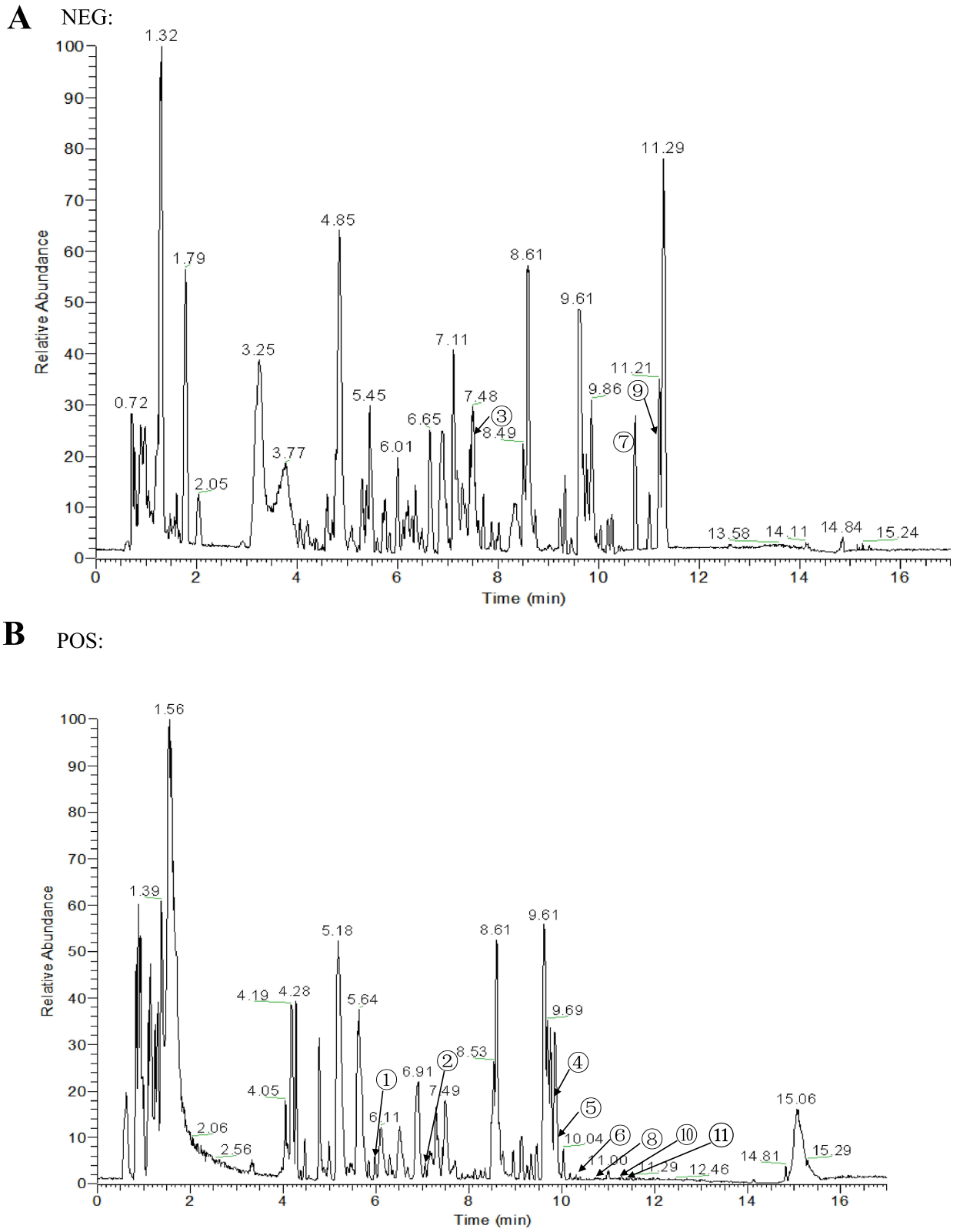

Figure 4 Identification of active compounds in DYY using UHPLC-MS/MS. (**A**) ESI- mode. (**B**) ESI+ mode.

Table 4 Chemical Characterization of Main Compounds in DYY

No.	Compound name	Molecular Formula	M/Z	Retention time (min)	Class
1	Quercetin	C15H10O7	303.04961	6.07225	Flavonols
2	Wogonin	C16H12O5	283.06154	7.4849833	Flavonoids
3	Formononetin	C16H12O4	269.08059	7.12975	Isoflavones
4	Glabrone	C20H16O5	337.10671	10.740933	Isoflavones
5	Anhydroicaritin	C21H20O6	369.13308	11.340883	Isoprene flavonoid derivatives
6	Magnolol	C18H18O2	265.12319	11.2761	Lignans
7	Glyasperin F	C20H18O6	337.10654	11.294383	Flavonoids
8	Oroxylin A	C16H12O5	285.07523	9.8511	Flavonoids
9	Honokiol	C18H18O2	265.12319	10.72155	Lignans
10	Lupiwighteone	C20H18O5	339.12235	9.8987	Isoflavones
11	Licochalcone A	C21H22O4	339.1589	10.256	Flavonoids

network pharmacology and vivo experiments to investigate how DYY works as an anti-ALI treatment. We confirmed 15 main targets out of 111 potential targets identified, including TP53, TNF, IL6, MAPK1, CASP3, and BCL2 associating with oxidative stress, inflammation, and apoptosis.

Oxidative stress causes the buildup of ROS in lung cells, impacting their function and triggering inflammation.[68] In acute lung injury, excessive NO production due to oxidative stress can lead to the formation of reactive nitrogen species (RNS),[69] disrupting pulmonary function,[70] which leads to induced NOS overexpression/activity and the release of pro-inflammatory cytokines.[71] In this study, we created an ALI rat model using LPS and treated it with DYY. Results showed that DYY effectively treated ALI by restoring normal W/D ratio, reducing inflammatory cell infiltration, and reconstructing alveolar structure. After LPS stimulation, pro-inflammatory cytokines like IL-6 and TNF-α were secreted in BALF,

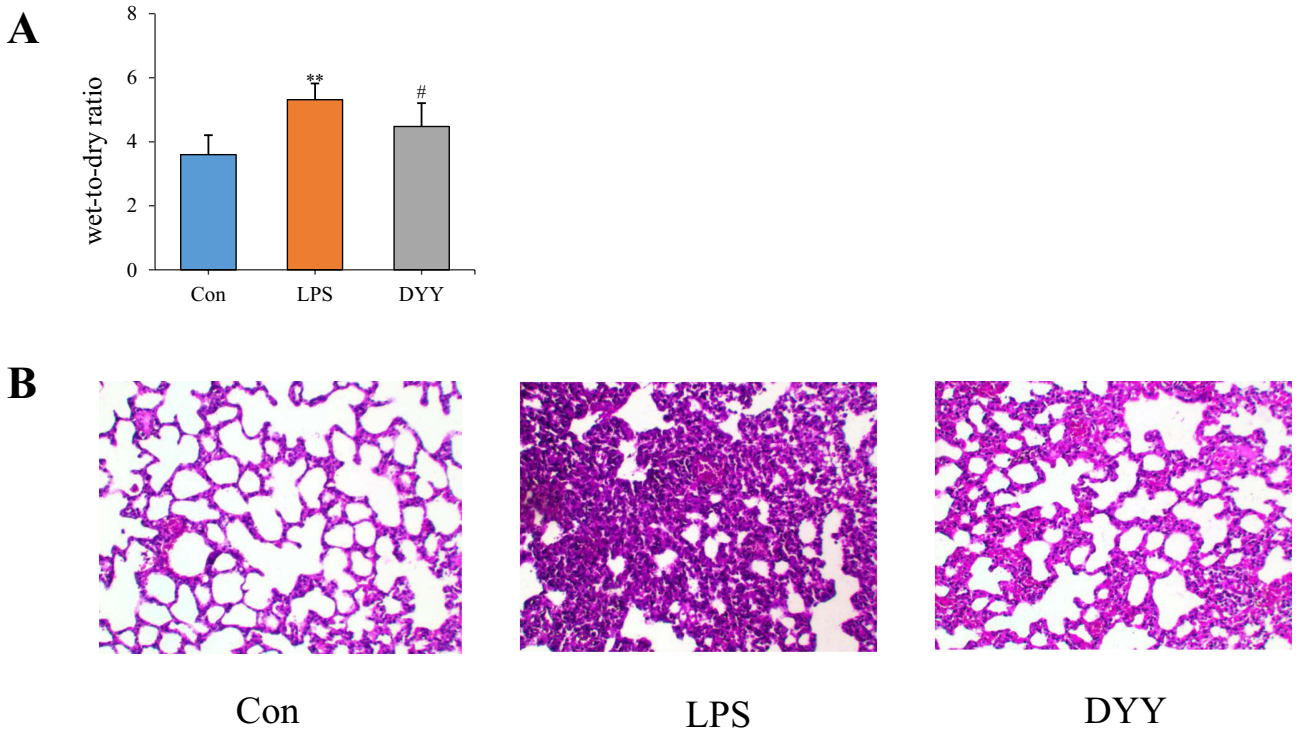

Figure 5 Effects of DYY on treating ALI rats. (**A**) Pulmonary wet-to-dry ratio (x±s, n= 6) compared with the Con group, **P < 0.01; compared with the LPS group, # p<0.05 . (**B**) Histopathological changes in LPS-induced lung tissues (×200).

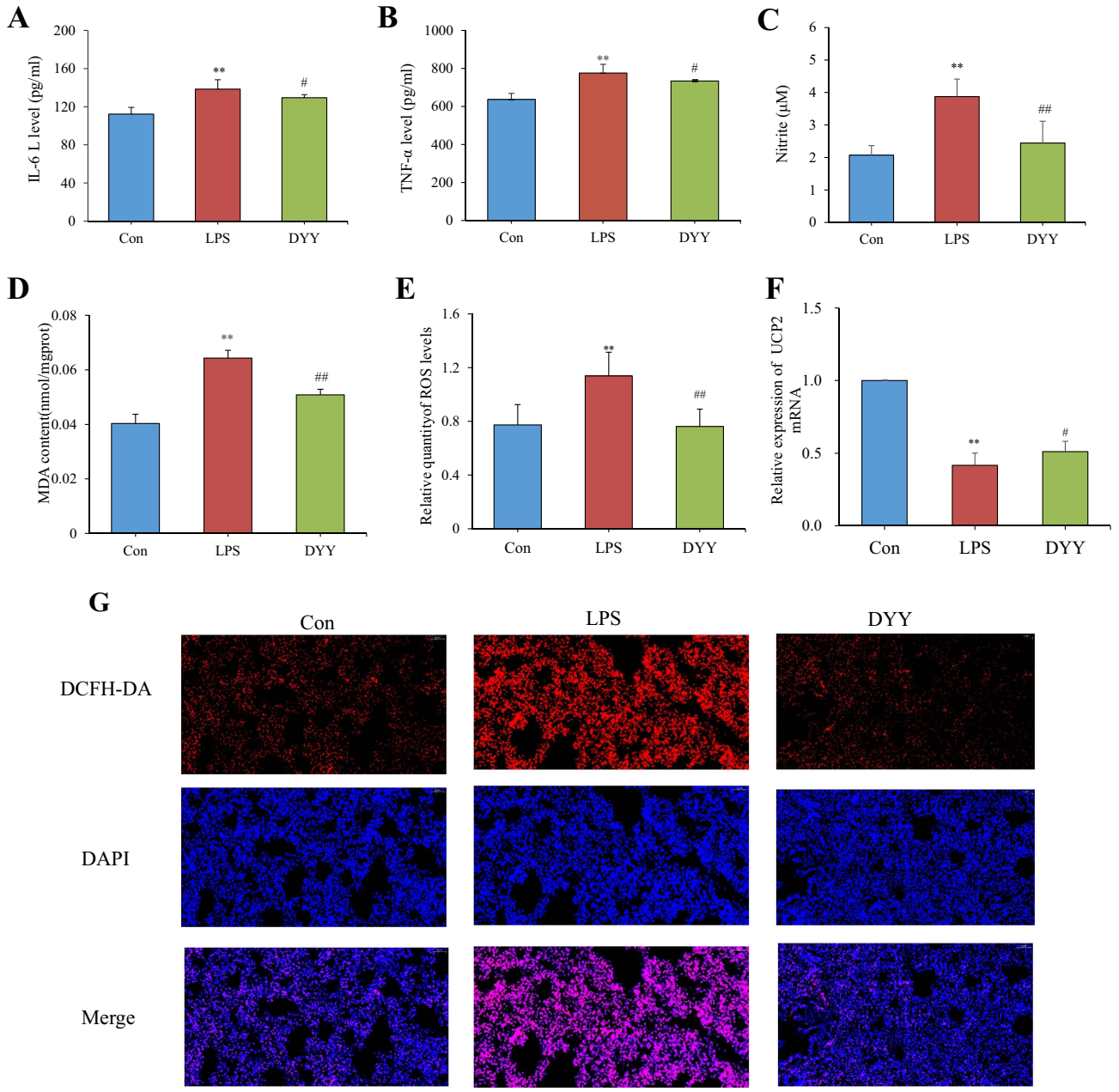

Figure 6 DYY alleviates LPS-induced inflammation and oxidative stress in ALI rats. (**A**) IL-6 level. (**B**) TNF-α level. (**C**) Nitrite level. (**D**) MDA level. (**E**) ROS level. (**F**) UCP2 expression. (**G**) DYY reduced ROS levels in ALI rats compared with the Con group, **P < 0.01; compared with the LPS group, # p < 0.05, # # p < 0.01.

indicating pulmonary inflammation. DYY reversed this by reducing cytokines, indicating inhibition of the inflammatory response.

It is well known that oxidative stress is caused by an imbalance between oxidants and antioxidants, leading to damage from ROS and depletion of antioxidants. This can worsen inflammation and harm mitochondria due to the production of oxides exceeding antioxidant defense.[72] MDA is a common biomarker for oxidative stress,[73] inducer NO synthase (iNOS) and endothelial nitric oxide synthase (eNOS) are enzymes involved in oxidative stress and can contribute to lung injury in ALI by increasing superoxide production such as NO and MDA.[74] New research suggests that oxidative stress is a key factor in the development of ALI, with ROS playing a crucial role in the process. Mitochondrial channels like mPTP and inner membrane anion channel (IMAC), their activation may be involved in intra- and intermitochondrial redox-environment changes leading to ROS release.[75] It should be noted that while ROS can protect cells from oxidative

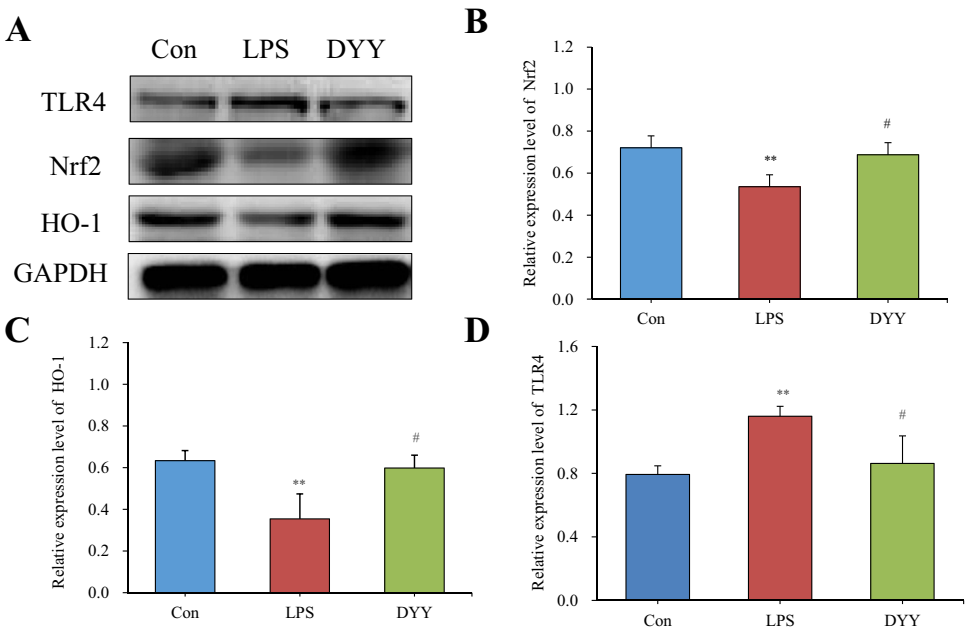

Figure 7 Effect of DYY on the expression of pathway proteins in lung tissue (x±s, n=3). (**A**) Overall expression of proteins. (**B**) Nrf2.(**C**) HO-1. (**D**) TLR4 compared with the Con group, **P < 0.01; compared with the LPS group, # p < 0.05.

stress, high levels can damage endothelial barriers and cause inflammation. Excessive ROS can deplete NO levels, and NO reacts with ROS to form excessive peroxynitrite, leading to oxidative damage and cell death through reacting with lipids, DNA and proteins.[72] UCP2, an anion transporter, helps maintain mitochondrial function, immune response and regulate oxidative stress, and protect against cell apoptosis.[49,76] Here, it may play a crucial role in protecting against LPS-induced ALI by stabilizing mitochondrial structure and reducing inflammation and oxidative stress induced by ROS, MDA and so on. To confirm DYY's inhibitory effect on ALI in rats, we observed changes in ROS, MDA, and NO levels. ROS levels increased significantly in ALI rats, indicating oxidative damage in lung tissue. MDA and NO levels also increased, indirectly reflecting tissue injury degree. DYY intervention effectively reduced intracellular ROS, inhibited ROS accumulation, decreased MDA and NO levels, tending to the Con group and reversed the decrease in UCP2 mRNA expression induced by LPS. This suggests that DYY can alleviate lung tissue inflammation and improve ALI by regulating oxidative stress processes.

We analyzed the mechanism of DYY in ALI through KEGG and GO enrichment, identifying the involvement of mitochondrial membrane, cell response to nitrogen compounds and pathways like PI3K-AKT and MAPK signaling in ALI development. By studying the signaling cascade, we aim to understand the key regulatory roles of upstream and downstream targets in inflammation and oxidative stress pathways in ALI. Numerous studies have demonstrated the significance of Nrf2 in controlling oxidative stress[77] and its role in iron apoptosis by regulating iron homeostasis and lipid peroxidation.[78] Furthermore, the Nrf2/HO-1 pathway helps regulate anti-inflammatory and antioxidant responses,

Table 5 Molecular Docking Predicted Results of Key Active Ingredient and Target Protein

Item	HMOX1	NF2L2	TLR4
Anhydroicaritin	−8.1	−5.7	−6.6
Honokiol	−7.5	−4.9	−5.9
LicochalconeA	−7.6	−5.4	−5.8
Quercetin	−7.1	−5.6	−5.9
Wogonin	−7.4	−5	−5.9

Note: The binding free energy is kcal/mol.

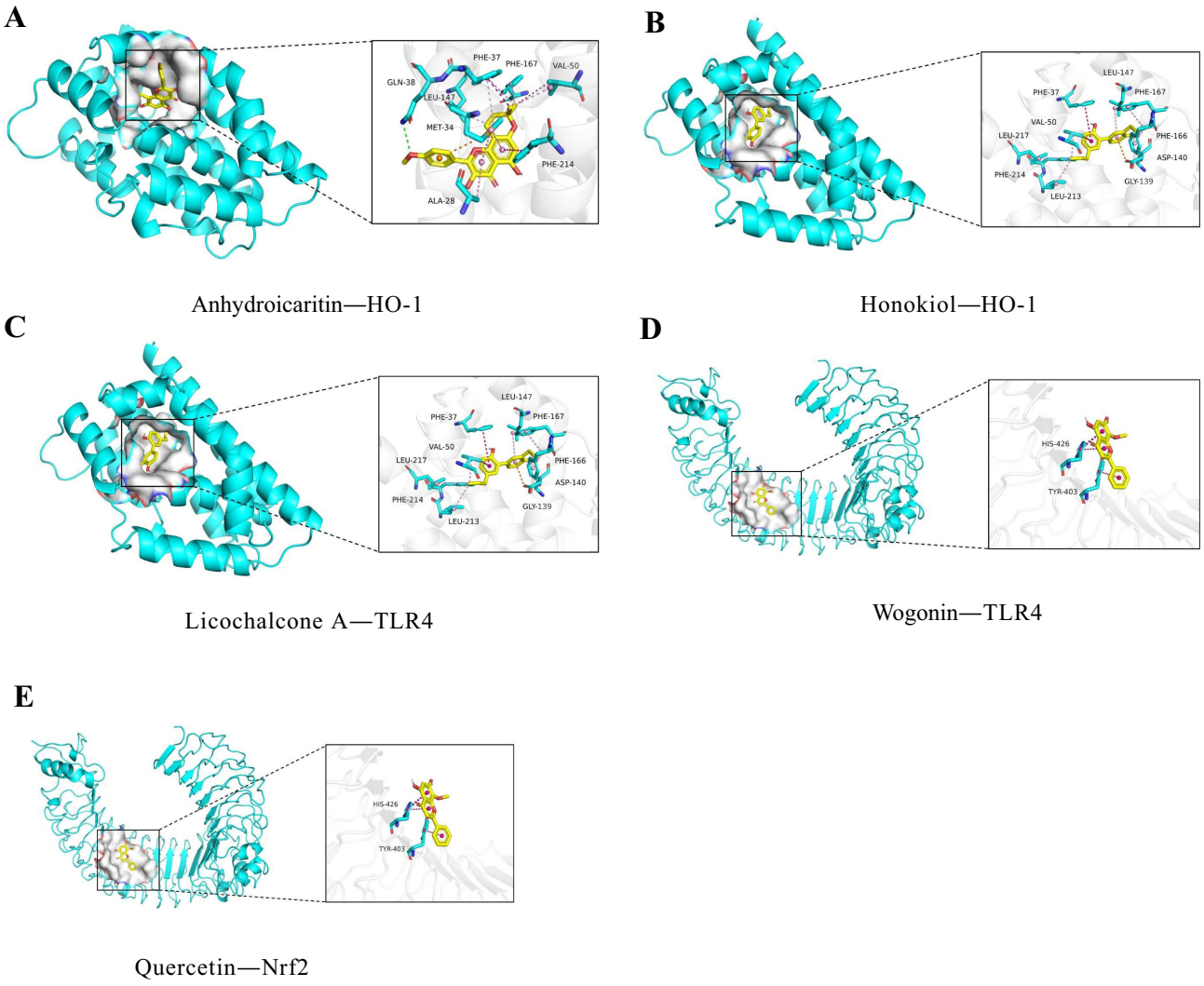

Figure 8 Molecular docking patterns between representative components and targets of DYY for treatment of ALI. (**A**) Anhydroicaritin-HO-1. (**B**) Honokiol-HO-1. (**C**) Licochalcone A-TLR4. (**D**) Wogonin-TLR4. (**E**) Quercetin-Nrf2.

providing multi-organ protection.[79,80] Recent reports suggest that natural drug ingredients protect against ALI through the Nrf2 signaling pathway.[81] Furthermore, through modulation of the Nrf2/HO-1 pathway, Shizukaol A exhibits anti-inflammatory properties,[82] Aucubin mitigates chronic obstructive pulmonary disease,[83] and Fraxetin reduces ferroptosis following myocardial infarction.[84] Collectively, these findings underscore the critical role of the Nrf2/HO-1 pathway in mediating anti-inflammatory and antioxidant effects. Our study shows that DYY activates the Nrf2/HO-1 pathway in ALI rats, reducing oxidative stress and inflammation in the lungs, such as the reduction of TLR4 expression and TNF-α, IL-6 levels, suggesting that DYY can prevent ALI by reducing inflammation and increasing antioxidant response. Nrf2 protects lung cells by influencing TLR4 signaling,[85] confirming its role in inflammatory lung injury, consistent with previous studies.[86]

Molecular docking techniques were used to predict the binding activity of core components to key targets. Results showed Honokiol had good binding activity to HO-1 and TLR4, while other components bound well to Nrf2, HO-1, and TLR4. Quercetin, a flavonoid compound, regulates oxidative stress and inflammation by inhibiting inflammatory factors and excessive release of ROS, proving effective in treating various diseases.[87] Wogonin is also flavonoids that reduce inflammation and oxidative stress by enhancing antioxidant capacity and counteracting inflammatory signals.[88] Honokiol is a natural polyphenol that has been shown to counteract oxidative stress and inflammatory signals in a variety of ways, including reversing elevated levels of inflammatory factors, increasing the production of antioxidants GSH and SOD in

the body, and alleviating LPS-induced apoptosis.[89,90] Prior research has demonstrated that Licochalcone A has antimicrobial and anti-inflammatory properties, protects against oxidative stress, and activates nuclear translocation of Nrf2 and enhancing HO-1 expression in cells.[91] Similar to the findings of many studies mentioned above, we predicted that DYY could alleviate acute lung injury in rats through various compounds. However, this study did not analyze specific active ingredients, which should be addressed in future research.

Conclusion

This study suggests that the acute lung injury induced by lipopolysaccharide in rats may serve as a model for understanding the mechanisms of lung inflammation and oxidative-antioxidant imbalance observed in humans. Our study indicates that DYY inhibits TLR4 signaling via the Nrf2/HO-1 pathway, thus reducing MDA, NO, IL-6, and TNF-α levels, thereby protecting against ALI in rats. While the LPS-induced ALI model used in this study is effective for examining inflammation and oxidative stress, ALI patients often experience worsening hypoxemia and respiratory distress. Further research is needed to determine the appropriate dosing to replicate lung dysfunction and to better align animal models of ALI with the clinical progression of the disease. While UHPLC-MS/MS technology clarifies DYY's main components, such as quercetin, Magnolol, and Wogonin. Further research is needed to pinpoint its key bioactive elements to better grasp the material basis of its anti-ALI mechanism. More importantly, current research on DYY for treating ALI has elucidated its mechanism of action; however, most studies remain confined to cellular and animal models, lacking corroborative clinical data. Given the complexity of ALI's etiology in patients compared to animal studies, future research should focus on large-scale clinical trials to assess DYY's efficacy in ALI patients.

In summary, the findings of this study endorse DYY as a potential therapeutic option for ALI and offer novel insights for future research and development of ALI treatment drugs.

Animal Ethics Statement

All surgical procedures on the experimental animals met all ethical requirements and were approved by the animal ethics committee of the Institute of Biology, Shandong Academy of Sciences (SWS20240130).

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Database Use Approval Statement

The use of database data in this study was reviewed and approved for implementation by the Institutional Review Committee of Kunshan Rehabilitation Hospital.

Consent to Publish

All authors consent to publish.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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