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Integrated network pharmacology and experimental validation reveal the anti-inflammatory mechanisms of Wuwei Shaji Powder in hepatic ischemia–reperfusion injury

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Abstract: **Background**: Hepatic ischemia–reperfusion injury (HIRI) is primarily mediated by oxidative stress and inflammatory activation, yet effective pharmacological interventions remain limited. **Objectives**: This study aimed to elucidate the key active ingredients, hub targets and molecular pathways through which Wuwei Shaji Powder may exert anti-inflammatory effects in HIRI using integrated network pharmacology and experimental validation. **Methods**: Active components of WSP and their predicted targets were obtained from TCMSP and HERB, while HIRI-related genes were obtained from GeneCards, OMIM and analysis of differential expression for the GEO dataset GSE228782. Differentially expressed genes were identified using the thresholds of $|\log2FC| \geq 1$ and adjusted P-value, also known as false discovery rate (FDR), < 0.05. Common targets underwent GO and KEGG enrichment, PPI network construction and molecular docking. Subsequently, functional verification was performed using a hypoxia/reoxygenation (H/R) model in AML12 cells treated with low- or high-dose WSP. Cell viability, inflammatory cytokines, ROS levels and protein expression of TLR4, AKT1, p-AKT (Ser473) and p-NF-κB were evaluated. **Results**: Network analysis highlighted inflammation- and immunity-related pathways, with AKT1, TNF, JUN, STAT3 and TP53 characterized as potential core regulatory targets. Docking results indicated favorable binding between selected WSP components and AKT1. Experimental data demonstrated that WSP significantly restored cell viability ($P < 0.001$), reduced IL-6, TNF-α and IL-1β secretion (all $P < 0.001$), attenuated ROS accumulation ($P < 0.001$) and suppressed activation of the TLR4–AKT1–NF-κB axis, as indicated by reduced protein levels of TLR4, p-AKT (Ser473) and p-NF-κB (all $P < 0.001$). **Conclusion**: WSP mitigates HIRI-induced inflammatory injury mainly by suppressing TLR4-mediated activation of the AKT1/NF-κB signaling cascade, thereby attenuating ROS accumulation and pro-inflammatory cytokine secretion (IL-6, TNF-α and IL-1β). The data furnish mechanistic evidence for its therapeutic potential against ischemia–reperfusion–triggered hepatic inflammation.

Keywords: Hepatic ischemia–reperfusion injury; Inflammation; Molecular docking; Network pharmacology; TLR4–AKT1–NF-κB pathway; Wuwei Shaji powder

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INTRODUCTION

Liver transplantation, major hepatectomy and trauma resuscitation frequently involve hepatic ischemia–reperfusion injury (HIRI), a complication that is common and often difficult to avoid (Zhao *et al*., 2025b). Clinical reports indicate that more than 80% of liver transplant recipients experience varying degrees of HIRI, which contributes to 20–40% of early graft dysfunction and significantly increases perioperative morbidity (Meurisse *et al*., 2023; Ito *et al*., 2021). Globally, over 250,000 liver-related surgeries each year carry a substantial risk of ischemia–reperfusion–associated injury, underscoring its critical impact on postoperative recovery and long-term outcomes (Pollok *et al*., 2023). Despite advances in surgical techniques and perioperative management, effective pharmacological interventions targeting the inflammatory cascade of HIRI remain limited, highlighting the urgent need to explore new therapeutic strategies.

At the mechanistic level, HIRI is primarily driven by a rapid and excessive inflammatory response triggered during the transition from hypoxia to reperfusion (Aboelez *et al*., 2024). Innate immune pathways—including Toll-like receptor 4 (TLR4), IL-17 signaling and NOD-like receptor activation—are rapidly engaged, amplifying oxidative stress and inflammatory cascades. Activation of these pathways stimulates downstream kinases and transcription factors such as AKT and NF-κB, thereby promoting cytokine production including IL-6, TNF-α and IL-1β (Guo *et al*., 2023; Sayaf *et al*., 2024). This inflammatory amplification loop is widely recognized as a key contributor to hepatocellular injury, yet modulating it safely and effectively remains challenging in clinical practice.

Against this biological landscape, multi-component herbal formulas have gained increasing attention for their capacity to modulate complex disease networks. The

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traditional formula Wuwei Shaji Powder (WSP), composed of *Hippophae rhamnoides* (berries), *Aucklandia lappa* (dry root), *Gardenia jasminoides* (fruit), *Glycyrrhiza uralensis* (root) and *Vitis vinifera* (dried fruit), contains multiple bioactive molecules with reported antioxidative and anti-inflammatory effects (Zhao *et al*., 2025a; Xiong *et al*., 2025). Several individual components—such as flavonoids from sea buckthorn and glycosides from licorice—have been shown to interact with inflammatory or stress-response signaling proteins (Fatima *et al*., 2024; Achary *et al*., 2025). However, the specific molecular targets through which WSP exerts potential protection in HIRI have not been elucidated.

Recent advances in network pharmacology provide an opportunity to dissect the multi-target regulatory potential of traditional formulas by integrating phytochemical data, disease-related gene networks and pathway enrichment analyses. When combined with molecular docking, this approach enables prediction of binding interactions between herbal components and key inflammatory mediators (Zhang *et al*., 2025). Such theoretical analyses, however, must be integrated with biological validation to establish mechanistic credibility and determine whether predicted targets truly participate in hepatocellular protection during ischemia–reperfusion.

Therefore, this study aimed to clarify how WSP exerts anti-inflammatory effects during hepatic ischemia–reperfusion injury (HIRI). A systematic mechanistic investigation was conducted using an integrated strategy comprising network pharmacology, protein–protein interaction (PPI) analysis, pathway enrichment, molecular docking and experimental validation in an H/R model of AML12 hepatocytes. Through this combined analytical–experimental framework, Crucial active ingredients, putative targets and inflammatory signaling pathways which involved in WSP-mediated protection could be identified, thereby providing a mechanistic basis for its potential application in hepatic ischemia–reperfusion–related inflammation.

MATERIALS AND METHODS

WSP active component screening and target prediction

The chemical constituents of WSP were retrieved from TCMSP and HERB. Specifically, compounds from *Hippophae rhamnoides, Aucklandia lappa, Gardenia jasminoides* and *Glycyrrhiza uralensis* were obtained from TCMSP, whereas Vitis vinifera-related components were supplemented using the HERB database. For TCMSP-derived compounds, oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥0.18 were adopted as cutoff values to identify candidate active components.

For each retained compound, the corresponding putative targets were retrieved from the "Related Targets" section of the TCMSP database. In addition, herb–target associations for grape-derived components were compiled using the HERB database (http://herb.ac.cn/) to ensure comprehensive coverage of all formula constituents. All protein targets collected from different sources were harmonized via the UniProt platform (https://www.uniprot.org/) to unify gene symbols and remove duplicates.

HIRI disease target collection

HIRI-related genes were retrieved from GeneCards (https://www.genecards.org/) and OMIM (https://omim.org/) using the keyword "hepatic ischemia–reperfusion injury," with a GeneCards Relevance Score ≥ 10 applied as the inclusion criterion. To incorporate transcriptomic evidence, the GEO dataset GSE228782 was downloaded, normalized and subjected to differential expression analysis between ischemia and reperfusion groups using the limma package in R. Differentially expressed genes were identified using the thresholds of $|\log2FC| \geq 1$ and adjusted P-value (FDR) < 0.05.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

Functional enrichment analysis of the intersecting targets was performed using the clusterProfiler package in R. Gene symbols were mapped to Entrez IDs with the org.Hs.eg.db annotation database prior to analysis. GO enrichment, including Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) categories, as well as KEGG pathways, were analyzed. Enrichment was evaluated using the hypergeometric test with an initial threshold of $P < 0.05$, followed by Benjamini–Hochberg correction for multiple testing. Terms were regarded as statistically significant if they had a false discovery rate (FDR) < 0.05. Bar plots and bubble plots were created in R to present the enrichment results graphically.

PPI network-based screening of hub regulatory genes

Targets shared by WSP and HIRI were uploaded to the STRING database (https://string-db.org/) for PPI network construction, restricting the analysis to *Homo sapiens* and applying an interaction score cutoff of ≥0.900. The "Multiple proteins" option was used to batch-submit the gene list and obtain interaction data containing protein nodes and edges. The generated network file was subsequently loaded into Cytoscape software for visualization and downstream analysis. Using the cytoHubba plugin, node degree was computed for each gene and the highest-ranked targets by degree were defined as hub genes.

Active component–target–disease–pathway network construction

Based on the identified active components, predicted

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targets, HIRI-related disease genes and significantly enriched KEGG pathways, an active component–target–disease–pathway network was further constructed in Cytoscape. This network served as an integrated framework to visualize the multicomponent–multitarget regulatory characteristics of WSP in the context of hepatic ischemia–reperfusion injury.

Molecular docking procedures

The top active components of WSP, screened based on oral bioavailability criteria, were retrieved from the TCMSP database in SDF format, while the corresponding protein structures for the selected targets were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). Protein structures were refined in PyMOL after deleting water molecules and native ligands, with subsequent hydrogen atom addition and structural optimization.

Molecular docking was then conducted using the CB-Dock2 platform (http://cao.labshare.cn/cb-dock2/), which automatically detects putative binding sites and conducts blind docking. Each compound–protein pair underwent five independent docking runs to ensure the stability of docking results. The workflow included cavity detection, grid box generation, affinity scoring and pose prediction. For each pair, the lowest-energy binding pose was used for further visualization. Final docking poses were examined in PyMOL to assess spatial orientation and interaction plausibility.

Cell culture and experimental grouping

Mouse hepatocyte AML12 cells (#GNM42; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM/F12 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin–streptomycin (Solarbio, China) and 1× ITS supplement (Gibco) at 37 °C with 5% CO_2. To establish the H/R model, cells at 70–80% confluence were first switched to serum-free DMEM/F12 and then placed into a hypoxia chamber (1% O_2, 5% CO_2, 94% N_2, 37°C; MIC-101, Billups-Rothenberg, USA) for 6 h. Subsequently, reoxygenation was initiated by returning cells to normoxic conditions (21% O_2, 5% CO_2, 37°C) for 18 h. WSP freeze-dried extract was dissolved in sterile PBS (pH 7.4) to prepare working solutions and was administered at the beginning of the reoxygenation phase. Before formal H/R intervention experiments, AML12 cells were treated with different concentrations of WSP and cell viability was evaluated using the CCK-8 assay. Based on the results showing no obvious cytotoxicity, 50 μg/mL and 150 μg/mL WSP were selected as the low- and high-concentration treatment conditions for subsequent experiments.

For experimental grouping, cells were allocated into four groups: (1) Control group, maintained under normoxic conditions without treatment; (2) Model group, subjected to H/R injury; (3) WSP-treated low-dose group (WSP-L), subjected to H/R injury and treated with WSP at 50 μg/mL; and (4) WSP-treated high-dose group (WSP-H), subjected to H/R injury and treated with WSP at 150 μg/mL.

Cell viability assay (CCK-8)

After H/R and drug treatments, cell viability was evaluated using a CCK-8 assay following the supplier's protocol. Each well was supplemented with 10 μL CCK-8 working solution (Dojindo, Japan) and incubated for 1–2 h at 37°C under normoxic conditions, after which absorbance at 450 nm was recorded using a microplate reader (BioTek, USA). Cell viability was calculated relative to the control group.

Enzyme-linked immunosorbent assay (ELISA) for cytokine detection

The concentrations of interleukin-6 (IL-6; #EK206/2), tumor necrosis factor-alpha (TNF-α; #EK282/2) and interleukin-1 beta (IL-1β; #EK201B/2) were measured using mouse ELISA kits purchased from MULTISCIENCES (Hangzhou, China). After completion of H/R and drug treatments, cell culture supernatants were harvested, clarified by centrifugation (1,000 × g, 10 min) to eliminate debris and kept at −80 °C until analysis. Pre-coated antibody plates received the standards and samples and were incubated as instructed by the manufacturer, after which detection antibodies and HRP-conjugated reagents were applied for further incubation. After color development with TMB substrate, absorbance at 450 nm was recorded on a microplate reader (BioTek) and cytokine levels were determined using analyte-specific standard curves.

Reactive oxygen species (ROS) fluorescence imaging

Following treatment, AML12 cells were rinsed with serum-free medium and loaded with 10 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate; Beyotime, Shanghai, China) diluted in serum-free DMEM/F12 for 20–30 min at 37 °C in the dark. Cells were then rinsed three times with PBS to remove excess probe. Fluorescence images were immediately acquired using an inverted fluorescence microscope (Nikon Ti-S, Japan) with excitation at 488 nm and emission at 525 nm. Mean fluorescence intensity per field was quantified using ImageJ with identical acquisition settings across all groups.

Western blotting

After treatment, AML12 cells were collected and processed in RIPA buffer (Beyotime) containing protease/phosphatase inhibitors. Total protein levels were quantified with a BCA assay (Beyotime) and samples of identical protein content were resolved by SDS–PAGE before being electrotransferred onto PVDF membranes

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(Millipore, USA), which were then blocked with 5% nonfat milk for 1 h at room temperature. Membranes were treated overnight at 4 °C with primary antibodies against TLR4 (A00017-3, Boster, 1:1,000), p-AKT (Ser473; ab81283, Abcam, 1:1,000), AKT1 (ab8805, Abcam, 1:1,000), p-NF-κB p65 (ab76302, Abcam, 1:1,000) and β-actin (ab8227, Abcam, 1:5,000), followed by incubation with HRP-conjugated goat anti-rabbit IgG (ab6721, Abcam, 1:5,000) for 1 h at room temperature. Immunoreactive signals were detected by an enhanced chemiluminescence kit (ECL; Millipore), captured using a ChemiDoc imaging system (Bio-Rad) and quantified by densitometric analysis with ImageJ software, with β-actin serving as the internal reference.Western blot analysis was performed using three independent biological replicates per group.

Statistical analysis

SPSS version 23.0 (IBM, USA) was applied for all statistical analyses. Continuous variables were described using mean ± standard deviation (SD). Normality was evaluated by the Shapiro–Wilk test. Group differences were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test when homogeneity of variance was met, or Dunnett's T3 test when variances were unequal. Statistical significance was defined as a two-tailed $P < 0.05$.

RESULTS

Shared targets of WSP and HIRI and their enriched biological functions

To clarify the potential biological processes through which WSP may influence HIRI, the shared targets between predicted drug targets and disease-associated genes were initially determined. A total of 127 overlapping targets were obtained (Fig. 1A), forming the basis for subsequent functional enrichment analyses.

GO enrichment analysis indicated that these genes were significantly enriched in biological processes related to inflammatory and stress responses, including reactions to lipopolysaccharide molecules of bacterial origin, xenobiotic stimuli, oxidative stress and hypoxia (Fig. 1B; adjusted $P < 0.05$). Several processes associated with apoptotic regulation and epithelial cell responses were also prominent, suggesting that the intersecting genes participate in cellular defense and stress adaptation during ischemia–reperfusion.

KEGG pathway analysis further highlighted inflammation- and immunity-related signaling pathways. IL-17 signaling, NOD-like receptor signaling, Toll-like receptor signaling and chemokine pathways were among the most significantly enriched (Fig. 1C; FDR < 0.05). These pathways play well-established roles in amplifying innate immune activation during HIRI.

Together, these findings indicate that the shared WSP–HIRI targets are strongly associated with inflammatory activation and immune regulation, providing a mechanistic framework for subsequent network and experimental validation.

Network analysis identifies core regulatory targets and validates ligand–protein interactions

To explore the key molecular nodes through which WSP may modulate the inflammatory response in HIRI, a protein–protein interaction analysis was conducted using the 127 shared targets. The resulting network displayed a densely connected structure centered around several genes with markedly high degree values (Fig. 2A). Among them, AKT1, JUN, STAT3, TP53 and TNF formed the most prominent interaction cluster, indicating their potential roles as central regulatory nodes involved in immune activation and stress signaling during ischemia–reperfusion. These hub genes were therefore prioritized for subsequent network interpretation and molecular docking analysis.

Building upon these findings, an integrated component–target–pathway network was constructed to visualize how major WSP constituents may converge on these core regulatory nodes (Fig. 2B). Active components such as quercetin, kaempferol, licorice chalcone A and naringenin showed extensive connectivity to hub genes and to pathways enriched in the KEGG analysis, including Toll-like receptor, IL-17 and NOD-like receptor signaling. This multi-layered mapping suggests that WSP exerts coordinated biological effects through parallel modulation of multiple inflammatory pathways.

To further support the biological significance of these network interactions, molecular docking was conducted between the five hub proteins and selected database-screened candidate compounds with relatively high oral bioavailability, including Glycyrol and 7,2',4'-trihydroxy-5-methoxy-3-arylcoumarin. These compounds were selected as putative candidates derived from TCMSP screening rather than chemically verified representative constituents of the experimental WSP extract. For each ligand–protein pair, five independent docking runs were conducted using CB-Dock2 and the lowest energy conformations were retained. The selected database-screened candidate compounds demonstrated favorable predicted binding to the catalytic or regulatory pockets of AKT1, JUN, STAT3, TP53 and TNF, forming hydrogen bonds or hydrophobic contacts with key residues. As a representative example, figure 2C shows the low-energy docking poses of selected candidate compounds with AKT1 (PDB ID: 3CQU), illustrating their ability to occupy the binding cavity and form favorable contacts with key residues.

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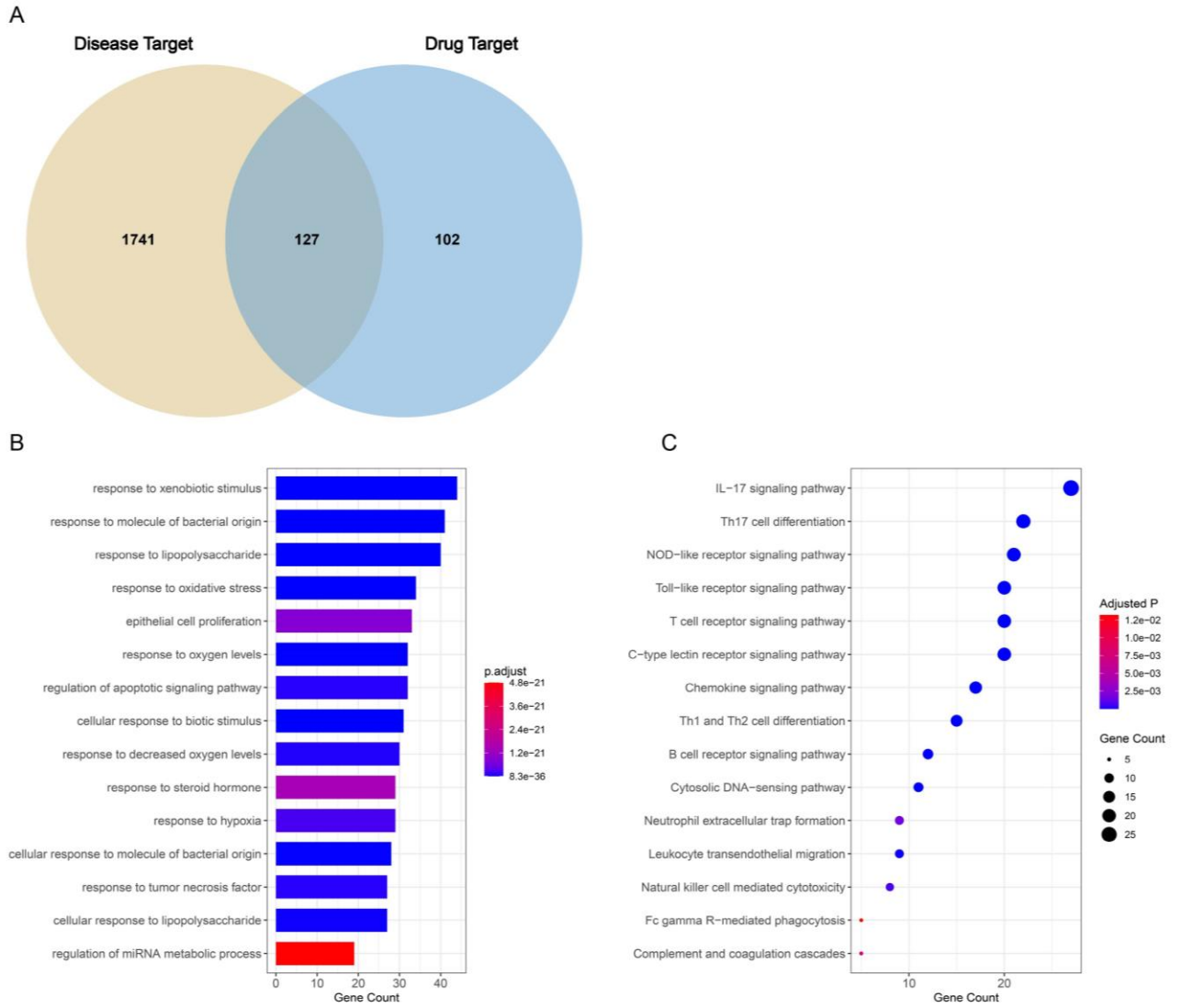

Fig. 1: Shared targets and enrichment analysis of WSP in HIRI. (A) Overlap between WSP-related and HIRI-related targets; (B-C) GO; (B) and KEGG; (C) enrichment of overlapping genes.

Overall, the integration of network topology and docking analyses indicates that WSP likely acts by targeting central inflammatory regulators such as AKT1, JUN, STAT3, TP53 and TNF. The ability of key active compounds to bind these proteins provides structural support for their involvement in modulating innate immune and stress-response pathways implicated in HIRI.

WSP protects hepatocytes against H/R-induced injury by improving viability, reducing inflammation and suppressing oxidative stress

To experimentally validate the protective effects predicted by the network and docking analyses, at first, cell viability following H/R injury was assessed. Figure 3A shows that H/R markedly reduced AML12 cell viability relative to the control group ($P < 0.001$), whereas WSP administration significantly restored viability in a dose-dependent manner ($P < 0.001$).

Consistent with the enriched inflammatory pathways, H/R stimulation caused substantial increases in IL-1β, IL-6 and TNF-α secretion (Fig. 3B, $P < 0.001$). Both WSP concentrations significantly suppressed these cytokines, with the high-dose group producing the most prominent reduction ($P < 0.001$), indicating that WSP attenuates H/R-induced inflammatory activation.

H/R also triggered marked intracellular ROS accumulation (Fig. 3C, $P < 0.001$). WSP treatment effectively decreased ROS levels in a dose-dependent fashion ($P < 0.001$), consistent with its predicted antioxidant effects.

Given that innate immune signaling emerged as a major regulatory axis in the network analysis, the TLR4–AKT–NF-κB pathway was examined next (Fig. 3D).

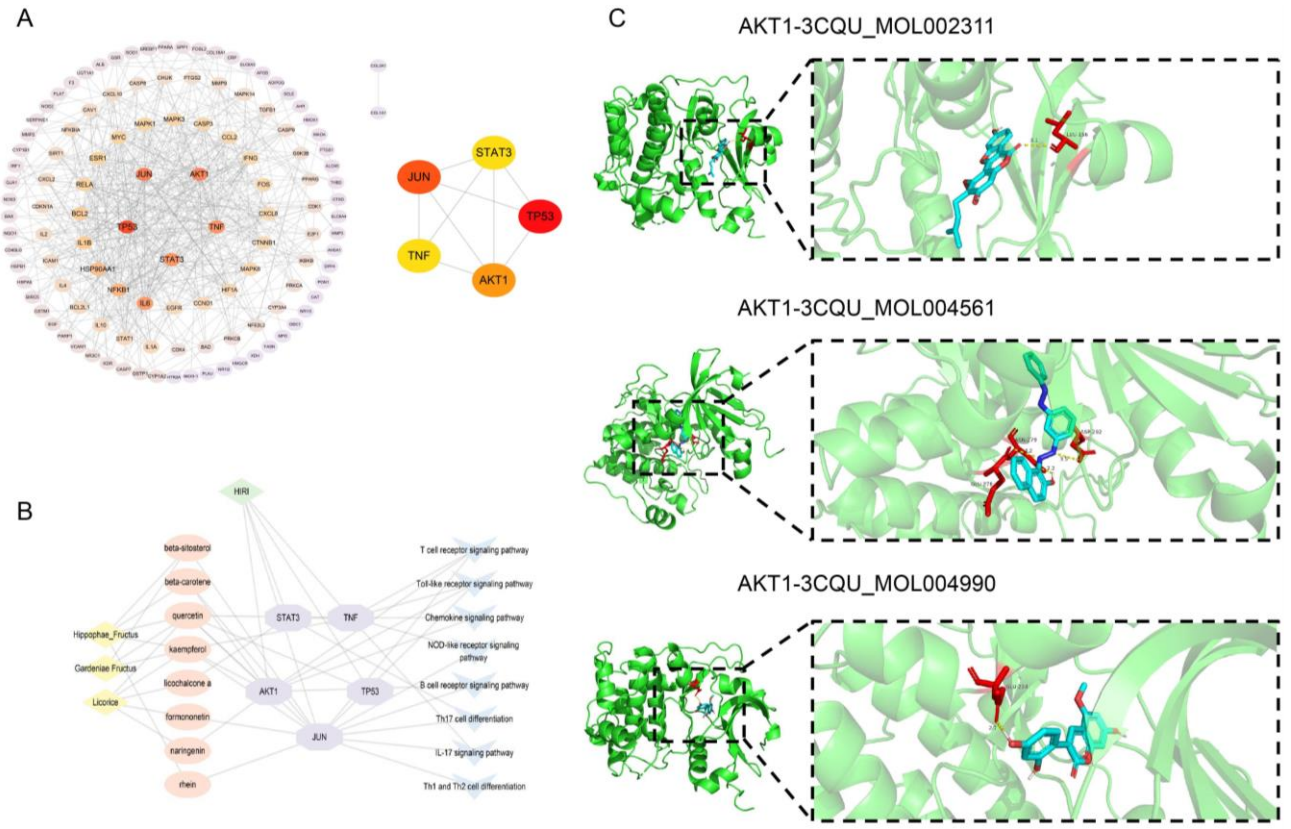

Fig. 2: Network analysis and molecular docking of WSP targets. (A) PPI network and hub gene identification based on degree values; (B) Component–target–pathway network linking WSP candidate compounds to hub genes and enriched immune pathways; (C) Molecular docking of selected database-screened candidate compounds with the five hub proteins.

H/R stimulation strongly upregulated TLR4 expression relative to the control group ($P < 0.001$), whereas WSP treatment significantly suppressed this increase, with the high-dose group showing a marked reduction relative to the H/R group ($P < 0.001$). Correspondingly, H/R significantly increased phosphorylation of AKT at Ser473 without altering total AKT1 expression. Both WSP concentrations significantly decreased p-AKT levels and high-dose WSP significantly reduced AKT activation relative to the H/R group ($P< 0.001$). Downstream of AKT signaling, phosphorylation of NF-κB p65 was also considerably elevated following H/R ($P < 0.001$). WSP intervention reversed this increase in a concentration-dependent fashion, with a significant decrease detected in the low-dose group ($P < 0.01$) and an even more pronounced effect evident in the high-dose group ($P < 0.001$).

In aggregate, these results suggest that WSP protects hepatocytes from H/R-induced injury by improving viability, reducing inflammatory cytokine release, limiting oxidative stress and effectively inhibiting activation of the TLR4–AKT–NF-κB inflammatory signaling axis.

DISCUSSION

Network pharmacology and molecular docking, together with cellular experiments, were applied in this study to demonstrate that WSP alleviates HIRI by mitigating inflammation and oxidative stress, potentially through inhibition of the TLR4–AKT–NF-κB signaling axis. The overlapping drug–disease targets were significantly enriched in pathways related to inflammatory activation, stress responses and innate immunity. PPI analysis identified AKT1, JUN, STAT3, TP53 and TNF as central regulatory nodes, while molecular docking suggested that selected database-screened candidate compounds showed favorable predicted binding to these hub proteins, especially AKT1. Importantly, these compounds were interpreted as putative pharmacological candidates derived from database screening rather than chemically verified representative constituents of the experimental WSP extract. Functional experiments confirmed that WSP improved cell viability, reduced secretion of key inflammatory cytokines, decreased ROS accumulation and suppressed activation of TLR4, p-AKT and p–NF-κB. The biological relevance of these findings can be interpreted within the context of established injury mechanisms.

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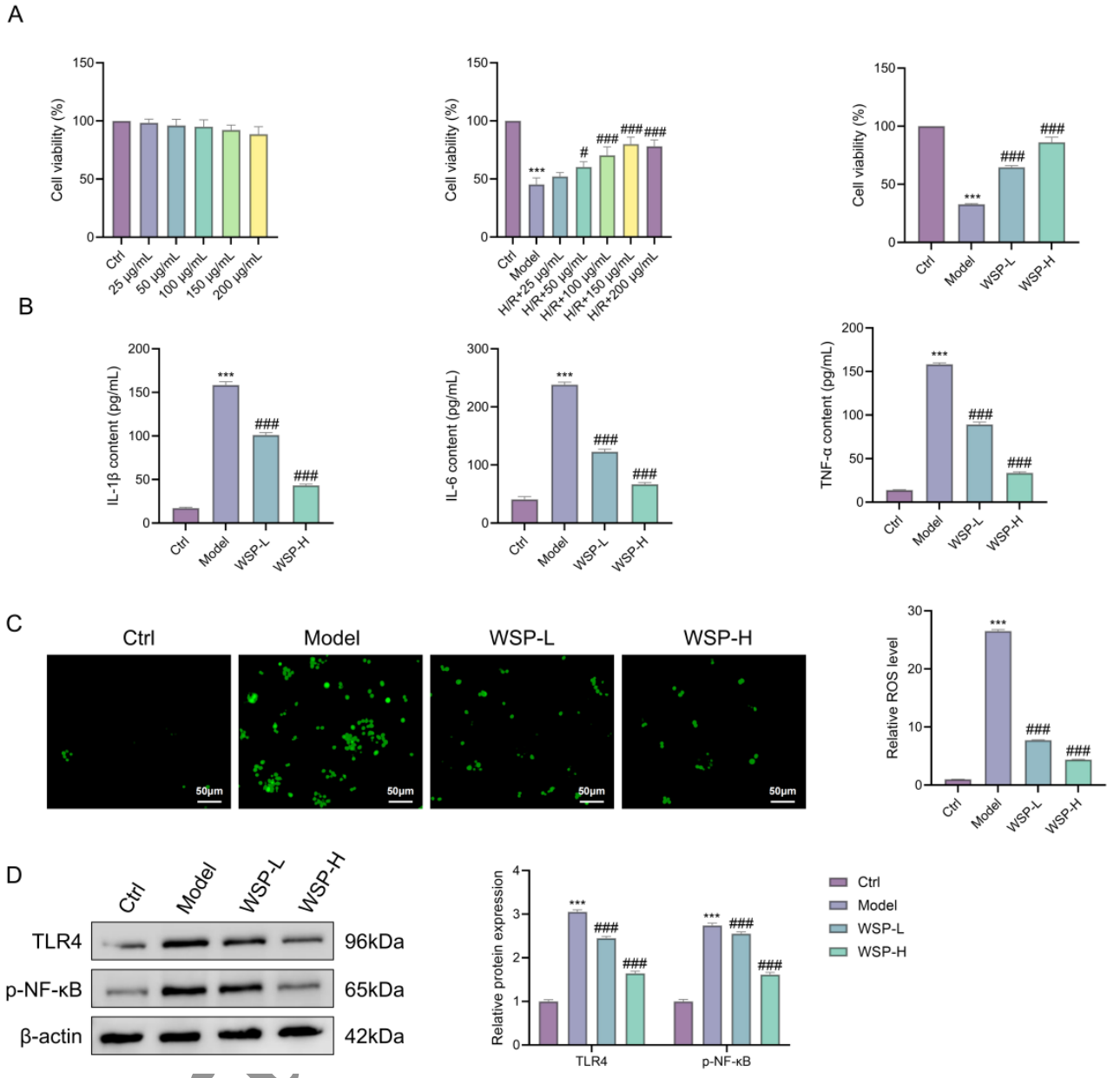

Fig. 3: Functional validation of WSP in H/R-treated AML12 cells. (A) CCK-8 assay measuring cell viability; (B) ELISA detecting IL-1β, IL-6 and TNF-α levels; (C) Intracellular ROS levels; (D) Western blot analysis of TLR4, p-AKT (Ser473), AKT1 and p-NF-κB expression. Data are presented as mean ± SD from three independent biological replicates.

HIRI is characterized by rapid inflammatory amplification and oxidative stress following reperfusion, with TLR4 serving as a critical upstream receptor sensing danger-associated molecular patterns (Wang *et al*., 2024). Prior studies have demonstrated that TLR4 activation drives MyD88-dependent signaling and downstream activation of AKT and NF-κB, contributing to inflammatory cytokine release and hepatocellular damage (Morsy *et al*., 2022). In this study, the marked elevation of TLR4, p-AKT and p–NF-κB following H/R stimulation aligns with these established mechanisms. The ability of WSP to suppress these molecules in a concentration-dependent fashion is consistent with the pharmacological profiles of its major herbal components—many of which, particularly flavonoids and sesquiterpenes, have been reported to attenuate TLR4 signaling and cytokine overproduction (Younis *et al*., 2023; Li *et al*., 2025). These parallel findings lend biological plausibility to the anti-inflammatory effects observed in this experiment.

AKT1 emerged as a particularly important regulatory node in this network analysis and recent studies also highlight its role in modulating both inflammatory transcription and oxidative stress during HIRI (Huo *et al*.,

2023). Ser473 phosphorylation of AKT is known to promote NF-κB translocation and amplify cytokine expression (Cui *et al*., 2020). Consistent with this, WSP reduced p-AKT without altering total AKT1 levels, suggesting modulation of kinase activation rather than protein abundance. This pattern matches the known behavior of many natural compounds, which often regulate kinase activity through noncovalent interactions with ATP-binding pockets. The docking results, showing stable ligand binding to AKT1, further support this interpretation. Meanwhile, the decrease in intracellular ROS levels detected in this study is consistent with the antioxidant properties of flavonoid-rich formulations, as several components of WSP—including quercetin derivatives—are known to scavenge reactive oxygen species and modulate PI3K/AKT activity (Liu *et al*., 2024). These converging lines of evidence reinforce the mechanistic coherence of the findings.

NF-κB acts as a terminal effector of inflammatory transcriptional amplification in HIRI and its persistent activation contributes to cytokine overproduction and hepatocyte injury (Deng and Zhou 2024). In this study, WSP significantly reduced p–NF-κB levels and markedly suppressed IL-1β, IL-6 and TNF-α secretion. These effects mirror the inhibitory trends observed for TLR4 and p-AKT, supporting the concept that WSP interferes with this entire upstream–downstream inflammatory cascade. While numerous reports have described anti-inflammatory properties of individual herbal constituents, few studies have systematically delineated the multi-target and multi-pathway interactions of a traditional formula within a defined signaling axis. By integrating network prediction, structural docking and functional validation, the present study offers mechanistic clarity and highlights WSP as a coordinated modulator of the TLR4–AKT–NF-κB axis, providing a fresh perspective on formula-based therapeutic strategies for HIRI.

In addition to pathway-level regulation, the multi-component nature of WSP should be considered when interpreting its biological effects. Unlike single-compound interventions, WSP may exert protective activity through coordinated regulation of multiple targets and signaling pathways. Candidate constituents such as flavonoids, coumarins, chalcones and other phytochemicals may jointly contribute to the inhibition of oxidative stress, inflammatory cytokine production and activation of the TLR4–AKT–NF-κB pathway. Therefore, the observed protective effect of WSP is more likely to reflect a coordinated multi-component and multi-target pharmacological pattern rather than the action of a single compound. Previous reviews on Chinese herbal medicine have suggested that such synergistic effects may arise from pharmacodynamic and pharmacokinetic interactions among multiple bioactive constituents, including complementary modulation of different molecular targets, enhanced bioavailability and reduction of adverse effects. Systems biology and network pharmacology have also been proposed as suitable approaches to dissect these multi-component and multi-target interactions (Zhou *et al*., 2016; Caesar and Cech, 2019). Consistently, studies of classical herbal formulas, such as Yinchenhao decoction, have demonstrated that combined active constituents can exert stronger hepatoprotective effects than individual components by acting on multiple targets in hepatic injury models. Nevertheless, the precise synergistic or antagonistic interactions among different WSP constituents remain unclear and require further experimental validation, including component-reconstitution studies and combination-index analyses (Jung and Cheon, 2024).

Despite the strengths of this integrated network pharmacology, molecular docking and cellular validation approach, several limitations should be acknowledged. First, although candidate active compounds of WSP were screened from TCMSP according to oral bioavailability and drug-likeness criteria, this database-based screening does not fully represent chemical standardization of the experimental WSP extract. The screened compounds should therefore be interpreted as putative pharmacological candidates rather than chemically verified marker constituents. In particular, Sudan III was retained only as a database-screened candidate compound and should not be overinterpreted as a representative bioactive marker of WSP. Future studies should use HPLC, UPLC, or LC-MS-based chemical fingerprinting and quantitative marker analysis to identify and verify the major active constituents of WSP. Second, molecular docking only provides predicted ligand-target interactions and known AKT inhibitors were not included as reference docking controls in the present study. Therefore, future docking and validation studies should include established AKT inhibitors as positive references and further confirm ligand-target interactions using biophysical techniques such as surface plasmon resonance or isothermal titration calorimetry. Third, although 50 μg/mL and 150 μg/mL WSP were selected based on CCK-8 cell viability results and showed no obvious cytotoxicity, only two concentrations were used in the subsequent functional experiments; thus, a complete dose-response relationship was not established. Additional concentration gradients are needed to determine the optimal effective concentration of WSP. Fourth, ROS levels were evaluated by fluorescence imaging rather than flow cytometry, which may limit the quantitative accuracy of oxidative stress assessment. Moreover, the involvement of the TLR4–AKT–NF-κB pathway was not confirmed using gene silencing or pharmacological inhibitors.

Future studies should employ siRNA-mediated knockdown or pathway-specific inhibitors targeting TLR4, AKT, or NF-κB to verify the causal role of this

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signaling axis in WSP-mediated protection. Finally, the present study mainly relied on an *in-vitro* H/R model using AML12 hepatocytes and focused on acute inflammatory and oxidative injury. This model cannot fully reproduce the complex immune microenvironment, inflammatory cell infiltration, sinusoidal endothelial dysfunction, hemodynamic changes and multicellular interactions involved in HIRI *in-vivo*. In addition, the long-term effects of WSP on sustained liver function recovery, fibrosis progression, chronic inflammation and tissue remodeling were not evaluated. Future studies should establish rodent HIRI models with extended observation periods to confirm the hepatoprotective effects of WSP and assess whether it can prevent chronic liver injury or fibrosis after ischemia-reperfusion injury.

In conclusion, this study suggests that WSP may attenuate H/R-induced hepatocellular injury by reducing inflammatory cytokine secretion, limiting ROS accumulation and suppressing activation of the TLR4–AKT–NF-κB signaling pathway. Network pharmacology and molecular docking further indicate that AKT1 and related inflammatory regulators may represent potential molecular nodes involved in the action of WSP. However, these findings are mainly based on computational prediction and *in-vitro* validation. Further studies involving chemical standardization, complete dose-response analysis, pathway-specific intervention experiments and long-term validation in rodent HIRI models are required to confirm the hepatoprotective potential and translational value of WSP.

CONCLUSION

Through the integration of computational prediction and *in-vitro* functional validation, this study suggests that WSP may exert protective effects against H/R-induced hepatocellular injury. WSP improved cell viability, reduced inflammatory cytokine secretion, attenuated oxidative stress and suppressed activation of the TLR4–AKT–NF-κB signaling cascade in AML12 cells. Network pharmacology and docking analyses further identified AKT1 and related inflammatory regulators as potential molecular nodes involved in WSP-mediated protection. However, these findings are mainly based on computational prediction and cellular experiments. Further studies involving chemical standardization, complete dose-response analysis, pathway-specific intervention, long-term observation and *in-vivo* validation are required to confirm the hepatoprotective potential and translational value of WSP.

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The authors declare that an AI-assisted language editing tool was used solely for English language editing and polishing of the manuscript to improve clarity and readability. The scientific content and data interpretation carried out were and the conclusions were drawn entirely by the authors.

Author's contributions
Jucun Huang and Dan Dai: Contributed to the conception and design of the study, execution of experiments, data acquisition, data analysis and interpretation. Both authors participated in drafting, revising and critically reviewing the manuscript, approved the final version for publication, 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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Data availability statement
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The transcriptomic data analyzed in this study were obtained from the Gene Expression Omnibus (GEO) under accession number GSE228782.

Ethical approval
Not applicable.

Conflict of interest
The authors declare no conflict of interest.

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