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Network pharmacology-based prediction and molecular docking-based strategy to investigate the potential mechanism of Leonurus japonicus Houtt. Against myocardial ischemia reperfusion injury

Xuan Liu[1], Zilian Zhan[1], Rui Zhang[1], Yadong Wang[1] and Qiang Xu[1*]

Abstract

Background Leonurus japonicus Houtt. (LJH) has multiple pharmacological effects.

Objective To investigate the potential mechanism of LJH in the treatment of myocardial ischemia-reperfusion injury (MIRI) using network pharmacology, molecular docking technology, and in vitro experiments.

Methods Herbs for ischemic heart disease were identified with the help of herb-disease databases. The TCMSP database was used to find the potential targets of LJH. Disease targets of MIRI were identified with the help of Disgenet, Genecard, Alliance of Genome Resources databases. The common targets were obtained with the help of VENN diagram, and the common targets were analyzed by GO function and KEEG pathway enrichment to predict the potential mechanism of action of LJH in treating MIRI. With the help of STRING database and Cytoscape software, we constructed a visual protein-protein interaction (PPI) network model to screen the core targets and then docked the core targets with the corresponding ligand molecules. AC16 cells were used to simulate MIRI by glucose-oxygen deprivation, and apoptosis was detected by Annexin V-FITC/PI double staining; protein expression was detected by Western blot.

Results LJH was one of the herbal remedies for the treatment of ischemic heart disease. LJH had 247 potential targets of action and 26 targets in common with MIRI. These 26 targets were enriched in the TNF signaling pathway and NF-kappa B signaling pathway, and the core targets screened by the PPI results included TNF, VCAM1, and MMP9. Molecular docking results showed that the compounds in LJH docked well with the core target proteins. In vitro experiments showed that LJH could inhibit the elevation of TNF, VCAM1, and MMP9 after MIRI, reduce apoptosis, and inhibit inflammation.

Conclusion The mechanism of LJH in the treatment of MIRI was mainly related to the activation of TNF signaling pathway and NF-kappa B signaling pathway, and the regulation of TNF, VCAM1, and MMP9 protein expression.

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Keywords Leonurus japonicus Houtt., Myocardial ischemia-reperfusion injury, TNF-α, NF-κB

Introduction

Acute myocardial infarction (AMI) is a serious clinical emergency [1]. AMI is a myocardial necrosis caused by acute and persistent ischemia and hypoxia of the coronary artery, characterized by acute onset, multiple complications, and a high mortality rate [2]. With the development of China's economy and changes in people's lifestyles, the mortality rate of AMI has shown an upward trend in recent years [3]. For patients with myocardial infarction, the preferred treatment method to reduce injury is thrombolytic therapy or one-stage percutaneous coronary intervention to achieve timely and effective myocardial reperfusion [4]. However, when the myocardium undergoes ischemia and re-establishes blood supply, local inflammation and increased production of reactive oxygen species can lead to secondary damage [5]. In many clinical practices, it has been found that AMI patients who recover blood flow reperfusion through percutaneous coronary intervention do not completely reverse the pathological process of AMI. Instead, further exacerbate myocardial injury and myocardial cell death usually results in enlarged myocardial infarction area, arrhythmia, and other symptoms, which is clinically defined as myocardial ischemia-reperfusion injury (MIRI) [6]. The recovery of blood flow and oxygen transport further activates the inflammatory signaling pathway usually induces inflammation during myocardial ischemia [5]. Currently, an increasing number of studies have found that interleukin (IL) and inflammasomes are key inflammatory mediators and processes leading to abnormal damage [7]. The pathogenesis of MIRI is complex. Effective prevention and treatment for MIRI are limited, so it is necessary to develop new treatment methods.

Traditional Chinese medicine has been used in China for thousands of years and has accumulated a long experience in treating cardiovascular diseases. At present, the databases on the ingredients, targets, and disease relationships of Chinese herbal medicine are relatively complete [8]. In recent years, traditional Chinese medicine has played an important role as an alternative therapy in the clinical treatment of MIRI. Herbs have become a research hotspot due to their high efficacy and few side effects. Herbs can participate in different signaling pathways and exert pharmacological effects through these different pathways, which is in line with the complex pathological mechanisms of MIRI [9]. The multi-target characteristics of Chinese herbal medicine stem from its complex chemical composition. Modern research suggests that the possible cardioprotective mechanisms of herbs may be multifactorial, including clearing inflammation, inhibiting cell apoptosis, and regulating vascular remodeling [10].

In current research, we explored frequently-used herbs in ischemic heart disease based on herb-disease databases. Then, using network pharmacology methods, Leonurus japonicus Houtt. (LJH) was used as the research object for in-depth target and pathway prediction. On the basis of PPI, we screened the hub gene and its corresponding components for molecular docking. Cell experiments validated the findings from network pharmacology.

Materials and methods

Searching for herb to be studied

Herbs related to ischemic heart disease were identified by Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (using 'ischemic heart disease' as a key word), HERB (using 'Acute ischemic heart disease' as keyword, P-value < 0.05), and TCM-Suite (using 'Ischemic Heart Disease' as keyword) databases. The obtained herbs from these three databases were subjected to VENN diagram for common herbs.

Searching for possible pharmacological components and potential targets for LJH

The TCMSP database was used to obtain the chemical compositions of LJH using LJH as the search term. For the chemical constituents of LJH, oral bioavailability (OB) ≥ 20% and drug-like properties (DL) ≥ 0.1 were set as the screening conditions to obtain the potential pharmacologically active components [11]. The corresponding target proteins were queried according to the active ingredient ID through TCMSP database. The collected target proteins were imported into UniProt database for name standardization, to obtain the target proteins corresponding to LJH.

MIRI-related genes were searched using the human disease-gene correlation databases, including DisGeNET, GeneCards, and Alliance of Genome Resources databases. The search term used for the Disgenet database was 'Myocardial Ischemia', the search term for GeneCards database was 'myocardial ischemia reperfusion' (Relevance score > 1), and the Alliance of Genome Resources database used the search term 'heart disease'. The obtained MIRI-related genes were imported into the UniProt database for name normalization and then intersected with LJH-targeting proteins. In this way, the LJH-targeting proteins against MIRI were obtained.

Construction of compound-disease target network and target annotation

LJH active compounds and the genes of target proteins targeted by these compounds were imported into Cytoscape software and visualized to construct a compound-MIRI target network. In the network diagram, compounds and targets were represented by nodes, and the interactions between nodes were represented by edges. Proteins were imported into the STRING database to analyze protein-protein interaction relationships at a high confidence of 0.700. Cytoscape software was used to screen the top three proteins using Closeness, Degree, MCC, and MNC methods. The target proteins were subjected to Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis by clusterProfiler and pathview R packages, with $P < 0.05$ as the screening condition. The proteins involved in TNF signaling pathway and NF-kappa B signaling pathway were traced for the compound source.

Molecular docking validation

The obtained hub proteins were used as receptors and their corresponding compounds as ligands for molecular docking analysis. Firstly, the 3D structure of compounds was obtained through PubChem database. The crystal structures of the proteins were obtained in the RCSB Protein Data Bank (PDB) database, with the H_2O and small molecule ligands removed using PyMOLWin.exe software. The processed receptor and ligand files were subjected to protein-ligand blind docking at the detected candidate pockets with AutoDock Vina. Protein-compound pairs with excellent Vina scores were validated for cellular experiments.

Preparation of lyophilizate from standard LJH decoction

LJH slices (200 g) were boiled twice with water. For the first boil, 12 times the amount of water was added and soaked for 30 min. After boiling on high heat, a slight boil was maintained for 30 min. The decoction liquid was filtered through a 350-mesh sieve. Then, 10 times the amount of water was added for the second boiling. The two filtrates were combined and concentrated under reduced pressure (temperature: 65℃, vacuum: -0.10 MPa) to 150 mL of liquid extract, which was divided into vials. After dispensing, the vials were transferred to a vacuum freeze-dryer for freeze-drying.

Cell culture

AC16 (ATCC, USA) were differentiated into mature cardiomyocytes using Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium, with the addition of 1× Insulin, Transferrin, Selenium Solution (Gibco, USA) and 2% Horse Serum (Gibco, USA) to make the medium complete. Hypoxia-glucose deprivation was performed on AC16 cells for simulating ischemia; then, oxygen-glucose supply was restored to AC16 cells for simulating reperfusion. Briefly, AC16 cells were incubated for 1 h using a pre-saturated ischemia-simulated solution of 95% N_2 + 5% CO_2 instead of medium. The simulated ischemia solution was then discarded and AC16 cells were incubated for 3 h using DMEM/F12 to simulate reperfusion solution. All groups except the control group received the ischemia-reperfusion procedure described above.

To investigate the effect of LJH on MIRI through TNF signaling pathway regulation, TNF-α was added to the culture medium to activate the TNF signaling pathway. LJH lyophilizate was used at concentrations of 20 (low), 50 (medium), and 100 (high) ng/mL. For pretreatment, the drug was used 24 h before MIRI modeling.

Western blot analysis

Protein expression in AC16 cells was determined by western blotting. After the cells were digested and lysed, the total protein concentration was determined using the Bradford protein assay (BioRad, USA). Aliquots of protein samples were loaded on 10–15% SDS polyacrylamide gel electrophoresis for separation and then transferred to polyvinylidene difluoride (PVDF) membranes. PVDF was blocked and incubated with primary antibodies overnight. The primary antibodies included anti-TNF-α (1:1000, Abcam), anti-MMP-9 (1:1000, Abcam), anti-VCAM1 (1:1000, Abcam), phospho-IKKα/β at its autophosphorylation site (Ser176/180) (Cell Signaling Technology), NF-κB p65 (1:1000, Cell Signaling Technology), anti-collagen type I (COL1) (1:1000, ThermoFisher Scientific), anti-αSMA (1:1000, Cell Signaling), and anti-osteopontin (OPN) (1:1000, Merck Darmstadt, Germany). After washing, the membrane was incubated with the second antibody conjugated with horseradish peroxidase. Imprint was incubated in Immunobilion ® Forte Western blotting of HRP substrates (Millipore, USA) to form luminescent bands for detection. The intensity of the bands was analyzed using ImageJ software and standardized to β-actin.

Enzyme linked immunosorbent assay (ELISA)

After treatment, the supernatant of cell culture was collected and centrifuged at 1000 g (3750 rpm) for 20 min at 4℃. The cell supernatant reacted and was tested using human TNF-α, IL-1β, and IL-6 ELISA kits according to each supplier's instruction.

Statistical analysis

All statistical analyses were conducted using GraphPad software. One-way Analysis of Variance with post-hoc Bonferroni test.

Results

Collection of active ingredients in LJH and construction of ingredient-target network

Based on TCMSP, HERB, and TCM-Suite database queries, LJH was one of the main herbs used to treat ischemic heart disease (Supplementary Fig. 1). After querying the TCMSP database, LJH contained 51 compounds. Among them, there were 18 compounds that complied with OB ≥ 20% and DL ≥ 0.1. Target prediction was performed on these active ingredients, and 231 target proteins were obtained after name normalization through UniProt. The network composed of these components and target proteins included 247 nodes and 445 edges (Fig. 1), with an average number of neighbors of 3.6. Therefore, LJH had the advantage of being multi-component and multi-target in the treatment of disease.

Annotation for targets of LJH

After searching through DisGeNET, GeneCards, and Alliance of Genome Resources, we collected MIRI-related genes. Then, LJH can target 26 MIRI-related genes (Fig. 2A, Supplementary Fig. 2). These targets were

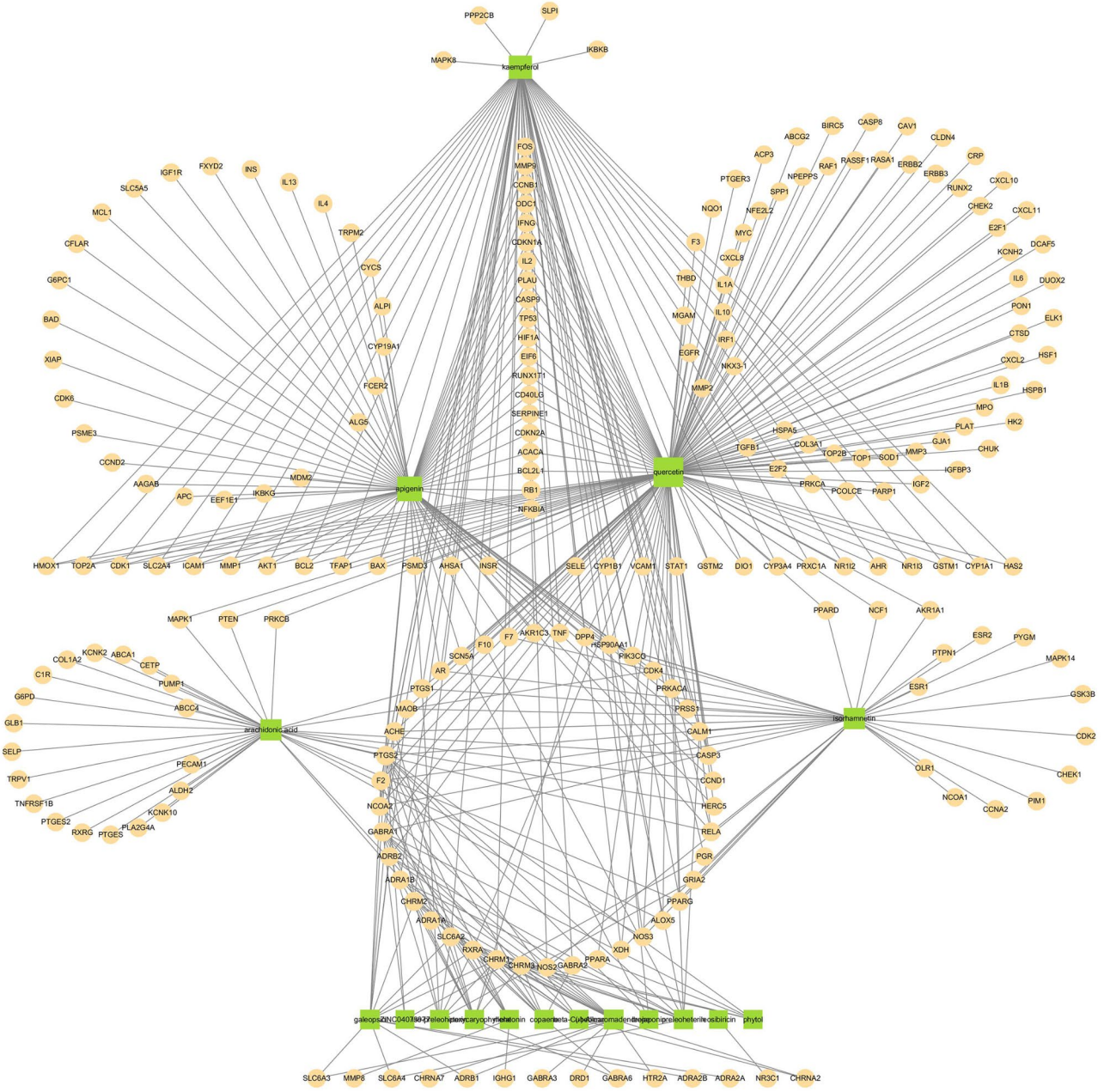

Fig. 1 The "compound-target" network of components and targets in LJH. Among them, the green square represents compounds; The yellow circle represents the target of action. After analysis, the network presents shapes of different sizes according to the degree values

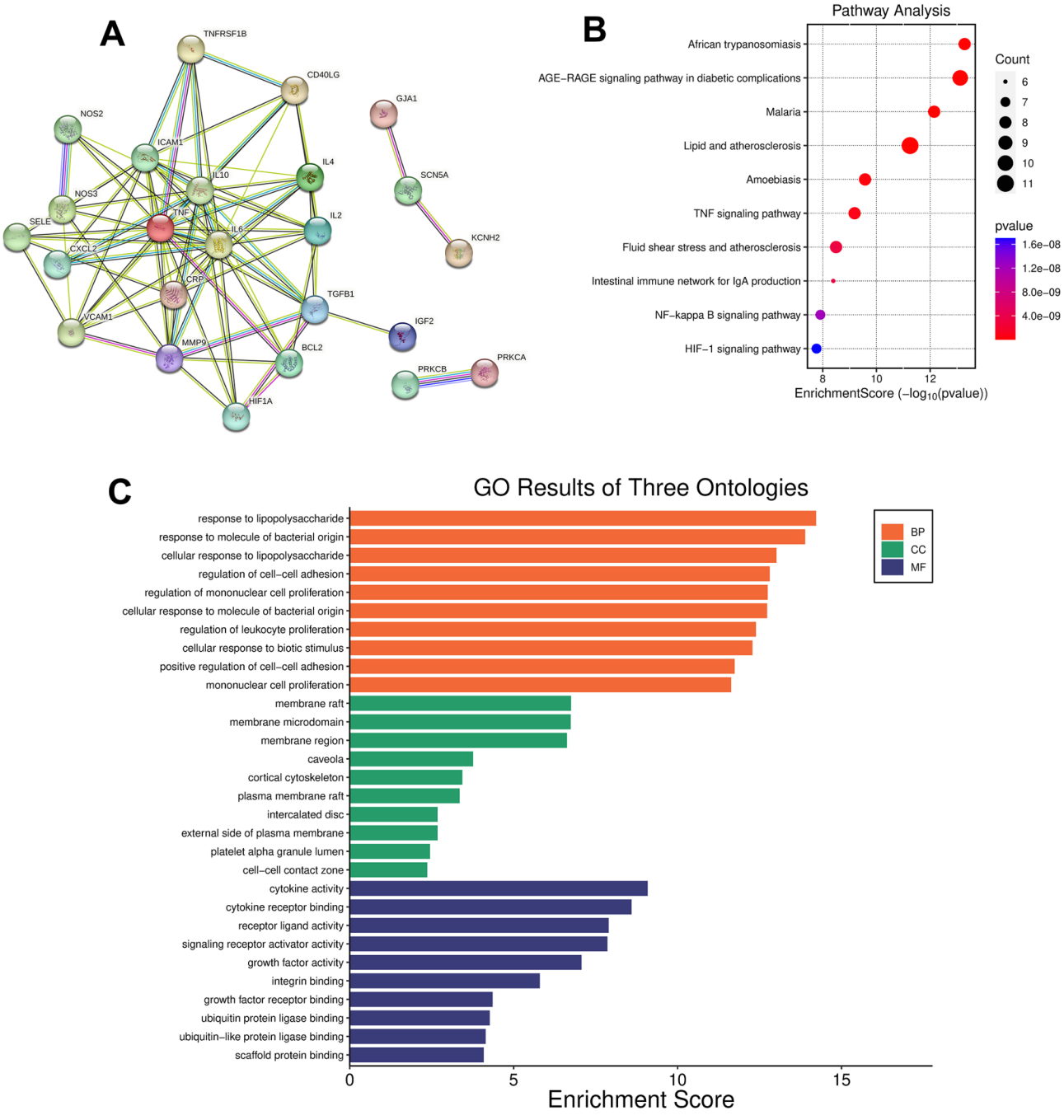

Fig. 2 Annotation of common targets between Leonurus japonicus Houtt. and MIRI. (**A**) A PPI network for common targets of Leonurus japonicus Houtt. and MIRI. (**B**) KEEG pathway enrichment analysis of common targets between Leonurus japonicus Houtt. and MIRI. (**C**) GO enrichment analysis of common targets between Leonurus japonicus Houtt. and MIRI

enriched in TNF signaling pathway, NF-kappa B signaling pathway, etc. (Fig. 2B). The GO terms enriched by these 26 targets were a response to lipopolysaccharide, regulation of cell-cell adhesion, regulation of mononuclear cell proliferation, cytokine activity, receptor ligand activity, growth factor activity, etc. (Fig. 2C).

Key pathways of LJH

The enriched TNF signaling pathway and NF-kappa B signaling pathway aroused our interest. We backtracked the involved targets and compounds in these two pathways. TNF, VCAM1, IL6, MMP9, ICAM1, CXCL2, TNFRSF1B, and SELE were enriched in TNF signaling pathway, while TNF, BCL2, PRKCB, VCAM1, CD40LG, ICAM1, and CXCL2 were enriched in NF-kappa B

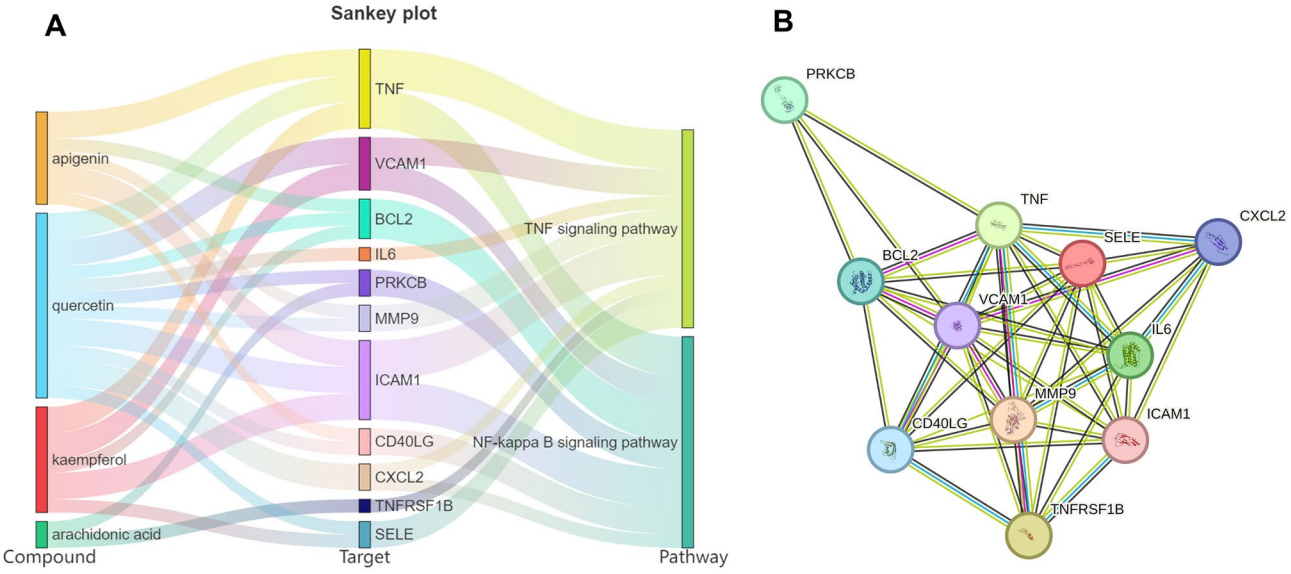

Fig. 3 TNF/NF-kappa B signaling pathway. (**A**) Sankey plots for the targets and compounds involved in TNF/NF-kappa B signaling pathway. (**B**) A PPI network for targets involved in TNF/NF-kappa B signaling pathway

Table 1 Molecular Docking results

Protein	Ligand	CurPocket ID	Vina score (kcal/mol)	Cavity volume (Å3)	Center (x, y, z)
MMP9	apigenin	C1	-9.7	705	39, 22, 141
		C2	-9.2	460	63, 31, 114
MMP9	quercetin	C1	-9.6	705	39, 22, 141
		C2	-9.4	460	63, 31, 114
TNF	apigenin	C1	-8.3	1593	-5, 84, 27
		C4	-7.6	243	-17, 72, 36
		C2	-7.1	337	8, 67, 12
TNF	quercetin	C1	-8.8	1593	-5, 84, 27
		C2	-7.7	337	8, 67, 12
		C4	-7.3	243	-17, 72, 36
TNF	kaempferol	C1	-7.9	1593	-5, 84, 27
		C4	-7.3	243	-17, 72, 36
		C2	-7.1	337	8, 67, 12
VCAM1	quercetin	C1	-8.4	1365	37, 15, 8
		C3	-7.8	1096	22, 8, 16
		C4	-7.7	691	15, 11, 3
		C5	-7.7	336	21, 18, 17
		C2	-7.5	1112	6, 15, 25
VCAM1	kaempferol	C3	-8.2	1096	22, 8, 16
		C1	-8.1	1365	37, 15, 8
		C5	-7.9	336	21, 18, 17
		C2	-7.5	1112	6, 15, 25
		C4	-7.4	691	15, 11, 3

signaling pathway (Fig. 3A). The protein-protein interactions among these eleven targets were shown in Fig. 3B. Using the hub gene screening methods, TNF, VCAM1, and MMP9 were identified as hub genes. After molecular docking, MMP9-apigenin pair showed the best Vina score (-9.7 kcal/mol) (Table 1).

LJH targeted to TNF, VCAM1, and MMP9

We conducted in vitro experiments using a human myocardial cell line (AC16) to further validate the network pharmacology prediction results of LJH in MIRI. Firstly, we stimulated AC16 cells with different concentrations of LJH and measured the expression TNF levels in cells and cell culture. The results showed that LJH

reduced the expression levels of TNF-α in the AC16 I/R model (Fig. 4A) and its release in the cell culture medium (Fig. 4B). LJH reduced the expression level of MMP9 protein in the AC16 I/R model, but the addition of TNF-α re-increased this level (Fig. 4C). LJH also reduced the expression level of VCAM1 protein in the AC16 I/R model, but the addition of TNF-α re-increased this level (Fig. 4D). Moreover, LJH regulated the protein levels in the NF kappa B signaling pathway, including IKKα/β (Figs. 4E) and NF-κB protein levels (Figs. 4F).

Effects of LJH on inflammatory response and vascular remodeling during MIRI

Our results indicate that compared to the control group, the apoptosis rate in the MIRI AC16 cell model was significantly increased; LJH can reduce the apoptosis rate of AC16 cells under MIRI state, but the addition of TNF-α can induce cell apoptosis again (Fig. 5A). Next, we investigated the levels of IL-1β in cell culture medium and the expression level of NLRP3 protein in cells using ELISA and Western blotting. Compared with the control group, LJH reduced the levels of IL-1β and NLRP3 protein expression induced by MIRI, while TNF-α could increase the levels of these factors (Fig. 5B and C). Moreover, LJH significantly inhibited the expression of vascular fibrosis marker COL1 in MIRI-induced AC16 cells (Fig. 5D). Furthermore, LJH significantly inhibited MIRI-induced OPN protein expression (Fig. 5E), but did not affect αSMA protein level (Fig. 5F), indicating that LJH inhibited phenotypic switching of AC16 cells under MIRI.

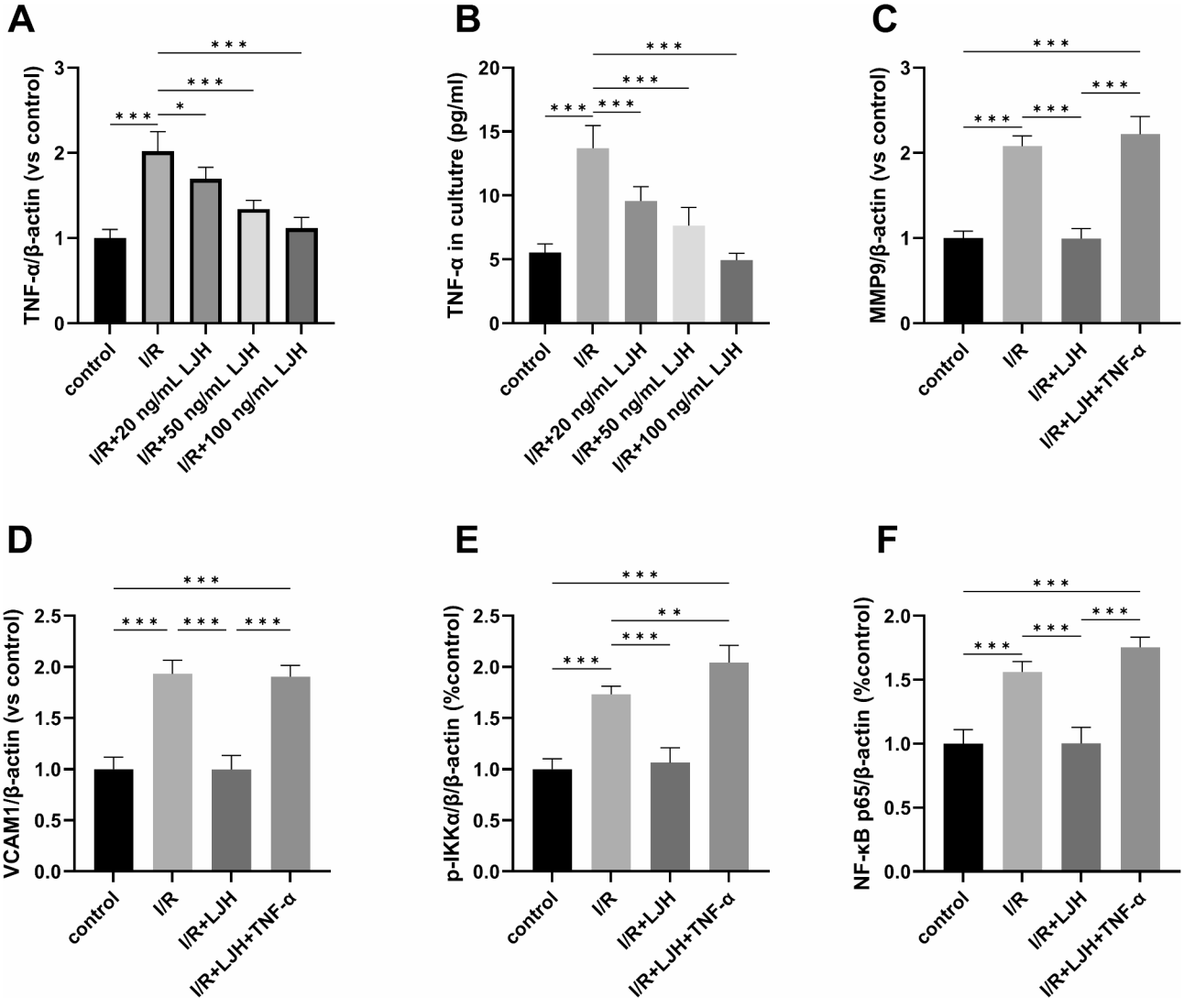

Fig. 4 Effects of Leonurus japonicus Houtt. on TNF-α, VCAM1, and MMP9 expression levels in AC16 cells. (**A**) TNF-α protein levels in AC16 cells were evaluated by western blot analysis with β-actin as a loading control. (**B**) TNF-α release in AC16 cell culture were evaluated by ELISA. (**C**) MMP9, (**D**) VCAM1, (**E**) p-IKKα/β, and (**F**) NF-κB protein expression in AC16 cells were evaluated by western blot analysis. ($n = 5$; ***$P < 0.001$, one-way Analysis of Variance)

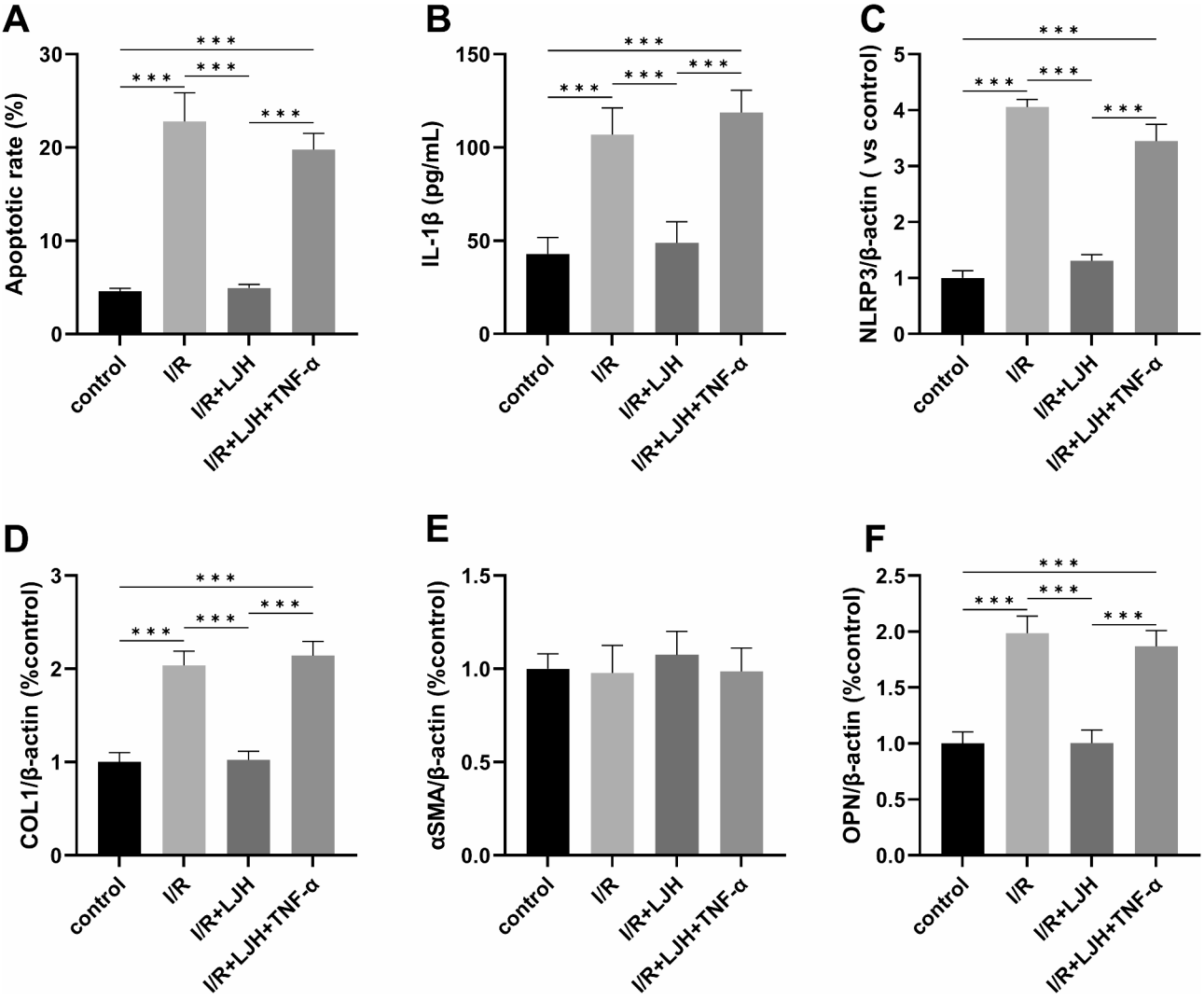

Fig. 5 Effects of Leonurus japonicus Houtt. on AC16 cell function. (**A**) AC16 cell apoptosis was determined using Flow cytometry. (**B**) IL-1β release in AC16 cell culture were evaluated by ELISA. (**C**) NLRP3, (**D**) COL1, (**E**) αSMA, and (**F**) OPN protein expression in AC16 cells were evaluated by western blot analysis. ($n = 5$; ***$P < 0.001$, one-way Analysis of Variance)

Discussion

In China, the incidence of AMI is gradually showing a younger trend. At present, the lack of effective treatment methods and drugs for MIRI after clinical treatment is an urgent problem that needs to be solved in clinical practice. Traditional Chinese medicine has certain advantages in preventing and treating MIRI. After database screening, this study found that LJH is one of the commonly used traditional Chinese medicines for the treatment of ischemic heart disease. LJH is a traditional Chinese medicine with a long history of development and application in China. Especially, recent studies have shown that LJH has various effects. The oxidative stress effects of LJH have been demonstrated in ischemic hearts [12]. LJH contains various active ingredients, including alkaloids, flavonoids, diterpenoids, and fatty acids. Recent studies have shown that these molecules play beneficial roles in coronary artery disease and cerebral ischemia [13, 14]. Therefore, these molecules may become new candidates for drug discovery and development. Based on database analysis, we found that LJH contained multiple pharmacologically active ingredients, including apigenin, leojaponin, melatonin, LJH, kaempferol, etc. These compounds targeted various targets and pathways, including TNF, VCAM1, MMP9, TNF signaling pathway, and NF-kappa B signaling pathway. We validated the regulatory effects of LJH on TNF, VCAM1, MMP9, and its rescue effects on MIRI cell damage through cell experiments.

Previous studies have found that an increase in TNF-α in the infarcted area after chronic myocardial infarction. In TNF-α-knockout mice, it has been observed that survival rate increased after chronic myocardial infarction, along with decreased heart rupture, inflammatory cell infiltration and cytokine expression [15]. Moreover, a

decrease in cytokine expression is also associated with an increase in matrix metalloproteinase-9 (MMP-9) in the infarcted myocardium [16]. TNF-α inhibition may be an effective strategy to reduce MIRI. It is known that pharmacological inhibition of NF-κB or TNF-α can significantly improve the formation of myocardial infarction [17]. VCAM1, TNF-α, and MMP-9 have been reported to be involved in inflammation and vascular remodeling [18]. Here, we found that LJH can inhibit TNF-α expression and release in AC16 under MIRI. During MIRI, excessive ROS leads to high permeability by disrupting the stability of the endothelial barrier. At the same time, adhesion molecules such as VCAM1 are upregulated, which leads to neutrophils blocking capillaries and infiltrating infarcted tissue [19]. We discovered that LJH can suppress the expression of VCAM1 in AC16 cells under MIRI. The phenotype transition of myocardial cells is considered a key mechanism of myocardial remodeling. The increased protein expression of the contraction marker αSMA and the synthetic marker OPN confirms that MIRI can induce phenotype transition in AC16 cells. LJH can significantly inhibit the expression of OPN protein and inhibit MIRI-induced phenotype transition.

Activation of inflammasomes is associated with myocardial I/R injury. The inhibition of NLRP3 by small interfering RNA can prevent inflammasome activation and cardiac cell death, thereby improving myocardial remodeling in MI mouse models [20]. Previous research has shown that NLRP3 is mainly localized in fibroblasts of the left ventricle and mediates its secretion of IL-1β for lipopolysaccharide, indicating that NLRP3 may be the initial receptor involved in inflammasome activation [19]. Here, LJH was found to inhibit NLRP3 in AC16 cells under MIRI, suggesting that LJH may be a promising therapeutic approach for preventing or reducing myocardial I/R injury. However, our study was limited to the in vitro experiments. In vivo validation is needed in future studies.

In summary, the results of this study indicate that LJH, as a commonly used herb for the treatment of ischemic heart disease, can exert pharmacological effects through multi-target and multi-pathway modes. LJH can reduce inflammation in MIRI state, which may be mediated by targeting TNF-α, VCAM1, and MMP9 to participate in the TNF/NF-κB pathway. Our study provides evidence for LJH as an effective therapeutic drug for MIRI.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13019-025-03425-7.

Supplementary Material 1: **Supplementary Fig. 1.** Common herbs of TCMSP, HERB, and TCM-Suite for treating Ischemic heart disease

Supplementary Material 2: **Supplementary Fig. 2.** Common targets of Leonurus japonicus Houtt. and MIRI

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Author contributions
Conceptualization, X.L. and Q.X.; Data curation, X.L. and Z.Z.; Formal analysis, R.Z. and Y.Z.; Funding acquisition, Q.X.; Investigation, R.Z. and Y.Z.; Methodology, X.L., Z.Z. and Q.X.; Project administration, Q.X.; Resources, R.Z. and Y.Z.; Software, X.L. and Z.Z.; Supervision, Q.X.; Validation, X.L. and Z.Z.; Visualization, X.L.; Roles/Writing - original draft, X.L.; Writing - review & editing, Q.X.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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