

The mechanism of *Epimedium* in the treatment of coronary atherosclerotic heart disease based on network pharmacology, molecular docking, and *in vitro* studies

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Abstract. – **OBJECTIVE:** There are many challenges related to the treatment of coronary atherosclerotic heart disease (CAD). Studies have confirmed that *Epimedium* extract inhibits myocardial injury induced by myocardial ischaemia, but the mechanism of action remains unclear. This study aimed at analysed the effective components and mechanisms of *Epimedium* in treating CAD based on network pharmacology and molecular docking studies and to verify the mechanism *in vitro*.

MATERIALS AND METHODS: The TCMSP and UniProt databases were used to filter for the active components and drug targets of *Epimedium*. The GeneCards database was used to screen disease targets associated with CAD. The intersection of the drug targets of *Epimedium* and the disease targets of coronary heart disease was studied to identify the targets of *Epimedium* in the treatment of CAD. Cytoscape software was used to establish and analyse an activity-target network. The STRING database was used to analyse a protein-protein interaction (PPI) network, and proteins in the PPI network were visualized in the R language. Bioconductor software was used for GO function and KEGG pathway enrichment analyses, and visualization analysis was performed in the R language. PyMOL software was used to verify the molecular docking between selected active components of *Epimedium* and the targets of CAD, and the potential key effective components of *Epimedium* in the treatment of coronary heart disease were identified. The involvement of the PI3K/Akt pathway was validated by Western blot analysis.

RESULTS: (1) Twenty-three active compounds, including *Epimedium* glycoside, quercetin, luteolin, and olive resin, were screened out. There were 68 common targets of *Epimedium* and CAD, including IL-6, ESR1, RELA, FOS, NCOA1, CCND1, EGFR, MAPK8, VEGFA, and CASP8. The potential signaling pathways involved in the treatment of CAD by *Epimedium* included the human cytomegalovirus infection pathway, the PI3K-Akt signaling pathway, the TNF signaling pathway, and the HIF-1 signaling pathway. (2) Luteolin, quercetin, sitosterol, and anhydroicaritin showed strong binding to targets of CAD based on molecular docking studies. (3) *Epimedium* extract increased the expression of PI3K, Akt and P-Akt but decreased the expression of IL-6 *in vitro*.

CONCLUSIONS: (1) Icariin, quercetin and luteolin may act on target proteins, including IL-6, ESR1, EGFR, MAPK8, VEGFA and CASP8, to participate in the regulation of the human cytomegalovirus infection pathway, the PI3K-Akt signaling pathway, the TNF signaling pathway and other signaling pathways in order to effectively treat CAD. (2) *In vitro* studies confirmed that *Epimedium* extract can treat CAD by upregulating PI3K, Akt and P-Akt protein expression and downregulating IL-6 protein expression in SD rat cardiomyocytes.

Key Words:

Epimedium, Coronary atherosclerotic heart disease, Molecular mechanism, Network pharmacology, PI3K/Akt.

Introduction

Coronary atherosclerotic heart disease (CAD), also known as ischemic heart disease, is characterized by chest distress, chest pain, dyspnea and other main clinical manifestations and is mostly caused by coronary artery obstruction and stenosis¹. At present, the standard treatments for CAD mainly focus on effects, such as improving coronary blood supply, reducing oxygen consumption, and improving the tolerance of myocardial tissue to hypoxia². However, there are approximately 11 million coronary heart disease patients in China, and the mortality rate of CAD is higher than that of other diseases, such as cancer³.

According to traditional Chinese medicine, CAD falls under the category of “chest obstruction” and is mainly related to Qi stagnation and blood stasis, cold coagulation and Qi stagnation, phlegm-dampness blocking collaterals, and Qi and Yin deficiency. Therefore, the principles for treating the disease involve activating Qi and blood, warming Yang and dispersing cold, resolving phlegm and clearing collaterals, and nourishing Qi and Yin. *Epimedium*, a traditional Chinese herb, has the functions of warming heart Yang, invigorating blood and clearing heart arteries and blood stasis. Previous clinical studies⁴⁻⁵ have also confirmed that *Epimedium* can inhibit myocardial injury caused by myocardial ischemia. However, due to the complex composition of *Epimedium* and the involvement of multiple regulatory genes and signaling pathways in the occurrence and development of CAD, single-target and single-drug research methods are not suitable for systematic analysis of the mechanism of *Epimedium* in the treatment of CAD.

Therefore, network pharmacology and molecular docking studies were used to analyze the multicomponent and multitarget mechanism of *Epimedium* in the treatment of CAD, and the mechanism was verified *in vitro*. The findings provide a reference for clinical applications and further research.

Materials and Methods

Network Pharmacology

Identification of the Active Components and Targets of *Epimedium*

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis

platform (<http://ibts.hkbu.edu.hk/LSP/tcmsp.php>) was searched with “*Epimedium*” as the keyword and “oral bioavailability OB 30% or higher, classes, medicinal DL acuity 0.18” as the filter conditions to screen the active components and drug targets of *Epimedium*. The species was defined as human, unmatched targets were eliminated, and the predicted target protein names were corrected to the official names according to the UniProt database (<http://www.uniprot.org>).

Screening of Coronary Heart Disease Targets

The GeneCards database (<https://www.genecards.org/>) was searched with “coronary atherosclerotic heart disease” as the keyword to identify CAD targets, and repeated targets were eliminated.

Identification of the Therapeutic Targets of *Epimedium* in the Treatment of CAD

The drug targets obtained from TCMSP and the disease targets obtained from UniProt database were intersected to identify the potential therapeutic targets of *Epimedium* in the treatment of CAD by using R software (version 3.5.3) (<https://www.r-project.org/>).

Construction of an Active Component-Target of Action Network

The active components of *Epimedium* and the common targets identified in section 1.1.3 were imported into Cytoscape software (3.7.1) (<https://cytoscape.org/>), which was used to construct and visualize an active component-target network. Topological analysis of the active component-target network was performed, with the degree and betweenness centrality reflecting the importance of the nodes. The higher the degree and betweenness centrality values were, the more important the node was in the network.

Protein-protein Interaction (PPI) Network Construction

The common targets of *Epimedium* and CAD were entered into the STRING database (<https://string-db.org/>). The parameters were set as follows: the species was set to *Homo sapiens*, disconnected nodes in the network were hidden, the minimum required interaction score was set to the highest confidence (0.900), and the rest of the parameters were set to the default settings. A PPI network was constructed and imported

into Cytoscape software (3.7.0) for visualization. An enrichment diagram of proteins in the PPI network was drawn in the R language (version 3.5.3).

GO Function and KEGG Pathway Enrichment Analysis

The coding genes of the targets were entered into Bioconductor open-source bioinformatics software (<http://www.bioconductor.org/>). The parameters were set as follows: p was set to less than 0.05, and the output was set to less than 20 to determine the main biological function of *Epimedium* and the potential mechanism of action of *Epimedium* in the treatment of CAD. The R language (version 3.5.3) was used to draw the bar graphs.

Molecular Docking

The mol2 files of the core components were downloaded from the TCMSP database, imported into AutoDock Tools 1.5.6, and saved in PDBQT format. The 3D structures of the key target proteins were downloaded from the PDB database (<https://www.rcsb.org>), and water molecules and redundant inactive ligands were removed from the proteins by PyMOL software. After the files were imported into AutoDock Tools 1.5.6, hydrogenation and charge processing were performed, and the files were saved in PDBQT format. Appropriate parameters were set, and semiflexible docking was performed in AutoDock Vina to obtain the optimal binding conformation. PyMOL was used to visualize the docking results. A lower binding energy of the ligand and receptor was associated with a more stable binding conformation and a greater possibility of interaction⁶.

In Vitro

Animals

Six- to eight-week-old male SPF-grade SD rats weighing 200±20 g, were obtained from Shanghai Slyke Experimental Animal Co., Ltd. [License No.: SCXK (Shanghai) 20124002]. Throughout the experiments, the rats were maintained in plastic cages at 21 ± 2°C on a 12 h light/dark cycle and provided free access to food and water. The experimental animal protocols were approved by the Nanjing University of Chinese Medicine Committee on Laboratory Animal Care, and all animals were treated humanely according to the

National Institutes of Health (USA) guidelines. All possible efforts were made to minimize the pain of the animals.

Extract Preparation

Epimedium (10 g) was pulverized and boiled twice. Ten times the volume of water was added, and the mixture was boiled for 1 h. The mixture was filtered, and the filtrate was collected. Eight times the volume of water was added, the mixture was boiled for 0.5 h, filtered, and the filtrates were combined. The water extract was concentrated to 1:1-1:2 (mL:g) and cooled, and ethanol was slowly added with stirring until the ethanol concentration was 75%. The extract was refrigerated for 24 h and filtered; the ethanol was removed; and the extract was purified, freeze-dried into a powder, and stored at -20°C.

Primary Myocardial Cell Culture

Primary cell isolation and culture were performed as described in the literature⁷ with some modifications. Isolated cultured cardiomyocytes were inoculated in 6-well plates at a density of 4×10⁵ cells/mL in 2 mL medium per well. When the cells had adhered to the substrate, the experiment was performed. Isolated cultured cardiomyocytes were inoculated in 96-well plates at a density of 5×10³ cells/mL in 100 μL medium per well. When the cells had adhered to the plate bottom, the cell activity test was performed.

Cell Grouping and Drug Administration

The cells were divided into the blank group, model group (1.0 μg/mL LPS), low-dose extract group (200 μg/mL), medium-dose extract group (400 μg/mL) and high-dose extract group (800 μg/mL). Extract was added to the medium of the cells in the extract groups, and vehicle was added to the medium of the cells in the blank control group and model group. After 1 h, 1.0 μg/mL LPS was added to the cells in the model and extract groups, and the same volume of vehicle was added to the cells in the blank control group. The cells were cultured at 37°C and 5% CO₂ for 45 min.

The cells were washed gently with PBS at 4°C 3 times. Two hundred microliters of cell lysate was added to each well, and the cells were shaken for 5 min at 4°C and centrifuged at 12000 r/min at 4°C for 10 min. The supernatant was collected, and protein loading buffer was added.

Cell Activity Measurement

Extract was administered at concentrations of 200 µg/mL, 400 µg/mL, and 800 µg/mL. A total of 10 µL CCK8 solution was added to each well of a 96-well plate. The culture plate was placed in an incubator for 2 h, and the absorbance at 450 nm was measured with a microplate reader.

Western Blot Analysis

Protein expression levels were measured by standard methods. A BCA Protein Assay Kit (Heart, Xi 'an, China) was used to measure the protein concentration. The following antibodies were used: IL-6 (1:1000, Bio-World, Ohio, USA), PI3K (1:2000, Cell Signaling, MA, USA), Akt (1:2000, Cell Signaling, MA, USA), pAkt (1:2000, Cell Signaling, MA, USA). Secondary antibodies (1:20000) were obtained from Merck Millipore (Darmstadt, Germany).

Statistical Analysis

Statistical analyses were performed using the SPSS 25.0 software package (IBM Corp., NY, USA). All values are presented as the mean ± standard error of the mean (SEM). The differences between groups were analyzed by one-way ANOVA. The results were considered statistically significant at $p < 0.05$.

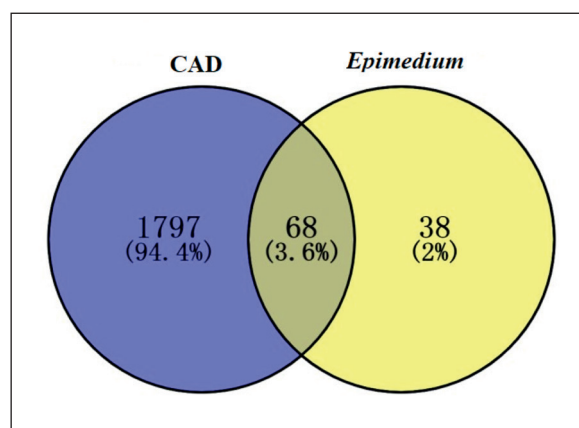


Figure 1. Venn diagram showing the intersection of *Epimedium* extract targets and CAD targets.

Results

Active Components and Targets

A total of 106 targets of *Epimedium* and 1865 targets of CAD were identified, and 68 common targets were found to intersect (Figure 1). A total of 23 active components of *Epimedium* were found to be involved in the treatment of CAD (Table I).

Table I. Active components of *Epimedium*.

Serial number	Coding gene	Name	OB (%)	DL
M1	MOL001510	24-Epicampestero	37.58	0.71
M2	MOL001645	Linoleyl acetate	42.1	0.2
M3	MOL001771	Poriferast-5-en-3beta-ol	36.91	0.75
M4	MOL001792	DFV	32.76	0.18
M5	MOL003044	Chrysoeriol	35.85	0.27
M6	MOL003542	8-Isopentenyl-kaempferol	38.04	0.39
M7	MOL000359	Sitosterol	36.91	0.75
M8	MOL000422	Kaempferol	41.88	0.24
M9	MOL004367	Olivil	62.23	0.41
M10	MOL004373	Anhydroicaritin	45.41	0.44
M11	MOL004380	C-Homoerythrinan-1,6-didehydro-3,15,16-trimethoxy -(3.beta.)	39.14	0.49
M12	MOL004382	Yinyanghuo A	56.96	0.77
M13	MOL004384	Yinyanghuo C	45.67	0.5
M14	MOL004386	Yinyanghuo E	51.63	0.55
M15	MOL004388	6-11,12-Hydroxy-dimethoxy-2,2-dimethyl-1,8-dioxo,3,4,8-tetrahydro-2-1 h-isochromeno [3, 4 - h] isoquinolin-2-ium	60.64	0.66
M16	MOL004391	8-(3-Methylbut-2-enyl)-2-phenyl-chromone	48.54	0.25
M17	MOL004394	Anhydroicaritin-3-O-alpha-L-rhamnoside	41.58	0.61
M18	MOL004396	1,2-bis (4-hydroxy-3-methoxyphenyl) propan-1,3-diol	52.31	0.22
M19	MOL004425	Icariin	41.58	0.61
M20	MOL004427	Icariside A7	31.91	0.86
M21	MOL000006	Luteolin	36.16	0.25
M22	MOL000622	Magnograndiolide	63.71	0.19
M23	MOL000098	Quercetin	46.43	0.28

Active Component-Target Network Analysis

46 nodes and 84 edges were obtained from the active component-target analysis of the action network (Figure 2). Screening of the core nodes showed that effective components such as icariin and quercetin had greater than the average degree and betweenness centrality values and that the targets PTGS1, AR, PPARG and ESR1 had greater than or equal to the average degree and betweenness centrality values.

PPI Network

The target PPI network had a total of 63 nodes and 284 edges (Figure 3). A diagram of the top 20 key nodes was drawn according to the degrees of the nodes; 7 nodes, i.e., ESR1, RELA, FOS, NCOA1, CCND1, EGFR and MAPK8, had degree values ≥ 10 (Figure 4).

GO Function and KEGG Pathway Enrichment Analysis

Epimedium may treat CAD by affecting a variety of biological functions, such as DNA transcription and transduction, receptor binding, enzyme activity and cytokine activity

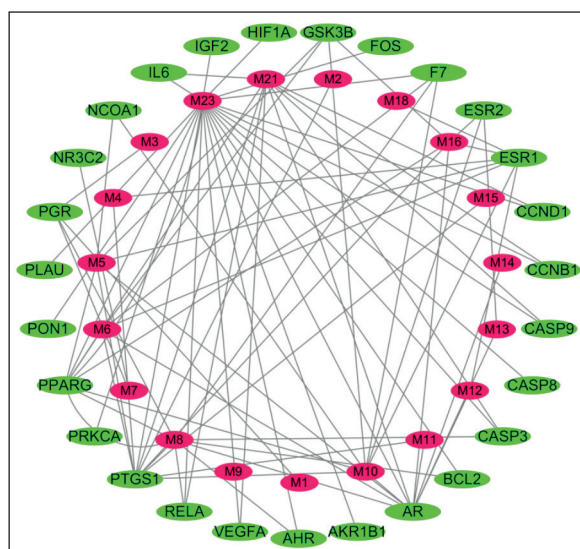


Figure 2. Active component-target network diagram (green = targets, red = active components).

(Figure 5). The main KEGG pathways related to coronary heart disease included the human cytomegalovirus infection pathway, the PI3K-Akt signaling pathway, the TNF signal-

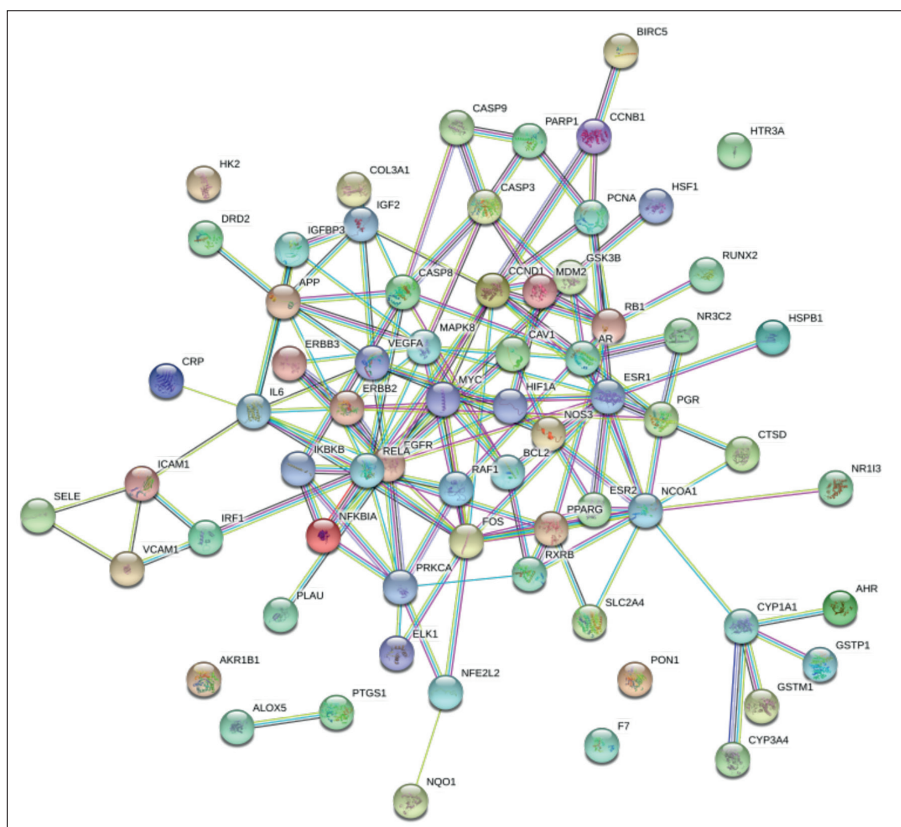


Figure 3. PPI network of common targets in the treatment of CAD with *Epimedium*.

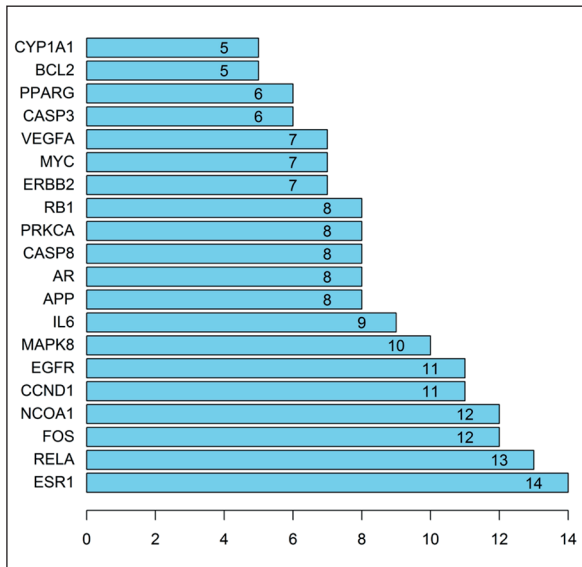


Figure 4. Sequencing of targets of action in the PPI network.

ing pathway and the HIF-1 signaling pathway, as determined by KEGG pathway enrichment analysis (Figure 6).

Molecular Docking Results

Icariin, quercetin, luteolin and other active components are the most likely key components

of *Epimedium* in the treatment of CAD (Table II). The molecular docking diagrams are shown in Figure 7 A-J.

Effects of *Epimedium* Extract on Cell Proliferation

The effects of *Epimedium* extract on myocardial cell proliferation in SD rats are shown (Table III). Compared with that of the blank group, the OD value of the model group was significantly decreased ($p < 0.01$). Compared with that of the model group, the OD values of the 200, 400 and 800 $\mu\text{g/mL}$ *Epimedium* extract groups were significantly increased ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). These results suggest that *Epimedium* extract can promote the proliferation of SD rat cardiomyocytes in a dose-dependent manner.

In Vitro Results

The target protein determination results are shown in Figure 8. Compared with that in the blank group, the expression of PI3K, Akt and P-Akt in the model group was significantly decreased ($p < 0.01$), and the protein expression of IL-6 was significantly increased ($p < 0.01$). Compared with that in the model group, the expression of PI3K, Akt and P-Akt in the low-dose, medium-dose and high-dose groups was significantly increased ($p < 0.01$), and the protein expression

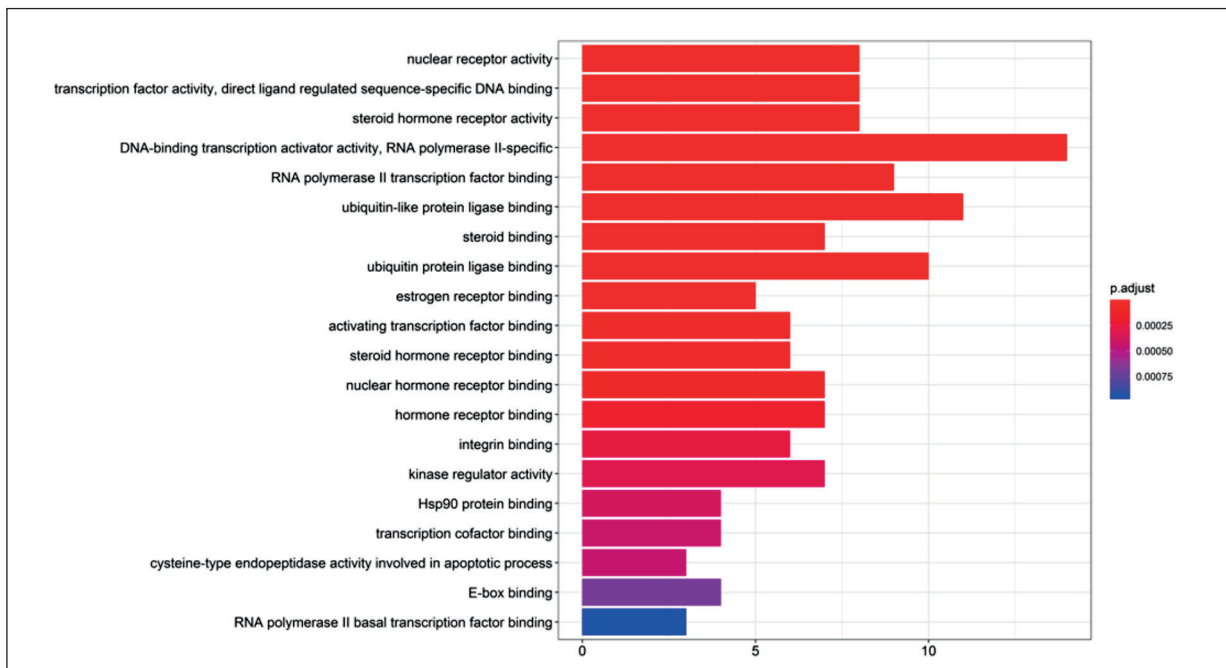


Figure 5. GO functional enrichment analysis.

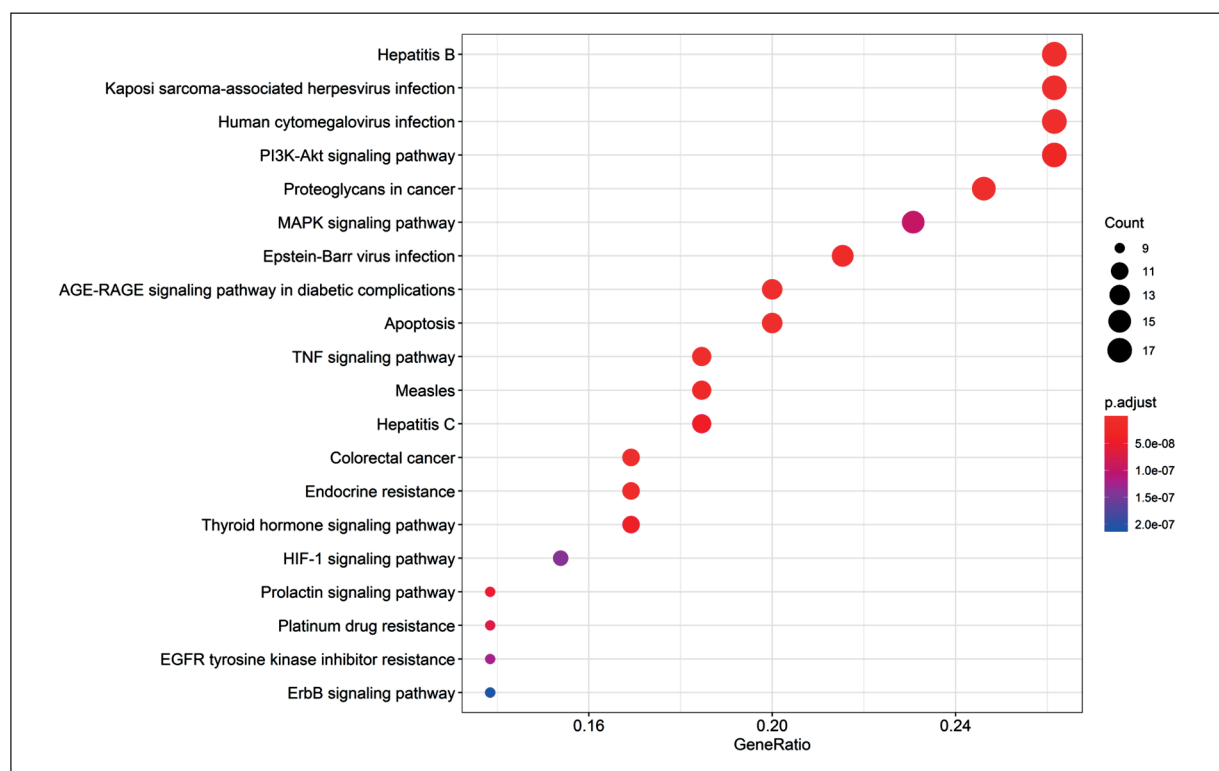


Figure 6. KEGG pathway enrichment analysis.

of IL-6 was significantly decreased ($p < 0.01$) (Figure 9 A-D). These results suggest that *Epi-medium* extract may be involved in the regulation of the PI3K-Akt-IL-6 pathway in the treatment of coronary heart disease.

Discussion

Epimedium, which was first described in Shen-nong Bencao Jing, has the functions of tonifying the kidneys, improving the brain, calming

the nerves, dispelling wind and dampness, and strengthening muscles and bones. It has a long medicinal history in China⁸. However, due to its complex components and many targets, it has not been widely used in clinical practice. Network pharmacology, which can predict the targets and pathways of drugs at the molecular level, has been widely applied in recent years. The application of network pharmacology combined with molecular docking can enable further exploration of the potential molecular basis and mechanism of *Epimedium* in the treatment of CAD.

Table II. Molecular docking energy.

Identifier	Mode	Compound	Affinity (kcal/mol)
A	MOL000006-CASP3	Luteolin	7.7
B	MOL000006-IL6	Luteolin	7.4
C	MOL000006-PPARG	Luteolin	8.8
D	MOL000098-HIF1A	Quercetin	8.5
E	MOL000098-RELA	Quercetin	7.5
F	MOL000359-NR3C2	Sitosterol	7.8
G	MOL004373-ESR1	Anhydroicaritin	7.0
H	MOL004373-GSK3B	Anhydroicaritin	8.7
I	MOL004373-PPARG	Anhydroicaritin	9.1
J	MOL004373-PTGS1	Anhydroicaritin	8.2

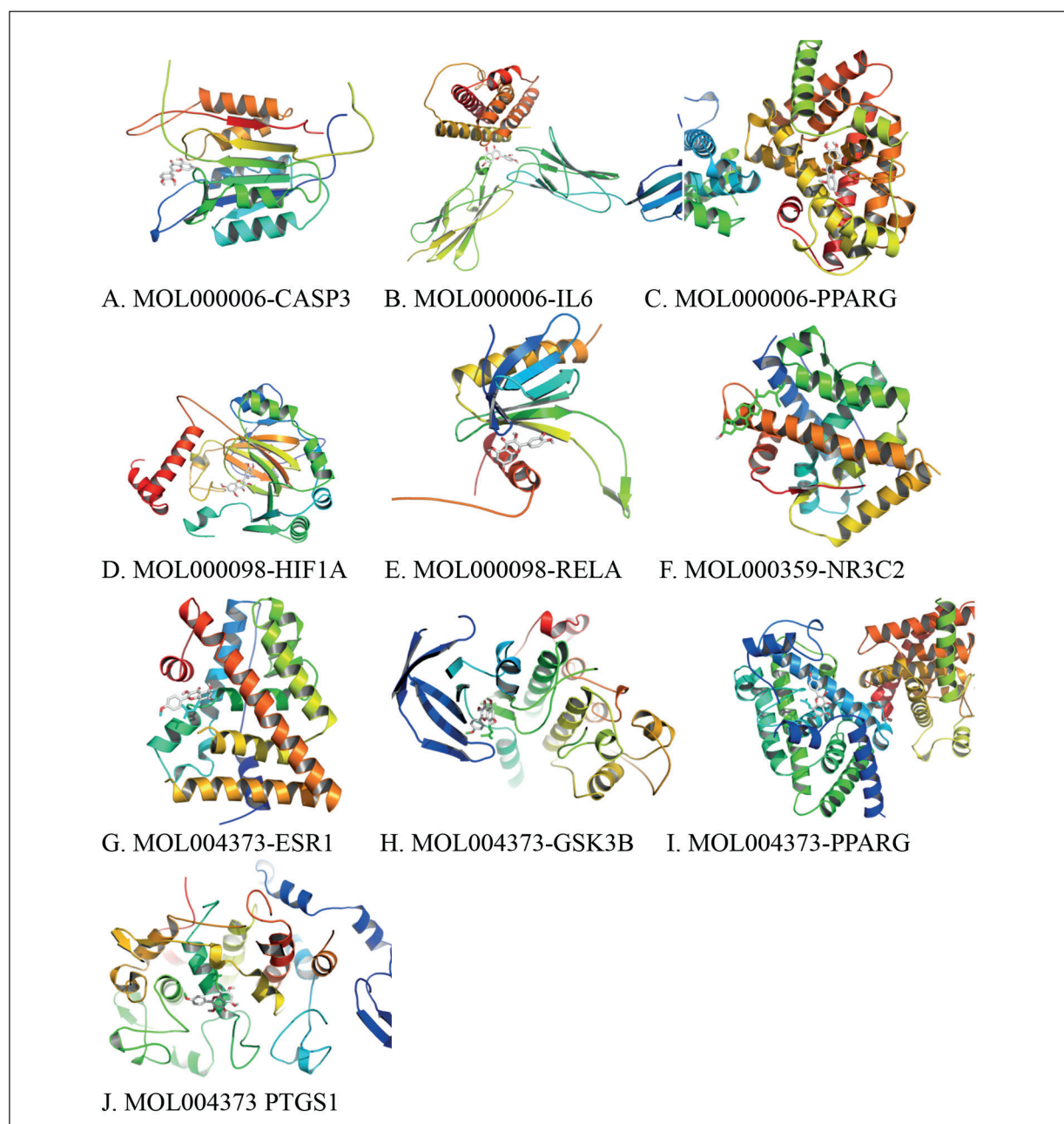


Figure 7. Molecular docking diagrams.

Table III. Cell viability analysis ($\bar{x} \pm s$, n = 6).

Group	Concentration ($\mu\text{g/mL}$)	OD value
Blank group	–	1.33 +/- 0.13
Model group	–	0.78 +/- 0.08**
Low-dose extract group	200	0.95 +/- 0.07#
Medium-dose extract group	400	1.14 +/- 0.10##
High-dose extract group	800	1.22 +/- 0.11##

Note: Compared with the blank group, ** $p < 0.01$; compared with the model group, # $p < 0.05$, ## $p < 0.01$.

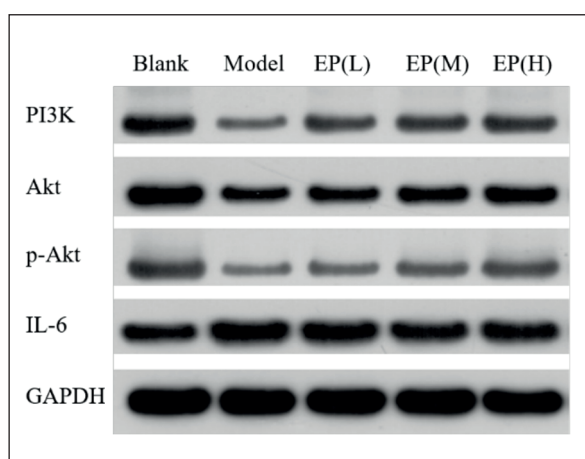


Figure 8. Target protein determination.

In this study, a component-disease target network was constructed to explore the mechanism of action of *Epimedium*. A total of 23 potential active components of *Epimedium*, i.e., anhydroicaritin, *Epimedium* glycoside, quercetin, luteolin, magnolia lactone, olive resin, etc., were

identified. Molecular docking revealed that anhydroicaritin, quercetin, and luteolin are the most likely active components of *Epimedium* in the treatment of CAD. A total of 68 potential targets, including IL-6, ESR1, RELA, FOS, NCOA1, CCND1, EGFR, MAPK8, VEGFA, and CASP8, were identified. The Hif-1 signaling pathway, AGE-RAGE signaling pathway, PI3K-Akt signaling pathway and MAPK signaling pathway were identified as the most highly enriched signaling pathways and these pathways are mainly involved in physiological and pathological processes, such as the inflammatory response and angiogenesis. *In vitro* experiments showed that *Epimedium* extract can increase the expression of PI3K, Akt and P-Akt in the PI3K-Akt signaling pathway ($p < 0.01$) and reduce the protein expression of IL-6 ($p < 0.01$) to treat CAD.

Studies have shown that icariin is a degradation product of icaritin, which can reduce the release of IL-6 and IL-10 by modulating immune function, promote the differentiation of embryonic stem cells into cardiomyocytes, promote the regeneration of cardiomyocytes, and exert a repar-

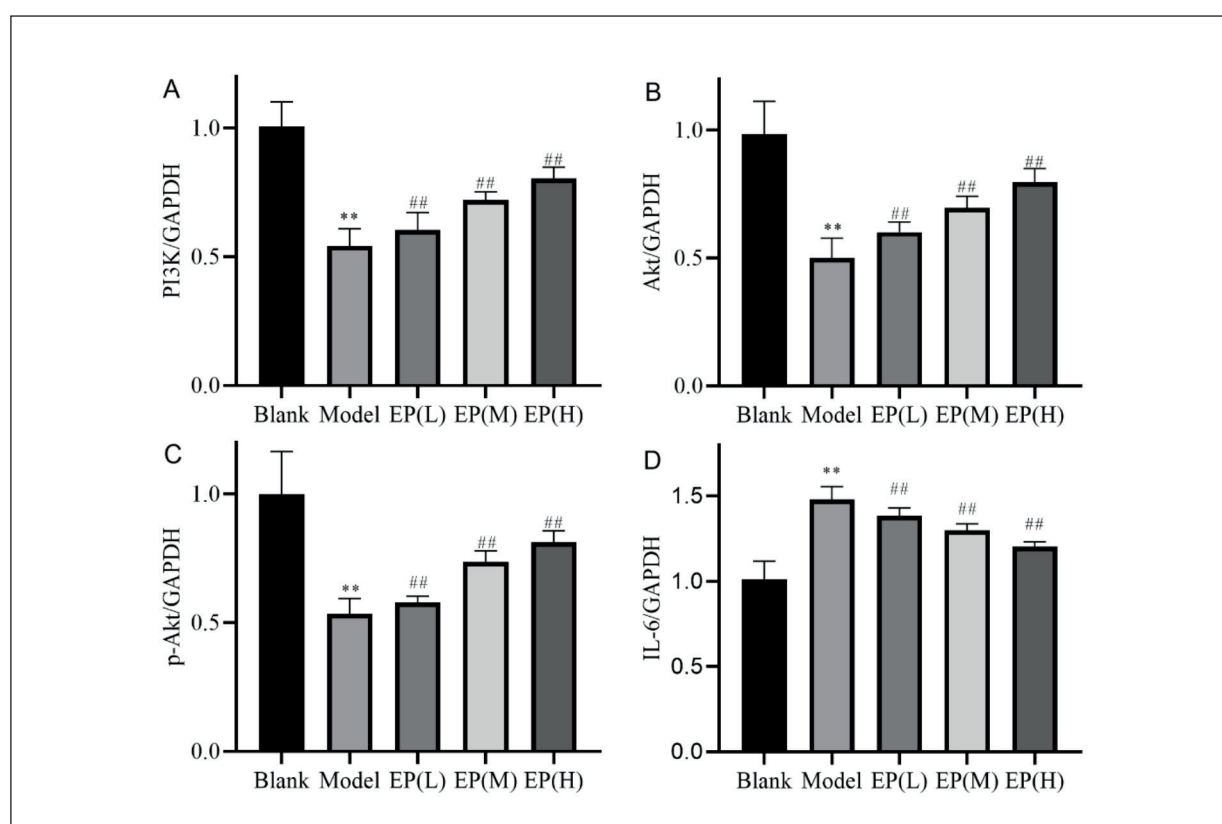


Figure 9. Western blot analyses (n = 6). Note: Compared with the blank group, ** $p < 0.01$; compared with the model group, ## $p < 0.01$.

ative effect on damaged myocardial cells⁹. Quercetin inhibits the release of the inflammatory mediators IL-6, TNF- α and IL-1 β ¹⁰ and regulates the PI3K-Akt pathway to prevent atherosclerosis and protect cardiomyocytes against ischemia-reperfusion^{11,12}. Luteolin can inhibit the release of IL-6, TNF- α and NO by inhibiting the AP-1 pathway¹³. Luteolin can also inhibit apoptosis and improve myocardial contractility by regulating the PI3K-Akt pathway¹⁴. Icariin can promote apoptosis of vascular smooth muscle cells, prevent excessive vascular smooth muscle cell proliferation, and delay atherosclerosis¹⁵. Related studies¹⁶ have also indicated that icariin can decrease the activity of matrix metalloproteinase-2/9, inhibit myocardial apoptosis and improve cardiac remodeling.

IL-6 is an important mediator of inflammatory and immune responses. It acts widely on the cardiovascular, endocrine and nervous systems and it is an important cytokine involved in the regulation of various biological functions^{17,18}. Co-activation of PI3K-Akt and JAK/STAT3 modulates proinflammatory responses mediated by IL-6 trans-signaling in human vascular endothelial cells¹⁹. The PI3K-akt pathway has been indicated to be one of the most important pro-proliferative and antiapoptotic signaling pathways in the myocardium and plays a key regulatory role in the survival and programmed death of cardiomyocytes²⁰. Liu et al²¹ further demonstrated that activation of the PI3K-Akt pathway can inhibit myocardial apoptosis after myocardial infarction.

Conclusions

Based on network pharmacology and molecular docking studies, we found that sitosterol, anhydroicaritin, quercetin and luteolin may be the key components of *Epimedium* in the treatment of CAD. *In vitro* experiments confirmed that *Epimedium* extract can increase the expression of PI3K, Akt and P-Akt and reduce the protein expression of IL-6 in SD rat cardiomyocytes by regulating the PI3K/Akt signaling pathway to treat CAD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Data Availability Statement

The data can be obtained in the article or its Supplementary Material; further inquiries can be directed to the corresponding author by email.

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Authors' Contribution

GX and GN contributed to the study design. SXM, MCH, ZM, and ZCB contributed to acquisition of the data. YYD and WXD contributed to the analyses and interpretation of the data. YYD and GX contributed to manuscript preparation.

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