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Mechanism of action of *Balanophora involucrata* polyphenolic compounds in the treatment of myocardial injury based on network pharmacology and molecular docking techniques

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Abstract The objective of this work was to investigate the mechanism of action of *Balanophora involucrata* polyphenolic compounds in the treatment of myocardial injury. In the present study, *Balanophora involucrata* was extracted by refluxing 75% of ethanol. The obtained extract was extracted with petroleum ether, ethyl acetate and n-butanol respectively. And the ethyl acetate layer was separated. The extract was prepared by silica gel column chromatography, sephadex LH-20 elution and thin layer chromatography. After that, the Swiss target prediction database was utilized to obtain the targets of *Balanophora involucrata*, and the Genecards, OMIM and TTD databases were used to predict and screen the targets of *Balanophora involucrata* for the treatment of myocardial injury. The active ingredient-target network was constructed using Cytoscape software, and the PPI network was mapped using String database and Cytoscape software. GO bioprocess enrichment analysis and KEGG pathway enrichment analysis were performed by Metascape software to predict the mechanism of action. Molecular docking was performed in Discovery Studio 2016 client software to verify the binding of *Balanophora involucrata* polyphenols to key targets. In this study, six polyphenolic compounds were isolated from *Balanophora involucrata*. By GO enrichment analysis, 1 614 biological processes (BP), 127 cellular compositions (CC), and 215 molecular functions (MF) were obtained; a total of 155 cross-targets were involved in the KEGG enrichment analysis. The PPI network showed that quercetin was the main active component of polyphenolic compounds against myocardial injury and that AKT1, EGFR, STAT3, SRC, ESR1, MMP9, HSP90AA1 and other related signals were associated with myocardial injury treatment. Finally, the multi-component-multi-target-multi-pathway action of *Balanophora involucrata* was concluded, which provided new ideas and methods for further research on the mechanism of action of *Balanophora involucrata* in myocardial injury.

Keywords: *Balanophora involucrata*; myocardial injury; phenolic compounds; network pharmacology; molecular docking

1 Introduction

Myocardial injury is one of the major diseases affecting human health, which is characterised by high morbidity and lethality [1]. Myocardial injury is a major problem in cardiovascular disease and can lead to arrhythmias, cardiac shock, heart failure and other major adverse cardiovascular events [2]. According to the U.S. Vital Statistics System, more than 30 million people have been diagnosed with myocardial injury in the U.S. alone, and more than 660 000

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people die each year from heart conditions such as coronary heart disease, cardiac arrest and hypertrophic cardiomyopathy. With the aging process intensifying in China, the number of deaths of residents due to myocardial injury showed a linear increase between 2008 and 2019, and the proportion of myocardial injury deaths in the total number of deaths reached about 25% [3]. Myocardial injury treatment occupies a large amount of healthcare resources, and has become the disease with the highest economic cost [1, 4]. Therefore, conducting research on myocardial injury treatment is of great practical significance for future healthcare management. Polyphenols are a large group of natural compounds of plant origin, characterised by the presence of multiple phenolic hydroxyl groups [5]. Based on their structural characteristics, polyphenols have in recent years shown great benefits in the treatment of cardiac disorders, and studies have demonstrated a high affinity for different cells and tissues, such as oesophageal mucus, colon, tumours and myocardial muscle, suggesting that polyphenols are more favourable for the body to absorb [6-8].

Balanophora involucrata is a traditional medicinal and food plant in China. Its main bioactive components include polyphenols, triterpenes, etc., which have antimyocardial damage, antioxidant and hypoglycemic effects [9]. Polyphenols are one of the main components of *Balanophora involucrata*, but the mechanism of their antimyocardial injury remains unclear.

In this study, we isolated the polyphenols of *Balanophora involucrata*, and predicted the mechanism of action of polyphenols in the treatment of myocardial injury from the viewpoint of network pharmacology. It indicates that polyphenols have multi-component, multi-target, and multi-pathway properties for the treatment of myocardial injury, and lays the foundation for further research on their molecular mechanisms.

2 Experimental materials and methods

2.1 Extraction and isolation of Balanophora involucrata

2.1.1 Experimental instruments

Rotary evaporator (Zhengzhou Great Wall Science, Industry and Trade Co); Circulating Water Vacuum Pumps (Zhengzhou Great Wall Science, Industry and Trade Co); Electronic Analytical Balance (Sartorius, Germany); Electrothermal constant temperature water bath (Beijing Changfeng Instrumentation Company); CNC Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co); Electrothermal blast thermostatic drying oven (Shaoxing Supe Instrument Co); Triple UV Analyser (Shanghai Li-Chen Bang Xi Instrument Technology Co); High Performance Liquid Chromatograph (Beijing Innovation Tongheng Technology Co).

2.1.2 Experimental material

Plant sources: The plant was extracted from the Shennongjia forest area of Enshi, Hubei Province, and was identified as the dried whole herb of *Balanophora involucrata* by professor Lu Jincai from the School of Traditional Chinese Medicine, Shenyang Pharmaceutical University.

Chromatographic packing: Chromatography silica gel 100−200 mesh, 300−400 mesh, Qingdao Ocean Chemical Factory; thin layer chromatography silica gel GF254, Qingdao Kangyexin Pharmaceutical Silica Gel Desiccant Co Ltd; Sephadex LH-20 packing, Pharmacia.

Drugs and reagents: Ethanol, petroleum ether, dichloromethane, n-butanol, ethyl acetate, methanol, acetone, formic acid, sulphuric acid are all analytically pure. Tianjin Lianlong Bohua Pharmaceutical & Chemical Co; Deuterium reagent: DMSO-D6, CD_3OD. Cambridge Isotope Laboratories, Inc.USA.

Sulfuric acid ethanol chromogenic agent: acid: ethanol = 1:9 preparation.

Thin-layer plate: mix thin-layer chromatography silica gel GF254 with 0.7% CMC-Na solution (1:4), grind it evenly, spread it uniformly on a clean glass plate, dry it naturally for 24 h, and collect it in a desiccator for spare.

2.1.3 Extraction and isolation

As shown in Fig 1, 10 kg of dried *Balanophora*

involucrata with 75% EtOH reflux twice. The extract was concentrated by rotary evaporator under reduced pressure and dried, obtaining 4 kg of EtOH extract. The EtOH extract (4 kg) was then separated in H_2O/EtOAc (1:1) to obtain EtOAc soluble fraction (580 g). The fraction (580 g) was fractionated by silica gel column (12 cm × 150 cm, 3 000 g) chromatography (eluted with ethyl acetate and ligarine in increasing polarity) to obtain seven fractions A−G. Fractions A (20 g), C (20 g), and D (25 g) were subjected to further chromatography on silica gel (6 cm × 70 cm, 250 g; eluted with ethyl acetate and ligarine in increasing polarity) respectively, yielding 15 fractions, A1−A15, 12 fractions, C1−C12, and 15 fractions, D1−D15. Fractions B (15 g) and E (12 g) were subjected to further chromatography on silica gel (6 cm × 70 cm, 150 g; eluted with ethyl acetate and ligarine in increasing polarity) respectively, yielding 16 fractions, B1−B16, 10 fractions, E1−E10. Fractions A5 (120 mg), A7 (50 mg), B8 (200 mg) and C5−C9 (300 mg) were subjected to further chromatography on Sephadex LH-20 (3 cm × 90 cm, 50 g), which was eluted (CH_2Cl_2/MeOH, 2:1). According to TCL detection, combining the same fractions, compound **3** (10 mg) and compound **5** (20 mg) were obtained from fraction A, compound **2** (35 mg) was obtained from fraction B, and compound **4** (50 mg) was obtained from fraction C. According to TCL detection, combining the same fractions, compound **1** (3 g) was obtained from fraction B, and compound **6** (400 mg) was obtained from fraction E.

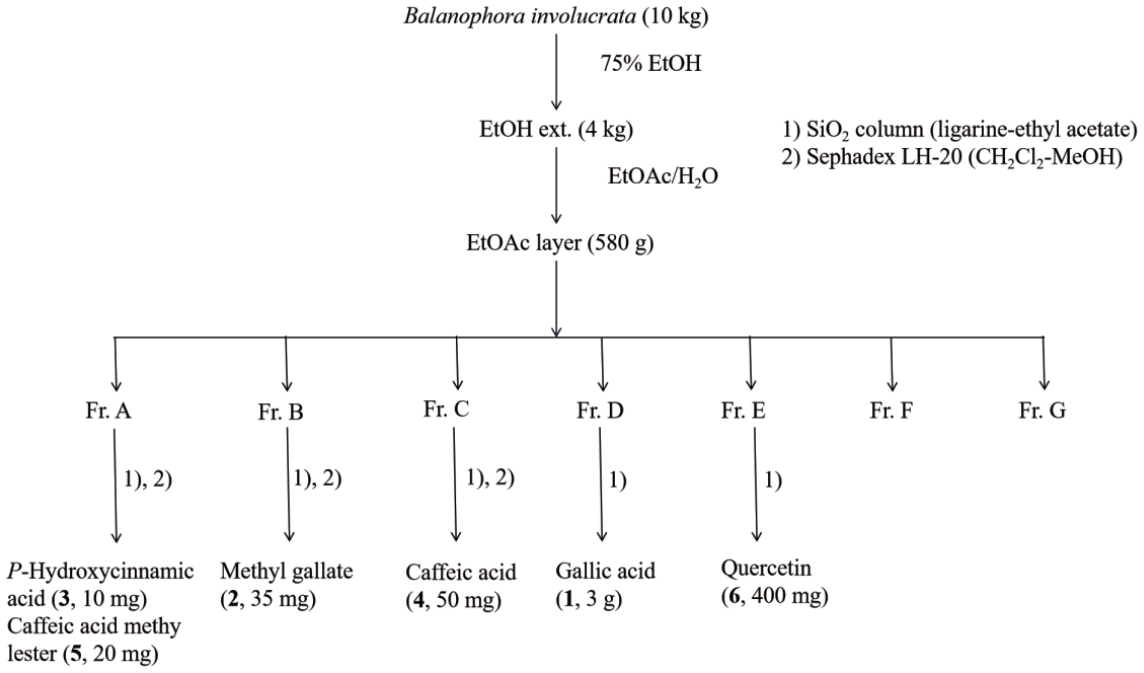


Fig. 1 Extraction and isolation flow schematic

2.2 Network pharmacology research

2.2.1 Screening of the active ingredients and their related targets of Balanophora involucrata

The six isolated compounds were searched in Pubchem database (https://pubchem.ncbi.nlm.nih.gov) to obtain the SMILES formula and 2D structure of the chemical compounds, and imported into the Swiss ADME database (http://www.swissadme.ch/), and the results were analyzed by gastrointestinal absortion (GIA). The result of gastrointestinal absortion was "HIGH", and two or more of the five drug properties (lipinski, ghose, veber, egan, muegge) were "YES". The resultant compounds are the active compounds. The SMILES or 2D structures of the screened active

compounds were imported into the Swiss Target Prediction database (http://swisstargetprediction.ch/), and the target was set as a Homo sapien, and the targets with a confidence level of ≥ 0.6 were screened. After removing the duplicates from the obtained targets, Cytoscape 3.9.0 software was used to construct the target network map of the compounds.

2.2.2 Screening of targets for myocardial injury

The keywords myocardial injury were analyzed in the Human Genetic Data Bank (Genecards, https://www.genecards.org), online Human Mendelian Inheritance Database (OMIM, http://www.omim.org), and therapeutic target database (TTD, http://bidd.nus.edu.sg/group/cjttd) to retrieve potential targets of myocardial injury. The targets obtained from the three databases were removed from duplicates to finalize the myocardial injury disease targets.

2.2.3 Acquisition of drug-disease common targets and construction of PPI networks

Combine compound targets and myocardial injury targets to remove duplicate values and obtain key targets. Venny plots were drawn using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools). String 11.0 database (https://string-db.org) was used to analyze the relationship between the common targets of drug-disease, and the organism was set as Homo sapiens, the minimum interaction threshold was highest confidence (> 0.9), while the unrelated nodes in the network were hidden, and the PPI graph was constructed using Cytoscape 3.9.0 software.

2.2.4 GO biofunctional analysis and KEGG pathway enrichment analysis

The drug-disease common targets were imported into the Metascape platform (https://metascape.org/) and analysed by GO biofunctional analysis and KEGG pathway enrichment analysis, $P < 0.01$, the obtained data results were visualised by applying the microbiotics platform (http://www.bioinformatics.com.cn) and the top 20 ranked entries were plotted as GO Biofunctional Analysis histograms and KEGG pathway enrichment analysis bubble diagrams.

2.3 Docking

Core active ingredients and core targets were selected for molecular docking validation using the PDB database (https://www.rcsb.org). The solvent molecules and ligands were removed and processed as well as the target proteins were subjected to hydrogen addition in Discovery Studio 2016 Client software and finally docking was analysed. Compound and receptor matching ability was evaluated based on LibDock score.

3 Results

3.1 Structural identification

Compound **1** is white crystal, soluble in methanol. ^{1}H-NMR (DMSO, 600 MHz) δ: **6.92 (2H, s, H-2, 6)**. ^{13}C-NMR (DMSO, 150 MHz) δ: 120.6 (C-1), 108.9 (C-2, 6), 145.6 (C-3, 5), 138.1 (C-4), 167.7 (C-7). The above data were in general agreement with the data reported in the literature [10], so the compound was identified as gallic acid.

Compound **2** is a white powder, soluble in methanol. ^{13}C-NMR (CD_3OD, 150 MHz) δ: 121.3 (C-1), 110.1 (C-2, 6), 146.3 (C-3, 5), 139.7 (C-4), 169.1 (C-7), 52.1 (C-8). The above data were in general agreement with the data control reported in literature [11], so the compound was identified as Methyl gallate.

Compound **3** is white crystal, soluble in methanol. ^{1}H-NMR (CD_3OD, 600MHz) δ: **7.54 (1H, d, *J* = 15.9 Hz, H-7), 7.41 (2H, d, *J* = 8.55 Hz, H-2, 6), 6.77 (2H, d, *J* = 8.64 Hz, H-3, 5), 6.25 (1H, d, *J* = 15.9 Hz, H-8)**. ^{13}C-NMR (CD_3OD, 150MHz) δ: 127.3 (C-1), 131.1 (C-2, 6), 116.8 (C-3, 5), 161.1 (C-4), 146.6 (C-7), 115.7 (C-8), 171.1 (C-9). The above data were basically consistent with the data control

reported in the literature [12], so the compound was identified as *p*-Hydroxycinnamic acid.

Compound **4** is light yellow crystal, soluble in methanol. ^{1}H-NMR (CD_3OD, 600MHz) δ: **7.54 (1H, d, *J* = 15.9 Hz, H-7), 7.05 (1H, d, *J* = 2.1 Hz, H-2), 6.95 (1H, dd, *J* = 8.2, 2.1 Hz, H-6), 6.79 (1H, d, *J* = 8.2 Hz, H-5), 6.22 (1H, d, *J* = 15.9 Hz, H-8)**. ^{13}C-NMR (CD_3OD, 150MHz) δ: 126.3 (C-1), 113.6 (C-2), 145.2 (C-3), 147.9 (C-4), 115 (C-5), 121.4 (C-6), 145.6 (C-7), 114.1 (C-8), 169.6 (C-9). The above data were in general agreement with the data reported in the literature [13], so the compound was identified as caffeic acid.

Compound **5** is white crystal, soluble in methanol. ^{1}H-NMR (CD_3OD, 600MHz) δ: **7.54 (1H, d, *J* = 16.0 Hz, H-7), 7.05 (1H, d, *J* = 2.0 Hz, H-2), 6.94 (1H, dd, *J* = 8.2, 2.0 Hz, H-6), 6.79 (1H, d, *J* = 8.2 Hz, H-5), 6.25 (1H, d, *J* = 16.0 Hz, H-8), 3.72 (3H, s, -OCH_3)**. ^{13}C-NMR (CD_3OD, 150MHz) δ: 127.6 (C-1), 115.1 (C-2), 146.7 (C-3), 149.4 (C-4), 122.9 (C-5), 116.4 (C-6), 146.8 (C-7), 114.8 (C-8), 169.7 (C-9), 51.9 (C-10). The above data are in general agreement with the data control reported in the literature [13], so the compound was identified as caffeic acid methy lester.

Compound **6** is a yellow powder, soluble in methanol. Co-thin layer chromatography of this compound with a standard of quercetin showed the same Rf value and display and no decrease in the melting point of the mixture. Therefore, compound **6** was identified as quercetin (as shown in Fig 2).

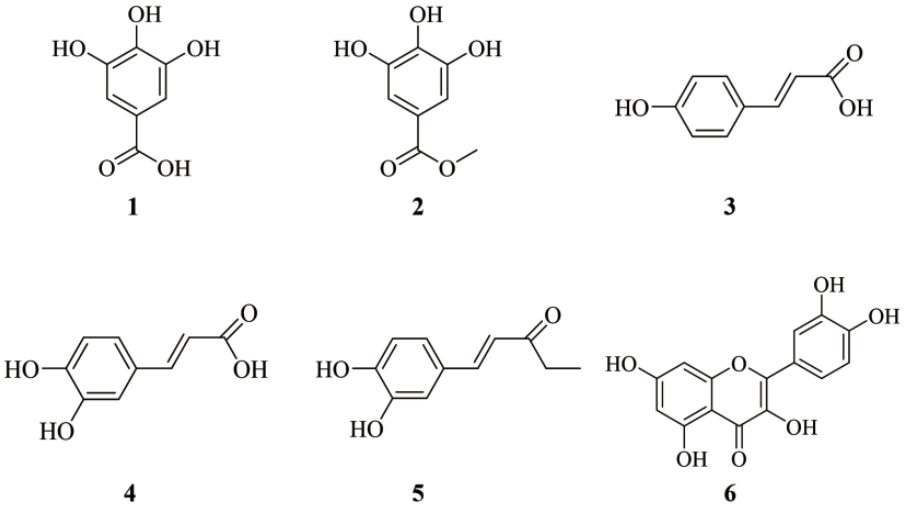


Fig. 2 Structures of compounds 1−6

3.2 Prediction of target, network and pathway of myocardial injury in Balanophora involucrata

3.2.1 Screening of drug and disease targets of Balanophora involucrata

The six compounds isolated from *Balanophora involucrata* should be screened in the Swiss Target Prediction database. In order to better demonstrate the relationship between active components and targets, Cytoscape 3.9.0 software was used to construct the compound-target network diagram of *Balanophora involucrate*. As shown in Fig. 3, GeneCards, OMIM, and TTD databases were searched to obtain 1 619, 182, and 7 potential targets for myocardial injury, respectively. After removing duplicate targets, a total of 1 680 targets related to myocardial injury were obtained. Compounds and disease targets were intersected on Venny 2.1.0, and 85 core gene targets were obtained, as shown in Fig. 4.

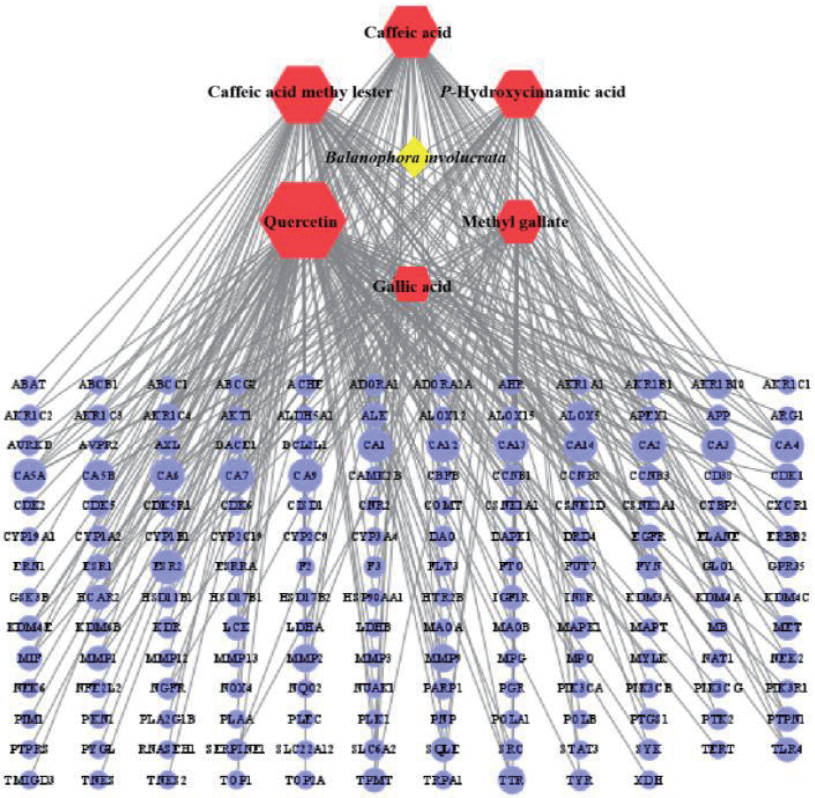


Fig. 3 “Compounds-targets” network

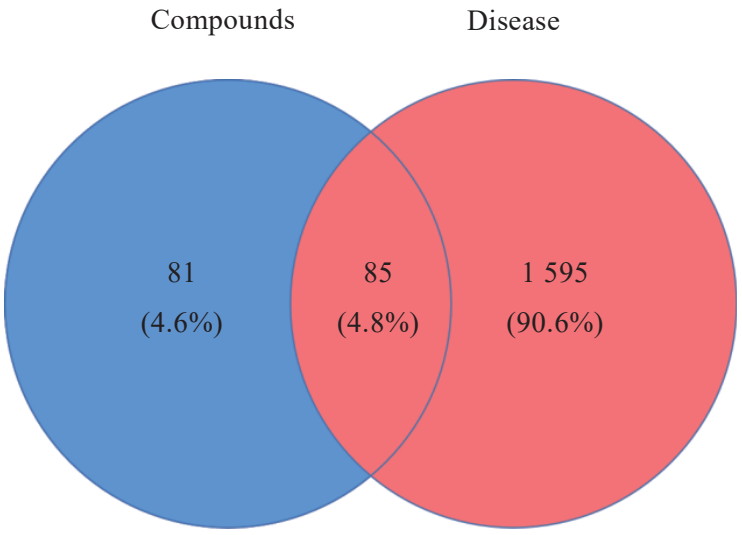


Fig. 4 “Disease-compound” target Venn diagram

3.2.2 PPI network construction

The information of compounds and corresponding targets were imported into the String database to map the interaction network. The core targets were saved and imported into Cytoscape 3.9.0, and the protein-protein interaction network of the core proteins was drawn, as shown in Fig 5. The greater the number of targets connected to each target, the larger the node area and the darker the color, the more important the target is in the protein interaction network.

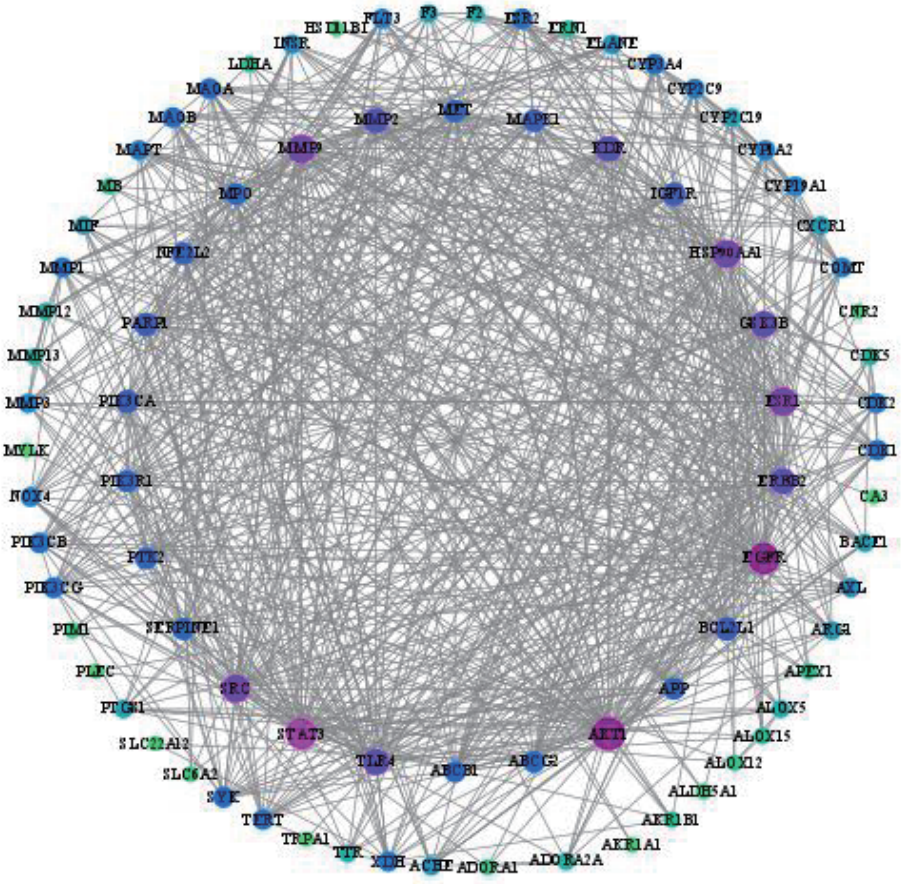


Fig. 5 PPI network

3.2.3 GO biofunction and KEGG pathway enrichment analysis

GO analysis and KEGG enrichment analysis were carried out on the Metascape database for the obtained core targets, and the related items of biological process (BP), cell component (CC) and molecular function (MF) in the GO analysis results were obtained. −lg*P* value was used to measure GO enrichment. The top 10 bars in each group were selected to draw bar charts, as shown in Fig. 6. Functional analysis of GO shows that response to inorganic substance, phosphorylation and response to peptide are mainly involved BP. Vesicle lumen, transferase complex, transferring phosphorus-containing groups and dendrite are the main CC. MF is mainly related to protein kinase activity, oxidoreductase activity and protein tyrosine kinase activity. 155 KEGG enrichment items were obtained by pathway analysis, with −lgP value as the condition, and the first 20 pathways, as shown in Fig. 7. KEGG enrichment showed that pathways in cancer, PI3K-Akt signaling pathway and proteoglycans in cancer were the main enrichment pathways.

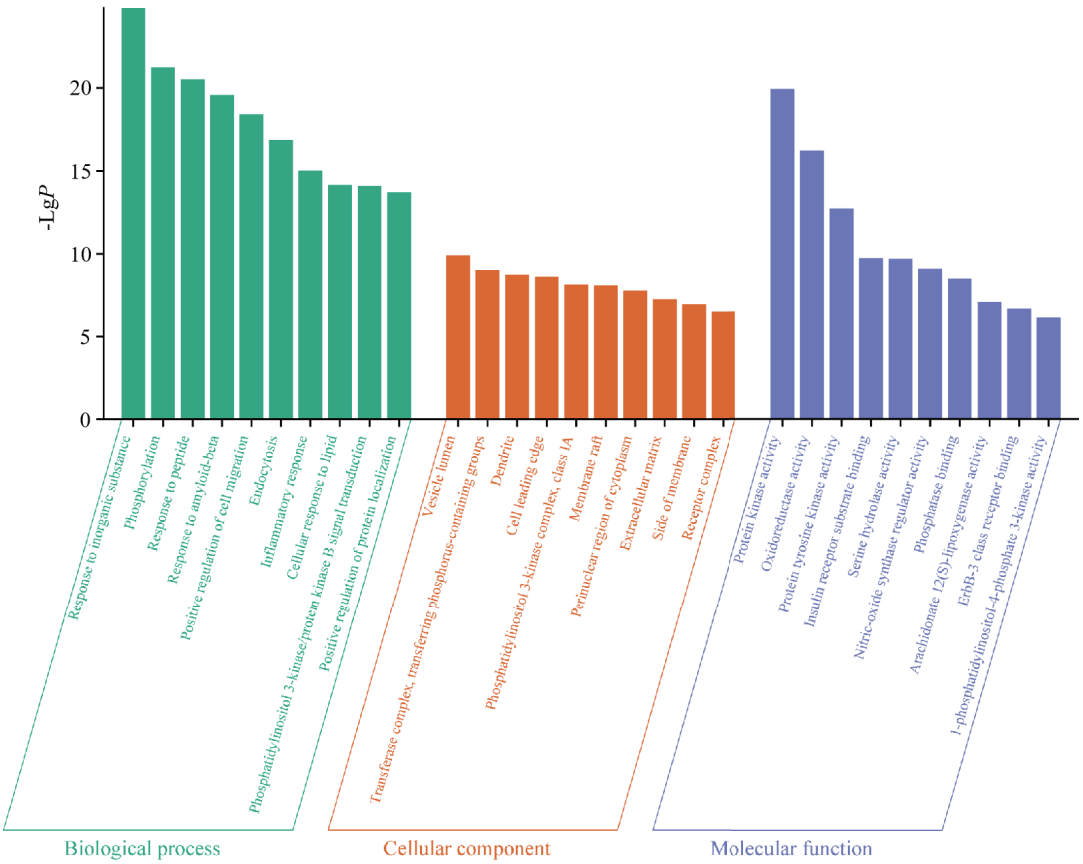


Fig. 6 GO analytics bar chart

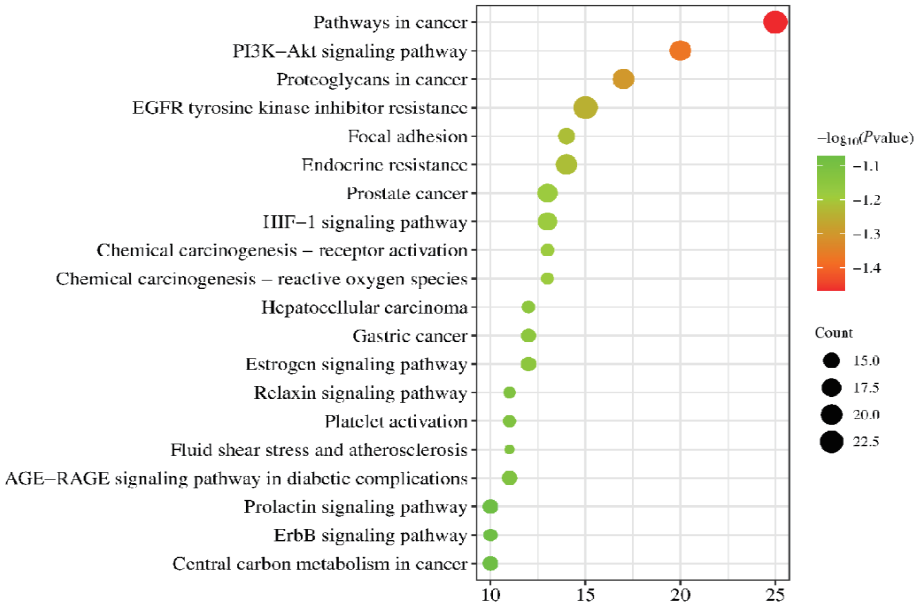
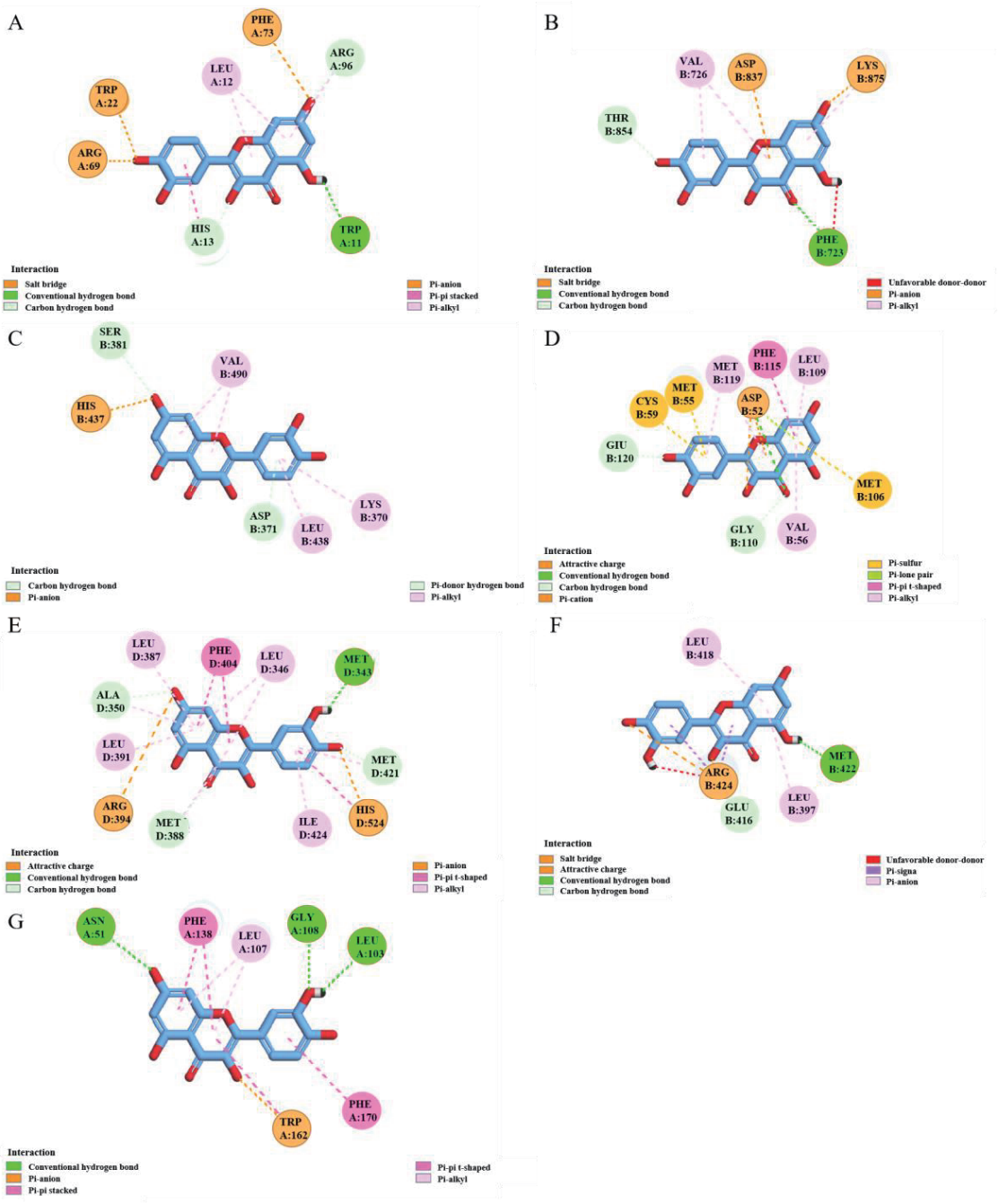


Fig. 7 KEGG pathway enrichment analysis bubble chart

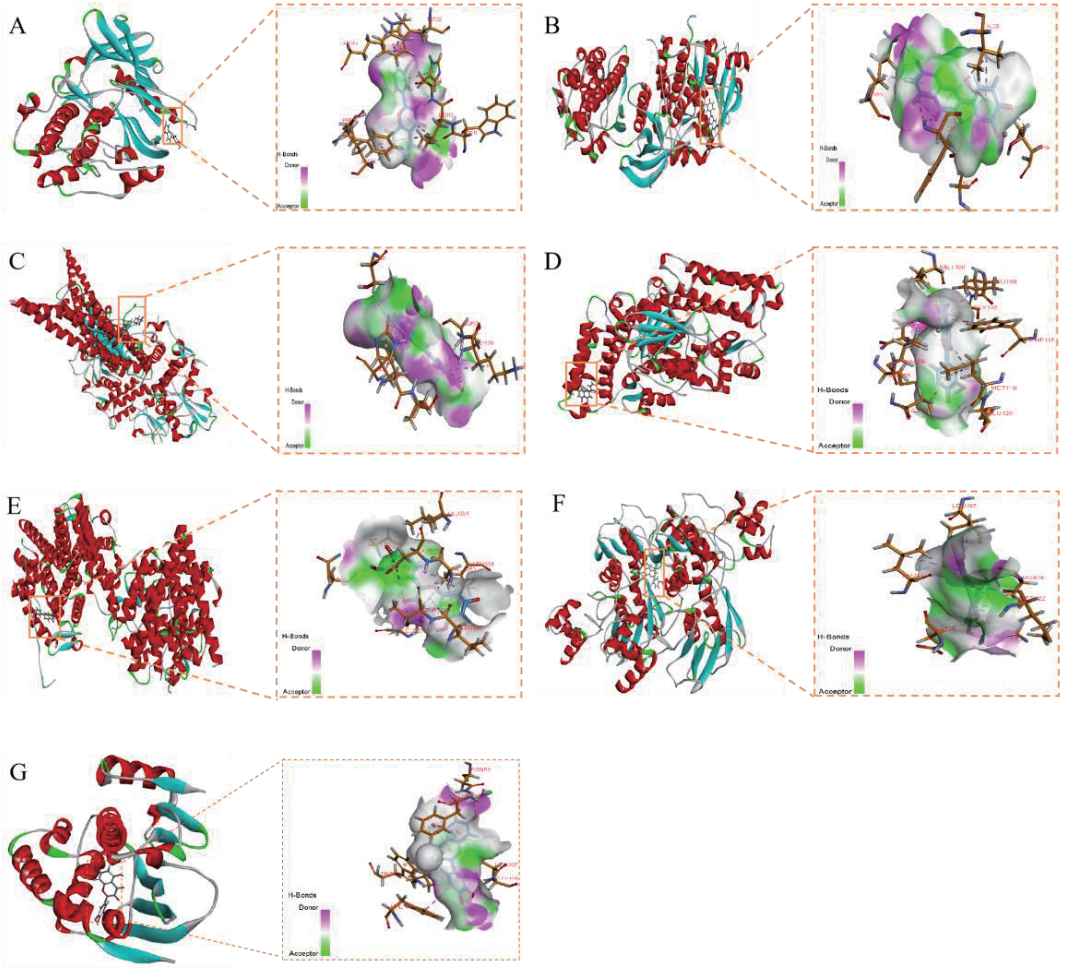
3.3 Molecular docking results

To further elucidate the binding effect between the key targets and active ingredients for the treatment of myocardial injury, the top 7 targets in the PPI network were selected for molecular docking analyses with 6 compounds. It is generally believed that the higher the LibDock score, the richer the form of force, the stronger the binding ability. In this study, seven target proteins were docked with six compounds, and the results showed that Quercetin had relatively good interaction with STAT3 (116.708) and Quercetin with HSP90AA1 (118.263). The binding mode of the target proteins to component docking is shown in Fig. 8 and Fig. 9.



A – AKT1; B – EGFR; C – STAT3; D – SRC; E – ESR1; F – MMP9; G – HSP90AA1

Fig. 8 Display of a 2D binding pattern of Quercetin interaction with target proteins



A – AKT1; B – EGFR; C – STAT3; D – SRC; E – ESR1; F – MMP9; G – HSP90AA1

Fig. 9 Demonstration of 3D binding H-binding interaction of Quercetin with target proteins

4 Discussion

In this study, six polyphenolic compounds were isolated from the ethyl acetate extract of *Balanophora involucrata*, based on which a drug-compound-target network was constructed, and 166 drug-disease cross-targets were obtained. The PPI network predicted that the targets of AKT1, EGFR, STAT3, SRC, ESR1, MMP9, and HSP90AA1 might be the key targets for the treatment of myocardial injury. Among them, AKT1, as a member of the PI3K-Akt signaling pathway, has a mechanism of action that may be related to the activation of this signaling pathway. Some studies have found that the PI3K-Akt signaling pathway has a role in resistance to myocardial injury [14]. EGFR is a cellular inflammatory factor that plays an important role in myocardial injury. STAT3, ESR1, SRC, MMP9, and HSP90AA1 are the confluence of multiple tumour pathways, which are consistently and abundantly expressed in tumour cells, and can promote tumour cell proliferation, anti-apoptosis, etc., as well as being important signal regulators in cardiovascular diseases. Their mechanism of anti-myocardial injury is to block the upstream molecules of these target signaling pathways or direct target proteins to inhibit the processes related to the signal transduction of these targets [15]. And GO analysis and KEGG enrichment showed that pathways in cancer, PI3K-Akt signaling pathway and proteoglycans in cancer were the main enrichment pathways. Analysis using Cytoscape showed that the active compound with the highest number of polyphenolic targets in *Balanophora involucrata* was quercetin. It was shown that most of the cardioprotective effects of quercetin were attributed to the antioxidant properties, which exerted cardioprotective effects by reducing oxygen radical

production and inhibiting neutrophil infiltration [16]. A large number of experimental data show that quercetin post-treatment can inhibit the occurrence of cardiomyocyte apoptosis and reduce cell damage by activating the PI3K/Akt signaling pathway and further interfering with the expression of apoptotic proteins Bcl-2 and Bax [17]. It can be hypothesized that the main active components of polyphenolic compounds in *Balanophora involucrata* may play a role in the treatment of myocardial injury through the inhibition of AKT1, EGFR, and STAT3 target activities and activation of the P13K/Akt pathway.

5 Conclusion

In this study, silica gel column chromatography and Sephadex LH−20 chromatography were used to separate the polyphenolic compounds within *Balanophora involucrata*, and then combined with network pharmacology to explore the active components, core targets, and signaling pathways of *Balanophora involucrata* for the treatment of myocardial injuries, and further verified the active components and core targets through molecular docking. core targets through molecular docking. The present study provides theoretical and experimental basis for the study of *Balanophora involucrata* in the treatment of myocardial injury.

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