Experimental Evidence and Network Pharmacology-Based Analysis Revealing the Effects and Mechanism of Branches of *Sophora Japonica* (L.) in Anti-Myocardial Ischemia

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Research

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Abstract

Background: Huai-Zhi (HZ), the fresh or dried branches of *Sophora japonica* (L.) are commonly used to treat several diseases such as heartache, red eyes, and metrorrhagia. The present study aimed to explore the potential mechanisms effects of HZ anti-myocardial ischemia by experiment and integrating network pharmacology.

Methods: Isoproterenol was used in this study to establish the myocardial ischemia model in mice. Different extraction processes were used to obtain different HZ extracts with a screening of their anti-myocardial ischemia activities. Furthermore, the network pharmacology methods together with molecular docking were utilized to explore the active components, targets, and mechanism of anti-myocardial ischemia of HZ.

Results: The ethyl acetate extract of HZ (HZ-EtOAc) significantly reduced the ST-segment elevation of mice in the preliminary test. The 95% ethanol fraction of the ethyl acetate extract of HZ (HZ-EtOAc-95) significantly reduced the ST-segment elevation, reduced the creatine kinase (CK) activity, reduced the levels of creatine kinase isoenzyme(CK-MB) and cardiac troponin I (cTnI) in serum, increased superoxide dismutase (SOD) activity, and decreased malondialdehyde (MDA) levels in the myocardial tissues. Moreover, these results indicated that HZ-EtOAc extract in mice ameliorates myocardial tissue injury. Additionally, network pharmacology demonstrated that nine active components and 177 protein targets are related to the anti-myocardial ischemic effects of HZ. Its underlying mechanism might be involved in multiple signaling pathways, including mitogen-activated protein kinase, Toll-like receptor, and PI3K-Akt.

Conclusion: This study used pharmacological experiments to determine the active site of HZ, and explored its potential mechanism in conjunction with network pharmacology.

Background

Myocardial ischemia, a pathophysiological state of insufficient blood and oxygen supply to the myocardium, occurs owing to coronary artery stenosis and spasmodic embolism [1]. Myocardial ischemia is categorized as coronary heart disease (CHD). According to the World Health Organization report, CHD is the leading cause of mortality in developed countries and one of the main causes of disease burden in developing countries, with an annual increase in the incidence of CHD [2]. The “China Cardiovascular Disease Report 2019” report demonstrates that as a common clinical disease, cardiovascular disease is the primary cause of death for residents, with high morbidity and mortality [3]. Currently, the treatment of this disease is primarily western medicine and interventional surgery. They act by modulating the role of antiplatelet aggregation, stabilizing atherosclerotic plaque, and improving blood supply to the heart. However, the clinical application is limited due to the challenges of antiplatelet drug resistance, poor myocardial perfusion, and in-stent restenosis [4]. Therefore, safe and effective drugs should be determined to prevent and treat myocardial ischemia.

Huai-Zhi (HZ), the fresh or dried branches of the leguminous plant *Sophora japonica* (L.), dispels blood stasis, clears the heart, and eliminates toxins [5]. It was first recorded in the Mingyi bielu and is currently included in the quality standard of traditional Chinese medicine (TCM) and ethnic medicine in Guizhou Province (2003 Edition). HZ is widely distributed in Guizhou Province and is commonly used in treating heartache, metrorrhagia, and other diseases. Modern studies found that HZ is rich in pharmacologically active substances, primarily flavonoids and volatile components [6–9]. Pharmacological studies have demonstrated significant effects of flavonoids and volatile components on myocardial ischemia injury in TCM [10–13]. HZ is used to treat vasculitis in modern clinical applications [14]. However, the anti-myocardial ischemic effect of HZ has not been reported. Therefore, the myocardial ischemia animal model combined with network pharmacology techniques were used in this study to explore the anti-myocardial ischemic effects and mechanisms of HZ.

TCM follows the holistic view in treating diseases; therefore, TCM has the characteristics of multi-component, multi-target, and multi-channel to treat human diseases [15]. Network pharmacology could establish the interaction between the molecular network of complex components in TCM and multiple targets. Moreover, the comprehensive analysis of drug action from the perspective of multiple targets may be realized. Therefore, the potential active components and their targets of TCM can be predicted [16, 17]. Since HZ has significant anti-myocardial ischemic effects, this study analyzed the anti-myocardial ischemic effects of HZ using network pharmacology techniques and molecular docking analysis.

Methods

Plant materials

Sophora japonica (L.). branches were collected from Longli County, Qiannan Buyi, and Miao Autonomous County, Guizhou Province, China. The plant specimen was identified by Professor Xiangpei Wang from the Guizhou Minzu University. The branches were shade-dried and grounded using a blender.

Reagents

Analytical grade n-butanol (Bu), ethyl acetate (EtOAc), petroleum ether, ethanol (EtOH), glacial acetic acid, and formaldehyde solution were purchased from Hanbang Science & Technology (Nanjing, China). Isoproterenol (ISO) was purchased from Shanghai Hefeng Pharmaceutical Co., Ltd. (Shanghai, China). Sodium pentobarbital was purchased from Chongqing Jinjing Fine Chemicals Co., Ltd. (Chongqing, China). Fufang danshen tablets were purchased from Guizhou Feiyunling Pharmaceutical Co., Ltd. (Guizhou, China). Creatine kinase (CK), superoxide dismutase (SOD), and malondialdehyde (MDA) assay kits were produced by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), while cardiac troponin I (cTnI) and CK myocardial isoenzyme (CK-MB) enzyme-linked immunosorbent assay (ELISA) kits were produced by the Shanghai Yuanye Biotechnology Co. (Shanghai, China). AB-8 macroporous resins were produced by Anhui Sanxing Resin Technology Co. Ltd. (Anhui, China). Silica gel (200–300 mesh size) was obtained from Qingdao Marine Chemical Factory (Qingdao, China).

Animals

Kunming mice (n = 304, male: female ratio = 1:1) were procured from Changsha Tianqin Biological Technology Co., Ltd. (license number: SCXK [Xiang] 2009-0013), weight (20 ± 2 g). They were maintained under standard laboratory conditions (temperature, $20 \pm 1^\circ\text{C}$; 12-h light/dark cycle) with food and water access ad libitum. This study was conducted as per the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Guiyang University of Chinese Medicine. All surgeries were performed using sodium pentobarbital anesthesia. All drugs were administered once daily orally at a dose of 4 or 8 g raw drug/kg bodyweight for 10 consecutive days according to TCM and ethnic medicine in Guizhou Province (2003 Edition) and pre-experiment results.

Preliminary extraction

Dried and powdered GZ (16.5 kg) was successively extracted using 95%, 70%, 50%, and 30% EtOH at room temperature and evaporated to dryness to obtain the HZ extract (yield: 9.65%). HZ was further purified from the extract by diluting with distilled water (1.5 L) and successive extraction using petroleum ether (4×2 L), EtOAc (4×2 L), and n-butanol (4×2 L). Each solvent extract was evaporated to dryness under reduced pressure to obtain the HZ-PE (yield: 0.60%), HZ-EtOAc (yield: 1.15%), and HZ-Bu (yield: 2.90%) fractions. The final aqueous phase extract was also evaporated to dryness to obtain the HZ-H₂O fraction (yield: 5.00%). All fractions were concentrated under vacuum and were stored at 4°C .

Activity and fractionation of the active extracts

According to the results of preliminary pharmacological screening, HZ-EtOAc and HZ-Bu extracts had remarkable effects on ISO-induced myocardial ischemia; therefore, the HZ-EtOAc fraction was further analyzed. To purify the extract, 10 g of HZ-EtOAc were separated using AB-8 macroporous resin columns (eluted consecutively with water, 50% EtOH, and 95% EtOH) to obtain the water (HZ-EtOAc-0, yield: 3.06%), 50% EtOH (HZ-EtOAc-50, yield: 33.69%), and 95% EtOH (HZ-EtOAc-95, yield: 62.04%) fractions. After the fractions were concentrated under vacuum, they were stored at 4°C .

Preliminary pharmacological screening

The mice were randomly distributed into 10 groups of 16 mice each. Two groups were administered HZ-PE (4 or 8 g/kg/day), two groups each received HZ-EtOAc (4 or 8 g/kg/day), HZ-Bu (4 or 8 g/kg/day), and HZ-H₂O (4 or 8 g/kg/day), one group received 0.58 g/kg Compound Danshen Tablets to serve as the negative control group, and another group received only distilled water to serve as the untreated model group. All treatments were orally administered. The mice were pretreated for 10 days, and then ISO was injected intraperitoneally (4 mg/kg, excluding the control group) for 2 consecutive days.

For determination of ST segment (which connects the QRS complex and the T wave) elevation in the electrocardiogram (ECG), the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (3%; 0.1 mL/10 g), 30 min after the last administration and were fixed on the bench in the

supine position. The BL-420S biological data acquisition and analysis system (Chengdu Thaimeng Technology Co., Ltd., Chengdu, China) was used to record the lead II ECG. ST-segment elevation was observed 30 s after the ECG had stabilized after intraperitoneal injection of ISO (2 mg/kg) as an indicator of myocardial ischemia.

Analyses of the activity of the extracts

The mice were assigned to nine groups of 16 mice each (equal number of both sexes) randomly. Two groups each received HZ-EtOAc-0 (4 or 8 g/kg/day), HZ-EtOAc-50 (4 or 8 g/kg/day), and HZ-EtOAc-95 (4 or 8 g/kg/day), and one group received 0.58 g/kg Compound Danshen Tablets to serve as the negative control group. Moreover, the mice in the ISO and normal control groups received distilled water. All treatments were orally administered. The mice were pretreated for 10 days and were then injected intraperitoneally with ISO (4 mg/kg, excluding the untreated model group) for 2 consecutive days.

Thirty minutes after the last intraperitoneal injection of ISO, the mice in each group were anesthetized using sodium pentobarbital (3%, 0.1 mL/10 g) intraperitoneally and were connected to the BL-420S biological data acquisition and analysis system. After stabilizing the lead II ECG, ISO was administered at a dose of 2 mg/kg, and the lead II ECG was recorded after 0.5, 1, 2, 5, 10, and 15 min. Following this, the ST-segment elevation or depression was measured as the change in amplitude relative to the value at 0.5 min.

The mice were euthanized, and the heart tissues were excised (excluding large blood vessels and connective tissues). The tissues were weighed after blotting using filter paper. The myocardial water content (MWC) was calculated as follows: $MWC = (\text{myocardial wet weight [MWW]} - \text{myocardial dry weight [MDW]}) / MWW \times 100$. The heart weight index (HWI) was calculated as follows: $HWI = \text{heart weight (HW)} / \text{body weight (BW)} \times 100\%$.

The CK activity was measured using assay kits. The CK-MB and cTnI expression levels were determined using ELISA. For determining SOD and MDA in the myocardial tissues, approximately 0.1 g of myocardial tissue was excised from the upper part of the heart, immersed in ice-cold saline solution (1:9 w/v), and homogenized. The homogenate was centrifuged at 3000 rpm for 15 min at 4°C, and the supernatant was utilized for the biochemical assays. The SOD and MDA levels were measured spectrophotometrically using diagnostic kits following the manufacturers' instructions.

The hearts were excised and fixed in 10% formalin solution immediately after euthanasia for histological examination of the myocardium. The standard histological methods were followed for sectioning and staining the heart tissues. Sections (5 µm) of the left ventricle were excised using a Leica RM2125 (Leica, Wetzlar, Germany) and were stained using hematoxylin and eosin (H&E), followed by examination using light microscopy (Nikon, Tokyo, Japan) at 200× magnification.

Network pharmacology and molecular docking research platform and software

1 CNKI (<https://kns.cnki.net/kns8/defaultresult/index>), 2 Guizhou digital library (<http://www.gzlib.org/>), 3 PubMed database (<https://pubmed.ncbi.nlm.nih.gov/>). 4 Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, <http://tcmospw.com/tcmosp.php>), 5 Genecard (<https://www.genecards.org>), 6 UniProt database (Universal Protein, <https://www.uniprot.org/>), 7 VENNY2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>), 8 Protein interaction platform String (<https://string-db.org/>), 9 Network image topology analysis and editing software Cytoscape 3.8.1, 10 PDB database (<http://www.rcsb.org>), 11 Human Genome Annotated Database DAVID (<https://david.ncifcrf.gov/>), 12 PyMOL 2.

Collection and screening of active compounds and related targets of HZ

The chemical constituents of HZ were searched from CNKI, Guizhou digital library, PubMed database.

The components from HZ were selected as active.

The active components from HZ were selected with literature report effects of anti-myocardial ischemia or $DL \geq 0.18$. The related target proteins of the active components were screened using TCMSP and transformed into gene names in the UniProt database.

Collection of anti-myocardial ischemia targets

Anti-myocardial ischemia-related targets were searched in the GeneCards database with the keyword “anti-myocardial ischemia.”

Construction of “component-target” network

The target genes of HZ active compounds and anti-myocardial ischemia were crossed, and the Venn diagram was drawn using the online tool VENNY2.1. The intersecting targets and active components were mapped to Cytoscape 3.8.1, and the “component-target” network was obtained.

Construction of PPI network and screening of core targets

The intersection targets were input into the string platform, species selection was set as homo, and the interaction score was 0.4 to obtain the PPI network diagram and Excel table. The Excel data was analyzed using Cytoscape 3.8.1 to obtain three topological parameters of each node, including Degree, Betweenness centrality, and Closeness centrality. The screening criteria of core targets of HZ against myocardial ischemia was its value greater than the median value of the three topological parameters.

GO classification enrichment analysis and pathway analysis

GO biological function and KEGG pathway of the core targets were analyzed in the David database, and the corresponding functional distribution and biological pathway of the target were obtained.

Construction of “active compound-core target-pathway” network

The “active compound-core target-pathway” network was constructed using Cytoscape 3.8.1.

Molecular docking

Among the core targets, the targets with the top five-degree values were selected to conduct molecular docking using HZ active compounds. The three-dimensional structure of the targets and the Mol2 structure of the active compounds were downloaded from the PDB and TCMSP databases, respectively. The proteins were added to PyMOL 2 to remove water and electrons. The drug molecules and proteins were introduced into the AutoDock Tool software for hydrogenation, charge calculation, and other operations to conduct docking. The docking results were analyzed and plotted using PyMOL 2.

Statistical methods

The data are presented as the mean $\pm$ standard deviation (SD). One-way analysis of variance followed by Bonferroni's multiple comparison test was applied for the data comparison between the groups. Statistical analyses were performed using the SPSS software v20.0 (SPSS, Inc., Chicago, IL, USA). Differences with p-values < 0.05 were considered statistically significant.

Results

The effects of HZ on ST-segment elevation assessed in the preliminary analysis

The ST segment was elevated 2 min after ISO administration in the untreated model group, indicating successful inception of myocardial ischemic damage. Treatment using HZ-Bu and HZ-EtOAc (4 and 8 g/kg, respectively) markedly decreased ST-segment elevation than that in the untreated model group (Table 1).

Table 1

The effect of Huai-Zhi (HZ) extracts on ST-segment elevation in mice with isoproterenol (ISO)-induced myocardial injury.

Treatment	ST D-value
ISO control	0.035 ± 0.009
HZ-H ₂ O L	0.030 ± 0.013
HZ-H ₂ O H	0.033 ± 0.015
HZ-Bu L	0.016 ± 0.006 ^{**}
HZ-Bu H	0.016 ± 0.004 ^{**}
HZ-EtOAc L	0.012 ± 0.003 ^{**}
HZ-EtOAc H	0.015 ± 0.006 ^{**}
HZ-PE L	0.029 ± 0.007
HZ-PE H	0.030 ± 0.010
Negative control	0.012 ± 0.002 ^{**}
ISO control refers to the isoproterenol model group; L: low dose, H: high dose. The data are presented as the mean ± standard deviation (SD), <i>n</i> = 16 in each group. ^{**} <i>P</i> < 0.01 versus the ISO control group.	

The effects of HZ-EtOAc on ST-segment elevation

The ST segment was elevated 30 s after ISO administration in the ISO control group than that in the normal control group (*P* < 0.01), demonstrating the successful inception of myocardial ischemic damage. After 15 min, ST-segment elevation persisted in the untreated group. However, ST-segment elevation was decreased in the negative control, HZ-EtOAc-95 H, and HZ-EtOAc-95 L groups than that in the ISO control group (*P* < 0.05, Table 2).

Table 2

The effects of the ethyl acetate extract of Huai-Zhi (HZ-EtOAc) extracts on ST-segment elevation in mice with isoproterenol (ISO)-induced myocardial injury.

Treatment	1–0.5 min (mV)	2–0.5 min (mV)	5–0.5 min (mV)	10–0.5 min (mV)	15–0.5 min (mV)
Normal control	0.00 ± 0.02	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
ISO control	0.08 ± 0.02 ^{##}	0.08 ± 0.02 ^{##}	0.09 ± 0.02 ^{##}	0.10 ± 0.03 ^{##}	0.10 ± 0.04 ^{##}
Negative control	0.01 ± 0.01 ^{**}	0.02 ± 0.01 ^{**}	0.01 ± 0.01 ^{**}	0.01 ± 0.01 ^{**}	0.01 ± 0.01 ^{**}
HZ-EtOAc-0 H	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
HZ-EtOAc-0 L	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
HZ-EtOAc-50 H	0.05 ± 0.03	0.05 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
HZ-EtOAc-50 L	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
HZ-EtOAc-95 H	0.01 ± 0.02 ^{**}	0.01 ± 0.01 ^{**}	0.01 ± 0.02 ^{**}	0.01 ± 0.02 ^{**}	0.00 ± 0.02 ^{**}
HZ-EtOAc-95 L	0.00 ± 0.01 ^{**}	0.01 ± 0.02 ^{**}	0.00 ± 0.02 ^{**}	0.00 ± 0.02 ^{**}	0.00 ± 0.01 ^{**}
ISO control refers to the isoproterenol model group; L: low dose, H: high dose. The data are presented as the mean ± standard deviation (SD), <i>n</i> = 16 in each group. ^{**} <i>P</i> < 0.01 versus the ISO control group; ^{##} <i>P</i> < 0.01 versus the normal control group.					

The effects of HZ-EtOAc on HWI

The HWI was higher in the ISO control group than that in the normal control group (*P* < 0.01). Mice pretreated using Compound Danshen Tablets or HZ-EtOAc-95 showed a dose-dependent decrease in HWI than that in the ISO control mice (Table 3).

Table 3

The effect of the ethyl acetate extract of Huai-Zhi (HZ-EtOAc) extracts on the MWC and HWI of mice with isoproterenol (ISO)-induced myocardial injury.

Treatment	MWC (%)	HWI (%)
Normal control	77.53 ± 0.87	0.45 ± 0.08
ISO control	77.62 ± 0.53	0.51 ± 0.02 ^{##}
Negative control	77.53 ± 1.17	0.44 ± 0.02 ^{**}
HZ-EtOAc - 0 H	77.23 ± 0.34	0.46 ± 0.02
HZ-EtOAc - 0 L	77.31 ± 1.79	0.48 ± 0.06
HZ-EtOAc - 50 H	76.96 ± 0.99	0.47 ± 0.04
HZ-EtOAc - 50 L	77.04 ± 0.89	0.47 ± 0.03
HZ-EtOAc - 95 H	77.09 ± 0.44	0.43 ± 0.06 ^{**}
HZ-EtOAc - 95 L	77.06 ± 0.83	0.44 ± 0.03 [*]
ISO control refers to the isoproterenol model group; L: low dose, H: high dose. The data are presented as the mean ± standard deviation (SD), $n = 16$ in each group. [*] $P < 0.05$, ^{**} $P < 0.01$ versus the ISO control group; ^{##} $P < 0.01$ versus the normal control group. MWC, myocardial water content; HWI, heart weight index.		

Determination of the cTnI and CK-MB expression levels and CK activity

At the end of the experiments, blood samples were collected and analyzed for CK activity and cTnI and CK-MB expression. The expression levels of cTnI and CK-MB were significantly increased in the ISO control group ($P < 0.01$) than those in the normal control group. However, pretreatment using HZ-EtOAc-95 decreased the cTnI and CK-MB expression levels than those in the ISO control group ($P < 0.01$). Moreover, the CK activity was significantly increased in the ISO control group ($P < 0.01$) than that in the normal control group. Pretreatment using HZ-EtOAc-95 decreased the CK activity than that in the ISO control group ($P < 0.01$, Table 4).

Table 4

The effect of the ethyl acetate extract of Huai-Zhi (HZ-EtOAc) extracts on the cTnl and CK-MB levels and CK, SOD, and MDA activity of mice with isoproterenol (ISO)-induced myocardial injury.

Treatment	cTnl (nmol/mL)	CK-MB (nmol/mL)	CK activity (U/mL)	SOD activity (U/mg)	MDA activity (U/mg)
Normal control	1.65 ± 0.51	2.59 ± 0.53	0.87 ± 0.06	294.699 ± 40.390	20.357 ± 3.850
ISO control	2.86 ± 0.16 ^{##}	3.49 ± 0.17 ^{##}	1.15 ± 0.06 ^{##}	143.148 ± 17.660 ^{##}	58.714 ± 3.785 ^{##}
Negative control	1.76 ± 0.52 ^{**}	2.61 ± 0.15 ^{**}	0.91 ± 0.04 ^{**}	249.784 ± 35.408 ^{**}	30.857 ± 2.777 ^{**}
HZ-EtOAc-0 H	2.37 ± 0.36	3.42 ± 0.78	1.10 ± 0.05	200.639 ± 56.236	54.857 ± 5.555
HZ-EtOAc-0 L	2.84 ± 0.18	3.43 ± 0.18	1.10 ± 0.04	191.540 ± 21.921	45.429 ± 5.731
HZ-EtOAc-50 H	2.22 ± 0.51	3.69 ± 0.42	1.07 ± 0.02 ^{**}	191.250 ± 40.543	45.643 ± 8.322
HZ-EtOAc-50 L	2.24 ± 0.40	3.03 ± 1.01	1.07 ± 0.01 [*]	195.017 ± 58.537	48.000 ± 4.991
HZ-EtOAc-95 H	1.30 ± 0.70 ^{**}	2.28 ± 0.36 ^{**}	0.93 ± 0.03 ^{**}	342.028 ± 31.623 ^{**}	27.000 ± 4.527 ^{**}
HZ-EtOAc-95 L	1.44 ± 0.42 ^{**}	1.91 ± 0.30 ^{**}	0.95 ± 0.02 ^{**}	309.245 ± 14.773 ^{**}	30.857 ± 3.637 ^{**}
ISO control refers to the isoproterenol model group; L: low dose, H: high dose. The data are presented as the mean ± SD, <i>n</i> = 16 in each group. * <i>P</i> < 0.05, ** <i>P</i> < 0.01 versus the ISO control group; ## <i>P</i> < 0.01 versus the normal control group. cTnl, cardiac troponin I; CK, creatine kinase; CK-MB, CK myocardial isoenzyme. SOD, superoxide dismutase; MDA, malondialdehyde					

The effects of HZ-EtOAc on SOD activity and the MDA levels in the myocardium

The activity of SOD in the ISO control group was significantly decreased (*P* < 0.01) than that in the normal control group. Additionally, pretreatment using HZ-EtOAc-95 increased SOD activity in a dose-dependent manner than that in the ISO control group (*P* < 0.01). However, the MDA levels were markedly increased in the ISO control group (*P* < 0.01) in comparison to the normal control group. Pretreatment with HZ-EtOAc-95 decreased the MDA levels in a dose-dependent manner than those in the ISO control group (*P* < 0.01, Table 4).

Histological examination of the myocardium

As illustrated in Fig. 1, mice in the normal control group exhibited neatly arranged myocardial fibers, interstitial connective tissues without inflammatory cell infiltration, absence of edema or hyperplasia of the connective tissues, with no apparent fiber/myocardial cell cross-striations. A large area of myocardial infarction was observed in the hearts of the mice in the ISO control group accompanied by intercellular edema. Additionally, myocardial cell degeneration, small blood vessel dilation, significant hyperemia, bleeding, and fibrous connective tissue proliferation were also observed in the myocardium. Notably, HZ-EtOAc-95 treatment markedly reduced myocardial cell damage; thereby, protecting against the myocardial injury (Fig.

Screening of active compounds of HZ

A total of nine HZ active compounds were screened, among which seven HZ compounds accorded with $DL \geq 0.18$, and another two compounds were reported to be effects of anti-myocardial ischemia. The nine active compounds corresponded to 374 targets. After omitting repetitions, 239 targets were obtained. The top three compounds with the most targets were quercetin, genistein, and kaempferol (Table 5).

Table 5
Active compounds and target number of Huai-Zhi (HZ)

MOL ID	Compound	DL	Number of targets
MOL000098	Quercetin	0.28	150
MOL000481	Genistein	0.21	78
MOL000422	Kaempferol	0.24	53
MOL000675	Oleic acid	0.14	40
MOL000415	Rutin	0.68	20
MOL003648	(+)-Maackiain	0.54	15
MOL000131	Linoleic acid	0.14	11
MOL000480	Genistin	0.75	4
MOL001506	Squalene	0.24	3

Collection of anti-myocardial ischemic targets

A total of 2230 anti-myocardial ischemia targets were obtained from the GeneCards database.

Construction of “component-target” network

Firstly, the targets of nine active compounds were intersected using the targets of anti-myocardial ischemia, and 174 intersected targets were obtained. Secondly, the Venn diagram was drawn using the online tool Venny (Fig. 2). Finally, the intersection target and active compound were mapped to Cytoscape 3.8.1 software to draw the network diagram of “component-target” (Fig. 3).

Construction of PPI network and screening of core targets

One hundred and seventy-seven intersection targets were imported into the String database for condition setting to obtain the PPI network diagram (Fig. 4). The Excel table data was downloaded from String and analyzed using Cytoscape 3.8.1 to derive topology parameters. Twenty core targets were screened by Betweenness Centrality > 0.00167425, Closeness Centrality > 0.55128205, and Degree > 86, among which the top five with large Degree values were INS, Akt1, IL6, TP53, and tumor necrosis factor (TNF) (Table 6).

Table 6
Information table of the target of Huai-Zhi (HZ) anti-myocardial ischemia

Betweenness Centrality	Closeness Centrality	Degree	Name
0.07896321	0.8	129	INS
0.04018997	0.77828054	123	AKT1
0.04593868	0.77828054	123	IL6
0.03260116	0.74458874	114	TP53
0.02305889	0.73191489	109	TNF

GO annotation analysis of HZ core target

The GO functional annotation analysis of 20 core targets of HZ was performed using David’s database. GO enrichment analysis included three parts: biological process (BP), molecular function (MF), and cell composition (CC). Moreover, $p \leq 0.05$ is considered to be significantly enriched. Based on this condition, 83, 10, and seven enrichment entries were obtained for BP, MF, and CC, respectively. Among them, biological processes have the strongest correlation with positive regulation of gene expression, transcription, DNA replication, and nitric oxide biosynthesis. Among the molecular functions, it has the strongest correlation with identical protein binding, protease binding, and transcription factor binding. It has the strongest correlation with extracellular space, protein complex, and extracellular region among the cellular components. The top 10 biological processes, molecular functions, and cell composition were plotted using a bar chart (Fig. 5). GO results demonstrated that the anti-myocardial ischemic effects of HZ were related to several biological processes.

KEGG pathway analysis and “active compound-core target-pathway” network analysis

The 20 key targets of HZ were analyzed using the David database, and 85 pathways were obtained using $P \leq 0.05$. The results demonstrated that the anti-myocardial ischemia target proteins of HZ were primarily related to the functions of signaling pathways such as hepatitis B, cancer pathways, and the TNF. The top 10 signaling pathways were plotted as a bar chart (Fig. 6). Cytoscape 3.8.1 was used to construct HZ “active compound core target pathway” network diagram (Fig. 7). Among them, the square represents the

pathway, the triangle represents the target, and the ellipse represents the active ingredient. The Figure illustrates that HZ anti-myocardial ischemia uses multiple components to act on multiple targets and affect multiple pathways.

Molecular docking

As shown in Table 7, the primary targets of HZ are INS, AKT1, interleukin (IL) 6, TP53, and TNF. AutoDock was used to generate grid coordinates. The nine compounds were molecularly docked with five targets, and the results are shown in Table 8. The binding energy of less than 0 indicates that the drug molecule can spontaneously bind to the receptor protein. Moreover, the smaller the binding energy, the better the docking. The results showed that the energy of the nine components was less than 0. Among them, (+)-Maackiain and kaempferol showed good binding energy with the targets INS, AKT1, IL6, TP53, and TNF. The molecular docking results are shown in Table 8, and part of the composite structure diagram is illustrated in Fig. 8.

Table 7
Generate grid coordinates

Target	x	y	z
TP53	10.828	95.874	87.795
TNF	-13.708	71.595	26.989
INS	10.511	3.009	-0.319
IL6	2.564	-20.107	9.255
AKT1	1.499	-0.752	25.256

Table 8
Molecular docking results

MOL ID	Compound	Binding free energy/(Kcal.mol ⁻¹)				
		INS	AKT1	IL6	TP53	TNF
MOL000098	quercetin	-4.62	-3.75	-4.31	-2.66	-4.15
MOL000481	genistein	-6.12	-4.53	-5.12	-3.57	-4.62
MOL000422	kaempferol	-6	-4.33	-4.64	-3.08	-3.95
MOL000675	oleic acid	-2.79	-1.78	-2.46	-1.62	-1.61
MOL000415	Rutin	-1.85	-2.03	-1.3	1.16	0.15
MOL003648	(+)-Maackiain	-6.49	-5.94	-5.82	-4.5	-5.87
MOL000131	linoleic acid	-2.99	-3.54	-3.15	-0.74	-3.24
MOL000480	genistin	-3.92	-3.47	-2.9	-1.73	-4.49
MOL001506	squalene	-2.39	-1.18	-3.13	-0.68	-3.42

Discussion

Studies have demonstrated that the subcutaneous injection of ISO can cause excitation of the heart, increase the oxygen consumption of the myocardium, aggravate myocardial ischemia and hypoxia, and lead to myocardial injury, currently a commonly used modeling method. Moreover, the myocardial ischemia injury model is relatively close to the natural human acute myocardial ischemia in the electrocardiogram, myocardial metabolism, and histopathological changes [18]. Therefore, this study uses the ISO-induced myocardial injury model to explore whether different extraction parts of HZ can interfere with myocardial injury.

With the decrease of blood flow and increase of oxygen consumption, myocardial tissue is in a state of ischemia and hypoxia; thus, possibly leading to a decrease in the stability of the myocardial cell membrane and an increase in permeability. This will further lead to leakage of myocardial enzymes from the inside and outside of the cell to the serum, causing the serum center to increase muscle enzyme activity [19]. CTnI, CK, and CK-MB are the primary myocardial marker enzymes, particularly cTnI is a protein specific to myocardial cells, released into the blood after myocardial injury, and is a “gold indicator” of myocardial injury [19, 20]. Therefore, the activity of cTnI, CK, and CK-MB in serum is used to judge the degree of myocardial ischemic injury. Moreover, SOD is an important antioxidant enzyme in myocardial tissues. Under hypoxia, free radicals can directly or indirectly cause cell structure damage or dysfunction through lipid peroxidation and deplete cell SOD and other free radical scavenging enzymes [21]. Oxygen-free radicals attack the polyunsaturated fatty acids in the biofilm, cause lipid peroxidation, and generate lipid peroxidation products, such as MDA, and further cause cell damage. Moreover, the content of MDA in the myocardium can reflect the degree of lipid peroxidation [22]. Therefore, the SOD

and MDA levels in myocardial cells can reflect the degree of myocardial peroxidation. The experimental results demonstrate that HZ-EtOAc-95 can resist the myocardial damage induced by ISO in mice. The results further demonstrated that HZ-EtOAc-95 could inhibit the myocardial injury induced by ISO in mice, reduce the abnormal elevation of ST-segment in ECG, decrease the content of cTnI and CK-MB in serum, increase the activity of SOD and decrease the content of MDA in myocardial cells. HE staining of myocardial tissue also depicted that HZ-EtOAc-95 can significantly reduce the damage of myocardial cells. Thus, the important approach of HZ to protect the myocardium is to enhance the ability of myocardial tissues to scavenge oxygen-free radicals and reduce the production of lipid peroxides in myocardial tissue.

The myocardial ischemic model is an effective method to screen and evaluate anti-myocardial ischemia drugs. Pharmacological experiments demonstrate that HZ-EtOAc-95 has an obvious anti myocardial ischemic effect on experimental mice. However, the target, effective material basis, and mechanism of action are not yet clear, with systematic research lacking. Therefore, the network pharmacology analysis method is used to assess it further. Nine HZ active components and 239 predicted targets were screened using network pharmacology. Some of the active components such as quercetin, kaempferol, oleic acid, and linoleic acid have been proven with certain anti-myocardial ischemic effects [23–25]. PPI shows the interaction relationship between target proteins, and 20 core targets are screened out. The first five targets with a higher degree value are INS, AKT1, IL6, TP53, and TNF. Clinical studies have demonstrated that INS can rapidly alleviate the hyperglycemia level after acute myocardial infarction, improve the energy metabolism of myocardial cells, and increase their glucose uptake to improve and accelerate the recovery of cardiac function [26]. AKT1 is abundantly expressed in the heart and brain tissues. Moreover, it can regulate nitric oxide production through the AKT1-eNOS signal axis to reduce damage to myocardial cells occurring owing to ischemia and reperfusion [27]. The increase of TNF and IL-6 in serum can cause myocardial fibrosis [28]. TP53 is a tumor suppressor gene protein, with the gene mutation possibly inhibiting apoptosis and leading to atherosclerosis, closely related to coronary heart disease [29]. The results of GO enrichment analysis demonstrated that the anti-myocardial ischemia effect of HZ was related to biological processes such as positive regulation of gene expression, transcription, and DNA replication. According to KEGG analysis results, combined with literature review, the predicted HZ anti-myocardial ischemia targets are primarily enriched in MAPK, Toll-like receptor, and PI3K-Akt signaling pathways. Studies have shown that the PI3K-Akt signaling pathway is closely related to myocardial ischemia-related diseases. When the pathway is activated, it can protect cardiomyocytes by improving the body's nutrient metabolism, reducing inflammation, and protecting vascular endothelial cells [30]. MAPK signaling pathway enhances the anti-apoptotic ability of cardiomyocytes by regulating EPK [31]. The results of molecular docking demonstrated that there was a good binding activity between the active component of HZ and the target, indicating that the target prediction was reliable.

Conclusion

In summary, this study explored the protective effects of different HZ extracts on myocardial injury through ISO-induced myocardial ischemia in mice. The results demonstrated that the HZ-EtOAc-95 site

can significantly inhibit myocardial injury in mice and is the main active site of HZ. Based on the preliminary discussion, combined with network pharmacology and molecular docking, the target and mechanism of HZ anti-myocardial ischemia were assessed, and the anti-myocardial ischemic effects of HZ were found to be possibly related to the regulation of multiple signal pathways by multiple chemical components.

Abbreviations

HZ

Huai-Zhi

ISO

Isoproterenol

HZ-EtOAc

Ethy acetate extract of HZ

HZ-EtOAc-95

95% ethanol fraction of the ethyl acetate extract of HZ

CK

Creatine kinase

CK-MB

Creatine kinase isoenzyme

cTnI

Cardiac troponin I

SOD

Superoxide dismutase

MDA

Malondialdehyde

CHD

Coronary atherosclerotic heart disease,

Bu

N-butanol

EtOAc

Ethyl acetate

EtOH

Ethanol

TCM

Traditional Chinese medicine

ECG

Elevation in the electrocardiogram

MWC

Myocardial water content

MDW
Myocardial dry weight
HWI
Heart weight index
MWW
Myocardial wet weight
TCMSP
Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform
BP
Biological process
MF
Molecular function
CC
Cell composition
INS
Insulin
IL6
Interleukin-6
TP53
Cellular tumor antigen p53
TNF
Tumor necrosis factor
MAPK
Mitogen-activated protein kinase
TLR
Toll-like receptor
Akt1
Serine/threonine-protein kinase
NO
Nitric oxide
eNOS
Endothelial nitric oxide synthase

Declarations

Ethics approval and consent to participate

This study was conducted as per the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Guiyang University of Chinese Medicine

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MZ and HL contributed equally to this work. XW and YZ designed the study; FX, YY, QD, PQ and WH contributed to the acquisition, analysis and interpretation of data, and writing of the article. MZ, HL and YY contributed to the acquisition and interpretation of data and also to the final critical revision of the article. All authors read and approved the manuscript.

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Figures

Figure 1

Pathological changes in the myocardial tissue (hematoxylin and eosin [H&E], 200× magnification).

Figure 2

Anti myocardial ischemia target and Hz target

Figure 3

“Drugs compound-Targets” Network Diagram

Figure 4

Interaction of target proteins in Huai-Zhi (HZ)

Figure 5

GO histogram of Huai-Zhi (HZ) anti-myocardial ischemia target

Figure 6

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

Figure 7

Network diagram of “active compound-core target-pathway”

Figure 8

Molecular docking pattern (A: (+)-Maackiain and AKT1, B: Genistein and INS, C: Kaempferol and INS, D: (+)-Maackiain and INS)