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Exploring the mechanism of action of Hypoglycaemic Shuxin Formula in the treatment of diabetes mellitus combined with chronic heart failure based on network pharmacology and experimental validation

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Abstract: The purpose of this paper is to investigate the mechanism of action of traditional Chinese medicine (TCM)compound hypoglycemic Shuxin formula in the treatment of diabetes mellitus(DM) combined with chronic heart failure(CHF) through network pharmacology methods and experimental verification. Methods: The network pharmacology database and related platforms were applied to retrieve the active ingredients of Jiang Tang Shu Xin Recipe(JTSX) and the targets related to diabetes mellitus combined with chronic heart failure.The PPI protein interaction network and compound drug-ingredient-target network were constructed. Molecular docking was performed to verify the affinity. DM and CHF rat models were established, HE staining was performed, and the levels ofC-reactive protein (CRP), tumor necrosis factor alpha (TNF -α), and interleukin-6 (IL-6) in rat serum were detected using ELISA, and the levels of TNF-α and IL-6 in myocardial tissues were detected by immunohistochemistry. Western blot method was used to verify the therapeutic efficacy of the network's core pharmacological targets. Results: A total of 171 active ingredients, 331 intersecting targets, and 196 signaling pathways were retrieved. The core components are: 1-dimethyl-2,3- dihydrophenanthren-4-one, kaempferol, miltionone I,Ginsenoside-Rh4, 3,9-di-O-methylnissolin,5,6-dihydroxy-7-isopropyl-1. The core targets are tyrosine kinase (SRC), epidermal growth factor (EGFR), serine/threonine protein kinase (AKT1),insulin (INS), and tumor necrosis factor (TNF).The molecular docking results show that there is a good binding ability between the core components and the core targets. The results of animal experiments hanve shown that: hypoglycemic Shuxin Fang can improve the degree of myocardial fibrosis, reduce the level of TNF-α, IL-6 in myocardial tissue and the expression of key protein AKT1 in myocardial tissue. Conclusion: JTSX treats diabetes mellitus combined with chronic heart failure through multi-components and multi-targets, and exerts anti-fibrotic effect on myocardial tissues.

Keywords: Chronic Heart Failure; Diabetes Mellitus; Network Pharmacology; Molecular Docking; Jiangtang Shuxin Recipe; Experimental Validation

With the steady improvement of people's living standards and the aging of the population, the number of patients with diabetes in the world has increased significantly, of which more than 95% are patients with type 2 diabetes (T2DM)[1]. Diabetes mellitus is an endocrine metabolic disorder caused by a variety of reasons, the main causes are mostly obesity, unhealthy eating habits and long-term high blood sugar[2].Heart failure is characterised by dyspnoea and limitation of physical activity and is prevalent in the elderly.When there is a lot of damage, death and loss of myocardial cells, then it leads to myocardial fibrosis, which in turn develops into heart failure.Heart failure is the terminal stage of various cardiovascular diseases, characterized by the inability of the heart to pump sufficient blood and oxygen to meet the metabolic needs of other organs[3-4].Heart failure is considered as a common complication of diabetes, and diabetes is an independent risk factor for heart failure[5].Compared with patients with chronic heart failure without diabetes, patients with chronic heart failure with diabetes have more severe exercise restriction and worse prognosis[6-7]. JTSX is composed of Ginseng,Schisandra,Ophiopogon japonicus, Astragalus, Raw rehmannia glutinosa, Chinese yam, Cornus officinalis,Rhubarb,Coptis chinensis,Salvia miltiorrhiza and other traditional Chinese medicines. Among them, ginseng, Ophiopogon japonicus, and Schisandra chinensis are the components of Shengmai San, which have the effects of nourishing qi, yin, and generating fluids. Raw Rehmannia glutinosa, Chinese yams and cornus officinalis are the components of Zuogui

Pill, which can cure the deficiency of true yin, and raw Rehmannia glutinosa can also fill the marrow.Astragalus can nourish qi and detoxify. Coptis chinensis and Rhubarb can clear heat and detoxify, and Salvia miltiorrhiz has the effect of promoting blood circulation and removing blood stasis. The whole formula is used to invigorate the body and dispel evil, with the effects of attacking and supplementing, nourishing yin and qi, promoting blood circulation, resolving phlegm, and detoxifying[8]. Network pharmacology collects and integrates resources from multiple databases, uses software to screen and systematically analyze the collected data, and constructs a relational network diagram. Revealed the interactions between drugs, diseases, and their related biomolecules. The core theory is to construct a target network, emphasizing the characteristics of "multiple genes and multiple targets", in order to explain the overall concept of traditional Chinese medicine[9].

1. Materials and Methods

2. JTSX active ingredient and target acquisition

Utilizing the Traditional Chinese Medicine System Pharmacology Database and Analysis Platform（TCMSP，https://old.tcmspe.com/tcmsp.php）[10]、ETCM database（http://www.tcmip.cn/ETCM/）、BATMAN-TCM database（http://bionet.ncpsb.org.cn/batman-tcm/index.php）、Search for 10 traditional Chinese medicines in JTSX, Screening of active ingredients based on oral bioavailability (OB) ≥ 30% and drug likeness (DL) ≥ 0.18[11]. The ETCM database screens the active ingredients of Schisandra chinensis and Ophiopogon japonicus based on Drug Similarity Grading as Moderate Good, and pre selects target genes with a confidence level greater than 0.8 as active ingredients[12]. The BATMAN-TCM database selects effective ingredients and targets of Rehmannia glutinosa based on a Score cutoff ≥ 20 and a corrected P-value less than 0.05[13]. Standardize protein gene names in the Uniport database. Search for SMILES number in PubChem database and screen in Swiss ADME using SMILES number, while meeting the following two conditions: 1. GI absorption score is "high"; 2. There are at least two "yes" in drug likeness. If the conditions are met, then use the Swiss Target database（ http://old.swisstargetprediction.ch ）Screening effective targets based on a probability>0.1.

3. Screening of disease-related targets

Gene Cards, OMIM, and TTD databases were used to search for 'Heart failure'and'Type 2 diabetes mellitus'[14-15], and the results were combined and duplicates were removed . The results were combined and duplicate values were removed. Due to the excessive number of Genecard values, the median was used for the cardinal values, and the final values were between 1000 and 2000.

4. Screening of common targets for diseases and drugs

The screened ingredient targets and disease targets of JTSX Formula were used to produce a Venn diagram using the Venny online platform to obtain the intersecting targets.

5. Constructing PPI protein interaction networks

The screened intersecting targets were imported into the STRING database (https://cn.string-db.org/), and the species was limited to 'homo sapiens', the interaction was ≥0.4, and the free nodes were hidden[16]. The key targets were screened using Centiscape 2.2 in Cytoscape 3.9.1 software, and the core targets were screened by three scores, namely, closeness, betweenness, and degree, with the larger degree value indicating the more important they are in the network[17].

6. GO and KEGG enrichment analysis

Through the DAVID database（https://david.ncifcrf.gov/）Conduct comprehensive bioinformatics analysis on the core targets of drugs and diseases. It covers gene ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The GO analysis is specifically divided into three parts: biological process (BP), cellular component (CC), and molecular function (MF)[20]. Using R studio 4.2.0 software, install R packages such as gglot2, openxlsx, tidyverse,the top 10 BP, CC, and MF rankings with the top 20 KEGG rankings were analysed for GO and KEGG visualisation.

7. Molecular docking

The five active ingredients with the highest degree values among the core targets were selected to be aligned with the top five core

targets with the highest degree values among the PPIs. The 2D structures of the five active ingredients with the highest degree values were downloaded from TCMSP and PubChem databases, and screened in the RSCB PDB database (https://www.rcsb.org/) according to the following conditions: ① originated from human (Homo sapiens); ② crystal resolution of the proteins is less than 3A; ③ Proteins were obtained by X-ray diffraction. The PDB files of the core targets were then obtained. Pymol 2.3.4 software was used to remove water and small molecule ligands from the proteins. The molecular docking of components and targets was performed by Auto Dock software, and visualised by Pymol software.

8. Experimental verification

9. Animals

Purchase 50 male SD rats with a body weight ranging from 220g to 250g from the Experimental Animal Center of Guangxi Medical University, license code: SCX (Gui) 2020-003. Feeding conditions: The temperature is maintained at 18 ℃ -22 ℃, the relative humidity is 50% -60%, and there is 12 hours of circulating lighting every day. Eating and drinking can be freely carried out.

10. Medications

Take 15g of astragalus,10g of ginseng, 10g of schizandra, 10g of Salvia miltiorrhiza, 15g of Ophiopogon japonicus, 8g of Coptis chinensis, 15g of Raw rehmannia glutinosa,, 5g of Rhubarb, 15g of yam, 10g of Cornus officinalis, and purchase them from the Youjiang University of Ethnic Medicine. Benazepril (Approval number: National Medical Products Administration Approval No. H20044840, Shanghai Xinya Pharmaceutical Minhang Co., Ltd.), Grequalone (Approval number: National Medical Products Administration Approval No. H20084004, Tianjin Jinshi Pharmaceutical Co., Ltd.).

11. Reagents and Instruments

Streptozotocin (STZ, batch no. 10099-141, Beijing Hua Yue Yang Biotechnology Co., Ltd.), HE staining kit (batch no. H1120, Shanghai Biyuntian Biotechnology Co., Ltd.), CRP kit, IL-6 kit, TNF-α kit (Wuhan PhD Bio-engineering Co., Ltd.), automatic biochemistry instrument (Shenzhen Myriad Biomedical Electronics Co., Ltd.), reverse transcription kit (batch no. K1622), BCA protein concentration test kit (batch no. C0038) were purchased from Thermo Fisher Scientific, USA. Ltd.), reverse transcription kit (batch no. K1622), BCA protein concentration detection kit (batch no. C0038) were purchased from Thermo Fisher Scientific, USA, RIPA lysate (Shanghai Biyuntian Biotechnology Co., Ltd.), and YKT-6300 optical microscope (Shanghai Yongke Optical Instrument Co., Ltd.). Anti-TNF antibody, anti-AKT1 antibody, IgG secondary antibody (Beijing Zhongsui Jinqiao Biotechnology Co., Ltd.), automatic enzyme immunoassay analyser (Beijing Huayueyang Biotechnology Co., Ltd.).

12. Grouping and Administration

Fifty rats were randomly divided into western medicine group, model group, control group, low-dose group of Jiang Tang Shu Xin Formula (JTSX1), and high-dose group of Jiang Tang Shu Xin Formula (JTSX2), with 10 rats in each group. The control group was injected with citric acid buffer and fed with regular feed. Make the hypoglycemic and soothing formula into a paste, with a daily dosage of 1 g/kg for the low-dose group and 1.5 g/kg for the high-dose group. The western medicine group, model group, JTSX1 and JTSX2 were given a single intraperitoneal injection of 50 mg/kg STZ, and tail vein blood glucose was detected by blood glucose test paper after 72 h. Diabetes mellitus modelling was considered to be successful if fasting glucose≥16.7 mmol/L on two consecutive occasions. Feed with high-fat feed (78.2% regular feed, 10% egg yolk powder, 10% lard, 0.3% pig bile salt, 1.5% cholesterol) for one month. Diabetes rats were anesthetized by intraperitoneal injection of 8% pentobarbital sodium (2mL/kg) after 8 hours of confinement. The abdominal cavity of diabetes rats was opened and the abdominal aorta was separated. A 7-gauge needle was placed adjacent to the abdominal aorta, and a 4-0 silk thread was passed through the separated abdominal aorta and the 7-gauge needle using a 4-0 silk thread, followed by a slow ligation and removal of the needle. Abdominal aortic constriction of 60% to 70% was treated with anti-infective therapy postoperatively and continued to be fed with high-fat diets. At 28 days postoperatively, cardiac function was examined using an ultrasound system, and when the ejection fraction (EF) was ≤45%, a successful heart failure model had been established[18]. The low-dose group of Jiangtang Shuxin Formula (JTSX1) was orally administered

with Jiangtang Shuxin Formula at a dose of 1.0g/(kg·d), while the high-dose group of Jiangtang Shuxin Formula (JTSX2) was orally administered with Jiangtang Shuxin Formula at a dose of 1.5g/(kg·d).The western medicine group was orally administered with an equal volume solution of benazepril and glitazone, while the control group and model group were orally administered with an equal volume of distilled water once a day for two consecutive months. During the gavage period, the control group continued to be fed with regular feed, while the other groups were fed with high-fat feed.

13. Pathological observation of myocardial tissue

Myocardial tissue was processed by performing hematoxylin-eosin (HE) staining using a standard procedure, followed by sealing, observation by microscopy and recording of images[19]. After the rats were executed, the hearts were quickly taken out on ice, and a part of myocardial tissues were extracted and dehydrated by ethanol step by step, followed by paraffin embedding, cutting 4um thin slices, oven baking at 60℃, de-waxing by xylene, ethanol dehydration step by step, staining by Weigert's hematoxylin staining for 5min, rinsing by tap water for 5min, ethanol differentiation of 1% hydrochloric acid for 30s, rinsing by tap water for anti-bluing, and placing in eosin solution for 2min, rinsing by tap water, ethanol dehydration step by step, randomly selected five non-overlapping fields were observed under microscope. 2min,tap water rinse,ethanol dehydration step by step,5 randomly selected non-overlapping fields of view were observed under the microscope. Image acquisition was performed using a 200x microscope and HE semi-quantitative analysis was performed. The remaining myocardial tissues were refrigerated at -80°C for immunohistochemistry and protein immunoblotting experiments.

14. Serological testing

According to the instructions of the kit, the concentration levels of C-reactive protein (CRP), tumor necrosis factor alpha (TNF - α), and interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assay (ELISA).

15. Immunohistochemical detection of TNF - α and IL-6 expression levels in myocardial tissue

After fixation with 4% paraformaldehyde, the myocardial tissue was dehydrated step by step in ethanol, embedded in paraffin, sliced into 4 μ m thin sections, dewaxed with xylene, dehydrated step by step in ethanol, and subjected to antigen repair. Endogenous peroxidase was inactivated with 3% H2O2 incubated for 10 minutes, and a blocking solution (5% BSA) was dropped. The primary antibody was added and incubated overnight at 4 ℃ in a wet box. The primary antibody was washed away with PBS the next day, followed by biotinylated secondary antibody streptavidin peroxidase conjugate, and PBS washed away the secondary antibody. Drip horseradish enzyme labeled streptavidin working solution, stain with diaminobenzidine (DAB), and counterstain with hematoxylin. Dehydrated, transparent, sealed with neutral gum, and randomly selected 5 non overlapping fields of view for observation and photography under a 10 × 40x microscope. The positive expression of TNF - α and IL-6 appeared brownish yellow, and the optical density values of positive expression were measured using Image Pro Plus Version 6.0 image analysis software.

16. Western blot detection of myocardial tissue related protein expression

Myocardial tissue was taken out from -80℃ refrigerator, thawed and cut with scissors, 1mL of cold Lysis Buffer was added, homogenised in a tissue homogeniser, and then placed on ice for 15 minutes. The homogenate was poured into a centrifuge tube, centrifuged for 5 minutes at 12,000 r/min, and the supernatant was removed and transferred to a cooled centrifuge tube, where it was detected by the BCA Protein Concentration Test Kit. Detect its concentration. After electrophoresis and membrane transfer by SDS-PAGE, the PVDF membrane was put into the incubation box, closed by adding 5% skimmed milk powder, and oscillated for 1.5 h. The membrane was washed with TBST for 3 times, and then put into the incubation box containing primary antibody, and incubated with oscillation at 4℃ overnight. The next day, the incubator was shaken at room temperature for 0.5 h. The primary antibody was aspirated, discarded, and the membrane was washed 3 times with TBST. 5% skimmed milk powder sealing solution was used to dilute the secondary antibody, and the incubator was shaken with a shaker for 1 h. The secondary antibody was then recovered and the membrane was washed 3 times with TBST. ECL developer was added to develop the film, pictures were taken using an ECL imager, the pictures were stored, and the grey values were determined using ImageJ software.

17. Statistical analysis

SPSS 26.0 statistical software was used to process and analyse the data. Measurement data were normally distributed and expressed in the form of mean±standard deviation ±S. Between-group data were analysed by one-way analysis of variance (ANOVA), and the difference was considered statistically significant if the value was $p<0.05$.

18. Results

19. Active ingredient screening and target prediction of JTSX

A search was conducted on the TCMSP, ETCM, and BATMAN-TCM databases of traditional Chinese medicine contained in JTSX, and a total of 171 active ingredients were obtained, including 14 Astragalus, 14 Ginseng, 10 Yam, 5 Cornus officinalis, 5 Rhubarb, 13 Coptis chinensis, 57 Salvia miltiorrhiza, 23 Ophiopogon japonicus, 26 Schisandra chinensis, and 4 Raw rehmannia glutinosa. These active ingredients were queried for their corresponding targets, and after eliminating the targets, a total of 1089 potential targets of action were obtained.

20. Active ingredient-disease target prediction results

Gene cards, OMIM and TTD databases were searched with the keywords 'Type 2 diabetes mellitus' and 'heart failure', and 1524 and 1621 disease targets were obtained respectively. The results showed that 1524 and 1621 targets were obtained, and 2623 disease targets were obtained by merging and deleting duplicates. The component targets and disease targets were entered into Venny2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html), and a total of 331 intersecting targets were obtained to create a Wayne diagram. See Figure 1.

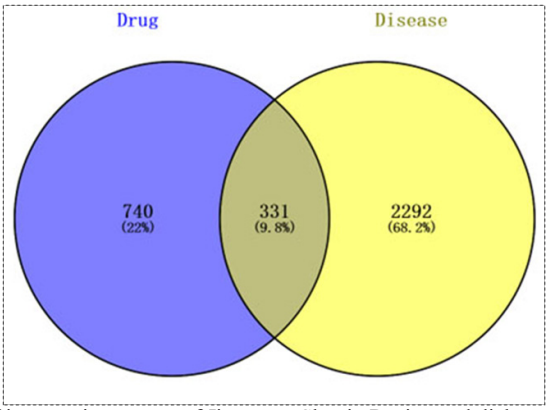


Figure 1 Venn diagram of intersection targets of Jiangtang Shuxin Recipe and diabetes with chronic heart failure

21. PPI protein interaction network analysis

The component-disease intersection targets were imported into the STRING database, free nodes were hidden, and the results were imported into Cytoscape 3.9.1 software for visualisation, obtaining 329 nodes and 6903 edges. Using the Centiscape 2.2 plugin, filtering was performed based on Closeness, Betweenness, and Degree values, resulting in 68 nodes and 1396 edges. Export the filtered file and rank it by degree value, with the top 5 key targets being AKT1, TNF, INS, SRC, and EGFR. The PPI protein interaction network diagram is shown in Figure 2. Note: The left image represents the intersection target, the middle is the key target, and the right image is the visualization of the key target.

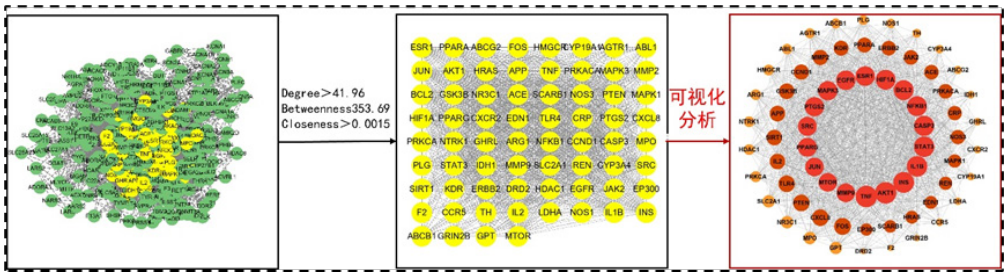


Figure 2 PPI protein interaction network

22. GO and KEGG Enrichment Analysis Results

Biological process annotation and metabolic pathway analysis (GO analysis and KEGG analysis) of drug and disease core targets using the DAVID database (https://david.ncifcrf.gov/tools.jsp), with a total of 1105 BPs, 123 CCs, 229 MFs and 195 KEGG signalling pathways obtained[20]. The data were sorted from largest to smallest according to Gene Radio, selecting the top ten BP, CC, MF, top 20 KEGG, and data with a P-Value value less than 0.05. GO analysis and KEGG enrichment analysis were performed using R studio 4.2.0 software. Refer to Figures 3 and 4. KEGG enrichment analysis mainly involves the following pathways: cancer pathway, metabolic pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, lipid and atherosclerosis, calcium signaling pathway, Rap1 signaling pathway, Ras signaling pathway, etc[21].

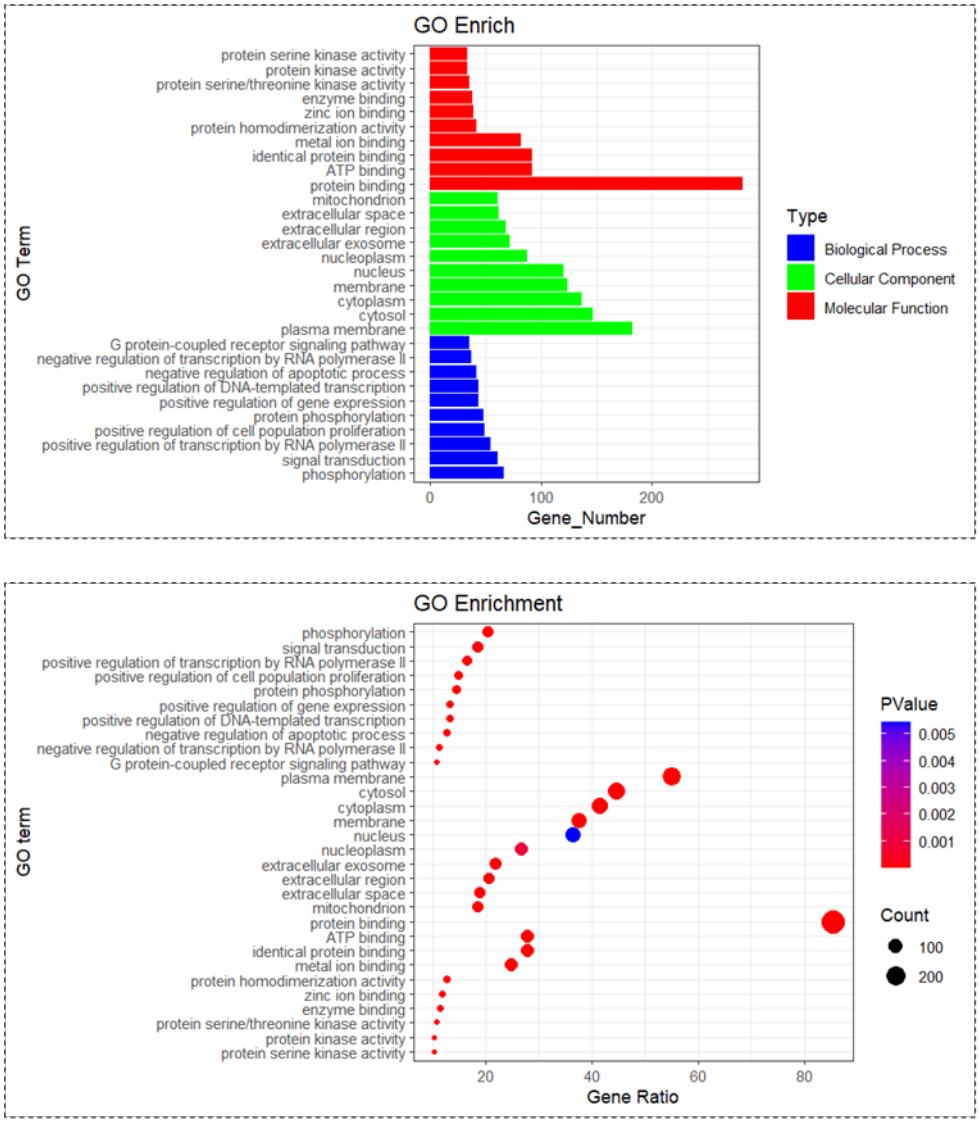


Figure 3 GO Function Enrichment Analysis

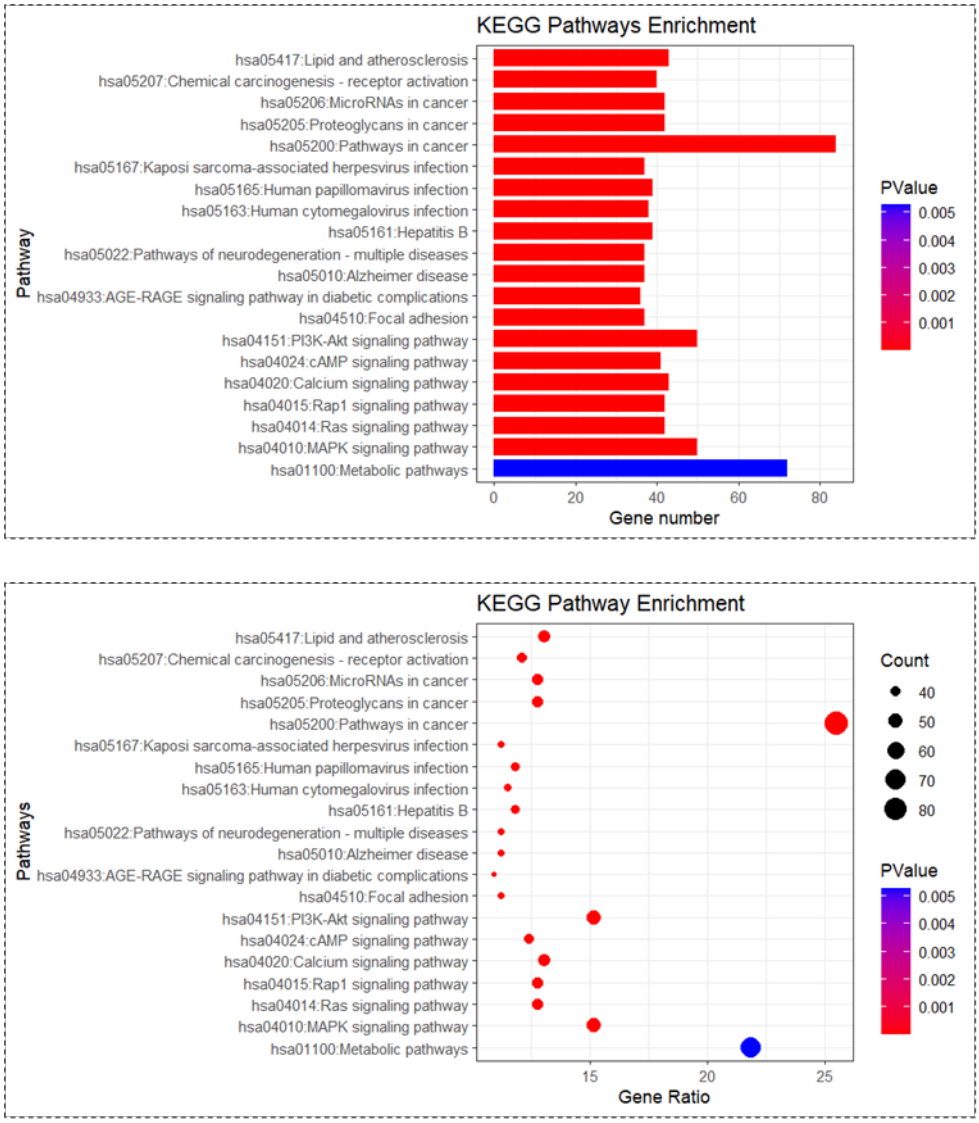


Figure 4 KEGG signalling pathway enrichment analysis

23. Construction of compound drug-component-target-pathway network diagrams

The name of the formula, the 10 Chinese herbal medicines composing the formula, the 171 core components obtained from screening, the 331 intersecting targets, and the pathways obtained from KEGG enrichment analysis were used to construct a disease-drug-component-target network by Cytoscape 3.9.1 software, and the results were analysed according to the topological structure of the network. The figure contains 329 nodes and 6903 edges. Refer to Figure 5. Analyze through Network Analyzer, export the file, arrange it in descending order of degree value, and select the top 5 components with the highest degree value as the main active ingredients for JTSX treatment of DM combined with CHF. MOL007036 (5,6-dihydroxy-7-isopropyl-1,1-dimethyl-2,3-dihydrophenanthren-4-one), MOL000422 (kaempferol), MOL007119 (miltionone I), MOL005348 (Ginsenoside-Rh4_qt), and MOL000371 (3,9-di-O-methylnissolin), respectively.

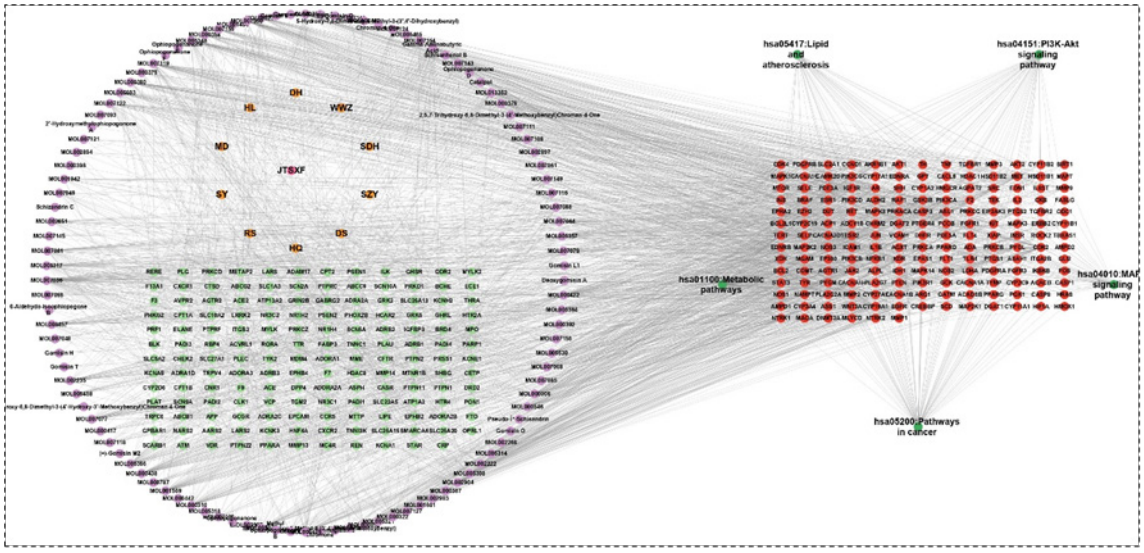
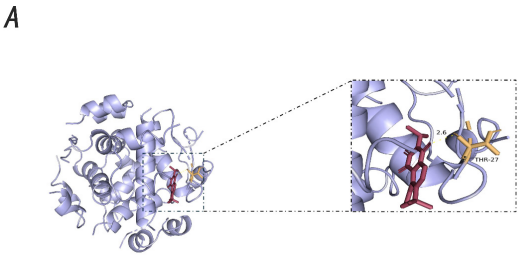


Figure 5 Drug-component-target-pathway Diagram

Note: The purple color in the figure represents the active ingredient, the orange color represents the drug name, the pink color represents the name of the hypoglycemic and comfortable formula, the light green color represents the intersection gene between the drug and the disease, the dark green color represents the pathway, and the red color represents the core target related to the pathway. The larger the node, the higher its degree value.

24. Molecular docking

Molecular docking of serine/threonine protein kinase (AKT1), tumour necrosis factor (TNF), insulin (INS), tyrosine kinase (SRC), and epidermal growth factor receptor (EGFR), screened by the active ingredient PPI protein interactions network, ranked among the top 5 active ingredient PPI proteins with the top 5 degrees of degreed value, was performed. Use Auto dock software to perform molecular docking between processed protein receptors and small molecule ligands. If the binding energy is less than -5.0 kcal/mol, it indicates that the small molecule ligand and protein receptor can effectively dock and the binding is relatively stable[22]. Refer to Table 1. Based on the binding energy, select the protein receptor and small molecule ligand with the lowest binding energy for molecular docking, and visualize it in Pymol2.3.4 software. See Figure 6.



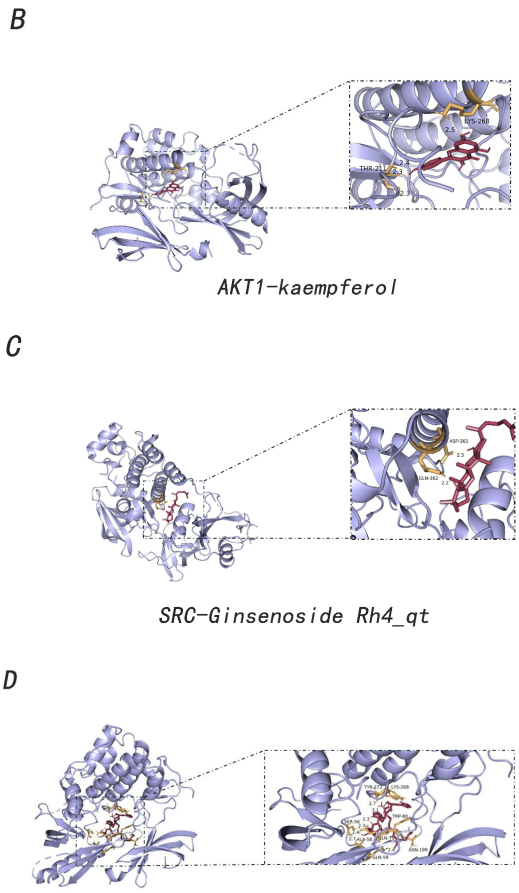


Figure 6 Molecular docking mode diagram

Table 1 Docking of Core Components and Key Target Molecules in Jiangtang Shuxin Formula

Component	Binding Energy (kcal/mol)				
	AKT1	TNF	INS	SRC	EGFR
5,6-dihydroxy-7-isopropyl-1,1-dimeth-yl-2,3-dihydrophenanthren-4-one	-7.2	-8.3	-6.5	-6.4	-8.3
kaempferol	-8.7	-6.5	-7.3	-7.6	-7.4
Miltionone I	-7.7	-7.3	-7.4	-6.8	-6.9
Ginsenoside Rh4_qt	-6.8	-6.6	-6.9	-7.3	-7.2
3,9-di-O-methylnissolin	-6.0	-7.3	-6.5	-6.4	-8.8

25. Animal experiment verification

26. HE Experiment

0 points: No inflammatory cell infiltration or myocardial necrosis; 1 point: The infiltration range of inflammatory cells and the area of myocardial necrosis are less than 25%; 2 points: The infiltration range of inflammatory cells and the area of myocardial necrosis are between 25% and 50%; 3 points: The infiltration range of inflammatory cells and the area of myocardial necrosis are between 50% and 75%; 4 points: Inflammatory cell infiltration range and myocardial necrosis area>75%.The HE staining results showed that the myocardial cells of the control group rats were arranged in an orderly manner, and the morphology of myocardial fibers and myocardial tissue was normal. The myocardial cell structure of the model group rats is disordered, swollen, and necrotic, and the myocardial tissue is severely damaged. The myocardial fibers are blurred, and some muscle filaments have necrosis, leading to the formation of vacuoles. JTSX1, JTSX2, and the Western medicine

group showed less myocardial cell damage compared to the model group. Refer to Figure 7 and Table 2.

Table 2 Comparison of scores for pathological alteration in rat myocardial tissue(± S,n=10)

groups	Control group	Model Group	Western medicine group	JTSX1	JTSX2
points	0.25±0.05	3.58±0.58a	2.82±0.54ab	1.98±0.35ab	1.70±0.28abc

Note: Compared with the control group, a P<0.01; compared with the model group, b P<0.05; compared with the western medicine group, c P<0.05.

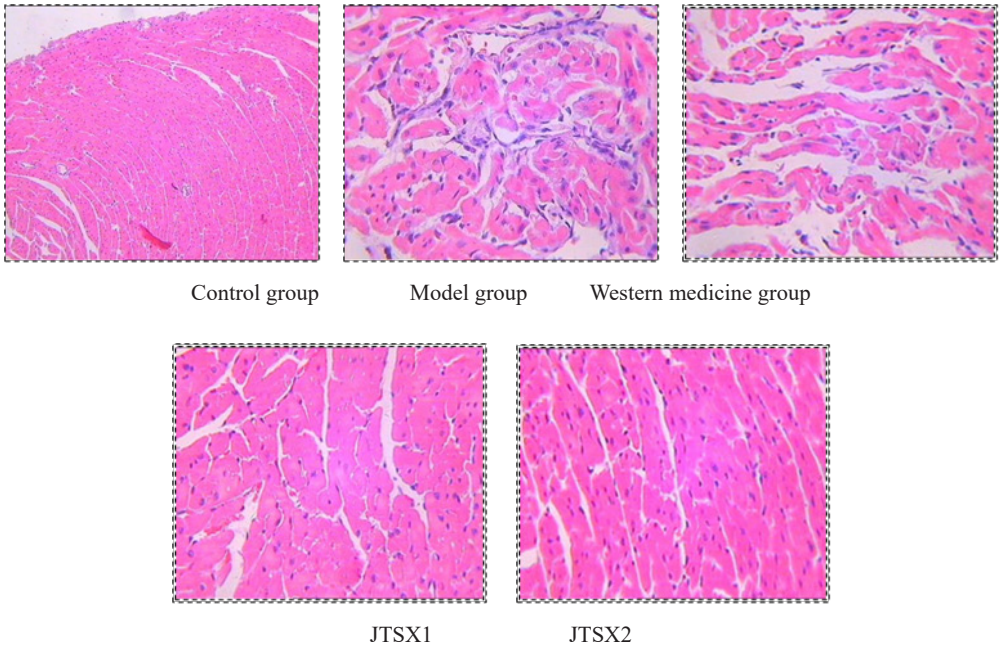


Figure 7 HE pathomorphology of rat myocardial tissue (HE staining, 400x)

27. Comparison of serum CRP, TNF - α, and IL-6 levels among different groups of rats.

Compared with the control group, the levels of serum CRP, TNF -α, and IL-6 in the model group rats were significantly increased (P<0.01); Compared with the model group, the levels of CRP, TNF-α,and IL-6 in the serum of each treatment group decreased (P<0.05)[23].In addition, when compared to the Western medicine group, it was observed that rats treated with a high-dose hypoglycemic and soothing formula exhibited reduced serum levels of CRP, TNF-α, and IL-6. The differences were statistically significant (P<0.05)[24]. Refer to Table 3.

Table 3 Comparison of serum CRP, TNF-α,and IL-6 levels among different groups of rats (±s)

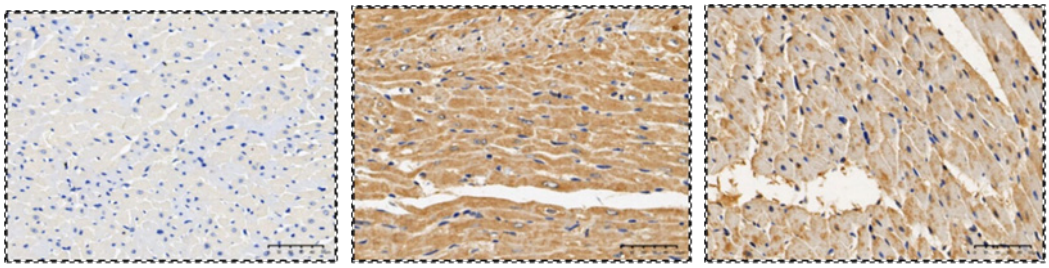
groups	n	CRP（mg/L)	TNF-α（ng/L)	IL-6（ng/L)
Control group	10	6.83±1.74	57.35±9.57	17.69±3.87
Model Group	10	15.45±3.12a	406.13±63.35a	85.47±8.18a
Western medicine group	10	10.53±2.25ab	138.65±40.16ab	59.65±7.75ab
JTSX1 组	10	9.57±2.15ab	131.29±41.24ab	55.35±6.63ab
JTSX2 组	10	7.64±2.28abc	109.77±33.87abc	48.87±6.82abc

Note: Compared with the control group, aP<0.05; Compared with the model group, bP<0.05; Compared with the Western medicine group, cP<0.05.

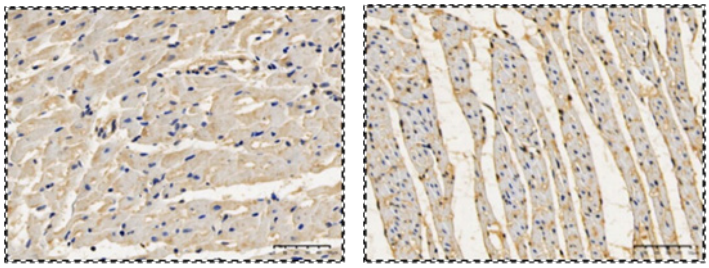
28. Effect of JTSX on TNF-α and IL-6 protein expression in myocardial tissue.

The results of immunohistochemistry and analysis showed that compared with the control group, the positive proteins in the model group were lamellar, cytoplasmically densely stained, and the expression was significantly increased (P<0.01); compared with the model group, the expression of TNF-α and IL-6 proteins was significantly decreased in all the drug-dosing groups (P<0.05); compared with the

western drug group, the expression of TNF-α and IL-6 proteins was decreased in JTSX2. See Figures 8 and 9, and Table 4.



Control group Model group Western medicine group



JTSX1 JTSX2

Figure 9 Immunohistochemical detection of IL-6 expression levels in rat myocardial tissue (×400)

Table 4 Comparison of functional protein expression of TNF - α and IL-6 in myocardial tissue by JTSX (±S, n=10)

groups	(IOD value)	TNF-α(IOD value)	IL-6(IOD value)
Control group	353±48	566±43	586±43
Model Group	8331±825a	8254±786a	8301±863a
Western medicine group	4316±526ab	3929±582ab	3853±461ab
JTSX1	4137±554ab	3764±489ab	3787±495ab
JTSX2	3898±546abc	3613±523abc	3688±536abc

Note: Compared with the control group, aP<0.01; compared with the model group, bP<0.05; compared with the western medicine group, cP<0.05.

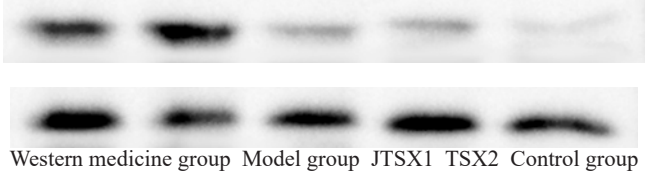
29. Comparison of AKT1 protein expression in myocardial tissue of rats in each groups.

Compared with the control group, the expression level of AKT1 protein in the myocardial tissue of the model group rats was significantly reduced. Compared with the model group, the expression level of AKT1 protein in the myocardial tissue of each treatment group increased. Compared with the Western medicine group, the high-dose group of hypoglycemic and soothing formula showed an increase in AKT1 protein levels in the myocardial tissue of rats. Refer to Table 5 and Figure 10.

Table 5 Effects of JTSX on AKT1 functional protein in myocardial lesions of rats (±S)

groups	Control group	Model Group	Western medicine group	JTSX1	JTSX2
AKT1 expression	0.958±0.075	2.926±0.076a	2.575±0.038ab	1.325±0.061abc	1.035±0.031abc

Note: Compared with the normal group, aP<0.01; compared with the model group, bP<0.05; compared with the western medicine group, cP<0.05.



Western medicine group Model group JTSX1 TSX2 Control group

Figure 10 AKT1 protein electrophoresis

30. Discussions

Diabetes mellitus belongs to the category of 'thirst' in traditional Chinese medicine, and it is common to have excessive thirst and drinking, excessive food and hunger, frequent urination, and emaciation, i.e. the symptoms of 'three more and one less'[25]. Thirst-quenching disease was first mentioned in the "Neijing", and its diagnosis and treatment based on syndrome differentiation are relatively comprehensive in Zhongjing's "Synopsis of the Golden Chamber"[26].Su Delin[27] believed that the deficiency of both qi and yin runs through the whole process of the disease of thirst, and that 'benefiting qi and nourishing yin, generating fluids and quenching thirst' should be the main method of treatment.Chronic heart failure belongs to the category of 'oedema and asthma' in traditional Chinese medicine, and the evidence belongs to 'deficiency', 'stasis' and 'water'. The main disease mechanism is 'deficiency', 'stasis' and 'water', and the main cause of the disease is qi deficiency and blood stasis due to the weakness of blood circulation, and the treatment is mainly based on benefiting qi, activating blood circulation, and inducing diuresis[28-29].The risk of heart failure is significantly increased in patients with diabetes mellitus, a diabetic state in which insulin resistance and hyperglycaemia lead to oxidative stress reaction, which leads to metabolic abnormalities and increased blood viscosity, inducing the development of heart failure[30].In this paper, we investigated the mechanism of action of Hypoglycemic Shuxin Fang in the treatment of diabetes mellitus combined with chronic heart failure through network pharmacology and experiments, constructed the 'drug-component-target-pathway network', and analysed the interactions among drugs, components, targets and pathways.

The results showed that the core components of Hypoglycaemic Soothing Formula for the treatment of diabetes mellitus combined with heart failure mainly included 5,6-dihydroxy-7-isopropyl-1,1-dimethyl-2,3-dihydrophenanthren-4-one, kaempferol ,miltionone I,Ginsenoside-Rh4_qt, and 3,9-di-O-methylnissolin. Kaempferol can alleviate the inflammatory reaction of adipose tissue and insulin resistance in diabetic rats[31-32], miltionone I has cytotoxic activity against human cancer cells in vitro and can mediate inflammatory responses [33-34].Ginsenosides are the main active ingredients in ginseng, which are widely used in traditional and modern medicine and have important biological and pharmacological activities, and ginsenoside-Rh4_qt has been shown to possess a wide range of anti-inflammatory, antidiabetic, antianaemic and anticancer properties, and to play an important role in the inhibition of aerobic glycolysis [35-36]. The PPI protein interaction network generates core proteins such as AKT1, TNF, INS, SRC, EGFR, etc.AKT1, also known as protein kinase B, is a member of the AGC kinase family, which regulates cell growth, metabolism and apoptosis by phosphorylating a range of downstream substrates[37].Any defects in the AKT/PKB pathway and its downstream molecules can trigger insulin resistance, and the loss of AKT1 can lead to heart failure, which occurs rapidly[38-39].The EGFR/PI3K/AKT signalling pathway inhibits the expression of cell cycle protein-dependent kinase inhibitory proteins and promotes cell proliferation and differentiation as well as tumour formation and progression[40].Tumour necrosis factor (TNF) is closely associated with insulin resistance, and its ligands and cognate TNF receptors are essential for the maintenance of immune homeostasis and execution of the immune response.TNF ligands and receptors selectively induce cell death and are directly involved in the immune response[41]. The SRC family, which consists of SRC-1, SRC-2 and SRC-3 together, regulates a variety of metabolic functions, including the inflammatory response as well as the regulation of energy[42].INS is a protein hormone, secreted by pancreatic β-cells, and its main physiological function is to regulate glucose metabolism.

The experimental results showed that Glucose Reducing and Heart Relaxing Formula was able to reduce the levels of TNF-α, IL-6 and CRP in serum, which proved that Glucose Reducing and Heart Relaxing Formula had anti-inflammatory effects and could inhibit the proliferation of collagen fibres and the expression of AKT1 protein in myocardial tissues, and verified the feasibility of the core targets screened by Network Pharmacology in the treatment of diabetes mellitus combined with chronic heart failure.

In conclusion, this present study preliminarily explored the mechanism of Jiangtang Shuxin Formula for the treatment of diabetes mellitus combined with heart failure through network pharmacology, molecular docking technology and experimental verification.Using network pharmacology and molecular docking technology, it shows that the combination between the core components of Jiangtang Shuxin Formula and the core target of the disease is relatively stable. On this basis, the experimental verification has been carried out, which shows that Jiangtang Shuxin Formula has a certain therapeutic effect on diabetes with heart failure.This study provides a new theory and idea for exploring the treatment of diabetes mellitus combined with heart failure by Jiangtang Shuxin Formula.

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