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Original Research Article

Identification of the components and molecular targets of Guanxin Danshen Dripping Pills for psycho-cardiology treatment based on network pharmacology and ultra-performance liquid chromatography-tandem mass spectrometry

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Abstract

Purpose: *To explore the molecular targets of Guanxin Danshen Dripping Pills (GXDS) in regulating psycho-cardiology disease through identifying its components by UPLC-MS/MS and network pharmacology approach.*

Methods: *Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was adopted to determine GXDS' main constituents. SwissTargetPrediction and the similarity ensemble approach were employed to predict possible molecular targets. Targets for psycho-cardiological disease were identified from the GeneCards database. The GXDS components overlapping these targets were identified using Venny web tool (version 2.1.0). Functional enrichment analysis was performed using Gene Ontology knowledgebase, while enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was undertaken. In Cytoscape, target-pathway, pathway-target, and compound-target networks were constructed.*

Results: *Among the 37 chemical components identified through UPLC-MS/MS, 19 were combined with 63 psycho-cardiological disease targets, which were subjected to gene ontology enrichment analysis to obtain terms corresponding to the sub-ontologies of cellular components, molecular functions, and biological processes (30, 84, and 1188, respectively). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed 109 related pathways.*

Conclusion: *This study identified GXDS components, combined them through network pharmacology analysis, and explored the material basis and targets of the therapeutic effect of GXDS on psycho-cardiological disease. Thus, GXDS is a potential candidate for use in the clinical treatment of psycho-cardiological diseases.*

Keywords: *Guanxin Danshen, Psycho-cardiology, Critical pathways, Molecular targeted, Network pharmacology*

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INTRODUCTION

Psycho-cardiology, a new concept for coronary heart disease (CHD), is the coexistence of cardiovascular disease and psychiatric disorders including anxiety and depression in one individual [1]. Psycho-cardiology, also known as psychological cardiology and behavioral cardiology, examines emotional, social, and behavioral problems associated with heart disease. Notably, high clinical efficacy has been achieved in this regard under traditional Chinese medicine (TCM) treatment. For example, the Buxinqi decoction led to favorable alleviation of depression and cardiological symptoms in one study [2].

Guanxin Danshen Dripping Pills (GXDS) has been established as a new drug for cardiovascular disease treatment by the China Food and Drug Administration (No. Z20010037). GXDS are composed of danshen (*Radix salviae miltiorrhizae*), sanqi (*Panax notoginseng*), and jiangxiang (*Dalbergiae odoriferae*). They reduce blood lipids and blood pressure, promote blood circulation, and alleviate blood stasis.

Guanxin Danshen Dripping Pills (GXDS) mitigate coronary symptoms and levels of anxiety and depression in individuals with angina pectoris [3]. Moreover, GXDS is a potential TCM antidepressant reported to regulate the antidepressant effect of the CamKII–CREB–BDNF signaling pathway [4]. Led by Keji Chen [5], a research group at Xiyuan Hospital of the China Academy of Chinese Medical Sciences studied the effect of GXDS on the cardiovascular prognosis and quality of life of patients with depression or anxiety undergoing percutaneous coronary intervention.

The Guanxin Danshen Dripping Pills (GXDS) have numerous chemical components. Among these, Danshen mainly contains lipid-soluble diterpenoid quinone compounds and water-soluble phenolic acids. The main components of sanqi are ginsenoside, ginsenoside triol type, and unique saponins such as *P. notoginseng* saponins. The principal chemical constituents of *D. odoriferae* oil are terpenoids, which contain high amounts of nerolidol and oxidized nerolidol [6]. These specific compounds of GXDS might be involved in psycho-cardiological therapy, but their targets remain unclear.

In network pharmacology, active molecule-target-disease relationships are constructed at the systemic level, which is undoubtedly of great relevance to the exploration of complex mechanisms of action and critical for the identification of active substances and TCM targets. The draft of the evaluation method of network pharmacology [7] ensures the development of this emerging discipline to improve the quality of network pharmacology research and to form a robust, scientific standard of evaluation.

Herein, the relationship between active molecules and psycho-cardiological targets of GXDS was established. The principal chemical constituents of GXDS were identified. Subsequently, network pharmacology analysis (target prediction, enrichment analysis, and network construction) was conducted sequentially (Figure 1).

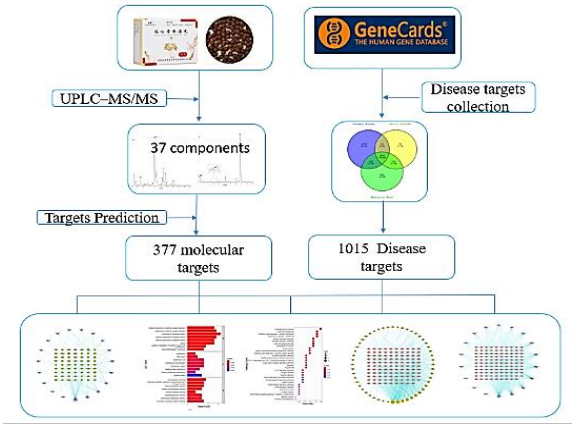

Figure 1: Study flowchart

EXPERIMENTAL

Chemical constituent identification

Materials

The Q Exactive Quadrupole-Electrostatic Field Track Well high-resolution mass spectrometry system and the Dionex Ultimate 3000 ultrahigh-performance liquid chromatography system were purchased from Thermo Fisher Scientific (San Jose, CA, USA). The ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, pore size 1.8 μm) was supplied by Waters. The Milli-Q UltraPure Water System was obtained from Millipore Corporation, USA. The Centrifuge 5427 R, analytical balance, and ultrasonic instrument were purchased from Eppendorf (Germany), Sartorius (Germany), and Kunshan Ultrasonic Instruments Co., Ltd., respectively.

The Guanxin Danshen Dripping Pills (GXDS) were supplied by Harbin Yerui Pharmaceutical Co., Ltd (batch number: 20180418). Formic acid was purchased from CNW Technologies (Germany). Acetonitrile and methanol (of a grade

suitable for high-performance liquid chromatography) were supplied by Thermo Fisher Scientific.

Test product preparation

The Guanxin Danshen Dripping Pills (GXDS) (approximately 0.2 g) were weighed and placed in a volumetric bottle (10 mL). Next, methanol was added. After 30 min of ultrasonic extraction (power = 250 W, frequency = 40 kHz), methanol was added at a constant volume. The mixture was centrifuged for 10 min at 12,000 rpm, after which the filtrate was passed through a 0.22-μm microporous membrane. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was subsequently performed.

Chromatography conditions

The mobile phase consisted of a 0.1 % formic acid solution (C) in acetonitrile (D). Gradient elution proceeded as follows: 0-9 min, 5-60 % C; 9-18 min, 60 % C; and 18-20 min, 60-95 % C. The column temperature and injection volumes were 45 ^{o}C and 3 μL, respectively, whereas the flow rate was 0.3 mL/min.

Mass spectrometry conditions

The ion source was HESI, with positive and negative ion detection modes. The settings were as follows: collision energy 6 eV, DdMS2 (resolution 17,500, NCE35, NCE50 %), ddMS^2 (resolution 17,500, NCE35, NCE50 %), collision energy 20 - 40 eV, and spray voltage: +3.2 kV / −2.8 kV. The auxiliary and sheath gas flow rates were 10 and 32 L/min, respectively. The capillary temperature was 320 ^{o}C, the voltage was 75.0 V, and the auxiliary gas heating temperature was 380 ^{o}C. The detection mode was full mass spectrometry. The scanning range and resolution were 80 – 1200 m/z and 70,000, respectively.

Spectral component analysis

Using Compound Discoverer 2.1 software (Thermo Fisher Scientific), compounds were analyzed and identified in accordance with their chromatographic characteristics (e.g., chromatographic peak retention time) and mass spectrometry information (e.g., accurate relative molecular weight, first-order mass spectrometry, and second-order mass spectrometry fragmentation).

Protein-protein interaction network

Protein-protein interactions (PPIs) of the GXDS' molecular targets were collected from a database called STRING (https://string-db.org/). The interaction score and organism were set to default (0.4) and *Homo sapiens*, respectively. Subsequently, the PPI network was constructed using Cytoscape3.8.2 software (https://cytoscape.org/), and topology analysis was conducted.

Target prediction of chemical constituents

A database on the similarity ensemble approach (SEA; http://sea.bkslab.org/) was employed to link proteins in accordance with their collective ligand-ligand chemical similarity. Under this method, large composite databases can be searched rapidly; moreover, graphs of cross-target similarity can be constructed. The simplified molecular input line entry system codes for small molecules were entered to match and obtain potential targets. The smaller the *p*-value, the more reliable the potential target. MaxTC values represent the degree of similarity between the database and the query molecule.

A web tool launched in 2014, SwissTargetPrediction (http://www.swisstargetprediction.ch/) examines small molecules and determines the protein targets with the greatest probability on the basis of reverse screening and the similarity principle. In accordance with how similar structures (whether two- or three-dimensional) were to established compounds, the compound targets were predicted. The results were sorted by probability, and the target name, Uniprot ID, and ChEMBL ID were obtained.

Target retrieval

Launched as the GeneCards project in 1997 by the Department of Molecular Genetics, Weizmann Institute of Science, Israel, the GeneCards database (http://www.genecards.org/) automatically mines and integrates human gene-related data from various sources. This information is categorized and employed in numerous projects. The Department of Molecular Genetics manages the database, aiming to integrate information fragments dispersed in specialized databases into a coherent picture. Herein, the database was searched for relevant gene targets by using the following keywords: coronary heart disease, anxiety disorders, and depressive disorder.

GO and KEGG pathway enrichment analysis

The GO database, a structured standard biological initiative developed by the Gene Ontology Consortium in 2000, provides data on

three sub-ontologies: the biological processes (BPs), molecular functions (MFs), and cellular components (CCs) of genes. A BP or KEGG pathway typically involves a cluster of genes, not an individual gene. The main basis of the enrichment analysis is that if a BP or pathway is determined to be abnormal, the probability of genes with a common function being included in relevant gene sets is high.

The clusterProfiler toolkit to examine the molecular mechanisms of GXDS' potential targets were employed. Specifically, the GO function and KEGG pathway enrichment of target proteins were investigated. The screening was performed according to a *p*-value of ≤ 0.05.

Network construction

To construct a compound-target network, bioactive components, and possible targets were linked. In accordance with the interactions between GXDS and protein targets and the KEGG enrichment analysis results, the target-pathway network was established. Compound-pathway networks comprised all compounds and signal transduction pathways. Network nodes indicated signaling pathways, candidate compounds, or potential targets. By contrast, edges denoted interactions between molecules and pathways, targets and pathways, or compounds and targets. A network diagram was generated using Cytoscape 3.8.2.

RESULTS

Chemical constituent identification

The chemical constituents of GXDS were analyzed using UHPLC-Q/Exactive technology. A total of 37 compounds were identified: (1) Azelaic acid, (2) Caffeic acid, (3) Cryptotanshinone, (4) Danshensu,(5) Dihydrotanshinone I, (6) Methyl rosmarinate, (7) Protocatechuic acid, (8) Salvianolic acid A, (9) Tanshinone IIA, (10) Salvianolic acid B, (11) Protocatechnic aldehyde, (12) Citric acid, (13) Germacrone, (14) Hyperoside, (15) Lithospermic acid, (16) Quinic acid, (17)Rosmarinic acid, (18) Pipecolic acid, (19) α-Boswellic acid, (20) α-Cyperone, (21) 20(R)-Ginsenoside Rh1, (22) 20(R)-Ginsenoside Rg2, (23) Ginsenoside F2, (24) Ginsenoside Rd, (25) Ginsenoside Re, (26) Ginsenoside Rg1, (27) Ginsenoside Rg2, (28) Gypenoside XVII, (29) Notoginsenoside R1, (30) Asiatic acid, (31) Lupenone, (32) Pulchinenoside A, (33) Kaempferol, (34) 3,5-Dimethoxy-4-hydroxybenzaldehyde, (35) Adenosine, (36) Nicotinamide, and (37) Valine. They include 11 organic acids, 3 quinones, 2 phenols, 6 terpenoids, 9 steroids, 2 flavonoids, 2 alkaloids, and 2 amino acids. Figure 2 displays the diagram of base peak intensity (considering both negative and positive ion modes).

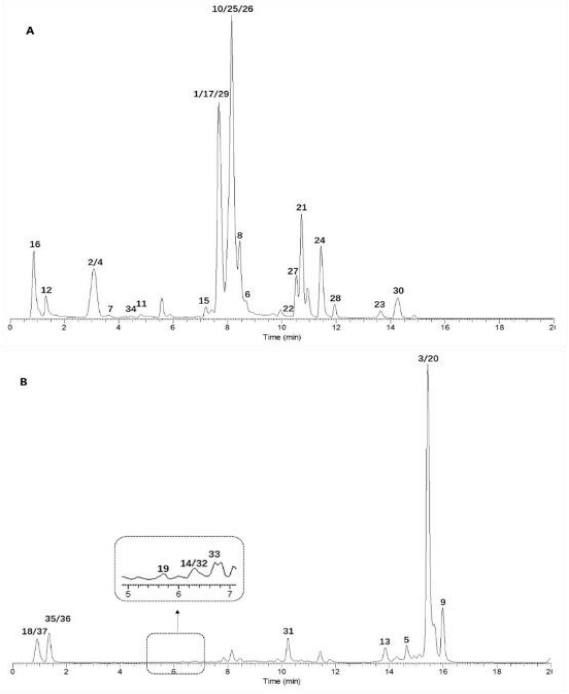

Figure 2: Base peak intensity diagram in (A) negative and (B) positive ion modes

Genetic information retrieval

From the GeneCards database, 2834, 1830, and 2817 CHD-related, anxiety-related, and depression-related targets with a relevance score of ≥ 5 were identified. Venn diagrams were created using Venny (version 2.1; https://bioinfogp.cnb.csic.es/tools/venny/), and the targets of each condition were intersected, revealing 1015 common targets.

Molecular target prediction

Among the 37 identified compounds, 205 targets with probability scores of ≥ 0.2 were determined using SwissTargetPrediction and 535 targets with MaxTC values of ≥ 0.5 were derived from the SEA database. After 740 duplicate genes were removed, 331 targets remained.

The 1015 disease genes and the 331 molecular targets were intersected to obtain 63 disease targets, which were associated with 19 types of GXDS molecules. Danshen, sanqi, and jiangxiang corresponded to 17, 4, and 1 component, respectively. Danshen and Sanqi had three constituents in common.

TCM exhibits pharmacological activity by acting on multiple targets. Therefore, the mechanism

underlying the effects of GXDS treatment on psychocardiological disease was investigated. In Cytoscape, a component-target network containing 82 nodes, 19 compound nodes, and 63 targets, forming 125 compound-target associations was constructed (Figure 3). The average number of neighbors was 3.288, and the network centralization and network heterogeneity values were 0.443 and 1.379, respectively. Nodes with the strongest correlations with other targets or compounds constituted the network hubs; they were designated potential compounds or targets. Kaempferol (MOL33, degree = 33), caffeic acid (MOL02, degree = 14), adenosine (MOL35, degree = 12), rosmarinic acid (MOL17, degree = 8), danshensu and methyl rosmarinate (MOL04 and MOL06, degree = 7), salvianolic acid B and hyperoside (MOL10 and MOL14, degree = 6), azelaic acid, and salvianolic acid A (MOL01 and MOL8, degree = 5) were the compounds with the highest degree of connectivity. Regarding the effects of GXDS treatment, these results indicate that multiple targets can be influenced by a single compound. In target analysis, of note, transthyretin was linked to nine compounds, and xanthine dehydrogenase, matrix metallopeptidase (MMP) 9 (MMP9), MMP2, and MMP1 were connected to seven compounds. Arachidonate 5-lipoxygenase was linked to six compounds.

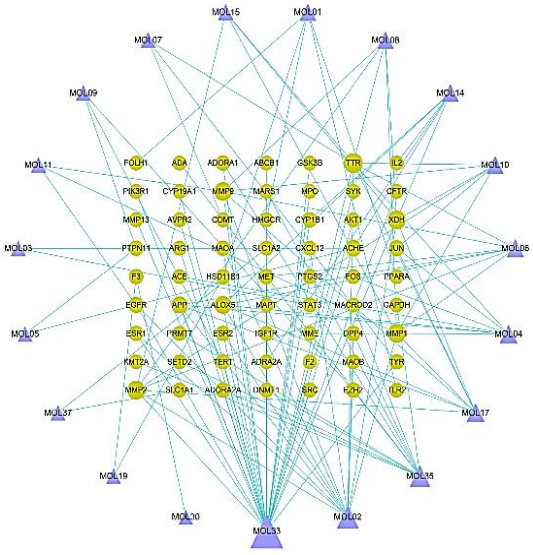

Figure 3: Compound-target network of psycho-cardiological disease. The triangle-shaped purple node represents a candidate active compound, the light green circular node denotes a potential protein target, and the edge indicates the interaction between the two. Node size and degree are positively linearly related

GXDS Target-Related PPI Network

To construct the PPI network, this study imported the 63 potential GXDS targets into the STRING database. The network analysis revealed 60 nodes, 890 edges, an average of 14.833 neighbors, a clustering coefficient of 0.597, a network centralization value of 0.459, and a network heterogeneity value of 0.682. Serine/threonine kinase (AKT) 1 (AKT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), epidermal growth factor receptor (EGFR), SRC, JUN, PTGS2, MMP9, FOS, ESR1, and STAT3 were the 10 nodes with the highest connectivity. Their identification may be of high value to the investigation and treatment of psychocardiological disease.

GO and KEGG Pathway Enrichment Analyses

GO enrichment analysis of the 63 genes of interest, undertaken using the clusterProfiler toolkit, revealed that 1302 GO terms fulfilled the criteria; the frequency count was set to ≥ 2 and adjusted according to a *p*-value of ≤ 0.05. In total, 1188, 30, and 84 items corresponded to BPs, CCs, and MFs, respectively. Figure 4 presents the top eight enrichment items in these three sub-ontologies. The BP-related enrichment items suggest a regulatory role of GXDS in psycho-cardiological disease through the responses of cells to aging, chemical and oxidative stress, neuroinflammation, and reactive oxygen species. Positive regulation of the proliferation of smooth muscle cells is also involved. The top eight CC-related items were axolemma, main axon, membrane raft, neuron projection membrane, membrane microdomain, membrane region, leading edge membrane, and vesicle lumen. The top eight MF-related items, listed in ascending order of frequency, were G protein-coupled receptor binding, serine-type peptidase activity, metallopeptidase activity, insulin receptor binding, protein phosphatase binding, serine hydrolase activity, action on phosphoric acid-nitrogen bonds, hydrolase activity, and phosphatase binding.

KEGG pathway analysis, performed using the clusterProfiler toolkit, identified 109 enrichment pathways ($p \leq 0.05$). Figure 5 present information on the KEGG pathways and the top 30 KEGG entries, respectively. These enriched genes were associated with numerous pathways, including 54 related to human diseases, 5 related to cellular processes, and 30 related to organismal systems as well as 13 signal transduction pathways and 7 metabolism-related genes.

To determine the GXDS' mechanism of action, a network of targets and pathways comprising 631 edges and 161 nodes was constructed (of which 109 were pathways and 52 were protein targets;

Figure 6). The network heterogeneity value was 1.344, whereas the network centralization value was 0.457. The average number of neighbors was 8.376.

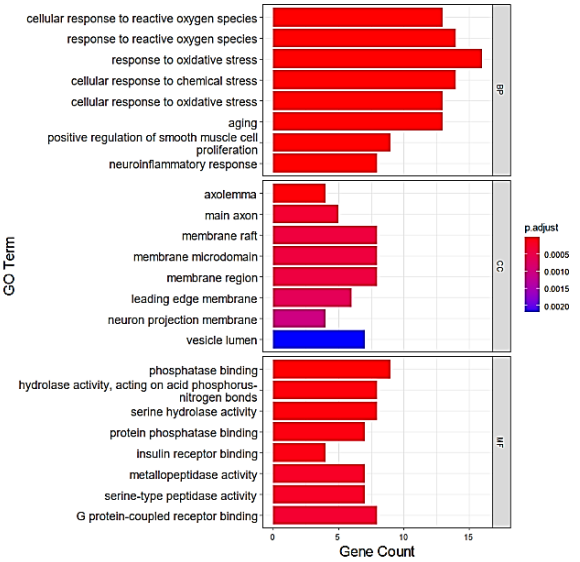

Figure 4: GO enrichment results, with the histogram color representing the level of *p* value adjustment. The lower the level, the redder the histogram.

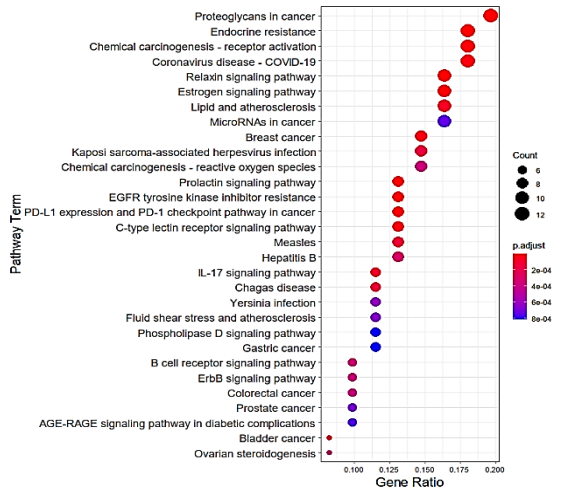

Figure 5: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment results, with the color of the solid circles representing the level of *p* value adjustment; the smaller the adjustment, the redder the circle. Solid circle size is positively linearly related to the number of enriched genes

A composite network of pathways to identify the compound-regulated pathways was constructed. Figure 7 reveals that the network contained 127 nodes (109 pathways and 18 compounds) and 605 edges. The network heterogeneity value was 1.501, and the network centralization value was 0.762. The average number of neighbors was 9.528. Kaempferol (MOL33) was associated with 104 pathways. Caffeic acid (MOL02), rosmarinic acid (MOL17), danshensu (MOL04), salvianolic acid B (MOL10), protocatechualdehyde (MOL11), methyl rosmarinate (MOL06), cryptotanshinone (MOL03), and azelaic acid (MOL01) were associated with 69, 60, 59, 55, 50, 41, 31, and 26 pathways, respectively. Other molecules each interacted with at least one molecule. In sum, through numerous pathways, multiple molecules contribute crucially to the therapeutic effects of GXDS on psychocardiological disease.

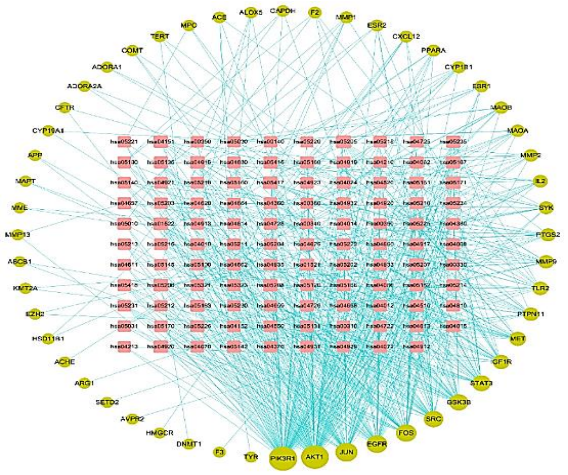

Figure 6: Relationship between KEGG pathway enrichment results and potential GXDS targets. Circular and square nodes correspond to targets and pathways, respectively, and edges indicate the target–pathway interactions. Node size and degree are positively linearly related

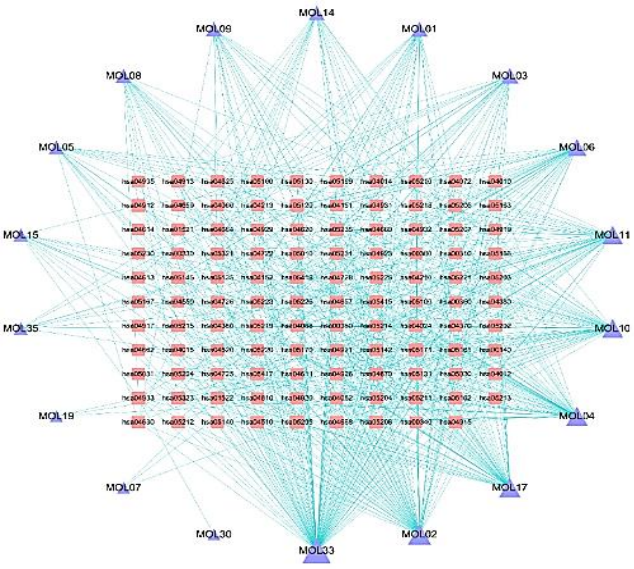

Figure 7: GXDS compound–pathway network. Edges represent the interaction between essential pathways (square red nodes) and candidate compounds (triangular blue nodes). Node size and degree are positively linearly related

DISCUSSION

The correlation between cardiovascular diseases and psychiatric disorders has received increasing scholarly attention. Numerous evidence-based medical studies have confirmed the clinical manifestations and incidence risks of the two diseases to be closely related. Challenging treatment and poor compliance are common among patients with both cardiovascular disease and psychiatric disorders. Depression is a notable CHD risk factor [8]. Moreover, anxiety is an independent CHD risk factor [9].

TCM theory systems involve syndrome differentiation and treatment on the basis of holistic concepts. Notably, TCM pays special attention to the psychosomatic treatment of cardiovascular disease and has made valuable contributions to psycho-cardiological treatment. The mechanisms of action of TCM compounds, which have multiple components, are complex and may act through numerous pathways and targets. Herein, GXDS' mechanisms of action by examining their chemical composition and performing network pharmacology analysis was investigated.

Danshen, an herb used in TCM, can improve microcirculation, relax the coronary arteries, inhibit thrombosis, inhibit the aggregation and adhesion of platelets, and protect against myocardial ischemia. It is employed extensively in patients with CHD or other cardiovascular diseases, whether by itself or alongside other herbs [10]. The therapeutic benefits of danshen are primarily attributable to tanshinone IIA, its lipid-soluble constituent. The antioxidant and anti-inflammatory effects of tanshinone II are mediated mainly through regulation of transcription factors such as β-catenin, Hif-1α, Smad 2/3, STAT 1/3, Nrf2, and NF-κB [11]. Sodium tanshinone IIA sulfonate is a National Medical Products Administration–approved drug for cardiovascular disease treatment (No. H31022558).

In GXDS, danshen is the monarch drug, calming the nerves, promoting blood circulation, and mitigating hemostasis. Sanqi, as a minister drug, promotes blood circulation, mitigates blood stasis, and alleviates pain. Furthermore, sanqi regulates coagulation and lipid metabolism and has anti-inflammatory, antiapoptotic, proangiogenic, and antiatherosclerotic effects. In addition, it prevents myocardial ischemia [12]. Jiangxiang, as an adjuvant drug, mitigates blood stasis, maintains hemostasis, regulates chi, and relieves pain. The Guanxin Danshen decoction has been reported to have preventive and therapeutic benefits on CHD with angina pectoris, asymptomatic myocardial ischemia, congestive heart failure, or other cardiac diseases [6].

Among the 37 chemical components examined herein, several have been noted to exhibit antidepressant and antianxiety effects. Lu et al. suggested that tanshinone IIA, the principal active constituent of danshen, mediated the antidepressant effects of the herb through the ERK–CREB–BDNF pathway [13]. In a murine model of depression induced by mild stress experienced in the long term, Zhang et al. observed that salvianolic acid B had a strong antidepressant-like effect. The mechanism concerned the suppression of apoptosis in cortical and hippocampal microglia [14]. Kwon et al. demonstrated that danshensu exerted antianxiety effects through dopaminergic signaling and neurotransmission [15]. Xia et al. indicated that α-cyperone has the potential to treat depression, which may be related to SIRT3 inhibiting the NLRP3 inflammasome to stimulate neuroplastic enhancement [16].

The present PPI network analysis revealed that AKT1, GAPDH, EGFR, SRC, JUN, PTGS2, MMP9, FOS, ESR1, and STAT3 were the top 10 GXDS targets. These targets may be strongly correlated with psycho-cardiological disease. AKT1, along with AKT2 and AKT3, regulates angiogenesis; cell growth, metabolism, proliferation, and survival; and other processes [17]. As long acknowledged, GAPDH is a crucial enzyme for intracellular energy metabolism and anaerobic glycolysis, which produces ATP and pyruvic acid. Its function and gene expression are related to tumorigenesis as well as to the proliferation of cells. Oxidative stress impairs the catalytic activity of GAPDH, leading to cell senescence and apoptosis [18].

Mechanisms underpinning the therapeutic effects of GXDS on psycho-cardiological disease, as identified through KEGG enrichment analysis, might be mediated by the MAPK, ErbB, Ras, Rap1, VEGF, JAK-STAT, AMPK, PI3K-AKT, phospholipase D, HIP-1, and TNF signaling pathways.

Regarding limitations of the study, limited GXDS components were determined, and only one constituent of Jiangxiang was identified. In the future, we will establish a method for examining the components of Jiangxiang oil. Furthermore, based on our prediction results, we will conduct target verification experiments *in vivo* and *in vitro*. Nevertheless, in current study, we established a theoretical foundation of the

multicomponent, multitarget, and multi-pathway treatment of psycho-cardiological disease by identifying the chemical constituents and potential targets of GXDS. The specific mechanisms warrant further investigation and experimental confirmation.

CONCLUSION

The bioactive components of GXDS as well as the pathways and targets of GXDS molecules in relation to psycho-cardiological disease have been identified in this study. The network pharmacology approach can be applied in laboratory research, and GXDS is a potential candidate for use in the clinical treatment of psycho-cardiological diseases.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jihan Huang and Tao Yang designed and conceived the study. Guanying Zhang and Yeqing Hu conceived the study and drafted the manuscript and contributed equally to this work. Wanqing liu, Zixuan Qian, Yuqing Huang, and Shirong Lin retrieved and analyzed the data. All authors read and approved the final manuscript for publication.

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