Screening and analysis of key active constituents in Guanxin-shutong capsule using mass spectrum and integrative network pharmacology

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[ABSTRACT] Guanxinshutong capsule (GXSTC) is an effective and safe traditional Chinese medicine used in the treatment of cardiovascular diseases (CVDs) for many years. However, the targets of this herbal formula and the underlying molecular mechanisms of action involved in the treatment of CVDs are still unclear. In the present study, we used a systems pharmacology approach to identify the active ingredients of GXSTC and their corresponding targets in the calcium signaling pathway with respect to the treatment of CVDs. This method integrated chromatographic techniques, prediction of absorption, distribution, metabolism, and excretion, analysis using Kyoto Encyclopedia of Genes and Genomes, network construction, and pharmacological experiments. 12 active compounds and 33 targets were found to have a role in the treatment of CVDs, and four main active ingredients, including protocatechuic acid, cryptotanshinone, eugenol, and borneol were selected to verify the effect of (GXSTC) on calcium signaling system in cardiomyocyte injury induced by hypoxia and reoxygenation. The results from the present study revealed the active components and targets of GXSTC in the treatment of CVDs, providing a new perspective to enhance the understanding of the role of the calcium signaling pathway in the therapeutic effect of GXSTC.

[KEY WORDS] Mass spectrum; Systems pharmacology; Guanxinshutong capsule; Cardiovascular diseases; Calcium signaling pathway

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Introduction

Cardiovascular diseases (CVDs) are the largest cause of mortality and morbidity in the world, accounting for about 20 million deaths a year worldwide [1]. Recently, the Guanxinshutong capsule (GXSTC) has attracted public attention because of its effectiveness in the prevention and treatment of CVDs as well as its safety profile [2].

GXSTC is a combination of the traditional herbs and Mongolian medicines, and composed of Choerospondiatis fructus, Salviae miltiorrhizae, Caryophylliflos, Borneolum and Concretio silicea bambusae. Pharmacodynamic studies have explored the mechanism of action for GXSTC in the treatment of CVDs. Liang et al. have investigated the protective effects of GXSTC against myocardial ischemia/reperfusion (MI/R) injury, and examined its role in controlling important factors that are involved in aggravating I/R injury [3, 4]. Previous reports have shown that the calcium signaling pathway plays a crucial role in the induction of cell death during the treatment of CVDs [5-7]. However, the targets and underlying molecular mechanisms of action for GXSTC in the treatment of CVDs are still unclear. It is necessary to carry out a systematic investigation and identify the mechanisms involved in the treatment of CVDs by GXSTC.

A traditional Chinese medicine (TCM) formula usually contains multiple components and has many targets involved...
in various pathways, which makes the process of delineating the molecular mechanism of action of the formula extremely difficult. Wang and coworkers have proposed systems pharmacology as a powerful new tool to overcome these challenges [8]. Systems pharmacology provides a platform for determining the mechanisms of a TCM formula at various levels, from molecular and cellular levels to tissue and organism levels, by integrating pharmacokinetic data with targets, pathways, and network analyses. This method has been successfully developed and applied to identify the rules of drug combinations in TCM, understand the mechanisms of action at molecular/system levels based on the TCM formula, predict potential new drugs and targets, and explore new drug combinations and so on [9-13].

In the present work, a systems pharmacology method was utilized to investigate the active ingredients of GXSTC, their corresponding targets and their roles in modulating the calcium signaling pathway. The method included LC-MS, GC-MS, systems pharmacology and classical pharmacological studies. The workflow is shown in Fig. 1. This work would not only significantly improve our understanding of the active compounds of GXSTC and their corresponding targets, but also reveal the role of the calcium signaling pathway in the treatment of CVDs by GXSTC.

Fig. 1 The workflow of screening and analysis of key active constituents in Guanxinshutong capsule

Materials and Methods

**Chemicals and materials**

GXSTC (batch no. 150816) was provided by Buchang Pharmaceuticals (Xi’an, China). LC-MS-grade acetonitrile was obtained from Merck (Darmstadt, Germany). MS-grade sodium formate and analytical reagent grade ethanol were obtained from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). Protocatechuic acid, cryptotanshinone, borneol, and eugenol were purchased from China Pharmaceutical Biological Products Analysis Institute (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). Collagenase II was obtained from MP Corp (Santa Ana CA, USA), and trypsin was purchased from Amresco (Solon, OH USA). Fluo-3/AM was provided by Biotium (Hayward, CA, USA). Prime Script™ RT reagent Kit and SYBR Premix Ex Taq™ II were purchased from Takara Bio, Inc. (Shiga, Japan). All primers used in the present study were obtained from AuGCT DNA-SYN Biotechnology (Beijing, China). Anti-F2R and NOS3 antibodies produced in rabbit were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Animals**

Neonatal Sprague-Dawley (SD) rats (five-days-old) were obtained from the Animal Centre of Xi’an Jiaotong University School of Medicine (Xi’an, China, Production Certificate No. SYSK (Shan) 2007-003). The rats were housed under controlled temperature and humidity conditions (23 ± 2 °C, 55% humidity) with free access to tap water and standard rat diet. All the animals were handled according to the recommendations and regulations of the experimental animal affairs administration, and the surgical procedures and experimental protocols were approved by the Ethical Committee of Xi’an Jiaotong University. (XJTU-(Shan)-2011-0045)

**UPLC-QTOF-MSE Analysis**

The GXSTC extracts were analyzed on a Waters Xevo G2-XSQ-TOF system coupled with H-Class UPLC system. Separations were accomplished on an ACQUITY UPLC BEH
Caco-2 cells are a popular model for studying epithelial transport [17], we obtained the Caco-2 values to predict the compound absorption and identifying substrates or inhibitors of transporters. With a robust in silico Caco-2 permeability prediction model [17], we obtained the Caco-2 values to predict the compound absorption. The threshold of Caco-2 permeability was set to 0.4. PreBBB model was used to predict whether the biologically active compounds can pass through the blood-brain barrier, and the threshold value was set to –0.3 [18].

Target prediction and analysis

Drug targeting

TCM formulae can effectively prevent complex diseases through the synergistic effects of multiple compounds and targets. To obtain the targets of the active ingredients for the treatment of CVDs, we have introduced the cross-species drug-target (CSDT) model in our previous work [19]. CSDT expands the predicted protein scope to all swiss-prot in UniProt database, including 549, 649 sequences involving 13, 241 species such as eukaryotes, procaryotes, and viruses. The algorithm is based on extraction of conserved patterns from subdivided drug-target interaction vectors. The advantage of this model lies in that it allows us to take proteins of different species into account and thus predict the targets of a broad spectrum of species on a large scale. The CSDT model performance well with sensitivity 77.1%, specificity 77.3%, accuracy 76.81% and AUC 0.86 (area under curve). Then, all obtained targets were mapped to UniProt for normalization with gene name and gene ID. Their corresponding diseases were obtained using Therapeutic Targets Database (TTD), Comparative Toxicogenomics Database (CTD), and Pharmacogenomics Knowledgebase (PharmGKB). Finally, all the data were verified in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) for further studies.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for targets

The KEGG pathway analysis was performed by mapping the targets using the Database for Annotation, Visualization and Integrated Discovery (DAVID) platform. The compounds and their corresponding targets related to the calcium signaling pathway involved in CVDs were used for constructing the network.

Network construction and analysis

The network graph was constructed using Cytoscape version 3.4.0, which is an open source software platform for visualizing complex networks and integrating these with any type of attribute data [20]. The compound-target (C-T) network was established. Then, the quantitative properties “degree” was analyzed by plugin Network Analyzer of Cytoscape to establish the effect of GXSTC on the calcium signaling pathway during the treatment of CVDs.

Experimental validation

Primary cell culture

Primary rat neonatal cardiomyocytes from 5-day-old SD rats were isolated and cultured as described previously [21-22]. Briefly, the mice were disinfected with 75% alcohol, and their hearts were harvested via thoracotomy and transferred to PBS. The atria were cut off, and the isolated ventricles were dissected into small pieces. The minced tissues were then subjected to trypsin (0.125%) and collagenase II (0.008%) digests in a balanced salt solution. The digestion was terminated by addition of serum-containing medium (90% DMEM, 10% fetal bovine serum, and 1% penicillin-streptomycin) and disaggregated cells were collected by centrifugation at 300 g for 10 min. The cells were filtered through a cell strainer (pore size 70 μm) and maintained for 1 h in the medium for sedimentation. Cardiac fibroblasts were enriched in the pellet and cardiomyocytes were present in the supernatant. The suspended cardiomyocytes were collected by centrifugation at 300 × g for 10 min, and the cell sediment was re-suspended in serum-containing medium (80% DMEM, 20% fetal bovine serum) and plated at a density of 1.67 × 10⁶ cells/well onto
In vitro hypoxia/reoxygenation (H/R) model

To establish the H/R model, the medium was changed to serum-free DMEM at 37 °C in a humidified atmosphere of 95% N₂ and 5% CO₂ and the cells were incubated for 20 h followed by reoxygenation for 4 h [23]. Then, the cells were incubated with different concentrations of four main active ingredients (FMAI) in the media for further 12 h.

Total RNA extraction and quantitative real-time PCR

Reverse transcriptase real-time PCR was employed to detect the expression of F2R and NOS3 mRNA in each group. Total RNA from cardiomyocytes was extracted using RNA fast 1000 kit, and 2 μg RNA was reverse transcribed to generate cDNA by Prime Script™ RT reagent kit, according to the manufacturer’s instructions. QPCR was carried out using SYBR Premix Ex Taq™ II. The primer sequences used for qPCR analyses were as follows:

F2R forward: 5-GCTACTACTTCTCCGGCACC-3,
F2R reverse: 5-ATGACACCGAGGCTGAAGGT-3;
NOS3 forward: 5-CCCAGGAGAGATCCACCTCA-3,
NOS3 reverse: 5-CCGGAAGGGTGCAATACCAGT-3;
β-actin forward: 5-ATGGTGAAAGTAGGTGTAACG-3,
β-actin reverse: 5-CGCTCTGGAAGATGGGTGATGG-3. The reaction solution consisted of 2.0 μL of diluted cDNA, 0.4 μmol-L⁻¹ of each paired primer, and 12.5 μL of SYBR Premix Ex Taq II. To determine the relative quantitation of gene expression of target genes, the comparative Ct (threshold cycle) method was used. Forty cycles of PCR amplification were performed for F2R, NOS3 and β-actin (95 °C for 30 s, 95 °C for 5 sec and 60°C for 30 s).

Determination of F2R and NOS3 protein expression

Western blot analyses were performed as described previously [21]. Proteins were extracted from the cells treated with or without FMAI using ice-cold RIPA buffer. Membranes were incubated with primary antibody raised against F2R (1 : 500), NOS3 (1 : 1 000) and internal control housekeeping gene β-actin (1 : 10 000) overnight at 4 °C. Then, the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody (1 : 50 000) for 1 h at room temperature. The protein expression was analyzed using Image-Pro Plus 7.0 analysis software.

Results and Discussion

QTOF-MS² analysis of GXSTC Extracts

For mapping the chemical profiles of the extracts of GXSTC, the data of mass fragmentation coupled with high-resolution spectrometry provided sufficient information. The data obtained from an online UPLC-Q-TOF and MS² satisfied the basic needs for the analysis of complex samples by recording exact mass data for each detectable compound and its structure.

Spectrometry analysis involved a three-step process. The initial step was to obtain the elemental composition using an isotope model with Masslynx, followed by search of online public databases such as the TCM Database@Taiwan, Massbank, and Met-frag for the identification of molecular ions of [M + Na]⁺, [M + H]⁺, or [M – H]⁻ in the MS data by comparing exact molecular weights and molecular formulas, and the last step was to confirm the identified molecules using MS² sub-structure data by Mass Fragment module of Masslynx and reported literature [25-28]. 50 herb components were detected and finally been confirmed by MS² sub-structure data of GXSTC extracts as shown in Fig 2.

The Base Peak Chromatogram (BPC) of GXSTC extracts in negative ion mode is shown in Fig 3A. GXSTC mainly contained phenolic acids, flavonoid glycosides. These compounds showed a better MS response in negative than in positive ion mode because carboxyl groups of the phenolic acid and tannin easily lose electrons in the negative ion mode and would not be capable of capturing a proton in the positive ion mode. More specifically, the identification of phenolic acids was easier owing to their characteristic and significant molecular ions. The dominant fragment ions of phenolic acid components 2, 4, 5, 6, 7, and 8 were produced by the loss of CO₂ from [M – H]⁻ in the negative ion mode, corresponding to the carboxylic group. These compounds were primarily derived from Choeropodiatis fructus.

Seventeen tannin components were identified from Salviae miltiorrhizae. The analysis of MS² data of tannins components was more difficult than that of phenolic acids, since weak molecular ion peaks were obtained with a high percentage of base unit phenolic acid fragments. For example, salvianolic acid B condensation from lithospermic acid and danshensu formed [M – H – 198]⁻ by loss of one molecule of danshensu and gave an m/z 519 [M – H – C₄H₆O₃]⁻ ion in the negative mode. The characteristic fragment ions of rosmarinic acid observed were m/z 197 [M – H – C₆H₄O₃]⁻ by loss of one molecule of caffeic acid and m/z 161 [M – H – C₄H₆O₃]⁻ by loss of one molecule of danshensu. Meanwhile, different MS BPC peaks had the same molecular ion peak, and MS² fragment peaks were of interest in the mass spectrometry data of tannins. Peaks 12 and 13 had the same molecular ion m/z 537.444 6, 537.451 2 [M – H]⁻. And they were obtained via three different fragment ways (fragment ion m/z 493.436 2, 493.437 6 [M – H – CO₂]⁻ by loss of one molecule of CO₂, m/z 295.267 7, 295.265 7 [M – H – CO₂ – C₄H₁₀O₂]⁻, by loss of one molecule of CO₂ and danshensu and m/z 185.154 9, 185.157 1 [M – H – CO₂ – C₆H₄O₂ – C₄H₁₀O₂]⁻ by loss of one molecule of CO₂ and danshensu coupled with catechol). The reason for the above phenomenon might be the presence of several chiral carbons in the tannins, and some of them are diastereoisomers having different retention properties in chromatography.

Among the diterpenoids from Salviae miltiorrhizae of GXSTC, 16 tanshinone compounds could be recognized by their [M + H]⁺ molecular ions, because the carbonyl group of the tanshinones captures electrons in the positive ion mode.
Fig. 2  The structures of the 50 identified components of GXSTC extracts using UPLC-QTOF-MS
Fig. 3 The representative chromatograms of GXSTC extracts in QTOF-MS\(^{−}\) and GC-MS. (A) The base peak chromatogram of GXSTC extracts in QTOF-MS\(^{−}\) negative ion mode; (B) The base peak chromatogram of GXSTC extracts in QTOF-MS\(^{+}\) positive ion mode; and (C) The total ions chromatograph of GXSTC extracts in GC-MS.

The BPC of GXSTC extracts in the positive ion mode is shown in Fig 3B. The MS data of tanshinones were analyzed by comparison with their accurate mass and the corresponding fragment mass ions as well as comparison with chemical markers. For example, tanshinone IIB yielded an \(m/z\) 311.349 7 \([M + H]\)^+\), the MS\(^{+}\) spectra showed base peaks at \(m/z\) 293.377 6 \([M + H – H_2O]\)^+ and \(m/z\) 275.321 9 \([M + H – 2H_2O]\)^+ through successive losses of H\(_2\)O and the fragment ion at \(m/z\) 247.313 2 \([M + H – 2H_2O – CO]\)^+ was observed owing to losses of CO. The results indicated that most peaks of tanshinones had \([M + H – 18]\)^+ and \([M + H – 28]\)^+ fragments corresponding to the loss of H\(_2\)O and CO, respectively. However, the isomers of cryptotanshinone and tanshinone IIA were identified on the basis of their retention behavior and frag-
GC-MS/MS analysis of GXSTC extracts

The total ion chromatography (TIC) of essential oil of GXSTC is shown in Fig. 3C. The large number of peaks in the plot showed that it is a complicated system. The majority of the peaks were separated from the baseline, and others were apparently overlapped. The analysis of GC-MS/MS data was performed using GC-MS solution software packages included with NIST 14 database. Using similarity searches in the NIST 14 mass library, 36 volatile compounds were identified. They were mainly from *Borneolum* and *Caryophylliflos*, and were composed of monocyclic monoterpenoids and bicyclic monoterpenoids, including borneol, eugenol, curcumene, bicyclogermacrene, and muurolene. The results of qualitative analysis are shown in Fig. 4.

**Active ingredients of GXSTC**

In total, 86 compounds were identified in GXSTC by QTOF-MS and GC-MS/MS. Then, the ADME studies, including OB, DL, Caco-2, and BBB, were performed to identify the active ingredients. 14 potential compounds with suitable ADME properties were obtained (Table 1). Among them, protocatechualdehyde and other compounds were selected as the active ingredients for further studies, since they have been reported as the active molecules for the treatment of CVDs, although they have relatively poor pharmacokinetic properties according to ADME prediction. For instance, although borneol and eugenol have poor DL, they are the active ingredients of *Borneolum* and *Caryophylliflos*, respectively, and...
have been reported to have potential therapeutic effects and can promote drug transport through the BBB \cite{29}. Protocatechualdehyde, danshensu, citric acid, and salvianolic acid B have poor ADME properties, but have been confirmed to be effective in the treatment of angina pectoris and myocardial ischemia \cite{30-31}. The compounds, such as 3, 4, 5-trihydroxybenzoic acid, rutin, protocatechuic acid, caffeic acid, and rosemary acid, have been shown to exhibit strong anti-inflammatory and anti-oxidant properties \cite{32-34}, which may play a significant role in the treatment of CVDs.

**KEGG pathway analysis**

The CSDT model predicted 281 targets for the 14 potential active compounds. We further screened the active ingredients and their targets for the treatment of CVDs by searching UniProt, TTD, CTD, and PharmGKB databases. During this process, protocatechualdehyde (MOL001452) and salvianolic acid B (MOL007074) were excluded. As a result, 12 active ingredients and 33 corresponding targets (Table 2) were determined for further KEGG pathway analysis.

Fig. 5 shows the results of the KEGG pathway analysis using the DAVID platform. The predicted targets are associated with 12 pathways, among which the calcium signaling pathway is one of the most important pathway and includes 8 targets EDNRA, ADRB2, ADRB1, NOS3, ADRA1D, F2R, CaM, and CaMKIIδ. Some of these targets have been shown to be associated with the calcium signaling pathway in the treatment of CVDs \cite{5, 7, 35-39}. For instance, a clinical research has indicated that downregulation of EDNRA significantly reduces blood pressure in patients with hypertension \cite{35}. Animal model study has demonstrated that activating F2R enhances the expression of VEGF and promotes angiogenesis, which indicates the significant role of F2R in blood vessel recruitment \cite{36}.

**C-T network construction and analysis**

Fig. 6 shows the network between the active ingredients of GXSTC and their corresponding targets. Remarkably, each target was connected with 3 or more ingredients in the C-T network. For example, ADRB2 was targeted by cryptotanshinone, danshensu, caffeic acid, tanshinone IIA, and eugenol; CaM and CaMKIIδ were targeted by cryptotanshinone, protocatechuic acid, borneol, and eugenol; ADRB1 was targeted by danshensu, caffeic acid, and eugenol; ADRA1D was the target of cryptotanshinone, danshensu, and eugenol; and F2R was targeted by danshensu, rosemary acid, and tanshinone IIA. Cryptotanshinone, danshensu, tanshinone IIA, and protocatechuic acid were from *Salviae miltiorrhizae*; eugenol, borneol and protocatechuic acid were obtained from *Caryophylliflos*, *Borneolum* and *Choerospondiatis fructus*, respectively. All these results indicated that multiple targets were linked with multiple compounds of different herbs, which might exhibit synergistic effects or additive effects of GXSTC in the treatment of CVDs.

The network was comprised of 45 nodes and 80 edges (12 compounds and 33 corresponding targets). The results showed that the average degree number of compounds was 6.67, and the average value was 2.42 per target. There were 5 compounds having degree value higher than the average, including cryptotanshinone (MOL007088, degree = 9), danshensu (MOL007134, degree = 12), rosemary acid (MOL011865, degree = 9), tanshinone IIA (MOL007154, degree = 10), and eugenol (MOL000254, degree = 14). Meanwhile, 11 targets exhibited high degree numbers. They...
were ADRA1D (degree = 3), ADRA2A (degree = 3), ADRB1 (degree = 3), ADRB2 (degree = 5), CaM (degree = 4), CaM-KIIδ (degree = 4), CASP3 (degree = 4), F2R (degree = 3), PTGS1 (degree = 7), PTGS2 (degree = 11), and RELA (degree = 16). Taking into consideration the potential active compounds, their targets, their degree values, and the herbs, four representative compounds (protocatechuic acid, cryptotanshinone, eugenol, and borneol) were selected to confirm their involvement of calcium signaling pathway.

Effects of four main active ingredients (FMAI) on F2R and NOS3 expression

According to the results of KEGG pathway analysis, F2R and NO3 were enriched on the calcium signaling pathway in treatment with CVDs for GXSTC. However, there are very few reports about the targets. To further verification, pharmacological experiments were carried out in the present study. Compared with the vehicle group, the F2R and NOS3 mRNA expression in administered group were declined and increased, respectively (Figs. 7A and 7B). As shown in Fig. 8, Western blotting analysis demonstrated that these compounds decreased the expression of F2R and increased the expression of NOS3. These changes further supported our previously findings that FMAI inhibited calcium overload and was beneficial to the injury cells. All the results indicated there was a good relationship between targets and calcium signaling molecules of F2R and NOS3.

Fig. 6  Compound-Target (C-T) network between the active ingredients of GXSTC and their corresponding targets. The purple triangles represent drug ingredients, the blue rounded rectangles denote the drug targets that are unrelated to the calcium signaling pathway, the red rounded rectangles represent the targets related to the calcium signaling pathway, and each edge is the interaction between the ingredient and corresponding target. The size is proportional to its degree

Fig. 7  Effects of FMAI on the calcium signal pathways of cardiomyocytes subjected to hypoxia and reoxygenation. The cells were incubated under hypoxia conditions for 20 h, followed by reoxygenation for 4 h, and another 12 h with different concentrations of four main active ingredients (FMAI). (A) F2R mRNA expression levels; (B) NOS3 mRNA expression levels. All the values are expressed as means ± standard error of means (SEM). Three independent experiments were conducted for each assay. *P < 0.05, **P < 0.01 vs vehicle group
Conclusions

In the present study, for the first time, we used a systematic approach to investigate the role of the calcium signaling pathway in the treatment of CVDs with GXSTC. First, QTOF-MS© and GC-MS/MS were performed to characterize the 86 ingredients in GXSTC. Then, 14 potential active compounds were selected based on an integrated ADME prediction model, which includes OB, DL, Caco-2, and BBB models. Next, target prediction, KEGG pathway analysis, and network pharmacology analyses were performed to evaluate the active compounds and their corresponding targets related to the calcium signaling pathway. Thus, 12 active ingredients and 33 corresponding targets were determined. Finally, FMAI (protocatechuic acid, cryptotanshinone, eugenol and borneol) and two targets were selected to validate the role of the calcium signaling pathway by using pharmacological experiments. This work would help enhance the understanding of the role of the calcium signaling pathway in the treatment of CVDs with GXSTC.

![Fig. 8 Effects of FMAI on expressions of F2R and NOS3 protein. The cells were incubated under hypoxia conditions for 20 h, followed by reoxygenation for 4 h. Then, the cells were incubated for further 12 h with different concentrations of four main active ingredients (FMAI). All the results were quantified by densitometry analysis of the bands and normalization to β-actin. (A) Western blotting bands of F2R, NOS3 and β-actin; (B) Quantitation data of F2R protein levels; (C) Quantitation data of NOS3 protein levels. All the values are expressed as means ± standard error of means (SEM). Three independent experiments were conducted for each assay. * P < 0.05, ** P < 0.01 vs vehicle group.](image-url)

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