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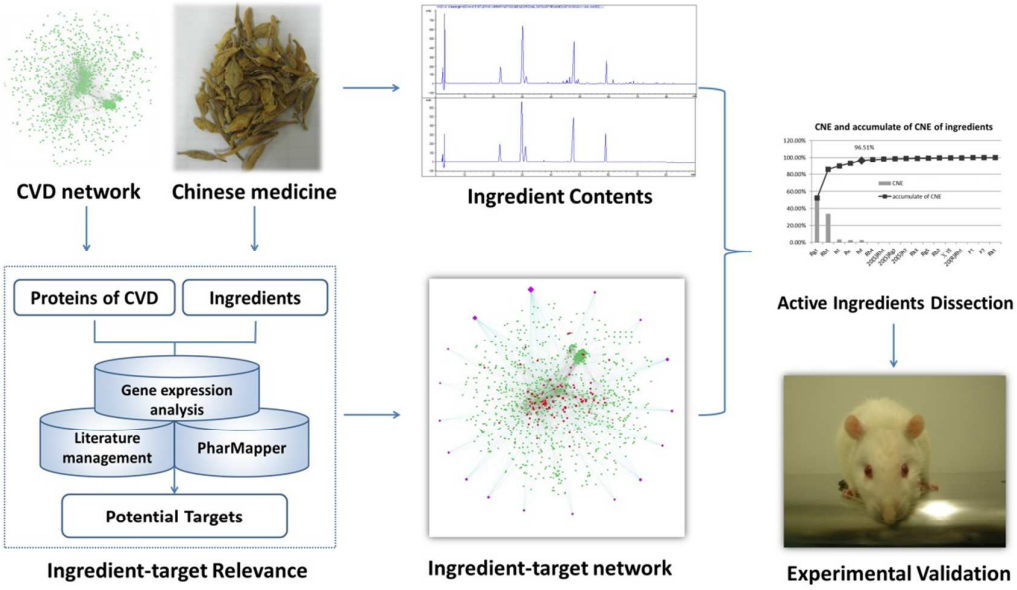

a novel approach integrating network pharmacology analysis with ingredient content and ingredient-target relationships to identify active ingredients of Chinese medicine

Dissecting active ingredients of Chinese medicine by content-weighted ingredient-target network

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ABSTRACT

Chinese medicine has been widely used in clinical practices, but its mode of action often remains obscure. This has seriously hindered further development and better clinical applications of Chinese medicine. Among the most critical questions to be addressed, the identification of active ingredients is an important one awaiting for more research. Existing methods only concerned the potential pharmacological effects of the individual purified chemical ingredients without consideration of the contents of these ingredients, which is critical to the comprehensive effect of Chinese medicine. A novel approach was proposed here to integrate network pharmacology analysis and ingredient content in Chinese medicine to identify active ingredients. The therapeutic action of Xuesaitong (XST) injection on myocardial infarction was analyzed as an example in this study. Firstly, we built a cardiovascular disease (CVD) related PPI network. Secondly, the potential targets of ingredients of XST were identified by integrating microarray data, text mining and pharmacophore model-based predictions. The target-ingredient relationships were then mapped to the network. Topological attributes related to the targets of these ingredients, together with the ingredients composition, were combined to calculate a composition-weighted index for integrative evaluation of ingredient efficacy. Our results indicated that major active ingredients in XST were notoginsenoside R1, ginsenoside Rg1, Rb1, Rd and Re, which was further validated on myocardial infarction rat models. In conclusion, this study presented a novel approach to identify active ingredients of Chinese medicine.

Introduction

Chinese medicine was developed through thousands of years of empirical applications and experience distillation. It has made important and significant contributions to the healthcare system in China.[1, 2] It was estimated that approximately sixty percent of the population in China takes Chinese medicine at least once a year for various health-related problems. Meanwhile its utilization in the western countries has also been steadily increasing.[3] In a word, the efficacy of Chinese medicine in various pathological conditions has been proven worldwidely through long time practices.[4, 5] However, its mechanisms at cellular and molecular levels remained elusive in spite of enormous research efforts from the academic community and pharmaceutical companies.[6, 7] This has become a major obstacle for further development and better clinical applications of Chinese medicine. To modernize Chinese medicine, active ingredients discovery and potential targets identification are two essential but daunting tasks with the following challenges to be addressed.[8, 9] First, Chinese medicines contain many ingredients covering diverse chemical classes. Secondly, there exists interactions between ingredients, which makes it difficult to evaluate the pharmacological effects of individual ones. Thirdly, a Chinese medicine can be formulated by multiple methods to treat several different diseases with varied underlying chemical ingredients due to formulation process.

Active ingredients of Chinese medicine are often thought to be the ones that can be separated with pharmacological activities confirmed *in vitro*.[10, 11] However, individual ingredients with good pharmacological activities *in vitro* may not absolutely extrapolate to the potent efficacies of Chinese medicines *in vivo* since the testing concentrations of *in vitro* experiments were not realistic under *in vivo* conditions due to the low concentration of those ingredients. Furthermore, the ingredient contents could vary widely due to the originating parts of the herbs, growth environment and other factors. Therefore, the information about ingredient contents and their pharmacological protein targets should be integrated to dissect active ingredients that are responsible for *in vivo* activities.

People have become more aware that Chinese medicine often acts through modulating multiple targets and pathways to balance biological systems. Network pharmacology[12-14] was proposed by Andrew L. Hopkins to investigate the complex interaction between drugs and targets, which models the interactions between drugs and the biological system in a view of network and aims to guide drug discovery by improving and restoring the delicate balance of biological networks. Its systems concept coincides with mechanisms of action for Chinese medicine. The development of network pharmacology has provided new perspectives in understanding the complex interactions between Chinese medicine and cellular proteins as well as how their interactions might influence the function and behavior of the system.[11, 15, 16] Most studies of network pharmacology in Chinese medicine focused on the molecular mechanisms, while its application in identifying active ingredients remains limited.

Xuesaitong (XST) injection[17] is a Chinese medicinal preparation consisting of the total saponins from *Panax Notoginseng* (Burk.) F. H. Chen (Sanqi). XST is one of the major varieties of Chinese medicine in the Chinese pharmaceutical market. It has been extensively used in the treatment of cardio-cerebrovascular diseases such as thrombosis, myocardial infarction, cerebral infarction, coronary heart disease in China, and the beneficial effect of XST has been proved through long-period clinical practices.[18-21] The total saponins that contain at least dozens of chemical ingredients are considered as the active constituents of XST. In our previous study, we developed a network-based approach combining differential expressed genes (DEGs) with lines of evidence from the literature mining to investigate the multi-compound, multi-target, and multi-pathway action of XST on anti-myocardial infarction.[15] To gain deeper understanding of molecular mechanisms of XST, the major active ingredients of the total saponins and their potential targets need to be further systematically elucidated.

In this article, we proposed an approach integrating ingredient content and multi-target effect with network pharmacology study to dissecting active ingredients of Chinese medicine. We applied this approach to study the action of XST to cardiovascular disease (CVD) on the basis of its multi-compound, multi-target research. Three important factors were considered by this novel method to determine

the efficacy of a Chinese medicine, including the ingredients' contents, their targets and the interaction between targets. A CVD network was firstly constructed from CVD-related proteins according to the published results and known protein-protein interactions. Analytical quantification of the total saponins was then taken to find the chemical ingredients, followed by the target exploration for these ingredients using gene expression analysis, text mining and computational approaches. Topological attributes related to the targets of these ingredients in the CVD network, together with the contents of ingredients, were combined to calculate a content-weighted index for integrative evaluation of ingredient efficacy. Based upon this analysis, we concluded that notoginsenoside R1, ginsenoside Rg1, Rb1, Rd and Re were main active ingredients of XST against myocardial infarction (MI). Results from *in vivo* validation of MI indicated the reliability of our approach in dissection of active ingredients from Chinese medicine.

Result and discussion

CVD network construction

We collected 1205 genes from rat genome database (RGD) and added another 182 from literature searching. Among them, 1360 genes can be converted into human entrez ID. The CVD network was constructed based upon PPI relationships, which contained 1153 proteins and 9395 interactions. Only the entities in CVD network were used for further study. The constructed CVD network provided a platform to study the correlation between entities associated with CVD.

Determination of the main saponins in the XST

Twelve saponins, namely 20(S) notoginsenoside R2, ginsenoside 20(S)-Rg2, 20(S)-Rh1, 20(R)-Rh1, Rb2, F1, F2, Rk3, Rh4, Rk1, Rg5 and gypenoside XVII were quantitatively determined from ten batches of XST lyophilized powder. The average contents of these saponins along with notoginsenoside R1, ginsenoside Rg1, Rb1, Rd and Re in the XST lyophilized powder were shown in **Table 1**. Ginsenoside Rg_1 was the major ingredient with a content of 33.84%, followed by ginsenoside Rb1

(32.03%), notoginsenoside R1 (9.24%), ginsenoside Rd (7.12%), and ginsenoside Re (4.63%). The average contents of these seventeen saponins were added up to 96.19% of the total ingredients in XST.

Ingredient-target relevence

551 genes in CVD network were differentially expressed after XST treatment on MI in microarray data. After literature mining, we acquired 223 compound-target interactions, shown in **Table 1.** Among the seventeen saponins, ginsenoside Rh4, ginsenoside F1 and gypenoside XVII did not return any target information, ginsenoside Rg1 possessed the most targets, followed by ginsenoside Rb1. To get a more comprehensive source of information, a computational target prediction process was applied for further target prediction to complement literature bias. It should be noted that 20(S)-ginsenoside Rh1 and 20(R)-ginsenoside Rh1 were not explicitly differentiated in literatures, and thus their targets were combined for ginsenoside Rh1. The ingredient-target relationships with no literature supporting evidences were selected for further analysis. 112 compound-target pairs were predicted as a result. Ginsenoside F1 hold fourteen targets and became the best winner in this analysis. After two step explorations, 113 targets and 335 compound-target relationships were established. Ginsenoside Rg1 and ginsenoside Rb1 remain on the top of the table, gypenoside XVII rolled into last place.

Dissect active ingredients by content-weighted ingredient-target network

The interactions between proteins are essential to all the biological processes in an organism. The protein-protein interaction network was one of the most important networks in systems biology research.[22] The degree of a node is one of the most important topology parameters to evaluate its significance in the network.[14, 23] The degrees of nodes in CVD network ranged from one to one hundred and sixty-four, representing the relative importance of these targets in CVD network. Instead of the number of targets interacting with an ingredients of XST, the sum of degrees (Network based Efficacy, NE) for these targets was used to assess the systemic effect on the network regulated by a particular ingredient. The scores of NE for all the ingredients were listed in **Table 1**. The saponins with the highest and lowest NE score

were ginsenoside Rg1 (1453) and gypenoside XVII (160), respectively.

As mentioned earlier, the influence by the contents of ingredients on *in vivo* efficacy couldn't be ignored during the process to identify the active ingredients in Chinese medicine. Based on the content and NE, the Content-weighted Network based Efficacy (CNE) of every ingredient was generated. NE value reflected the influence of the ingredient to the CVD network and could be used to describe the potential pharmacological effect of the ingredient itself. After incorporating information of content, CNE could be a good indicator for the potential effect of the ingredient in a Chinese medicine by addressing the influences by the ingredient content and target-target interactions in a biological system (disease networks) simultaneously. Based on our approach, only ingredients with reasonable level of content and remarkable impacts on the network could be selected as the potential active ingredients. In this work, if the sum of CNEs for the top N ingredients was more than 95%, these N ingredients were considered to be the potential active ingredients. According to our results, five potential active ingredients of XST were identified, which were ginsenoside Rg1, ginsenoside Rb1, notoginsenoside R1, ginsenoside Re and ginsenoside Rd, as the sum of their CNEs was 96.51%. The CNE and cumulative CNE of main sapains were shown in **Fig.2**.

Noteworthy, there were 88 validated potential targets that were annotated CVD relevant and associated with the five potential active ingredients of XST, while only 25 targets were associated with other ingredients. The XST influenced CVD network was shown in **Fig.3**. The topology information for CVD network and XST influenced network were also calculated. In CVD network, the average degree of targets of XST was 22.54. For the eighty-eight targets associated with active ingredients, the average degree was 23.24 while the corresponding value was 16.30 in the whole network.

***in vivo* experimental validation**

Myocardial infarction (MI) is a disease of myocardial necrosis resulting from sustained insufficient blood supply to the heart, which could be caused by coronary atherosclerosis, thrombosis, coronary branch block. MI is a major cause of cardiac death in patients with coronary heart disease.[24] It is also the major clinical indication

for XST.[25, 26] We used myocardial infarction rat models by left coronary artery ligation to compare the pharmacological effect of XST versus combination of five potential active ingredients (shown in **Fig.4**). In the absence of XST, The percentage ratio of infarction of the MI model was 27.1±6.0% of the area at risk (n = 6). With the administration of XST (150 mg kg^{-1}), infarct size was significantly reduced to 17.1±4.8%. The percentage ratio of infarction was 19.1±5.5% when five active ingredients (130 mg kg^{-1}) were given together. There were no significant differences in term of the average infarction ratio between potential active ingredients and XST. We concluded that notoginsenoside R1, ginsenoside Rg1, Rb1, Re and Rd was the active ingredients responsible for the *in vivo* efficacy of XST in reducing the damage of MI. In addition, ginsenoside Rg1[27], Rb1[28], Re[29] and Rd[30] were all reported to attenuate myocardial ischemia (or ischemia/reperfusion) injury in literatures, which gave indirect evidences to our results.

Based on our results, this new method could be a good approach for reliable dissection of active ingredients from Chinese medicine. In particular, the identification of active ingredients and their potential targets provides essential information to explain the complex mechanisms. In addition, the elucidation of active ingredients could also provide guidance for quality control of Chinese medicine. As the future work, we will focus on the further verification of the potential targets for XST and keep improving the accuracy of CNE in evaluating pharmacological effects of ingredients in Chinese medicine. Besides, the strength of ingredient-target interactions and interaction effects between ingredients have not been taken into account, which could be additional factors affecting the efficacy of an ingredient.

Experimental

Material and reagents

Methanol and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Acetic acid glacial (HPLC grade) was obtained from ROE Scientific Inc. (Newark, USA). Chloral hydrate was purchased from Tianjin Kemiou Chemical

Reagent Co. (Tianjin, China). 2, 3, 5-Triphenyltetrazolium chloride (TTC) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). All other reagents were of analytical grade. Reference compounds ginsenoside 20(S)-Rg2, 20(S)- Rh1、20(R)-Rh1, Rb2, F1, F2 and gypenoside XVII were purchased from Ronghe Pharmaceutical Technology Development Co. Ltd. (Shanghai, China). 20(S)-notoginsenoside R2, ginsenoside Rk3, Rh4, Rk1 and Rg5 were isolated in our laboratory. The XST lyophilized powders, one of the major types of XST in clinical practices, were provided by Zhenbaodao pharmaceutical Co. Ltd. (Heilongjiang, China).

CVD network construction

The basic gene list associated with CVD were collected from Rat Genome Database (RGD, http://www.rgd.mcw.edu/), one of the core resources for rat genomics.[31, 32] To supplement the list, we manually collected CVD related protein entities from papers published in the journal "Circulation" from year 2006 to 2011. The merger of the two list comprise CVD related entities. All the entities were converted to human Entrez ID, then protein-protein interaction (PPI) relationships between CVD related entities were collected from an open-access source integrating seven PPI knowledge bases.[33] The CVD network was constructed based on CVD related entities and their PPI relationships. We analyzed the CVD network using Cytoscape[34] to calculate the degree of every node. CVD related entities appeared in the CVD network were used for further study.

Determination of the main saponins in XST

In total, the contents of 17 saponins in XST were studied in this paper. Five of them, notoginsenoside R1, ginsenoside Rg1, Rb1, Rd and Re, were adopted from our previous research. For the rest of 12 saponins, the determination analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, USA) with an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm). The sample injection volume was 20 μL. The mobile phase flow rate was 1 mL min^{-1} and the column temperature was at 28 °C. Detection wavelength was set at 203 nm. Mobile phase A was water-acetic acid (100:0.01, v/v) and B was acetonitrile-acetic acid (100:0.01,

v/v). The solvent gradient adopted was as follows: 19–21% B at 0–30 min, 21–28% B at 30–35 min, 28–32% B at 35–41 min, 32% B at 41–52 min, 32–53% B at 52–70 min, 53–90% B at 70–80 min. Re-equilibrium time was 10 min. The total run time was 90 min.

Mixed standard solutions containing appropriate concentrations of reference ingredients were prepared in 70% aqueous methanol (v/v) to establish the calibration curves. The XST lyophilized powders were prepared by dissolving them in 50% aqueous methanol (v/v) to obtain total saponins at a concentration of about 10 mg mL^{-1}. Prior to analysis, the sample solutions and standard solutions were centrifuged at 10000 rpm for 10 min at room temperature. The supernatant fluid was collected and injected for HPLC analysis. The method has been validated with acceptable linearity, specificity, sensitivity, accuracy and precision and stability over the relevant concentration range.

Ingredient-target relevance

DEGs of XST on MI

Gene expression data of XST was collected from our previous study.[15] Briefly, the experiment was performed in a rat myocardial infarction model with occlusion of the left anterior descending coronary artery. 5% ethanol-saline solutions (v/v) of XST (150 mg kg^{-1} body wt) were given to XST treatment group by intravenous injection once daily and consecutively for 7 days, respectively. 5% ethanol-saline solutions were given to control group and MI group. At the end of experiment, the risk region in rat heart was collected for further microarray experiment. The differentially expressed genes (DEGs) associated with CVD were calculated by the same algorithm[15] that RR > 0.5 and FC >1.1 with the updated CVD gene list. Then the DEGs were hereby validated by literature and target prediction results, and assigned to the ingredients.

Target validation by literature

We manually collected the potential targets of the main saponins in XST from PUBMED (http://www.ncbi.nlm.nih.gov/pubmed). Once a target was explicitly mentioned to be influenced by an ingredient then the ingredient-target relationship

was established. For this study, we were only interested in the targets from DEGs. For the ingredient-target relationships with no literature supporting evidence, we used a computational method to predict the potential interactions.

Target prediction

All the chemical structures of saponins determined in the previous step were downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov/) or TCM@Taiwan35 and their 2-Dimensional (2D) structures were converted into the corresponding 3-Dimensional (3D) structures by CORINA online demo service (http://www.molecular-networks.com/online_demos/corina_demo). They were further optimized by ACD/ChemSketch (Freeware). Then the chemical structures were converted to the proper format for pharmacophore searching by OpenBabel.[36]

Target prediction was performed by PharmMapper Server[37], a web server to identify potential drug targets based on a large pharmacophore database (PharmTargetDB) extracted from Target-Bank, DrugBank, BindingDB and PDTD. PharmMapper Server consisted of 7302 receptor-based pharmacophore models (2241 are "Human protein targets"). When a molecule is submitted, it automatically queries all the models and generates the best mapping poses for this compound. In this work, the target set was only limited to the human targets and other parameters were kept to the default setting. Fit score over 4.00 was selected as the threshold for the potential ligand-protein interactions.

Dissect active ingredients by content-weighted ingredient-target network

DGEs from gene expression data analysis were all converted to human Entrez ID based on their names. The text-mined proteins and predicted targets of ingredients were mapped the the CVD network to analysis their topological attributes to dissect active ingredients. Degree is the number of edges connecting between a node and other nodes in a network.[38] It is an important characteristic parameter for identifying significant network nodes, since highly connected nodes are more likely to be of critical importance in protein-protein interaction networks.[23, 38] We chose degree as the topological attributes to evaluate the ability of an ingredient to regulate the CVD network, namely Network based Efficacy (NE). NE was calculated by summarizing

the degree values for all the targets in CVD network associated with the ingredient by Eq. (1):

$$\mathrm{NE(j)} = \sum_{i=1}^{n} d_i \qquad (1)$$

where n is the number of targets associated with ingredient j, d_i is the degree of target i associated with ingredient j.

We proposed an index based on the content and NE to evaluate the contribution of an ingredient to the overall efficacy, namely Content-weighted Network based Efficacy (CNE) calculated by Eq. (2):

$$\mathrm{CNE(j)} = \frac{c_j * \mathrm{NE(j)}}{\sum_{i=1}^{m} c_i * \mathrm{NE(i)}} \times 100\% \qquad (2)$$

where c_i is the content of ingredient i, m is the number of ingredients.

We assumed that if the sum of the CNE for top N ingredients was more than 95 percent, these relevant N ingredients were considered to be the potential active ingredients.

Based on the targets exploration and active ingredients dissection, the topology of CVD network and targets of XST active ingredients was calculated using Cytoscape.

***In vivo* experimental validation**

Male Sprague-Dawley rats (230-295g) were purchased from Weitong-Lihua Experimental Animal Co. Ltd. (Beijing, China). All experiments were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (ALAAAC)-approved facility. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC, China) and the experimental animal protocols were approved by the Institutional Animal Ethical Committee of Tianjin Centre for Drug Safety Assessment (Tianjin, China). Myocardial infarction was produced by occlusion of the left anterior descending coronary artery. The model construction was assumed to be successful if the ST-segment elevation was more than 0.2 mV after 15 min. Rats were randomly assigned to four groups: control group (the ligation suture was placed in the heart, but without ligation), myocardial infarction group (MI), XST treatment group (MI +XST) and potential active ingredients treatment group (MI + AXST). 5%

ethanol-saline solutions (v/v) of XST (150 mg kg^{-1} body wt) and potential active ingredients (130 mg kg^{-1} body wt, the proportions were based on the content) were given to the XST group and potential active ingredients treatment group by intravenous injection once daily for consecutively seven days. 5% ethanol-saline solutions (v/v) were given to control group and MI group accordingly. The administration procedure for rats in this study is in accordance with clinical use.

At the eighth day, 1 mL of 1% Evans blue was injected into the femoral vein after taking blood sample, followed by the removal of the left ventricle 1 min later. The left ventricles were sliced transversely into 1.5-2.0 mm thick slices. The sliced sections were then counterstained with TTC to delineate infarcted myocardium for 10 min. As the gold standard for assessing ischemic burden,[39] infarct size was measured by MP150 biomedical data acquisition system (Biopac Systems Inc, USA) and calculated as the percentage ratio of infarction to total left ventricular circumference.

Conclusion

Chinese medicine is a very complicated system, and it is difficult to identify active ingredients and study the mechanisms systematically. We proposed a novel approach integrating network pharmacology analysis with analytical ingredient content and ingredient-target relationships to identify active ingredients. A new index CNE was proposed in this work to evaluating the potential integrated pharmacological effect of the ingredient by considering both the ingredient content and the multi-target effect of Chinese medicine at the network level. By this approach, we found notoginsenoside R1, ginsenoside Rg1, Rb1, Re and Rd were the active ingredients of XST, a widely used Chinese medicinal preparation for CVD. In the follow up validation experiments, treatment with a combination of five active ingredients achieved fairly similar performance to reduce the infarction ratio on myocardial infarction rat models as XST.

Conflict of Interests

The authors claim no conflict of interests.

Acknowledgements

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Figure legend

Fig.1. The flowchart illustrates the framework for dissecting active ingredients of Chinese medicine by content-weighted ingredient-target network.

Fig.2. The CNE and cumulative CNE of ingredients. The cumulative CNE of ginsenoside Rg1, ginsenoside Rb1, notoginsenoside R1, ginsenoside Re and Rd was 96.51%, which were concluded to be the active ingredients of XST.

Fig.3. XST influenced CVD network. The diamond nodes represented the ingredients. The round square nodes represented the targets of XST from our prediction, while the round ones were targets associated with CVD from literature mining. The node size was proportional to the number of ingredients that the target was associated with.

Fig.4. The effect of XST and active ingredients on infarct size in myocardial infarction. XST and active ingredients both significantly reduced infarct size in myocardial infarction. But there were no significant differences between active ingredients and XST. Values are means ± SD (n = 6), *P<0.05 versus MI, **P<0.01 versus MI

Figures

Fig.1. The flowchart illustrates the framework for dissecting active ingredients of Chinese medicine by content-weighted ingredient-target network.

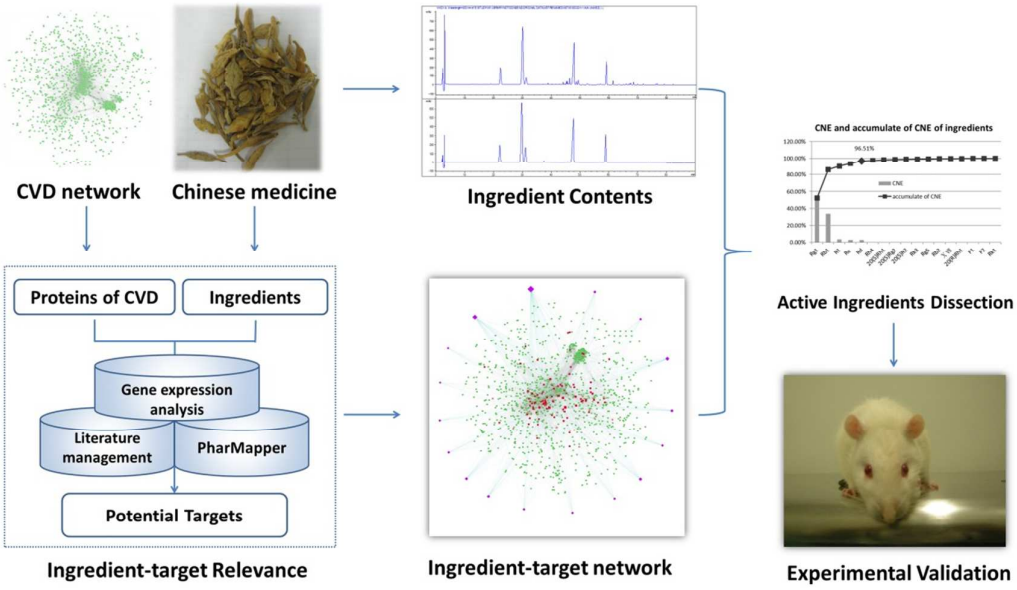


Fig. 2. The CNE and cumulative CNE of ingredients.

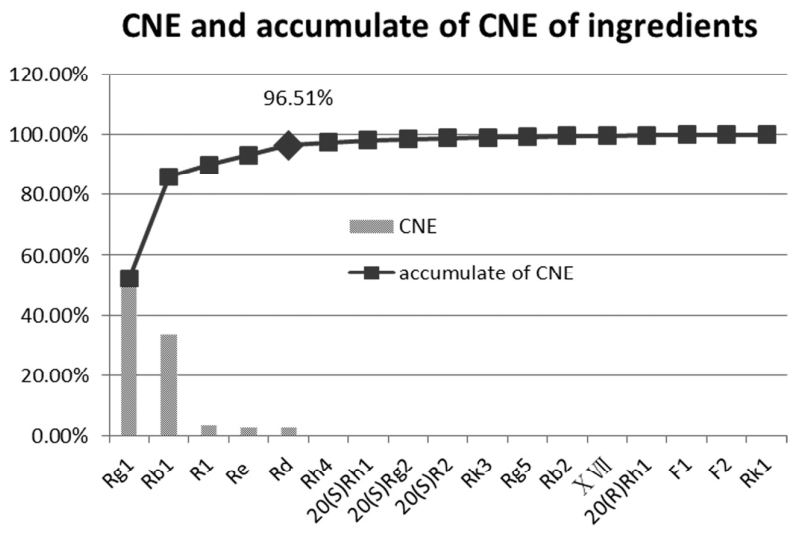


Fig. 3. XST influenced CVD network.

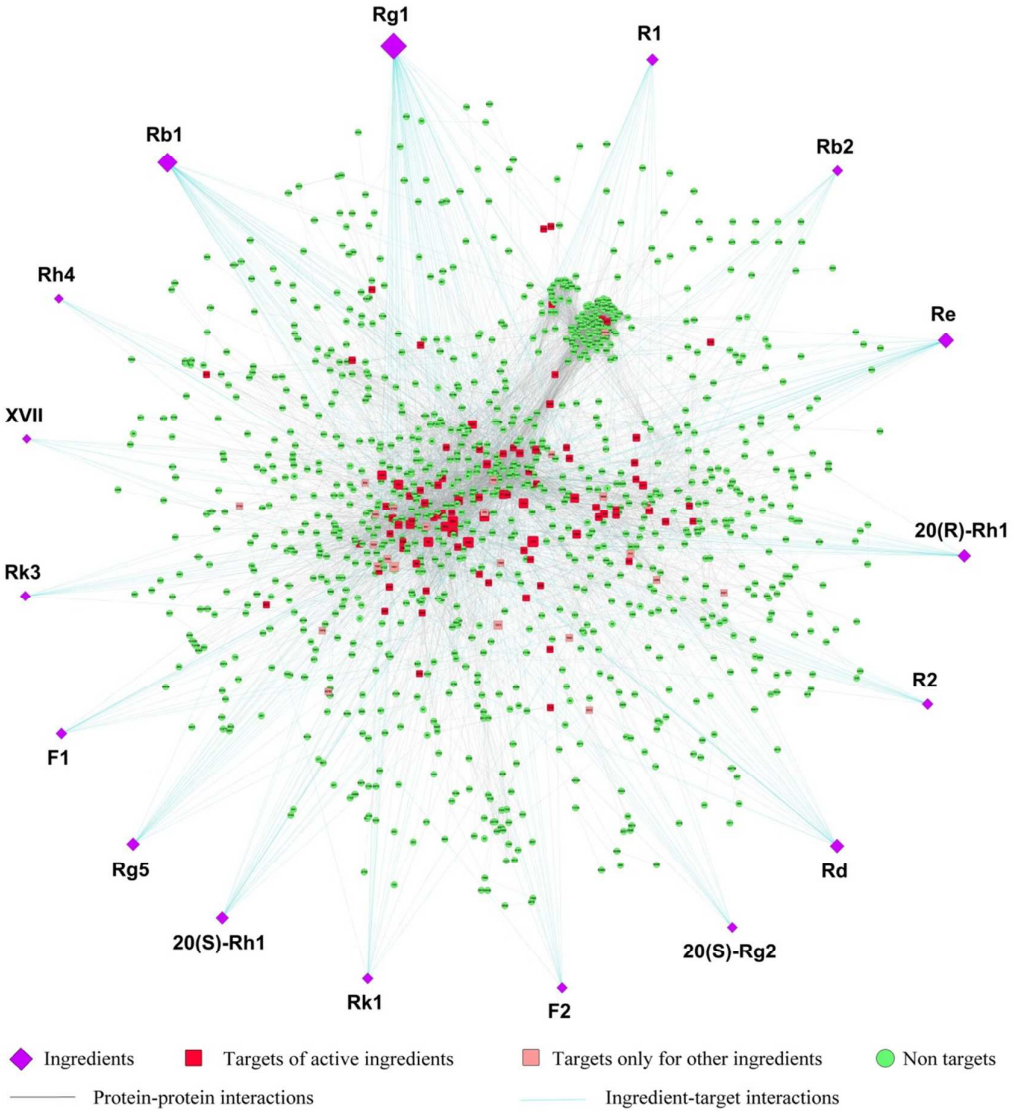
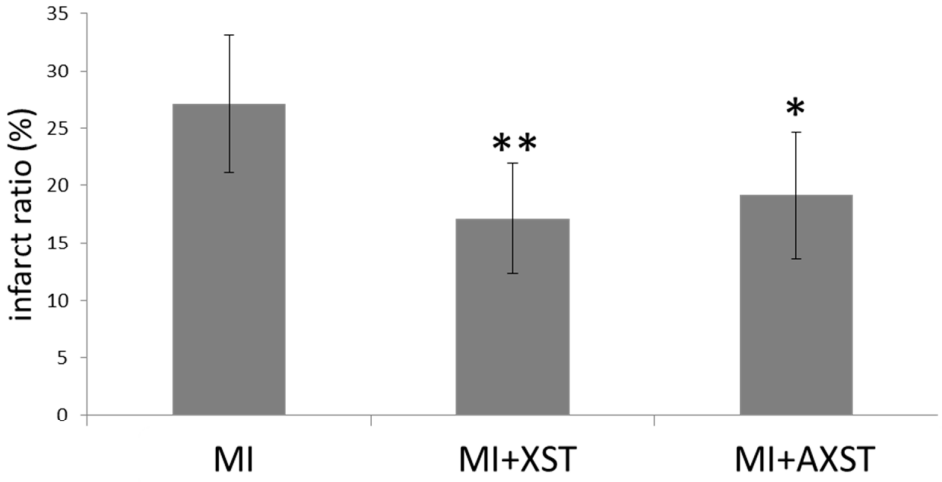


Fig. 4. The effect of XST and active ingredients (AXST) on infarct size in myocardial infarction



Tables

Table 1. The content, number of targets, and NE of seventeen ingredients in XST

Ingredient	content (%)	NO. Targets in literatures	NO. Targets by prediction	NE
Ginsenoside Rg1	33.84±0.70	55	4	1453
Ginsenoside Rb1	32.03±1.36	39	1	998
Notoginsenoside R1	9.24±0.30	11	6	406
Ginsenoside Rd	7.12±0.28	21	2	429
Ginsenoside Re	4.63±0.19	25	3	681
Ginsenoside Rh4	2.45±0.44	0	9	338
20(S)-Ginsenoside Rh1	1.34±0.14	13	6	502
Notoginsenoside R2	1.21±0.09	2	12	279
20(S)-Ginsenoside Rg2	0.88±0.05	9	3	395
Gypenoside XVII	0.88±0.07	0	8	160
Ginsenoside Rk3	0.66±0.11	2	10	366
Ginsenoside Rg5	0.60±0.11	11	9	373
Ginsenoside Rb2	0.54±0.05	12	1	383
20(R)-Ginsenoside Rh1	0.25±0.06	13	7	513
Ginsenoside F1	0.22±0.05	0	14	317
Ginsenoside F2	0.20±0.02	4	9	332
Ginsenoside Rk1	0.10±0.02	6	8	330