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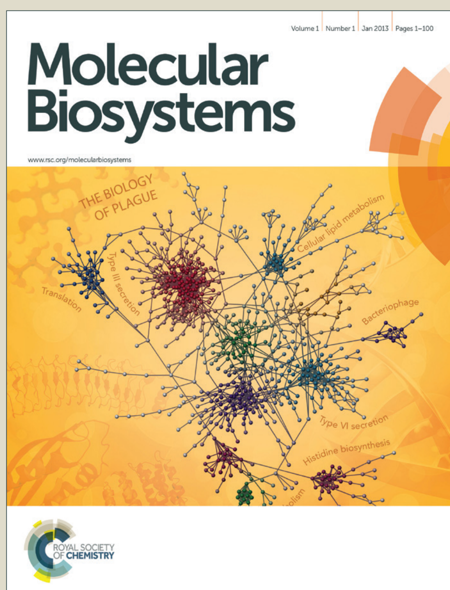

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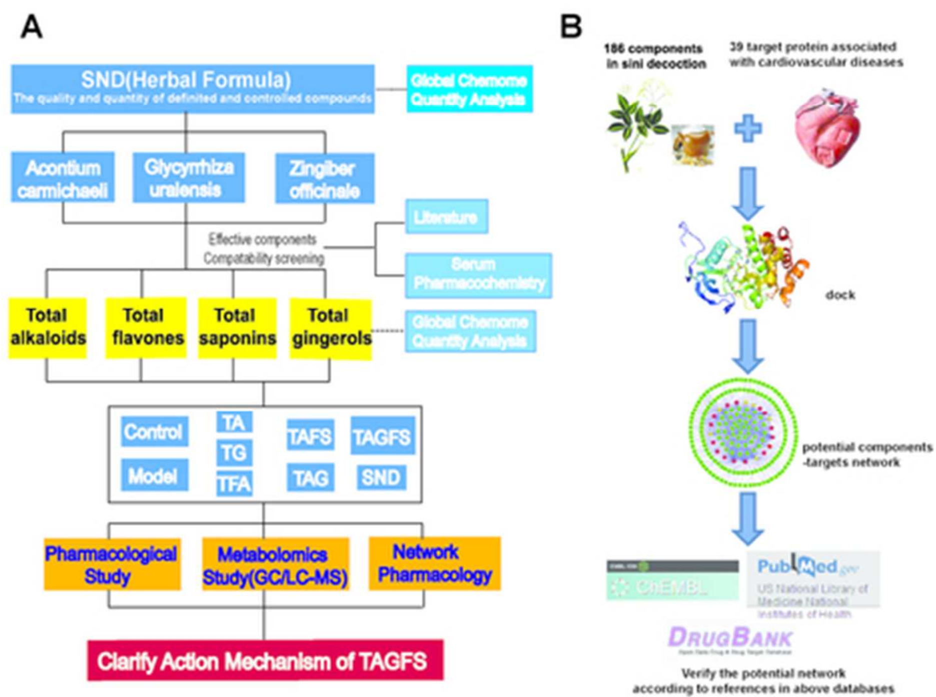

Investigation of therapeutic effectiveness of active components in Sini decoction by a comprehensive GC/LC-MS based metabolomics and network pharmacology approaches
39x30mm (300 x 300 DPI)

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Investigation of therapeutic effectiveness of active components in *Sini* decoction by a comprehensive GC/LC-MS based metabolomics and network pharmacology approaches

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Abstract

As a classical formula, *Sini* decoction (*SND*) has been fully proved to be clinically effective in treating doxorubicin (DOX)-induced cardiomyopathy. Current chemomics and pharmacology proved that the total alkaloids (TA), total gingerols (TG), total flavones and total saponins (TFS) are major active ingredients of *Acontium Carmichaeli, Zingiber Officinale* and *Glycyrrhiza Uralensis* in *SND* respectively. Our animal experiments in this study demonstrated that above active ingredients (TAGFS) were more effective than formulas formed by any one or two of the three individual components and nearly the same as *SND*. However, very little is known about the action mechanisms of TAGFS. Thus, this study aimed to use for the first time the combination of GC/LC-MS based metabolomics and network pharmacology for solving this problem. By metabolomics, it was found that TAGFS worked by regulating six primary pathways. Then, network pharmacology was applied to search specific targets. 17 potential cardiovascular related targets were found through molecular docking and 11 of which were identified by references, which demonstrated the therapeutic effectiveness of TAGFS by network pharmacology. Among these targets, four targets, including phosphoinositide 3-kinase gamma, insulin receptor, ornithine aminotransferase and glucokinase, were involved in the pathways TAGFS regulated. What is more, phosphoinositide 3-kinase gamma, insulin receptor and glucokinase were proved to be targets of active components in *SND*. In addition, our data indicated TA as the principal ingredients in *SND* formula, whereas TG and TFS served as adjuvant ingredients. We therefore suggest that dissecting the mode of action of clinically effective formulae with the combination use of metabolomics and network pharmacology may be a good strategy in exploring action mechanisms of Traditional Chinese Medicine.

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Key words: Traditional Chinese Medicine; *Sini* decoction; active components; metabolomics; network pharmacology; action mechanisms

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1 # 1. Introduction

2 A paradigm shift has occurred in pharmacy by laying the focus on agents that
3 simultaneously modulate multiple targets rather than working at the level of
4 individual protein molecules.[1, 2] Multi-target approaches have recently been employed
5 to design medications that are used to treat chronic and multifactorial diseases.[3]
6 During the past few years, the pharmaceutical industry has seen a shift from the "one
7 disease-one target-one drug" and "one drug fits all" strategies to the pursuit of
8 combination therapies that include more than one active ingredient for multifactorial
9 diseases.[4, 5] Due to the complexity of medicine, treatment protocols need to be
10 carefully designed and prescriptions need to be carefully developed in the successful
11 fight against these diseases.

12 Interestingly, traditional Chinese medicine (*TCM*) as a unique medical system has
13 successfully assisted ancient and contemporary Chinese people in dealing with
14 diseases by using combinatorial therapeutic strategies with traditional prescriptions
15 called formulae for more than 2,500 years.[2] For instance, *Sini* decoction(*SND*),
16 officially recorded in the Chinese Pharmacopoeia 2010 edition, is composed of three
17 medicinal plants: *Acontium carmichaeli, Zingiber officinale* and *Glycyrrhiza uralensis*.
18 Previous studies have confirmed that *SND* could treat DOX-induced
19 cardiomyopathy.[6, 7] And Cardiomyopathy is a group of cardiovascular diseases with
20 multiple risks.[3] Moreover, extensive studies have shown that total alkaloid (TA), total
21 gingerols (TG), total flavones and total saponins (TFS) are the main active
22 components in each single-herb of *SND* responsible for curing cardiovascular
23 diseases.[8-10] Our previous studies about SND also provided evidence from the view of
24 chemome and serum pharmacochemistry.[11-13] We thus wonder whether the
25 combination of above three-herbs active components (TAGFS) has a clinical efficacy
26 on DOX-induced cardiomyopathy. If so, as the complex nature and holistic treatment
27 concept of *SND*, it is meaningful to simplify it with the combinational use of all the
28 active components for more stringent quality control. Apart from the demonstration of
29 clinical efficacy of TAGFS, there is also an urgent need to further investigate its

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1 action mechanisms of it. Although many researches have been done to elucidate the
2 action mechanisms of *SND*,[14-16] the mechanisms have not been comprehensively
3 clarified yet. The mechanisms of TAGFS were not clarified neither.
4 The emergence of metabolomics provides a new strategy to investigate the action
5 mechanisms of TAGFS. Metabolomics employ metabolic profiling methods for the
6 comprehensive analysis of biological fluids and tissues,[17, 18] providing insights into
7 the global metabolic state of entire organisms, which is well coincident with the
8 integrity and systemic feature of *TCM*.[19] Though our previous study had applied
9 GC-MS based metabolomics to find biomarkers related with DOX-induced
10 cardiomyopathy.[6] Some pivotal circulating metabolites, which cannot be derivatized,
11 including unsaturated fatty acids and glycerophospholipids, are still not detected, due
12 to the limitation of GC-MS method in detecting involatile substances. In this study,
13 the combination of GC-MS and LC-MS were used for separation and detection of
14 myocardium metabolites in DOX-induced cardiomyopathy mouse, which provides a
15 more comprehensive view of metabolites to elucidate action mechanisms of TAGFS.
16 With primary regulated pathways of TAGFS found by metabolomics, the
17 appearance of network pharmacology, which is a newly developed strategy firstly
18 mentioned by Andrew L Hopkins[20] and focuses on searching relationship of active
19 ingredients and potential targets,[21] were further applied to find targets in pathways of
20 TAGFS. Studies have been recently reported to apply network pharmacology to study
21 action mechanisms of *TCM*.[21-23] However, the analysis of *TCM* based on network
22 pharmacology concept is still in its infancy stage,[24] and few drug–target interaction
23 network approaches have been specifically explored for *TCM*. Therefore, the aim of
24 network pharmacology in our case is to construct an herb component-target network
25 and find potential targets definitely involved in metabolomics results to clarify the
26 action mechanisms of TAGFS. To data, *in silico* methods developed to address the
27 issues of drug-target interaction prediction can be categorized into ligand-based,[25]
28 receptor-based,[26] chemogenomics-based,[27] biological network-based,[28] drug side
29 effects-based[29] and gene expression profile-based ones.[30] As the aim of our study was
30 to identify potential targets in some cardiovascular related targets, and enough targets

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1 with known three-dimensional structures were provided, receptor-based methods were
2 the most appropriate.
3 In dissection of the action mechanisms of active components in *SND*, here we
4 applied the treatment of DOX-induced cardiomyopathy with TAGFS as a
5 experimental model. The efficacy and mechanisms of TAGFS counteracting
6 DOX-induced cardiomyopathy were tested in mice with pharmacological test and
7 metabolomics. Molecular docking was conducted to find potential targets of TAGFS
8 in network pharmacology. Our purpose was to investigate the action mechanisms of
9 TAGFS with the combination of metabolomics and network pharmacology for the
10 first time. The roadmap of this study is shown in Fig. 1.
11
12 **2. Materials and Methods**
13 **2.1 Ethics statement**. All animal experiments were approved by the Administrative
14 Committee of Experimental Animal Care and Use of Second Military Medical
15 University (SCXK (Hu) 2007-0005), and conformed to the National Institute of
16 Health guidelines on the ethical use of animals.
17
18 **2.2 Materials and reagents.** The assay kits for creatine kinases (CK) and lactate
19 dehydrogenase (LDH) were purchased from Wohong biotechnology co.
20 Methoxylamine hydrochloride,N-methyl-N-(trimethylsilyl)-
21 trifluoracetamide(MSTFA), pyridine, trimethyl-chlorosilane (TMCS), n-heptane,
22 acetone were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol and
23 acetonitrile were chromatography pure (Merk, Germany). The following compounds
24 were obtained from Shanghai Jingchun Reagent Co.: ribitol, lactate, L-alanine,
25 phosphate, glycine, malate, L-proline, L-glutamine, glucose, stearic acid.
26 *Acontium carmichaeli* (collection in Sichuan, China), *Glycyrrhiza uralensis*
27 (collection in Xinjiang, China) and *Zingiber officinale* (collection in Guizhou, China)
28 were purchased from Shanghai Dekang Medicine Corp. (Shanghai, China) and were
29 authenticated by Lianna Sun(Department of Pharmacognosy, School of Pharmacy,

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1 Second Military Medical University, Shanghai, China). Total alkaloids were prepared
2 according to previous study.[31] Total gingerols were provided by Kaiping Healthwise
3 Health Food Co., Ltd. Total flavone and Total saponins were provided by Nanjing
4 Zelang Medical Technology Co., Ltd. Tissue pathological test was conducted in
5 Shanghai shunbai biotechnology Co.
6
7 **2.3 Construction of chemical database of active components in each single-herb**
8 ***in SND***. The database was created using Agilent software
9 'Formula-Database-Generator'. The database contained one table with 11 searchable
10 fields: Structure, Formula, Accurate mass, Name, Chinese name, Original plant,
11 Chemical Abstracts service registry number, UV, Mass spectrum data, References,
12 and Notes. Records of 185 compounds were input into the database according to the
13 phytochemical and pharmacological literature of *SND* and its individual herbs and the
14 Combined Chemical Dictionary. Among the database, we collected 38 alkaloids (S),
15 52 gingerols (J), 55 flavones (H) and 28 saponins (Z). Detailed informations were
16 shown in the Supplementary Information Table S1.
17
18 **2.4 Preparation of *SND* and quality control of active components in each**
19 **single-herb**. Procedures of the preparation of *SND* (1g/ml) were the same as before.[6]
20 HPLC-Q-TOF analysis of *SND*, Total alkaloids, Total gingerols, Total flavones and
21 Total saponins were performed according to previous study.[32] Based on acquired
22 fingerprints, we conducted non-target compounds identification, the formulas were
23 proposed based on the mass spectra and other rules, such as the general rule of the
24 number of nitrogen atoms, the double bond equivalent (DBE) index and 'show
25 isotopic' function. As a result, 32 alkaloids, 52 gingerols, 22 saponins, 51 flavones
26 and 138 components tentatively identified in TA, TG, TS, TF and *SND*, respectively.
27 Then, according to previous study, HPLC/UV analysis was conducted to quantify
28 major active components in *SND* and TAGSF. The detailed descriptions for the
29 studies of global chemome fingerprint of materials above were given in the in the
30 Supplementary Information Fig. S1-S5 and Table S2-S6. Quantity results were shown

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in Supplementary Information Table S7.

2.5 Animal experiments. Our previously described methods were used to copy the mouse model of DOX-induced cardiomyopathy,[6] while there were tiny changes occurring in the following operation. Firstly, the animals were randomly divided into nine groups(n = 10),including: control(n=8), DOX, *SND*, Total alkaloids(TA), Total flavone and Total saponins (TFS), Total gingerols（TG）, Total alkaloids and Total flavones and Total saponins (TAFS), Total alkaloids and Gingerols (TAG), Three active components (TAGFS), as shown in Fig. 1. Secondly, Control group: 200μL of normal saline were injected i.p.，DOX group: animals of the DOX group received a single dose of DOX (15 mg/kg, i.p.). DOX plus medicated group: Apart from receiving a single dose of DOX (15 mg/kg, i.p.), *SND* 10g/kg/BW， TA（10ml/kg/BW），TFS（10ml/kg/BW），TG（10ml/kg/BW）, TAFS（10ml/kg/BW），TAG（10ml/kg/BW），TAGFS（10ml/kg/BW. The TA, TG and TFS were combined at a ratio of which corresponding to the *SND* 3:2:3 according to Chinese Pharmacopoeia (2010 Edition). When compatibility groups contained any one or two of active components, same volume of water replaced the other components. The medicines which were not soluble in water, including total flavones, total saponins and total gingerols, were dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution. Thirdly, blood samples collected were used for biomedical measurement including CK and LDH, hearts were rapidly excised and frozen in liquid nitrogen for metabolomics analysis, and two hearts in each group were quickly excised and fixed in the paraformaldehyde for pathologic analysis.

2.6 Sample preparation, GC/LC-MS analysis and data preprocessing. The obtained myocardial tissues above were divided into two parts: one was used for GC/MS analysis, the other LC/MS analysis. Methods of preparation, analysis of myocardial tissue and data preprocessing in GC-MS were the same as before.[6] Methods of preparation of myocardial tissue for LC/MS were as follows: myocardial tissue (~35 mg) was homogenized in 200 μL saline and then ultrasounded in the iced

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1 bath for 15 min.500 μL ice cold acetonitrile was added into the tube. After vigorous
2 shaking for 5 min and centrifuged at 12,000 g for 15 min, 0.5 mL aliquot of the
3 supernatant was transferred into the tube and evaporated to dryness under N_2 stream at
4 room temperature. 100 μL acetonitrile: H_2O (1:1) was added to redissolve it and the
5 80 μL supernatant was used for LC-MS analysis. Our previously described procedures
6 [32] were used in the LC/MS analysis and data preprocessing.
7
8 **2.7 Validation of GC/LC-MS method**. In order to validate the stability of the
9 GC/LC-MS system, QC samples were prepared from a representative subset of
10 subjects, subaliquoted to minimize freeze-thaw cycle effects and stored frozen until
11 required.[33] The QCs were processed as real samples and then was randomly inserted
12 amongst the real sample queue to be analyzed fourteen times each accordingly, the
13 detailed information are shown in Fig.S6. The system stability was expressed as the
14 relative standard deviation (RSD) of the relative peak areas, i.e., the ratios of peak
15 areas of metabolites to that of the internal standard. Twelve common extracted ion
16 chromatograms (EICs) shared by these injections were selected based on their
17 relatively high abundance levels and wide retention time distribution range in the
18 chromatogram. The positive mode and negative mode in LC/MS and GC/MS result
19 were 2.13% -14.18%, 1.35% -10.89% and 3.45% -17.57% respectively,
20 demonstrating the robustness of the methods. The results meant that differences amid
21 the test samples from different individuals were more likely to reflect varied
22 metabolite profiles rather than analytical variation.
23
24 **2.8 Multivariate statistical analysis.** The data preprocessed was introduced to
25 SIMCA-P V 13.0(demo, Umetrics, Sweden) for partial least squares discriminant
26 analysis (PLS-DA) after mean centering and pareto scaling. The quality of the models
27 were evaluated with the relevant R^2 and Q^2 as discussed elsewhere.[32] Univariate
28 statistical analyses were performed using SAS 9.0(SAS Institute Inc.).Statistically
29 significant differences in mean values were tested by one-way ANOVO and the
30 Tukey post hoc test for comparisons of multiple groups. The difference were

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1 considered significant when $p<0.05$.The significant peak changes between samples
2 were confirmed by manual quantification by calculating the area under the peak from
3 raw chromatograms.
4
5 **2.9 Molecular docking and network analysis.** According to previous study,[34] we
6 collected 39 kinds of therapeutic target proteins related with cardiovascular diseases.
7 The structure of these target proteins were collected from PDB (http://www.rcsb.org).
8 In all, we get 39 protein targets and 186 active components in *SND*. Then, molecular
9 docking was conducted: firstly, the X-ray crystal structures of protein targets were
10 preprocessed. Hydrogen was added to the model, and its orientation was optimized
11 using the CHARMm force field energy minimization while all non-hydrogen atoms
12 were not allowed to move. The active sites of each protein were defined by the
13 residues around the cocrystallized ligands. Secondly, docking protocol was performed
14 to show the interaction of 185 active components in *SND* with protein target using
15 LibDock. The dockscore of the protein with cocrystallized ligands was used as the
16 cutoff value in this protocol.[35] The whole work was conducted using commercial
17 software Discovery Studio 2.5 (http://www.accelrys.com). As Libdock can provide
18 10-100 predicted dockscores from different docking poses for each compound in a
19 binding pocket of a protein, and we only consider the best dockscore. To facilitate
20 scientific interpretation of complex relationships between active components in *SND*
21 and cardiomyopathy related protein target, network analysis was performed by
22 connecting active compounds and their potential targets with higher docking score
23 than cutoff value. The network was generated by Cytoscape
24 (http://www.cytoscape.org/), which is an open source software project for integrating
25 biomolecular interaction networks with high-throughput expression data and other
26 molecular states into a unified conceptual framework.[36]
27
28 **2.10 Validation of potential components-targets interactions.** 186 components in
29 *sini* decoction were input into Drugbank (http://www.drugbank.ca/), ChEMBL
30 (https://www.ebi.ac.uk/chembldb/) and PubMed

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1 (http://www.ncbi.nlm.nih.gov/pubmed) databases, some experimental data for *SND*
2 active components were searched. References which can validate potential
3 components-targets interactions were retained.

4 3. Results and Discussion

5 3.1 Pharmacological Test

6 3.1.1 Serum enzymes measurement

7 In this study, a significant elevation of LDH and CK levels in the DOX group was
8 detected compared with control group, as shown in Fig. S7, Both the levels of LDH
9 and CK could be reversed statistically significantly to control levels in *SND* group and
10 compatibility groups containing total alkaloids, while the level of LDH in TA group
11 was an exception. The results demonstrated that *SND* and compatibility groups
12 containing total alkaloids played a therapeutic role, while TFS and Gin groups had no
13 curative powers.
14

15 3.1.2 Histological assay

16 As shown in Fig. S8, fibrosis and myocytolysis were observed clearly in the DOX
17 group, coupled with obvious edema. Dead myocardium (DOX group) appeared dark
18 as opposed to intact myocardium (control group). The histopathology sections of *SND*
19 treated group and TAGFS treated group were closed to the control
20 group. Inflammation and myocytolysis were apparently reversed to normal tissue
21 (control group). Although myocytolysis could still be observed, they were much less
22 than DOX group. In addition, myocytolysis, fibrotic and inflammation could be
23 clearly observed in the remaining five medicated groups, particularly in the TFS, Gin
24 and TA groups. In TAFS and TAG groups, a proportion of normal myocardium could
25 be observed.

26 It was concluded that the single-herb components showed little therapeutic effects,
27 while *SND* and TAGFS treated groups obtained better results in protecting a
28 cardiomyopathy heart. In addition, medicated groups containing total alkaloids could
29 play a certain pharmacodynamics .It demonstrated that total alkaloids in Acontium

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1 carmichaeli may be the major effective ingredients for curing DOX-induced
2 cardiomyopathy in *SND*, which was in accordance with previous study.[9] At last,
3 TAFS, TAG and TAGFS showed better therapeutic effect than TA, which indicated
4 that total flavones, total saponins and total gingerols had auxiliary fuction for total
5 alkaloids in curing diseases.
6
7 **3.2 Metabolomics study**
8 **3.2.1 Multivariate statistical analysis of GC/LC-MS data**
9 Before multivariate statistical analysis, peaks with a retention time less than 0.5 min
10 (near to the dead time) in LC/MS were excluded due to a high degree of ion
11 suppression.[37] There were 701 variables in positive mode and 1625 variables in
12 negative mode detected in the LC-Q-TOF/MS data, 188 variables detected in the
13 GC/MS data. To determine whether the metabolite fingerprints in myocardium
14 differed between the control and DOX groups in our metabolomics approach, we
15 constructed partial least squares linear discriminant analysis (PLS-DA) models which
16 had been widely used in metabolomics study.[32, 38] As Fig. 2 shows, there is a
17 distinguished classification between the clustering of the control and DOX groups.
18 Commonly, R^2Y provides an estimate of how well the model fits the Y data, whereas
19 Q^2Y is an estimate of how well the model predicts the Y.[32] In order to gain high
20 predictive ability, the values of R^2Y and Q^2Y should be close to 1. The related
21 parameters of three PLS-DA models are given in Fig. 2, which indicate that three
22 models all have good quality and prediction characteristics. To validate the model,
23 permutation tests with 100 iterations were further performed. Permutation tests
24 compared the goodness of fit of the original model with the goodness of fit of
25 randomly permuted models. As shown in Fig. 2, the validation plot indicates that the
26 original model is valid.
27
28 **3.2.2 Identification of biomarkers related to cardiomyopathy and their function**
29 Among the 2326 signals detected in LC/MS and 188 signals in GC/MS, variables that
30 significantly contributed to the clustering and discrimination were identified

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1 according to a threshold of variable importance in the projection (VIP) values
2 (VIP >1), which could be generated after PLS-DA processing these variables.
3 According to the VIP value, the 629 variables and 30 variables were selected as the
4 candidates of potential biomarkers in LC/MS and GC/MS respectively. Next,
5 metabolites that differed significantly between control and DOX groups (false
6 discovery rate $q<0.05$) were identified as candidate biomarkers. Moreover, the criteria
7 were further restricted to features with an average intensity difference of 1.5-fold
8 between control and DOX. Among metabolites acquired above, some did not match
9 the database contents, some were peptide segments, some were exogenous
10 compounds from food, and we also need to merge the variables from identical
11 metabolites in GC/MS. Finally, 20 metabolites (12 in positive mode, 8 in negative
12 mode) in LC-MS were identified by searching MS/MS fragments in Biofluid
13 Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database
14 (http://www.hmdb.ca), and were confirmed by commercial standards as previous
15 study.[32] 18 metabolites in GC/MS were identified with the match of database and
16 confirmed by commercial standards. In addition, 24 unidentified variables in LC/MS
17 which were strongly up/down regulated were also listed in Table S8. In conclusion,
18 Table S8 shows identified 38 biomarkers and 24 unidentified variables of DOX and
19 their metabolism pathways.

20 To analyze related regulated pathways, we only considered 38 identified
21 biomarkers. As shown in Table S8, 10 of the 38 biomarkers identified were
22 up-regulated and 28 of them were depressed in the DOX group. The names of
23 biomarkers were used to search related pathways in KEGG Pathway Database
24 (http://www.genome.jp/kegg/) and HMDB (http://www.hmdb.ca) in pathway column.
25 A network of 32 biomarkers was established after merging the related pathways of
26 biomarkers as Fig. 3 showed. These 32 potential biomarkers related with
27 DOX-induced cardiomyopathy primarily involving glycolysis, α-amino acids
28 metabolism, glycerophospholipid metabolism, fatty acids metabolism, citrate cycle,
29 urea cycle and energy metabolism.

30 Among these metabolites, lactate, D-Glucose and D-glucose-6P are related with

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glycolysis, indicating the modulation of glycolytic pathway in cardiomyopathy. The low level of lactate was observed in DOX-induced group. Recent studies showed that lactate dehydrogenase B (LDHB) was up-regulated in DOX treated cardiomyocytes,[39, 40] which could lead to lactate reduction through the conversion of lactate to pyruvate. In addition, the build-up of D-Glucose and D-glucose-6P could result from decreased glycolysis.[41-43] Reasons can be concluded in the following two parts: firstly, DOX may effects on glucose supply and/or the ability of cells to stimulate it;[42] secondly, impairment of phosphofructokinase (PFK), the rate-limiting enzyme of glycolysis, could happen.[44] As D-glucose-6P is an intermediate of D-Glucose and β-D-Fructose-1,6P2 (Fig. 3), up-regulating of glucose and suppression of PFK definitely could lead to high levels of D-glucose-6P.

It is known that α-amino acids are important energy metabolism precursors. In this study, low levels of L-Alanine, Glycine, L-Aspartic acid and glutamine, high levels of L-proline were observed in DOX group, one possible speculation was that oxidative stress caused by DOX lead to the metabolic remodeling of α-amino acids to meet energy requirement in myocardium.[45] In addition, a large number of studies have shown that DOX reduces ATP concentrations in cultured cardiomyocytes,[44, 46-49] which leads to the accumulation of phosphate. Urea is formed in a cyclic pathway known simply as the urea cycle. In this cycle, amino groups donated by ammonia and L-aspartate are converted to urea. As α-amino acids, including L-aspartate decreased significantly, which result in low levels of urea.

Lysophospholipids (LPLs), including lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and so on, participates in the pathophysiological change of myocardial tissue.[50] LPC and LPE is formed by hydrolysis of phosphatidylcholines (PC) and phosphatidylethanolamine (PE) by the enzyme phospholipase A2. As Table S8 showed, an increase of PC and PE, decrease of LPC and LPE in our study indicated an inhibition of phospholipase A2, which is confirmed by a lot of study.[51, 52] As glycerol is a component of glycerophospholipid metabolism as Fig. 3 shows, we can deduce that the low levels of LPC could lead to decrease of glycerol.

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Fatty acids are important constituents of all cell membranes including endothelial and myocardial cells. In this study, the levels of ten fatty acids, including Palmitic acid, Stearic acid, Hydroxyphenyllactic acid, Hexadecenoic acid, Stearidonic acid, Pinolenic acid, 5, 6-dehydro Arachidonic acid, Tetracosahexaenoic acid, 7-Hexadecenoic acid, Pinolenic acid, EPA and 29:3 were significantly down-regulated .It is known that DOX caused the peroxidation of fatty acids,[53, 54] which should be the main cause of decreased level of fatty acids. The abnormal oxidation status contributed to excessive oxidation damage on myocardial mitochondrial. Moreover, several studies have reported that the level of malondialdehyde (MDA), the end product of fatty acids peroxidation, was increased in DOX-treated mice, which also indirectly confirmed the supposition.[55, 56] At last, fatty acids could also be released from phospholipids by phospholipase A2, inhibition of phospholipase A2 could also explain the decrease of fatty acids.

In addition, as creatinine is a breakdown product of creatine phosphate in muscles, low levels of creatine phosphate might result in the down-regulating of creatinine.[57] Moreover, malate in citrate cycle was significantly down-regulated .It has been reported that DOX-induced cardiomyopathy is related with a decreased utilization of substrates, fatty acids and glucose,[41] which could, at least in part, lead to the reduction of malate synthesis.

Finally, the level of palmitic amide、N-Lauroylglycine、3-ketosphingosine、1-methoxy-1,3-propanediol、purine、1-Monopalmitin and 1-Monostearin was also changed in myocardial tissue, which was perplexing due to lack of detailed information about their biology pathways awaiting for further interpretation.

3.2.3 Metabolomics study of *SND* and TAGFS

A PLS-DA model including control, DOX, TAGFS and *SND* have been established in order to evaluate the therapeutic effectiveness of TAGFS and *SND*. As shown in Fig. 4A and 4B, *SND* and TAGFS groups were both away from the DOX group, which means that *SND* and TAGFS have positive therapeutic effectiveness to DOX-induced cardiomyopathy. Furthermore, the *SND* and TAGFS group were nearly

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overlapping, suggesting that the effect of TAGFS treatment was nearly the same as *SND* treatment on DOX mice.

Mean levels of the 38 identified biomarkers were also used to evaluate therapeutic effects of *SND* and TAGFS on DOX. As shown in Table S9, 29 biomarkers could be reversed by *SND*. After mapping these biomarkers into related pathway, we find that main action mechanism of *SND* is bound up with metabolic remodeling of α-amino acids, glycolysis, urea cycle, energy metabolism, fatty acids metabolism and glycerophospholipid metabolism.

TAGFS could also reverse 29 biomarkers (Table S9). Among these biomarkers, 23 biomarkers also could be reversed by *SND*, involving metabolic remodeling of α-amino acids, glycolysis and glycerophospholipid metabolism, which indicate that TAGFS has the effect of inhibiting metabolic remodeling of α-amino acids, promoting glycolysis and glycerophospholipid metabolism, thus providing a positive therapeutic effect on DOX-induced cardiomyopathy. Additionally, there were 3 biomarkers including glutamine, hydroxyphenyllactic acid, N-glycine which couldn't be reversed by TAGFS but could by *SND*. As these biomarkers were mainly related with amino acids metabolism and fatty acids metabolism, it was demonstrated that the reversed effects on amino acids metabolism and fatty acids metabolism in the *SND* treated group were superior to the TAGFS treated group. Meanwhile 1-methoxy-1, 3-propanediol, L-proline and creatinine could significantly be reversed toward the control level by TAGFS but not by *SND*. Though the corresponding biological meanings in cardiomyopathy pathology of 1-methoxy-1, 3-propanediol and creatinine not known, they are still important biomarkers for the discrimination of the *SND* and TAGFS treated groups.

3.2.4 Metabolomics study of TAG, TAFS, TA, TG and TFS

A PLS-DA model including control, DOX, TAG, TAFS and TA have been established in order to evaluate the therapeutic effectiveness of three medicated groups. As shown in Fig. 4C, the TAG, TAFS and TA groups were away from the DOX group, meanwhile TAG and TAFS groups were nearer to the control group than

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1 TA group, which means that TAG, TAFS and TA groups have positive therapeutic
2 effectiveness to DOX-induced cardiomyopathy and double-herbs components were
3 more effective than single-herb components. In order to study the holistic therapeutic
4 effectiveness of single-herb components, another PLS-DA analysis was performed
5 including control, DOX, TA, TG and TFS groups. As shown in Fig. 4D, TG and TFS
6 were not clearly separated from the DOX group, which indicated that both of them
7 have little therapeutic effectiveness to DOX-induced cardiomyopathy. As TA group
8 was closer to the control group than TFS and TG groups, the importance of TA was
9 better than TFS and TG in *SND*, which was in accordance with previous compatibility
10 rule of "junchenzuoshi".

11 Mean levels of the 38 identified biomarkers and 24 unidentified variables were
12 also used to evaluate therapeutic effects of five medicated groups on DOX. As shown
13 in Table S9, 27, 22, 19, 14 and 17 biomarkers could be reversed toward the control
14 level by TAG, TAFS, TA, TG and TFS respectively. Among these biomarkers, 5, 3, 1,
15 1 and 2 biomarkers could be reversed toward the control level significantly by TAG,
16 TAFS, TA, TG and TFS meanwhile, which showed that regulation effect of
17 double-herbs active components medicine was superior to single active components
18 and the importance of TA was better than TFS and TG in *SND*. According to
19 pathways biomarkers involved in, TAG, TAFS and TA mainly regulates the disorder
20 of glycolysis, amino acid metabolism and glycerophospholipid metabolism to cure
21 DOX-induced cardiomyopathy. Biomarkers, which are mainly involved in glycolysis
22 and glycerophospholipid metabolism, could be reversed by TG and TFS.

23 Moreover, palmitic acid, stearic acid, 7-Hexadecenoic acid, palmitic amide,
24 pinolenic acid, 5, 6-dehydro arachidonic acid, PC (18:1/0:0), 29:3, 3-ketosphingosine,
25 which were mainly fatty acids, can be significantly reversed by TAGFS but not by
26 any of the single active components, which indicated that there may exist some
27 compatibility principle in TAGFS. Multi-herbs components groups (*SND* and TAGFS)
28 showed better therapeutic effectiveness than mono-herb and double-herbs active
29 components groups. The reason may be that *TCM* was famous for preventing diseases
30 in an integrative and holistic way and the pathogenesis of DOX-induced

1 cardiomyopathy involves multi-factor risks, single active components in Acontium
2 carmichaeli, Glycyrrhiza uralensis and Zingiber officinale broke its synergism and
3 could hardly fight the disease. For the treatment of complex diseases and simplifying
4 the components in *TCM*, the combination of active components in *TCM* such as
5 TAGFS should be needed.
6

7 **3.3 Network pharmacology**

8 Fig. 5 illustrates interaction between the active components in *SND* and potential
9 target protein in cardiovascular disease. In total, this network consists of 197 nodes
10 (176 active compounds and 21 potential drug targets) and 764 edges. The compounds
11 (green circle) in the outer circle show much less interactions with potential
12 biomarkers than those in the inner circles. This indicates many compounds can hit
13 multiple potential targets, while some can only hit less potential biomarkers.
14 Compounds which can hit multiple potential targets are thought to be major active
15 compounds in *SND*. These compounds include hypaconine, glycyrrhizic acid etc,
16 some of which have been proved to have cardiovascular activity.[58, 59] Detailed
17 compounds and targets information have been concluded in the Supplementary
18 Information Table S1 and S10. Such large numbers of compounds, which are related
19 with cardiovascular target protein, demonstrates that *SND* is a reasonable compound
20 prescription and the combination of its three single-herb active components (TAGFS)
21 is on the assurance to cure cardiovascular disease. As same proteins have different
22 PDB ID, there are 17 potential targets (red and yellow hexagon), which means that
23 TAGFS may treat cardiovascular diseases through regulating these 17 targets.
24 According to experimental data from CHEMBL, Drugbank and PubMed database,
25 some experimental data for *SND* active components and their potential targets were
26 found as Table 1 showed. This table demonstrated that 11 out of 17 potential targets
27 were identified to be exact targets of active components in *sini* decoction, which
28 proved the reliability of molecular docking.
29

30 **3.4 Combination of metabolomics and network pharmacology**

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1 Among 17 targets (Fig. 5), 4 of which (in yellow) found by network pharmacology
2 are in consistence with former metabolomics results (Fig. 3). Specifically, 2a4z,
3 showing dockscores > 103.44 (cutoff value of all protein can be seen in Table S11)
4 with active components in TAGFS (Fig. 6A), refered to phosphoinositide 3-kinase
5 gamma (a type of class Ⅰ phosphoinositide 3-kinase). Our metabolomics study
6 showed that TAGFS can significantly reverse DOX-induced glycerophospholipid
7 metabolic disturbance to cure cardiomyopathy. As class Ⅰ PI3K (phosphoinositide
8 3-kinase) has an important role in glycerophospholipid metabolism (Fig. 3), thus
9 action on PI3K can indirectly influence glycerophospholipid metabolism. As previous
10 studies had demonstrated that class Ⅰ PI3K played important roles in cardiovascular
11 function and disease,[60, 61] a deduction could be made that class Ⅰ PI3K is a potential
12 target of active components in *SND*. Interestingly, previous study had verified our
13 deduction that active components in *SND* can suppress DOX-triggered oxidative
14 stress and apoptosis in cardiomyocytes via upregulation of PI3K/Akt pathway.[8] Thus,
15 we can conclude that TAGFS played its pharmacodynamics through activating
16 phosphoinositide 3-kinase gamma. Insulin receptor, i.e. 1ir3 (Fig. 6B), can activate
17 phosphatidylinositol 3-kinase (PI3K) either directly by binding to the p85 regulatory
18 subunit, or indirectly via IRS1. As the importance of PI3K in DOX-induced
19 cardiomyopathy,[8] we deduce that insulin receptor is a potential target of TAGFS,
20 which is verified by experimental data in CHEMBL database as Table 1 shows.

21 Glucokinase (1v4s) can intervene in the regulation of glycolysis. As Fig. 3 shows,
22 glucokinase can transform D-Glucose to D-Glucose-6p. Our metabolomics results
23 showed that TAGFS can reverse the disorder of D-Glucose and D-Glucose-6p. As
24 glucokinase has dockscores>118.948 with active components (Fig. 6C), it is also
25 deduced to be a potential target. Experimental data from CHEMBL database (Table 1)
26 verified that glucokinase was definitely a target of TAGFS.

27 1oat, 2can and 2oat are the same kind of enzyme associated with amino-acid
28 biosynthesis, i.e. ornithine aminotransferase, which has higher dockscores than cutoff
29 value with active components in TAGFS (Fig. 6D). And our metabolomics study (Fig.
30 3) showed that TAGFS could significantly adjust amino-acid biosynthesis to cure

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1 DOX-induced cardiomyopathy. Thus, we deduce that ornithine aminotransferase is a
2 potential target of TAGFS.
3 To sum up, phosphoinositide 3-kinase gamma, insulin receptor and glucokinase
4 found by metabolomics and network pharmacology are targets of TAGFS. Ornithine
5 aminotransferase are the potential targets of TAGFS. It demonstrated the viability of
6 combination of metabolomics and network pharmacology. In this study, small sizes of
7 cardiovascular related targets were used to demonstrate the viability of combination of
8 metabolomics and network pharmacology. It's our future plan to conduct a more
9 comprehensive research which will include much more cardiovascular related targets.
10

11 4. Conclusion

12 In this study, pharmacological test, GC/LC-MS based metabolomics and network
13 pharmacology have been carried out to investigate the therapeutic effectiveness and
14 action mechanisms of active components in *SND*. The results demonstrated that
15 multi-therapy groups (*SND* and TAGFS groups) provide better therapeutic
16 effectiveness than double-herbs components and mono-herb components groups.
17 Among the new formula TAGFS, TA was the principal active ingredients, whereas
18 TG and TFS exerted as adjuvant active ingredients. By metabolomics, 38 metabolites
19 had been identified as potential biomarkers in the myocardium of DOX mice. Six
20 pathways including glycolysis, α-amino acids metabolism, fatty acids metabolism,
21 glycerophospholipid metabolism, urea cycle and energy metabolism, contributed to
22 the dysfunction of DOX-induced cardiomyopathy. TAGFS can cure DOX-induced
23 cardiomyopathy through reversing above pathways. 17 protein targets of active
24 components in *SND* were found by network pharmacology and 11 of which were
25 verified by previous references. Among these targets, four targets, including
26 phosphoinositide 3-kinase gamma, insulin receptor, ornithine aminotransferase and
27 glucokinase, were involved in the pathways TAGFS regulated. Phosphoinositide
28 3-kinase gamma, insulin receptor and glucokinase were proved to be targets of active
29 components in *SND* according to previous study. Taken together, the combination of

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1 metabolomics and network pharmacology in our study, as a promising approach,
2 demonstrates the action mechanisms of active components in *SND*, which realizes
3 both of quality control and effectiveness of *SND* formula, providing a novel clue for
4 combination therapy of multi-risk diseases.
5
6

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5

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1 **Table 1.** Component-target interactions verified by references.

Target	PDB ID	Components in *sini* decoction	Components ID[1]	Reference	Database
Insulin receptor	1ir3	Aconitine	S25	CHEMBL1909046	CHEMBL
Angiotensin-converting enzyme	1o86 1uze	Aconitine	S25	CHEMBL1909046	CHEMBL
Mitogen-activated protein kinase p38 alpha	1wbv	Aconitine	S25	CHEMBL1909046	CHEMBL
Glucokinase	1v4s	Quercetin	H11	CHEMBL12018	CHEMBL
Phosphoinositide 3-kinase gamma	2a4z 1e8z	Quercetin	H11	8	drugbank
Mitogen-activated protein kinase p38 alpha	1wbv	Quercetin	H11	[62]	CHEMBL
Thrombin	1awh	Quercetin	H11	[63] CHEMBL2424526	CHEMBL
c-Jun N-terminal kinase 1	2no3	Quercetin	H11	[62, 64]	CHEMBL
Mitogen-activated protein kinase p38 gamma	1cm8	Quercetin	H11	[62]	CHEMBL
Neprilysin	1r1i	Quercetin	H11	[65]	CHEMBL
Angiotensin-converting enzyme	1o86 1uze	Isoquercetin	H12	[66]	CHEMBL
Basic fibroblast growth factor receptor 1	1agw	Isoliquiritigenin	H29	[67]	CHEMBL
Mast/stem cell growth factor receptor	1t46	Isoliquiritigenin	H29	[67]	CHEMBL
Insulin receptor	1ir3	Glycyrrhizic acid	Z1	CHEMBL1909046	CHEMBL
Mitogen-activated protein kinase p38 alpha	1wbv	Glycyrrhizic acid	Z1	CHEMBL1909046	CHEMBL

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Angiotensin-converting enzyme	1o86 1uze	Glycyrrhizic acid	Z1	CHEMBL1909046	CHEMBL

1 [1] Components ID corresponds to NO. in Table. S1.

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1 ## Conflict of interest

2 The authors declare that no conflict of interests exists.

3 ## Abbreviations

4 TAGFS, total alkaloids, total gingerols, total flavones and total saponins

5

6

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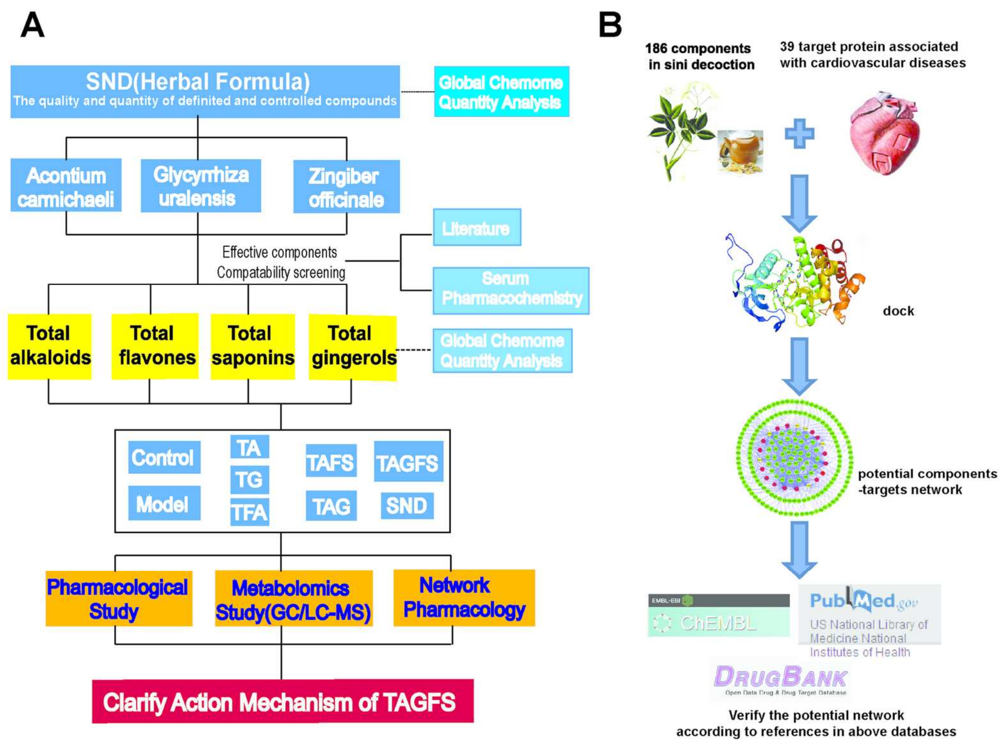

Fig. 1 A: Flow chart of the whole study; B: Process in network pharmacology.
160x121mm (300 x 300 DPI)

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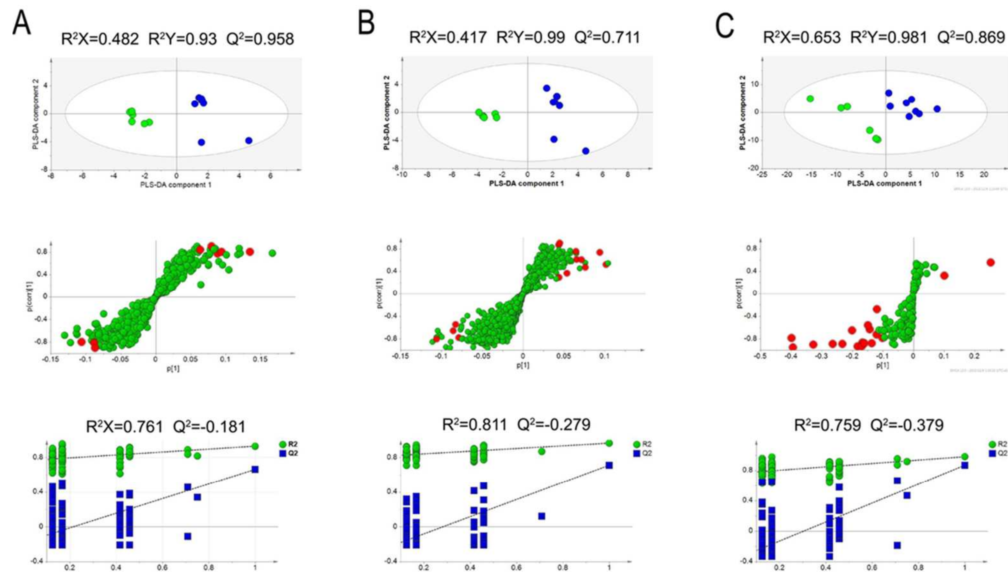

Fig. 2 PLS-DA scores plot (top panel), S-plot (middle panel) and 100-permutation test (bottom panel) of LC/GC-MS spectral from control group (blue) and cardiomyopathy group (green). (A:ESI (+); B:ESI (-); C:GC/MS).
96x55mm (300 x 300 DPI)

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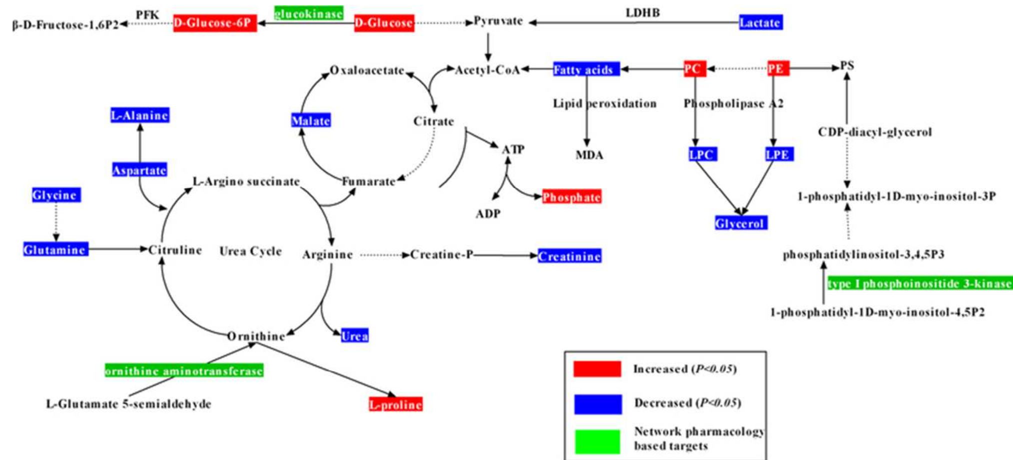

Fig. 3 The integrative plot of the metabolites and the relevant pathways changing for DOX-induced cardiomyopathy in circulation system. Metabolites with red dashed area represent significant increase in DOX group compared to control group. Metabolites with blue dashed area represent significant decrease in DOX group compared to control group. Proteins with green dashed area represent potential targets found by network pharmacology.
70x32mm (300 x 300 DPI)

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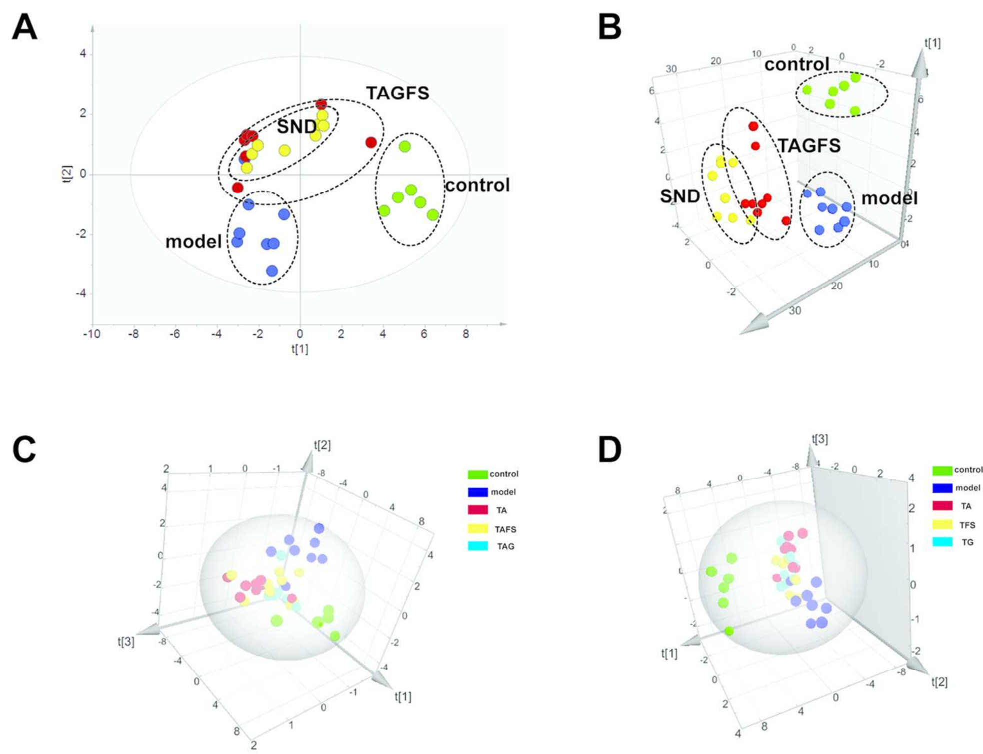

Fig. 4 PLS-DA score plots of the control, model and medicated groups. A 2D score plot, B 3D score plot, Q2Y=0.45, R2X=0.801, R2Y=0.485, C 3D score plot, Q2Y=0.361, R2X=0.812, R2Y=0.441, D 3D score plot, Q2Y=0.339, R2X=0.883, R2Y=0.484.
130x99mm (300 x 300 DPI)

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Fig. 5 Network of 176 compounds predicted to have 20 potential protein targets. The green circles represent the compounds, while red hexagons delineate the proteins and yellow hexagons stand for protein targets involved in pathway of DOX-induced cardiomyopathy. Detailed informations of these compounds and targets were concluded in the Supplementary Information Table S1 and S10.
63x56mm (300 x 300 DPI)

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Fig. 6 Network of 4 potential targets (red hexagon) connected to respective compounds (green circles) in TAGFS.
121x87mm (300 x 300 DPI)

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