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Deciphering the combination principles of Traditional Chinese Medicine from a systems pharmacology perspective based on Ma-huang Decoction

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ABSTRACT

Ethnopharmacology relevance: The main therapeutic concept in Traditional Chinese Medicine (TCM) is herb formula, which treats various diseases via potential herb interactions to maximize the efficacy and minimize the adverse effects. However, the combination principle of herb formula still remains a mystery due to the lack of appropriate methods.
Methods: A systems pharmacology method integrating the pharmacokinetic analysis, drug targeting, and drug–target–disease network is developed to dissect this rule embedded in the herbal formula. All these are exemplified by a representative TCM formula, Ma-huang decoction, made up of four botanic herbs.
Results: Based on the deep investigation of the function and compatibility of each herb, in a molecular/systems level, we demonstrate the different pharmacological roles that each herb might play in the prescription. By the way of enhancing the bioavailability and/or making the pharmacological synergy among different herbs, the four herbs effectively combine together to be suitable for treating diseases.
Conclusions: The present work lays foundations for a more comprehensive understanding of the combination rule of TCM, which might also be beneficial to drug development and applications.

1. Introduction

Traditional Chinese Medicines (TCMs) have been used to treat diseases for over 4000 years whose applications have been raised dramatically around the world in recent decades due to their moderate treatment effects and lower side effects (Tang et al., 2009). In China, a flood of herbs have been employed in the therapeutic treatment of common cold (X. Zhang et al., 2007), cancer (Liu et al., 2011), cardiovascular disease (Mashour et al., 1998), etc. Actually, TCMs are identified as a treasure of natural herbal products and used to restore overall healthful balance and normal body function in a holistic way.

In TCM, Herb Formula ("Fu-Fang" in Chinese, also termed as "Fang Ji") is the main therapeutic concept, which customarily purports a multiple components combination of herbs (Li, 2009). It is not just a simply pile of medicinal herbs, but is organized by the TCM theory and follows the rule of drug compatibility and combination to treat a specific disease (Jia et al., 2004). The combination principle of TCM is based on the rule of "*Jun–Chen–Zuo–Shi*", known as the Four Responsible Roles, which reveals the fact that each herbal ingredient performs its specific function organized and arranged integrally (Lin and Li, 2009). In the TCM theory, the "*Jun* (emperor)" herb is the principal active herb in the formula, treating the main disease or principal syndrome. The "*Chen* (minister)" herb assists the "*Jun*" drug to promote the curative effect. The "*Zuo* (adjuvant)" herb modulates the effects of *Jun* and *Chen*, alleviating the toxicity, or generally improving the drug efficacy. The "*Shi* (messenger)" herb plays an indispensable conciliation role in harmonizing the action of all the other ingredients and enhancing their functions. Based on this rule, over 100,000 herbal formulae have been produced during the last 2000 years, and a mass of modern TCM products obtained

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from the TCM formulae have been successful applied into the treatment of various diseases, such as the Compound Dan-shen Pill (Jia et al., 2004), Shuang-Huang-Lian oral liquid (Cao et al., 2006), Huo-Xiang-Zheng-Qi liquid (He et al., 2006) etc.

It is generally known that TCM has a luxuriant diversity of components, and the structure and biological activity of each component is significantly dissimilar in different herbs or even in one herb. This characteristic evidently poses a big challenge to analytical chemistry and pharmacology for the quality control and application of TCMs. However, a growing body of evidence shows that only a few active ingredients make for the therapeutic effects (Zhao et al., 2010). Nowadays, despite the fact that a number of modern chemical analysis techniques have been successfully used to separate and characterize the active ingredients (Tang et al., 2009) it is still difficult to reveal the pharmacological mechanisms of TCM by molecular biology methods (Gu et al., 2009).

Chinese herbal formulae have two vital fundamental concepts: the holistic treatment and synergy strategies. Speculation as to these reasons, the TCM theory cries out for a thorough understanding to determine whether it involves synergy, enhanced bioavailability, reduced toxicity or just a simply overlying of the constituents. However, how and why these herbs can be prescribed in one formula still remains a mystery, and there is still a lack of appropriate methods to decipher the rule for herb combination (Li et al., 2010). Fortunately, a territory which might provide useful concept for resolving the complexity of TCM both in theory and practice is emerging, such as systems biology (Li et al., 2009).

Systems biology describes the complex and multi-levels interactions by various networks and clarifies the underlying mechanisms of biological systems (Zhao et al., 2010). Combining with pharmacology and pharmacodynamics, the development of systems biology has given birth to a new discipline, i.e., systems pharmacology (van der Greef and McBurney, 2005; Berger and Iyengar, 2009; van der Graaf and Benson, 2011; Huang et al., 2013). Systems pharmacology-based study of TCM may open up the possibility to understand the TCM theory and the mechanisms of action in the context of a molecular network. Previously, we have developed a set of systems-pharmacology methods and applied them for the study of the synergistic effects and the underlying mechanisms of actions of several Chinese herbal medicines (B. Li et al., 2012; X. Li et al., 2012; Tao et al., 2013).

In the present work, we apply systems pharmacology to dissect the rule of drug combination for TCM, which is exemplified by Ma-huang Decoction (also known as Ephedra Decoction, MHD). MHD is one of the most famous and rigorous TCM formulae developed in *Treatise on Cold Pathogenic Diseases* (*Shang Han Lun* in Chinese) during the East Han Dynasty in China. It is made up of four botanic drugs: *Herba Ephedrae* (Eph, Ma-huang), *Ramulus Cinnamomi* (RC, Gui-zhi), *Semen Armeniacae Amarum* (SAA, Xing-ren), and *Radix Glycyrrhizae* (RG, Gan-cao), in a weight ratio of 3:2:2:1. In virtue, this herbal formula can dispel pathogenic chills cold from the exterior by inducing sweat and relieving cough, asthma, headache, arthralgia, etc. as early symptoms of colds.

In this formula, Eph is considered as "*Jun*" herb involved in promoting the sweating to release the exterior, diffusing the lung and stabilizing the panting. RC as the "*Chen*" herb, is considered to assist Eph in treating cold. SAA ("*Zuo*" herb) and RG ("*Shi*" herb) work together to improve the drug delivery and therapeutic effects of *Jun* and *Chen* herbs. However, up to now, there is still no sufficient evidence to support the hypothesis of the drug combination principles of TCM from either the molecular or the systems levels. In order to clarify the combination rule, for the first time, this work provides a systematic analysis of TCM combination by using systems pharmacology methods on this typical Ma-huang Decoction, which will also be important to novel drug development and applications.

2. Materials and methods

In order to disclose the combination principle of TCM, an integrated systems pharmacology approach including the druglikeness evaluation, drug targeting and network analysis is introduced to the Ma-huang formula. The protocol includes five main steps as follows:

(1) Molecular database building. The chemicals of all four herbs in the Ma-huang formula are collected from literatures and online databases.
(2) Herb ingredient comparison. The physicochemical properties and structural features of compounds in the four herbs are measured and compared to investigate the difference and similarity between herbs.
(3) Oral bioavailability screening and druglikeness evaluation. This step is introduced to screen out the druglike chemicals in the whole compound database of Ma-huang formula.
(4) Evaluation of interactions with ADME key proteins. This is applied to evaluate if the compounds are involved in drug–drug interactions in pharmacokinetics.
(5) Drug targeting. Comprehensive identification of compound–target interactions by *in silico* model.
(6) Network construction and analysis. Construction of Drug–Target–Disease Network based on the obtained drug–target interactions and elucidation of the underlying action of mechanisms of TCM and the drug combination rule through network analysis.

2.1. Molecular database building

In order to obtain the known chemical ingredients in these four herbs, a total of 728 compounds (Supporting information Table S1) are collected from the Chinese academy of sciences Chemistry Database (http://www.organchem.csdb.cn) and literatures (details are displayed in Section 3.2), which have been uploaded to our previously developed database: Traditional Chinese Medicine Systems Pharmacology Database (TcmSP™, http://tcmspnw.com). It is a unique systems pharmacology platform designed for herbal medicines. TcmSP™ provides detailed, up-to-date and accurate structural and physicochemical properties like molecular weight, oral bioavailability, druglikeness, intestinal epithelial permeability and aqueous solubility, drug targets and their relationships with diseases. Since those compounds with glycosyl groups may be deglycosylated into their products by the rule of glycosidase hydrolysis reaction, 21 aglycons in the four herbs labeled by _qt are also included in the compound databases.

2.2. Herb comparison

In order to investigate whether the four herbs are similar or different in chemicals, herbal ingredient comparisons based on chemical properties are performed. Four important pharmacology-related descriptors based on Lipinski's rule of five including the molecular weight (MW), the number of donor atoms for H-bonds (nHDon), the number of acceptor atoms for H-bonds (nHAcc) and Moriguchi octanol–water partition coeff. (log *P*) (MLOGP) are calculated by the DRAGON soft (version 5.6; Talete SRL: Milano, Italian, 2006). These four parameters, which describe the molecular properties important for orally bioavailable drugs, can reflect the basic characteristics of a molecule (Lipinski et al., 1997). Hence, by considering these four parameters, oral bioavailability (OB) and druglikeness indices, the physicochemical properties and pharmacological features of compounds for each herb are investigated. Thereafter, the histogram of the physicochemical properties of all

Table 1
Comparison of molecular properties between Eph, RC, SAA and RG.

INDEX	Eph (mean ± SD)	RC (mean ± SD)	SAA (mean ± SD)	RG (mean ± SD)
MW	177.62 (76.45)	191.84 (72.45)	273.46 (123.7)	355.84 (158.51)
nHDon	0.68 (1.24)	0.59 (1.00)	0.78 (1.38)	2.57 (2.44)
nHAcc	1.52 (1.78)	1.46 (1.45)	1.70 (2.13)	5.28 (3.93)
MLogP	2.83 (1.93)	3.17 (2.95)	4.81 (3.15)	2.48 (1.91)
OB	38.62 (20.63)	36.29 (18.17)	27.85 (18.24)	32.31 (22.5)
DL	0.06 (0.09)	0.08 (0.12)	0.17 (0.17)	0.41 (0.25)

SD, standard deviation; Eph, *Herba Ephedrae*; RC, *Ramulus Cinnamomi*; SAA, *Semen Armeniacae Amarum*; and RG, *Radix Glycyrrhizae*.

chemicals in the four herbs accompanying with *t*-test is carried out to analyze the variables in the property spaces.

2.3. Oral bioavailability screening and druglikeness evaluation

Although a TCM formula usually contains hundreds even thousands of components, only a few bioactive compounds in the TCM contribute to its therapeutic effects. It is known that poor pharmacokinetics accounts for about 30% attrition in drug development process (Kola and Landis, 2004). For the purpose of identifying the potential active compounds from a TCM recipe, one method integrating the oral bioavailability screening and druglikeness evaluation is used presently.

2.3.1. Oral bioavailability (OB)

As a subcategory of absorption, OB is the fraction of an administered dose of unchanged drug that reaches the systemic circulation, and then plays pharmacology effects in the organism (van de Waterbeemd and Gifford, 2003). It is one of the principal pharmacokinetic properties of drugs. In this work, a robust *in silico* model, i.e., OBioavail1.1 (Xu et al., 2012b), is applied to calculate the OB value for drugs. This model is efficient in screening out the potential ADME-favorable molecules from the large number of compounds in a TCM receipt. The OB threshold in this work is defined as 30% since, normally, the first-pass extraction ratio for the gut and liver were 43% and 44% respectively (Paine et al., 1996). Thus, those compounds with OB ≥ 30% are adopted as the candidate compounds here.

2.3.2. Druglikeness

The Tanimoto coefficient is applied to measure the structural similarity between herbal ingredients and the drugs in Drugbank database (http://www.drugbank.ca/), and help us to select the compounds in herbs deemed to be chemically and pharmacologically suitable for drugs (Ma et al., 2011). This database-dependent druglikeness evaluation approach is shown as the following

$$T(A,B) = \frac{A \cdot B}{\|A\|^2 + \|B\|^2 - A \cdot B} \quad (1)$$

where A represents the molecular descriptors of herb compounds, B is the average molecular properties of all compounds in Drugbank database. In this work, the druglikeness index ≥ 0.18 (average value for Drugbank) is defined as the criterion to select those druglike compounds (Table 2). In short, molecules are regarded as potential active compounds in the following case: OB ≥ 30% and DL ≥ 0.18. In addition, several ingredients which are omitted from these screening criteria, but supported by the literature evidence are also kept as candidate compounds for further analysis.

2.4. Interaction with ADME key proteins

To determine whether herbal drugs can interact with ADME key factors such as the drug-efflux pump P-glycoprotein (P-gp) and drug-metabolism enzyme cytochrome P450 3A (CYP3A) or not, molecular docking simulations are performed between *Zuo–Shi* herbal compounds with these two proteins. The two proteins might be functionally linked and synergistic in limiting the oral availability of xenobiotics for their substrate specificity and locations similarity in human body (Watkins, 1997). Disclosing the relationships between herbs with these proteins should be important for understanding the potential drug–drug interactions.

P-gp is extensively distributed and expressed in the intestinal epithelium, hepatocytes, and capillary endothelial cells, which plays important roles in blood–brain and blood–testis barrier for most xenobiotic compounds (Varma et al., 2003). And the structure–activity relationship analyses indicate that inhibitors display a fairly strict requirement for the least value of ClogP (2.9, the lipophilicity), N_{lc} (18, the length of the chain of the molecule) and E_{homo} (−9.6, the energy of the highest occupied orbital) (Wang et al., 2003). In this work, the Mopac program (version 7.0) is used to calculate E_{homo} with the semi-empirical molecular orbital AM1 method (Dewar et al., 1985), and ClogP values are calculated by a web-based system, the Osiris Property Explorer (OPE; organic chemistry portal., http://www.organic-chemistry/.org).

CYP3A is the most abundant P-450 expressed in human liver and small intestine, which contributes to the metabolism of about 1/3rd orally administered drugs (Guengerich, 1999). In this work, to discriminate the inhibitors and substrates, the structure-based descriptors are used to develop classification models on the basis of linear discriminant analysis (LDA) (Ford et al., 2004). The DRAGON soft (version 5.6; Talete SRL: Milano, Italian, 2006) is applied to calculate the descriptors. The training set, which is used to generate classification model, is composed of 36 CYP3A4 substrates and 11 inhibitors obtained from the online database: Psychresidentonline (http://www.Psychresidentonline.com) and the chemical structures are obtained from the Chemical Book (http://www.chemicalbook.com). The optimal model is used to predict the bioactive ingredients from SAA and RG.

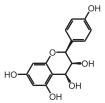
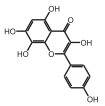
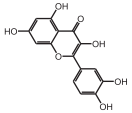
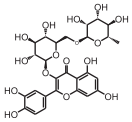
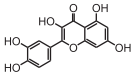
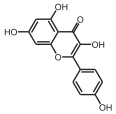
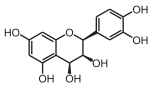
The molecular docking is performed by the widely used GOLD soft (www.ccdc.cam.ac.uk/prods/gold/). GOLD employs a genetic algorithm (GA) to explore the full range of ligand conformational flexibility and the selected receptor hydrogens flexibility (Joy et al., 2006). The crystal structure of CYP3A4 (pdb code: 3NXU) is downloaded from the RCSB Protein Data Bank (http://www.pdb.org/), the homology model of P-gp is obtained from our previous work (Xu et al., 2012a), and the binding pocket of each adjuvant/messenger drug is determined according to the coordinates of ligands in the crystal structure. Docking simulations are performed under the "Standard default settings": mode-number of islands is 5, population size is 100, and number of operations is 100,000.

Ligands are regarded as the potential inhibitors of P-gp or P-450 if they meet the following conditions: (1) the docking score is larger than 35 and (2) they are predicted as inhibitors by the models for P-gp and CYP3A as mentioned above.

2.5. Drug targeting for potential active compounds

Comprehensively determining the compound–target interaction profiles is necessary for elucidating the mechanisms of action of drugs. To predict the multi-targets of potential active drugs, another robust *in silico* model which efficiently integrates a large scale of chemical, genomic and pharmacological data, was developed previously (Yu et al., 2012). The dataset used in building these models included 6511 drugs and 3987 targets with known compound–protein interactions in Drugbank database. The

Table 2
Chemical information of 45 candidate compounds and their network parameters.

ID	Compound	OB	DL	Degree	BC	Structure	Source
Eph.1[a]	***Ephedrine***	***45.2***	***0.034***	***50***	***0.015***		Database; Zhou et al. (2008)
Eph.2[a]	Norephedrine	66.8	0.028	28	0.009		Database; Zhou et al. (2008)
Eph.3[a]	Norpseudoephedrine	74.1	0.028	28	0.009		Database; Zhou et al. (2008)
Eph.4[a]	***Pseudoephedrine***	***40.8***	***0.034***	***50***	***0.015***		Database; Zhou et al. (2008)
Eph.5[a]	N-methylephedrine	37.8	0.041	45	0.011		Zhou et al. (2008)
Eph.6[a]	***Leucopelargonidin***	***58.0***	***0.237***	***80***	***0.026***		Zhou et al. (2008)
Eph.7[a]	***Herbacetin***	***37.5***	***0.271***	***81***	***0.034***		Database; Zhou et al. (2008)
Eph.8[a]	***Quercetin***	***46.4***	***0.275***	***82***	***0.034***		Zhou et al. (2008)
Eph.9[a]	Methylephedrine	32.9	0.041	45	0.011		Database
Eph.10[a]	Methylpseudoephedrine	40.3	0.041	45	0.011		Database
Eph.11	Rutin	11.7	0.683	13	0.022		Zhou et al. (2008)
Eph.11_qt[a]	***rutin_qt***	***46.7***	***0.275***	***82***	***0.034***		Zhou et al. (2008)
Eph.12[a]	***Kaempferol***	***42.0***	***0.241***	***87***	***0.040***		Database; Zhou et al. (2008)
Eph.13	cis-Piperitol	37.5	0.271	19	0.011		Ji and Xu (1997)
Eph.14[a]	***Leucocyanidin***	***41.0***	***0.271***	***79***	***0.024***		Zhou et al. (2008)

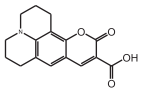
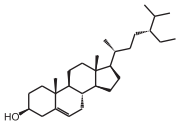
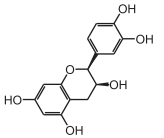
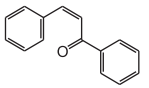
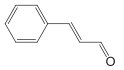
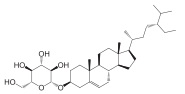
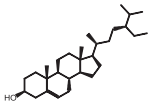
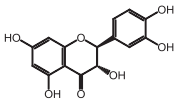
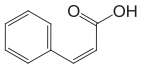
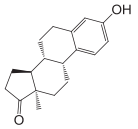
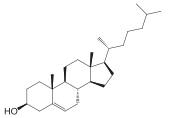
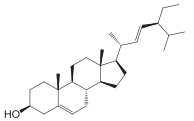
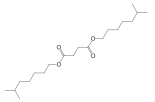
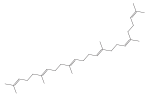
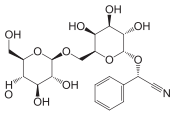
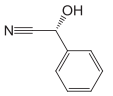
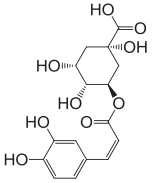
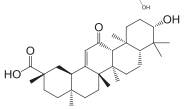
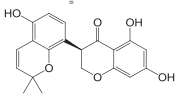
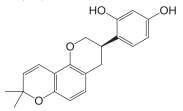
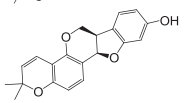
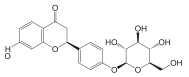
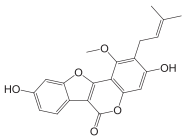
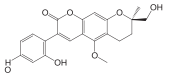
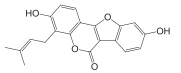
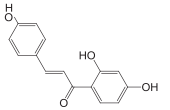
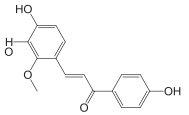
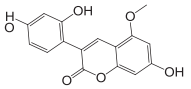
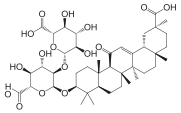
RC.1[a]	***Coumarin***	***24.0***	***0.378***	***94***	***0.089***		Database; Xu et al. (2001), Ding et al. (2002)
RC.2	Coumarin 343	29.7	0.043	10	0.0007		Database
RC.3	β-Sitosterol	36.9	0.751	34	0.012		Ma et al. (1999), Gupta et al. (2008)
RC.4[a]	***Epicatechin***	***49.1***	***0.242***	***64***	***0.013***		Schroeter et al. (2006)
RC.5[a]	***trans-Chalcone***	***62.0***	***0.084***	***67***	***0.042***		Ding et al. (2002)
RC.6[a]	***Cinnamaldehyde***	***27.2***	***0.023***	***22***	***0.035***		Database; Xu et al. (2001), Ding et al. (2002)
RC.7	Daucosterol	20.6	0.624	0	0		Database
RC.7_qt	daucosterol_qt	36.9	0.751	3	0.0002		Database
RC.8[a]	***Taxifolin***	***74.5***	***0.273***	***77***	***0.023***		Trouillas et al. (2006)
RC.9[a]	Camphor	67.3	0.053	2	0.01		Ma et al. (1999)
RC.10[a]	***Cinnamic acid***	***38.2***	***0.029***	***36***	***0.071***		Ding et al. (2002), Ma et al. (1999)
SAA.1[a]	***Estrone***	***53.6***	***0.319***	***61***	***0.019***		Database
SAA.2	Cholesterol	37.9	0.677	45	0.03		Database
SAA.3	Stigmasterol	43.8	0.757	43	0.019		Database

Table 2 (*continued*)

ID	Compound	OB	DL	Degree	BC	Structure	Source
SAA.4	Di-iso-octyl-succinate	31.6	0.232	4	0.002		Han (2008)
SAA.5[a]	Eicosadienoic acid	40.0	0.200	9	0.015		J. Zhang et al. (2007)
SAA.6	Squalene	33.5	0.424	0	0		Yao et al. (2007)
SAA.7[a]	Amygdalin	7.3	0.612	37	0.020		Database; Wei and Liu (2007)
SAA.7_qt	amygdalin_qt	56.8	0.024	14	0.007		Database; Ames et al. (1981)
SAA.8[a]	Chlorogenic acid	31.3	0.326	44	0.023		Database
SAA.9[a]	***Eicosenoic acid***	***30.7***	***0.197***	***11***	***0.042***		J. Zhang et al. (2007)
RG.1	18β-glycyrrhetic acid	35.0	0.862	12	0.0009		Database; Liu et al. (2013)
RG.2[a]	Glyasperin F	75.8	0.522	29	0.002		Liu et al. (2013)
RG.3[a]	***Glabridin***	***53.3***	***0.446***	***62***	***0.014***		Database; Liu et al. (2013)
RG.4[a]	Shinpterocarpin	80.4	0.664	42	0.006		Liu et al. (2013)
RG.5[a]	***Liquiritin***	***65.7***	***0.633***	***69***	***0.045***		Database; Liu et al. (2013)

RG.6[a]	***Neoglycyrol***	***98.9***	***0.619***	***66***	***0.031***		Liu et al. (2013)
RG.7[a]	***Licopyranocoumarin***	***80.4***	***0.583***	***78***	***0.057***		Database; Liu et al. (2013)
RG.8[a]	***Phaseol***	***78.8***	***0.535***	***59***	***0.014***		Liu et al. (2013)
RG.9[a]	***Isoliquiritigenin***	***89.5***	***0.183***	***90***	***0.061***		Database; Liu et al. (2013)
RG.10[a]	***Licochalcone B***	***76.8***	***0.234***	***77***	***0.039***		Database; Liu et al. (2013)
RG.11[a]	***7,2′,4′-Trihydroxy-5-methoxy-3-arylcoumarin***	***83.7***	***0.316***	***76***	***0.023***		Liu et al. (2013)
RG.12	Glycyrrhizic acid	19.6	0.143	0	0		Database; Liu et al. (2013)

_qt: the compound with glycosyl groups is deglycosylated by the rule of glycosidase hydrolysis reaction.
Database: The Chinese Academy of Sciences Chemistry Database.
Bold and italic figure: Compounds with both high degree and betweenness centrality (BC).

[a] Compounds which are selected as hub drugs.

optimal models by Support Vector Machine (SVM) and Random Forests (RF) show impressive performance of prediction for drug–target interactions, with a concordance of 82.83%, a sensitivity of 81.33% and a specificity of 93.62%. In this work, the compound–target interactions with a SVM score ≥ 0.8 and a RF score ≥ 0.7 are selected as candidate targets for further analysis. Then the obtained target proteins are further applied as baits to fish their related diseases in the back ground of TTD database (http://bidd.nus.edu.sg/group/cjttd/).

2.6. Network construction and analysis

Furthermore, we construct the Drug–Target–Disease Network in order to study the drugs in the context of targets and diseases networks, as well as to understand the combination principle of TCM. Presently, the "network" is a mathematical and computable representation of various connections between drugs, targets and diseases, in which the biological components (i.e., drugs, proteins and diseases) are represented by nodes, and the interaction between two nodes is represented by an edge. Due to that the therapeutic effectiveness of a TCM formula is achieved through collectively modulating the molecular network by its active ingredients, two key topological parameters, i.e., degree and betweenness centrality (Azuaje et al., 2011) are analyzed to specify the importance of each node in the net. The "degree" of a node is the number of edges connecting to the node, and the highly connected nodes (half of the maximum degree of nodes) are referred to as hubs. And the betweenness centrality of a node is its capacity to be located in the shortest communication paths between different pairs of nodes in the network. The nodes which have high betweenness centrality are also regarded as network bottlenecks. All the topological properties of these networks are analyzed using Network Analysis plugin and CentiScaPe 1.2 of Cytoscape (Shannon et al., 2003).

3. Results

TCMs mixtures normally contain many active constituents which generally act upon multiple targets (Li et al., 2008). This poses a big challenge to modern analytical chemistry and pharmacology analysis (Qiu, 2007; Tang et al., 2009). The present work tries to perform a systems study on a representative TCM formula to illuminate the mechanism of drug action and the basic combination principle of TCM.

3.1. Herbal ingredient comparison

In the first step, ingredients in the four herbs (Eph, RC, SAA and RG) are compared based on four important drug-associated descriptors including the MW, nHDon, nHAcc, and MLogP: (I) From the average number of MWs (Fig. 1 and Table 1), it is seen that the values are similar ($p=0.09$) for Eph (177.62) and RC (191.84), while RG is significantly higher than that of Eph ($p=7.81E-47$), RC ($p=4.44E-24$) and SAA (273.46, $p=1.06E-05$). (II) The average number of H-bond donors of RG (2.57) is larger than that of other herbs (Eph is 0.68, $p=3.13E-29$; RC is 0.59, $p=7.78E-19$; and SAA is 0.78, $p=3.20E-12$). (III) The average number of H-bond acceptors (nHAcc) of RG (5.28) is larger than all other three herbs with Eph of 1.52 ($p=9.90E-37$), SAA of 1.70 ($p=2.94E-22$) and RC of 1.46 ($p=4.72E-15$). (IV) The compounds in SAA are most hydrophobic with the average number of MLogP of 4.81, while for other three herbs, their averages are only 3.17 for RC ($p=3.05E-06$), 2.83 for Eph ($p=3.98E-11$) and 2.48 for RG ($p=4.71E-16$).

The oral bioavailability and druglikeness analysis also indicate the differences among these four herbs (as seen in Fig. 1 and Table 1). For OB, Eph possesses the highest average value of 38.62, followed by RC of 36.29 ($p=0.3$), RG of 32.31 ($p=0.00095$), and SAA (27.85, $p=2.53E-05$). Thus Eph is significantly different from RG and SAA, but not from RC. However, RC ($p=0.09$) and SAA ($p=0.09$) are not significantly different from RG. Whereas for DL analysis, unlike OB distribution Eph reveals the lowest average DL index (0.06) which displays no significant difference ($p=0.06$) from RC.

In summary, all the results show that in properties of the chemical ingredients RG is obviously different from Eph, RC and SAA. However, to a certain extent, Eph has somewhat similar properties with RC (MW, $p=0.9$; nHDon, $p=0.5$; nHAcc, $p=0.8$; MLogP, $p=0.1$; OB, $p=0.3$; DL, $p=0.06$).

3.2. Oral bioavailability screening and druglikeness evaluation

Due to the fact that TCMs are often orally administered, the analysis of their ingredients' oral bioavailability capability dependent on the absorption, distribution and liver metabolism conditions *in vivo*, as well as the druglikeness property based on the Tanimoto coefficient, are crucial for finding out those compounds which are deemed to be active in an herb. In this work, it is discovered that 48 compounds accounting for 6.59% of all the 728 chemicals in the Ma-huang Decoction recipe are potential active molecules (Table 2) details of which are listed as follows:

3.2.1. Eph (Ma-huang)

Herba Ephedrae belongs to the family of Ephedraceae which includes the *Ephedra sinica Stapf*, *Ephedra intermedia Schrenk et C. A. Mey.* and *Ephedra equisetina Bge.* (Bensky et al., 2004). It has 237 reported chemicals (Ji and Xu, 1997; Miyazawa et al., 1997; Wang et al., 2006; Zhou et al., 2008) in which 63.3% compounds have appropriate OB values ($\geq 30\%$) and 8.4% ones are much druglike ($DL \geq 0.18$). For further study, those compounds satisfying both the OB and DL criteria are selected, including leucocyanidin (Eph.6), herbacetin (Eph.7) and quercetin (Eph.8), etc. Ephedrine (Eph.1) is the major reported pharmacological compound, followed by norephedrine (Eph.2), norpseudoephedrine (Eph.3), pseudoephedrine (Eph.4), methylephedrine (Eph.9), and methylpseudoephedrine (Eph.10) (Andraws et al., 2005; Pellati and Benvenuti, 2008). Notably, the OB of ephedrine (Eph.1) is about 50% (Takagi et al., 2006), which is basically supported by the current study (about 45.2%). As a matter of fact, the present data show that almost all ephedra alkaloids are orally bioavailable (with $OB \geq 30\%$) but less druglike (< 0.04). By considering the potential pharmacological effects of alkaloids (Pellati and Benvenuti, 2008), they are also included for further targeting. The detailed results are shown in Table 2.

3.2.2. RC (Gui-zhi)

Belonging to the family of lauraceae, *Ramulus Cinnamomi* is the dry twig of *Cinnamomum cassia PRESL* and other species of the same genus (Hwang et al., 2009a). Among the 117 ingredients in RC (Ma et al., 1999; Qiu et al., 2000; Xu et al., 2001; Ding et al., 2002), 57.3% of them have reasonable OB values ($\geq 30\%$) and 7.7% of them have appropriate druglikeness indices (≥ 0.18), including most of the active compounds such as β-sitosterol (RC.3) (Gupta et al., 2008), taxifolin (RC.8) (Trouillas et al., 2006) and epicatechin (RC.4) (Schroeter et al., 2006).

Another active compound cinnamaldehyde (RC.6) has an OB of 20% (Feng et al., 2004), which is basically consistent with the current prediction (about 27.2%). The possible reason for this relative low OB is that this compound is initially oxidized to

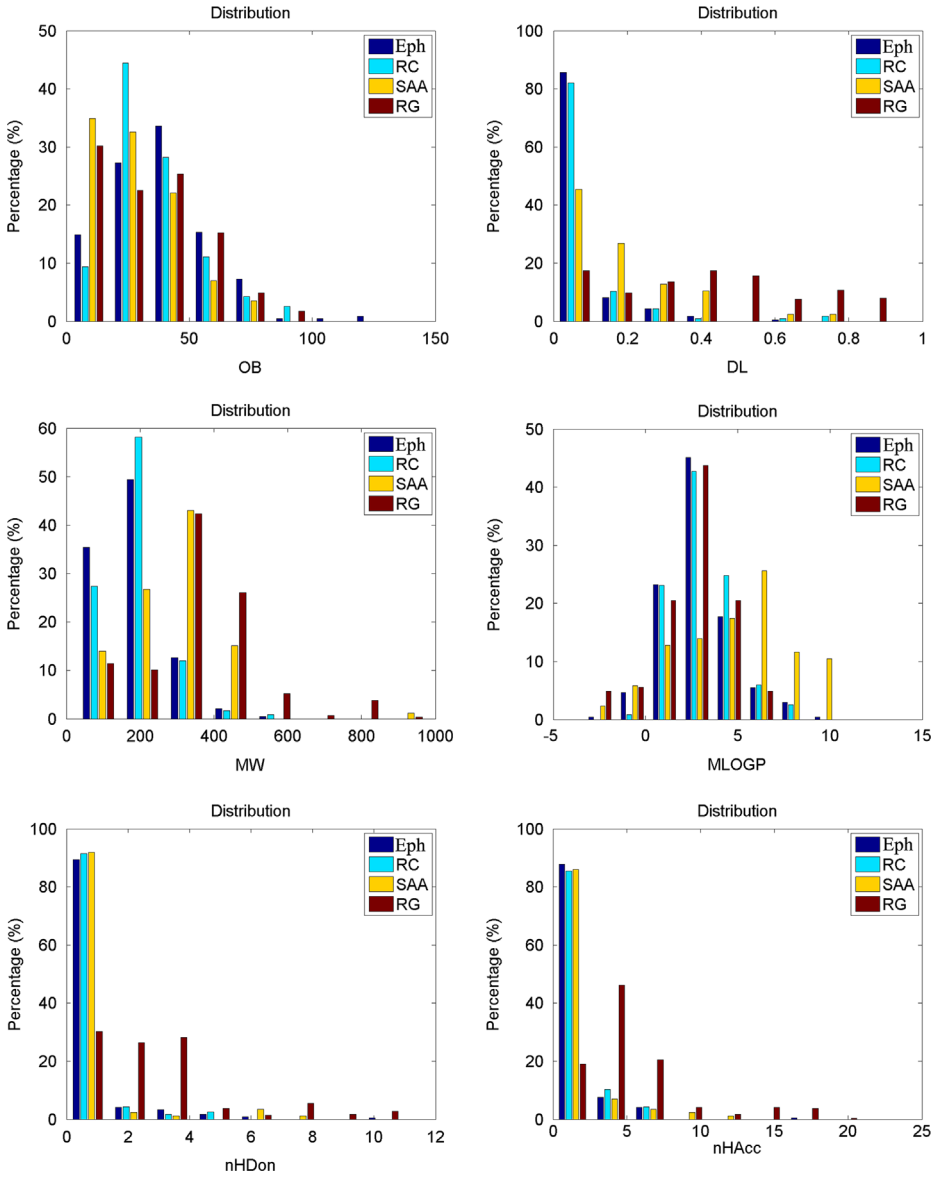


Fig. 1. The profile distributions of six important molecular properties for all ingredients from *Herba Ephedrae* (Eph, Ma-huang), *Ramulus Cinnamomi* (RC, Gui-zhi), *Semen Armeniacae Amarum* (SAA, Xing-ren), and *Radix Glycyrrhizae* (RG, Gan-cao) to analyze the molecular diversity of compounds. Molecular properties consist of oral bioavailability (OB), druglikeness (DL), molecular weight (MW), number of donor atoms for H-bonds (nHDon), number of acceptor atoms for H-bonds (nHAcc) and Moriguchi octanol–water partition coeff. log *P* (MLogP).

cinnamic acid (RC.10, OB = 38.2%) which could be quickly eliminated in human body (Chen et al., 2009). Both camphor (RC.9) and trans-chalcone (RC.5) are orally available (OB > 60%), which are also reported pharmacologically active (Xu et al., 2005; Liu et al., 2007). Coumarin (RC.1), as the main active ingredient in RC (Jung et al., 2011), exhibits low druglikeness value (OB = 24.0%, DL < 0.04). Based on all these considerations, finally, 11 molecules including especially the β-sitosterol (RC.3), epicatechin (RC.4), cinnamaldehyde (RC.6) and camphor (RC.9) are selected as potential active compounds (Table 2).

3.2.3. SAA (Xing-ren)

Semen Armeniacae Amarum is the seed of the genus Prunus and the subgenus Amygdalus within the Rosaceae family (Yada et al., 2011). SAA contains the least amount (86) of molecules (Yao et al., 2007; J. Zhang et al., 2007; Han, 2008) in which 40.7% have appropriate OB values (≥ 30%), and 33.7% are druglike (≥ 0.18). Table 2 shows that nine ingredients meet the selection criteria, such as the estrone (SAA.1), stigmasterol (SAA.3) and squalene (SAA.6). Amygdalin (SAA.7), the main active ingredient, accounts for 2–3% in this herb (Wei and Liu, 2007). However, its OB is relatively low (7.3%) which might be due to the fact that this compound is hard to be absorbed (Rauws et al., 1982). We propose that it is not the amygdalin molecule itself, but is its metabolite that makes the compound exert the pharmacological function, since this compound can be quickly hydrolyzed into a more bioavailable compound, mandelonitrile (SAA.7_qt, OB = 56.8%) *in vivo* (Ames et al., 1981). Thus amygdalin and its hydrolysate (SAA.7_qt) are also added as candidate drugs for further analysis.

The inhibition of P-gp has been reported as one of the main causes of drug–drug interactions, which may result in an increase in the systemic bioavailability of poorly absorbed drugs (Lin, 2003). After docking all active compounds of SAA into P-gp structure, SAA.2 (cholesterol), SAA.3 (stigmasterol), SAA.6 (squalene), SAA.8 (chlorogenic acid) and SAA.9 (eicosenoic acid) yield the expected good docking scores (47.6, 44.0, 67.7, 46.4 and 57.8 respectively) with a fit close to the proposed drug-binding pocket

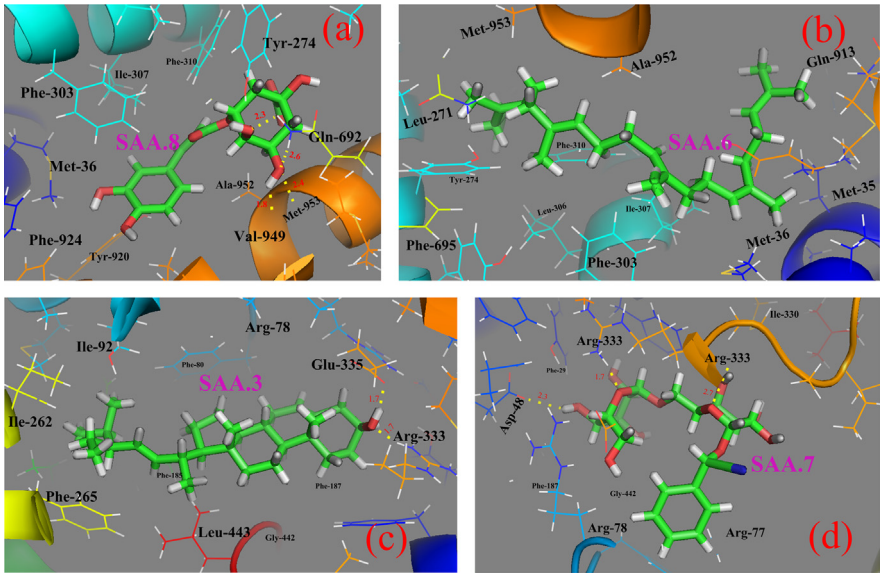


Fig. 2. Molecular models of ingredients from SAA in the binding sites of P-gp and CYP3A4. The dashed lines show the formation and distance of the hydrogen bonds. Active site amino acid residues are represented as lines. (a) Representative interactions between SAA.8 (chlorogenic acid) and P-gp. (b) Representative interactions between SAA.6 (squalene) and P-gp. (c) Representative interactions between SAA.3 (stigmasterol) and CYP3A4. (d) Representative interactions between SAA.7 (amygdalin) and CYP3A4.

(as shown in Fig. 2a). Among these five ingredients, cholesterol is a known P-gp modulator (Troost et al., 2004), stigmasterol a P-gp substrate (El-Readi et al., 2010), and chlorogenic acid (SAA.8) a P-gp inhibitor (Najar et al., 2010) which forms at least three hydrogen bonds (H-bonds) with Gln-692, Val-949 and Met-953 (Fig. 2a and b). In addition, SAA.6 and SAA.9 might also be P-gp inhibitor candidates, which possess ClogP values of 12.88 and 8.844, 24 and 21-atom-long molecular axis, and high E_{homo} values of −9.67 and −7.67, respectively.

Another common cause for the documented drug interactions is the inhibition of CYP enzymes, particular CYP3A4 (Lin, 2003). The present analysis shows that SAA.2, SAA.3, SAA.5 (eicosadienoic acid), SAA.7 (amygdalin), SAA.7_qt (amygdalin_qt), SAA.8 and SAA.9 in *Semen Armeniacae Amarum* can bind to CYP3A4 with high binding affinities (score=57.5, 58.0, 61.3, 60.7, 36.6, 54.2 and 69.6 respectively). The binding model shows SAA.2 forms two H-bonds with Tyr-25 and Asp-33 in the binding pocket. SAA.3 is H-bonded to Glu-335 and Arg-333 (as seen in Fig. 2c); SAA.5 interacts with Arg-102, SAA.7_qt to form H-bonds with Arg-77 and Arg-401, while SAA.9 with Arg-77, Arg-401 and Trp-98. Moreover, both SAA.7 and SAA.8 also interact with the benzene ring of Arg-77 via π–π stacking. The present LDA model also predicts that the above six ingredients are potential inhibitors, interestingly, among which molecule stigmasterol (SAA.3) has been demonstrated as a CYP3A4 inhibitor in this herb (Nair et al., 2007).

3.2.4. RG (Gan-cao)

Being the rhizome of *Glycyrrhiza uralensis Fisch.*, *Glycyrrhiza inflata Bat.* or *Glycyrrhiza glabra L* (Leguminosae) (Shen et al., 2007), *Glycyrrhizae radix* (called Gan-cao in China, which means "sweet weed") belongs to the family of galegeae and genus glycyrrhiza. RG has 288 reported molecules (Liu et al., 2013) in which over 50% have proper OB values (≥ 30%) and druglikeness indices (≥ 0.18). The weight ratio of Eph to RG is 3:1, thus the OB screening threshold is raised to 75% to assure that sufficient amount of ADME favorable compounds in RG can be included. Finally, 12 ingredients are selected, such as glabridin (RG.3), liquiritin (RG.5), isoliquiritigenin (RG.9) and licochalcone B (RG.10) which have been reported as bioactive compounds in RG (Rauchensteiner et al., 2005; Zhan and Yang, 2006; Furusawa et al., 2009). In addition, the intestinal bacteria metabolite 18β-glycyrrhetic acid (RG.1, OB=35.0%) (Goto et al., 2005) is also added although its precursor, the glycyrrhizic acid (RG.12, OB=19.6%) (Shibata et al., 2001), is quite non-orally bioactive.

Docking results show that RG.2 (glyasperin F), RG.3 (glabridin), RG.4 (shinpterocarpin), RG.5 (liquiritin), RG.6 (neoglycyrol), RG.8 (phaseol), and RG.12 (glycyrrhizic acid) are of high binding affinities (score=44.8, 42.3, 42.4, 50.4, 51.9, 50.9 and 38.0 respectively). Glabridin (Cao et al., 2007) and liquiritin (Chen et al., 2012) have been demonstrated as P-gp substrates, and glycyrrhizic acid exhibits inhibitory effect on P-gp (Nabekura et al., 2008) (Fig. 3a). These seven ingredients mainly bind to P-gp by interacting with the key amino acids including Leu-32, Met-36, Ile-307, Phe-310, Gln-692, Val-949, Ala-952, Met-953, etc. as shown in Fig. 3a and b. In addition, the structure–activity relationship analyses indicate that RG.2 (CLogP=4.02, N_{lc}=15, E_{homo}=−5.99), RG.4 (CLogP=4.41, N_{lc}=13, E_{homo}=−7.77), RG.6 (CLogP=5.04, N_{lc}=15, E_{homo}=−7.55) and RG.8 (CLogP=5.09, N_{lc}=15, E_{homo}=−7.51) are potent inhibitors.

In addition, RG.1 (18β-glycyrrhetic acid), RG.3, RG.5, RG.6, RG.7 (licopyranocoumarin), RG.8 and RG.12 can bind to CYP3A4 with high affinity (score=56.1, 53.2, 60.0, 59.3, 53.6, 59.5 and 54.1, respectively). Glabridin (RG.3) (Kent et al., 2002), liquiritin (RG.5) (Tsukamoto et al., 2005) and glycyrrhizic acid (RG.12) (Kimura et al., 2010) are known as CYP3A4 inhibitors, while the others are potential inhibitors predicted by the LDA model. The –OH groups of RG.5, RG.6 and RG.12 form H-bonds with the guanidine group of Arg-77. Similarly, RG.1 and RG.7 form H-bonds with Glu-335, and RG.3 interacts with the benzene ring of Arg-78 via π–π stacking. In addition, RG.1 can bind to Ser-91, RG.6 makes at least two hydrogen bonds (−OH···Thr−271, OH···Leu−44), and RG.8 can form H-bonds with Ala-331, Glu-269 and Leu-444 (as seen in Fig. 3d).

3.3. Drug targeting for potential active compounds

Based on our previous drug targeting technique (Yu et al., 2012), a total of 156 candidate targets of all the above 45 active compounds are obtained. The candidate drugs, targets and diseases for each herb are provided as follows.

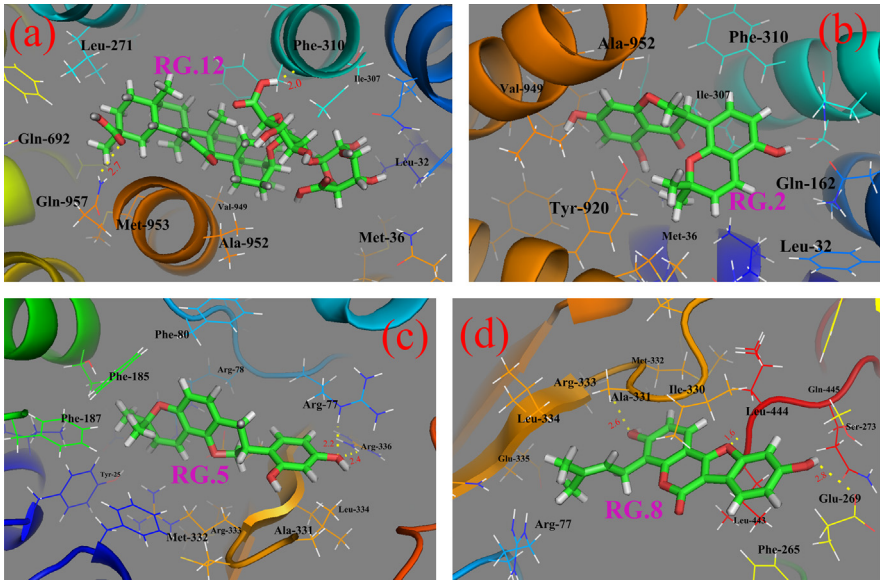


Fig. 3. Molecular models of ingredients from RG in the binding sites of P-gp and CYP3A4. The dashed lines show the formation and distance of the hydrogen bonds. Active site amino acid residues are represented as lines. (a) Representative interactions between RG.12 (glycyrrhizic acid) and P-gp. (b) Representative interactions between RG.2 (glyasperin F) and P-gp. (c) Representative interactions between RG.5 (liquiritin) and CYP3A4. (d) Representative interactions between RG.8 (phaseol) and CYP3A4.

3.3.1. *Herba Ephedrae*

Herba Ephedrae has been used to mainly treat bronchial asthma, cold and flu, chills, fever, lack of perspiration, headache, edema, nasal decongestion and cough (Abourashed et al., 2003; Soni et al., 2004). One hundred and twenty-three target proteins of Eph are predicted, most of which are related to the above mentioned diseases like the 5-hydroxytryptamine 1B/2A receptor (HTR1B/ HTR2A), beta-secretase 1 (BACE1), cell division protein kinase 2 (CDK2), delta-type opioid receptor (OPRD1), histamine H1 receptor (HRH1) and so forth. Moreover, ephedrine alkaloids are predicted to act on α- and β-adrenergic receptors, which are supported by the fact that these compounds are the primary active ingredients which target adrenergic receptors (Blechman et al., 2004). It is also known that these receptors are related to the cardiovascular, respiratory, central nervous systems (Blechman et al., 2004) as well as the inflammatory diseases (Abourashed et al., 2003).

3.3.2. *Ramulus Cinnamomi*

Many experiments have proved that the extracts of *Ramulus Cinnamomi* might be effective in the prevention or therapeutic treatment of inflammation and inflammation-mediated neurodegenerative diseases (Hwang et al., 2009b). The current work predicts 127 targets for RC, in which many of them have relationships with inflammation and other diseases. For example, the main ingredients essential oil cinnamaldehyde (RC.6) and cinnamic acid (RC.10) are highly connected with Trypsin-1 (PRSS1), which are involved in various pathological processes including inflammation, abnormal blood coagulation, tumor invasion, and atherosclerosis (Koshikawa et al., 1998).

3.3.3. *Semen Armeniacae Amarum*

Ninety-eight targets are predicted for this herb, many of which are related to coronary artery disease (Jenkins et al., 2002) such as the 5-hydroxytryptamine 2A receptor (5HT2A) (Coto et al., 2003) and estrogen receptor beta (ESR2) (Rexrode et al., 2007). Interestingly, the two targets interact with the same compound estrone (SAA.1), indicating that SAA may reduce the risk of heart disease (Chen et al., 2005). In addition, it is also proposed that amygdalin (SAA.7) has antitumor effects (Rauws et al., 1982) by interacting with targets like lysozyme (E) and androgen receptor (AR) (Grossmann et al., 2001).

3.3.4. *Radix Glycyrrhizae*

This botanic drug interacts with 112 targets covering a wide range of diseases such as cardiovascular diseases, cancer, tussis and inflammation, etc. (Shen et al., 2007; Asl and Hosseinzadeh, 2008). For example, targeted by isoliquiritigenin (RG.9), Prostaglandin G/H Synthase-1 and -2 (PGHS-1 and -2) are involved in inflammation and neoplastic transformation (Kargman et al., 1995). Moreover, isoliquiritigenin (RG.9) can also modulate inflammation and tumor by interacting with Endothelial Nitric Oxide Synthase (eNOS) (Grossmann et al., 2001). Prothrombin (F2) can be regulated by licochalcone B (RG.10), which is responsible for the treatment of bleeding (Poort et al., 1996).

3.4. *Network construction and analysis*

A general understanding of drug action requires a systems-level view to uncover the underlying interactions between drugs, targets, and complex diseases. Mapping the polypharmacology network to the Ma-huang Decoction network reveals that drugs commonly act on multiple targets, while targets are often involved with multiple diseases. Over 80% of drug targets can map to more than one disease. The Ma-huang Decoction network consists of 281 nodes and 2366 edges. Among the edges, there are 2151 drug–target interactions, and 215 drug–disease interactions.

3.4.1. *Drug–Target Network (D–T Network)*

A graph of drug–target interaction is built by candidate drugs and their related targets, and the network analysis method is used for analyzing properties of the network. In this D–T Network (**Fig. 4**), out of 45 drugs with 156 protein targets, 42 have at most 10 links with other targets, and seven drugs have the largest number of bound components with at least 80 links.

Among the 45 chemicals, coumarin (RC.1) has the largest number of interactions (94 interactions), followed by isoliquiritigenin (RG.9), kaempferol (Eph.12), rutin without glycosyl group (Eph.11_qt), quercetin (Eph.8), herbacetin (Eph.7), leucopelargonidin (Eph.6), leucocyanidin (Eph.14), and so forth. The

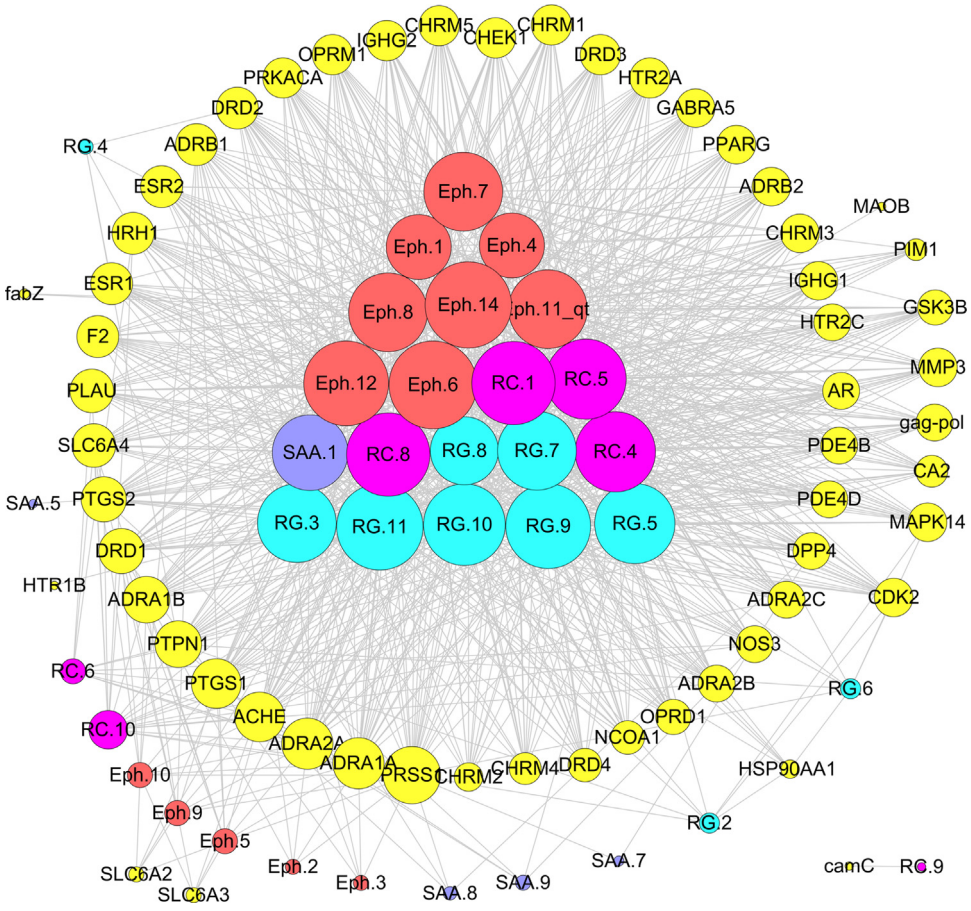


Fig. 4. Drug–Target Network (D–T Network). The D–T Network is built by linking the candidate drugs and all their candidate targets, which consists of 201 nodes and 2151 edges, with 45 active drugs and their predicted 156 targets. The area of the node is proportional to the degree value. (Eph, *Herba Ephedrae*; RC, *Ramulus Cinnamomi*; SAA, *Semen Armeniacae Amarum*; RG, *Radix Glycyrrhizae*.).

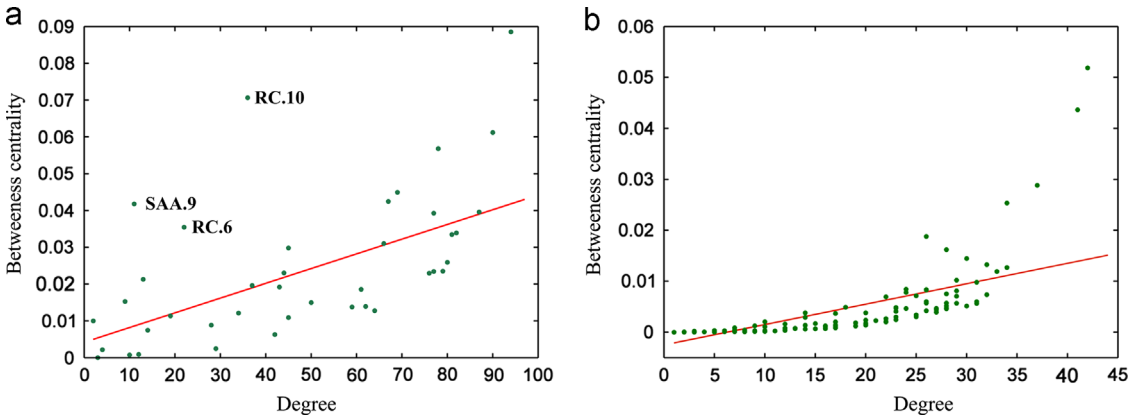


Fig. 5. (a) Relationship between degree and betweenness centrality in the drug set. The "degree" of a node is the number of edges connecting to the node, and the "betweenness centrality" of a node is the capacity to be located in the shortest communication path between different pairs of nodes in the network. Three molecules RC.6 (cinnamaldehyde, degree=22, betweenness centrality=0.036), RC.10 (cinnamicacid, degree=36, betweenness centrality=0.07) and SAA.9 (eicosenoic acid, degree=11, betweenness centrality=0.042) have low degrees but high centralities. (b) Relationship between the degree and betweenness centrality in the target set. This figure illustrates that the distribution of degree and betweenness centrality is strongly correlated and the most highly connected vertices have high centrality scores.

protein with the highest degree (59 interactions) is Trypsin-1 (PRSS1), then is acetylcholinesterase (ACHE), which is followed by Prostaglandin G/H synthase 2 (PTGS2), Alpha-2A adrenergic receptor (ADRA2A), Prostaglandin G/H synthase 1 (PTGS1), Alpha-1A adrenergic receptor (ADRA1A), etc. In addition, we observe that the distribution of degree and betweenness centrality is strongly correlated as seen in Fig. 5. However, there are still three molecules (Fig. 5), i.e., cinnamaldehyde (RC.6, degree=22, betweenness centrality=0.035), cinnamic acid (RC.10, degree=36, betweenness centrality=0.071) and eicosenoic acid (SAA.9, degree=11, betweenness centrality=0.042) which have low degrees but high centralities, implying that they might serve as bridges in the network to strengthen the relations in this drug–target community.

Based on the above analysis, 24 active ingredients (eight from Eph, six from RC, two from SAA and eight from RG) and 49

Table 3
The potential targets and their network parameters and related diseases.

Target	Gene Name	Degree	BC	Related Diseases
5-Hydroxytryptamine 1B receptor	HTR1B	20	0.002	Mental disorders, nervous system diseases, respiratory tract diseases
5-Hydroxytryptamine 2A receptor	HTR2A	32	0.007	Behavior and behavior mechanisms, cardiovascular diseases, endocrine system diseases, female urogenital diseases and pregnancy complications, male urogenital diseases, mental disorders, nervous system diseases, substance-related disorders
5-Hydroxytryptamine 2C receptor	HTR2C	27	0.004	Mental disorders, pathological conditions, signs and symptoms, substance-related disorders
Acetylcholinesterase	ACHE	41	0.044	Cardiovascular diseases, mental disorders, nervous system diseases, pathological conditions, signs and symptoms, psychological phenomena and processes
Alpha-1A adrenergic receptor	ADRA1A	33	0.012	Cardiovascular diseases, urogenital system
Alpha-1B adrenergic receptor	ADRA1B	29	0.007	Cardiovascular diseases, nervous system diseases
Alpha-2A adrenergic receptor	ADRA2A	34	0.013	Cardiovascular diseases
Alpha-2B adrenergic receptor	ADRA2B	26	0.006	Cardiovascular diseases
Alpha-2C adrenergic receptor	ADRA2C	23	0.005	Cardiovascular diseases, nervous system diseases
Amine oxidase [flavin-containing] B	MAOB	18	0.005	Mental disorders, nervous system diseases
Androgen receptor	AR	26	0.008	Male urogenital diseases, musculoskeletal diseases, neoplasms, nervous system diseases
Beta-1 adrenergic receptor	ADRB1	22	0.002	Cardiovascular diseases, eye diseases, immune system diseases, mental disorders, pathological conditions, signs and symptoms, respiratory tract diseases
Beta-2 adrenergic receptor	ADRB2	26	0.006	Behavior and behavior mechanisms, cardiovascular diseases, eye diseases, immune system diseases, mental disorders, musculoskeletal system, nervous system diseases, respiratory tract diseases, pathological conditions, signs and symptoms
Carbonic anhydrase II	CA2	24	0.008	Digestive system diseases, endocrine system diseases, eye diseases, male urogenital diseases, neoplasms
Cell division protein kinase 2	CDK2	23	0.005	Cardiovascular diseases, hemic and lymphatic diseases, immune system diseases, neoplasms, virus diseases
D(1A) dopamine receptor	DRD1	28	0.008	Nervous system diseases
D(2) dopamine receptor	DRD2	31	0.006	Behavior and behavior mechanisms, digestive system and oral physiological phenomena, male urogenital diseases, mental disorders, nervous system diseases, psychological phenomena and processes, respiratory tract diseases, pathological conditions, signs and symptoms, substance-related disorders
D(3) dopamine receptor	DRD3	27	0.004	Mental disorders, nervous system diseases, respiratory tract diseases, substance-related disorders
D(4) dopamine receptor	DRD4	27	0.005	Nervous system diseases, respiratory tract diseases
Delta-type opioid receptor	OPRD1	29	0.0057	Chemical actions and uses, musculoskeletal and neural physiological phenomena, nervous system diseases, pathological conditions, signs and symptoms, respiratory tract diseases
Dipeptidyl peptidase IV	DPP4	23	0.00408643	Endocrine system diseases, immune system diseases, neoplasms, nutritional and metabolic diseases, pathological conditions, signs and symptoms, physiological phenomena
Estrogen receptor	ESR1	29	0.01	Cardiovascular diseases, congenital, hereditary, and neonatal diseases and abnormalities, digestive system diseases, musculoskeletal diseases, neoplasms, nervous system diseases, skin and connective tissue diseases, wounds and injuries
Estrogen receptor beta	ESR2	28	0.006	Cardiovascular diseases, neoplasms, nervous system diseases, skin and connective tissue diseases
Glycogen synthase kinase-3 beta	GSK3B	22	0.003	Immune system diseases, mental disorders, neoplasms, nervous system diseases, pathological conditions, signs and symptoms, wounds and injuries
Histamine H1 receptor	HRH1	26	0.008	Cardiovascular diseases, hemic and lymphatic diseases, immune system diseases, mental disorders, neoplasms, nervous system diseases, otorhinolaryngologic diseases, pathological conditions, signs and symptoms, respiratory tract diseases, skin and connective tissue diseases
Mitogen-activated protein kinase 14	MAPK14	21	0.002	Amino acids, peptides, and proteins, bacterial infections and mycoses, cardiovascular diseases, digestive system diseases, immune system diseases, male urogenital diseases, mental disorders, musculoskeletal diseases, neoplasms, nervous system diseases, nutritional and metabolic diseases, pathological conditions, signs and symptoms, physiological phenomena, respiratory tract diseases, skin and connective tissue diseases
Muscarinic acetylcholine receptor M1	CHRM1	23	0.002	Mental disorders, nervous system diseases, psychological phenomena and processes, respiratory tract diseases
Muscarinic acetylcholine receptor M2	CHRM2	28	0.005	Cardiovascular diseases, chemical actions and uses, circulatory and respiratory physiological phenomena, mental disorders, musculoskeletal and neural physiological phenomena, nervous system diseases, pathological conditions, signs and symptoms, respiratory tract diseases
Muscarinic acetylcholine receptor M3	CHRM3	22	0.007	Female urogenital diseases and pregnancy complications, male urogenital diseases, pathological conditions, signs and symptoms, respiratory tract diseases
Muscarinic acetylcholine receptor M4	CHRM4	28	0.005	Chemical actions and uses, mental disorders, musculoskeletal and neural physiological phenomena, nervous system diseases, pathological conditions, signs and symptoms
Muscarinic acetylcholine receptor M5	CHRM5	26	0.004	Mental disorders
Mu-type opioid receptor	OPRM1	31	0.006	Chemical actions and uses, musculoskeletal and neural physiological phenomena, nervous system diseases, pathological conditions, signs and symptoms, respiratory tract diseases
Nitric-oxide synthase, endothelial	NOS3	26	0.019	Bacterial infections and mycoses, cardiovascular diseases, digestive system diseases, mental disorders, neoplasms, pathological conditions, signs and symptoms
Peroxisome proliferator activated receptor gamma	PPARG	28	0.016	Cardiovascular diseases, congenital, hereditary, and neonatal diseases and abnormalities, endocrine system diseases, female urogenital diseases and pregnancy complications, immune system diseases, male urogenital diseases, neoplasms, nervous system diseases, nutritional and metabolic diseases, pathological conditions, signs and symptoms, physiological phenomena, respiratory tract diseases, skin and connective tissue diseases
Prostaglandin G/H synthase 1	PTGS1	34	0.025	Cardiovascular diseases, pathological conditions, signs and symptoms

Table 3 (*continued*)

Target	Gene Name	Degree	BC	Related Diseases
Prostaglandin G/H synthase 2	PTGS2	37	0.029	Cardiovascular diseases, chemical actions and uses, digestive system diseases, female urogenital diseases and pregnancy complications, immune system diseases, male urogenital diseases, mental disorders, musculoskeletal and neural physiological phenomena, musculoskeletal diseases, neoplasms, nervous system diseases, pathological conditions, signs and symptoms, skin and connective tissue diseases, wounds and injuries
Prothrombin	F2	31	0.01	Cardiovascular diseases, hemic and lymphatic diseases, neoplasms, pathological conditions, signs and symptoms
Serine/threonine-protein kinase Chk1	CHEK1	23	0.002	Neoplasms
Sodium-dependent dopamine transporter	SLC6A3	17	0.004	Nervous system diseases
Sodium-dependent noradrenaline transporter	SLC6A2	14	0.001	Behavior and behavior mechanisms
Sodium-dependent serotonin transporter	SLC6A4	25	0.007	Mental disorders
Stromelysin-1	MMP3	28	0.005	Cardiovascular diseases, digestive system diseases, endocrine system diseases, female urogenital diseases and pregnancy complications, male urogenital diseases, musculoskeletal diseases, nervous system diseases
Urokinase-type plasminogen activator	PLAU	32	0.013	Neoplasms
Cytochrome P450-cam	camC	1	0	ND
Gamma-aminobutyric-acid receptor subunit alpha-5	GABRA5	25	0.003	ND
Gag-Pol polyprotein	gag-pol	24	0.008	ND
(3R)-hydroxymyristoyl-acyl carrier protein dehydratase	fabZ	11	0.0002	ND
Heat shock protein HSP 90-alpha	HSP90AA1	19	0.002	ND
Ig gamma-1 chain C region	IGHG1	29	0.008	ND
Ig gamma-2 chain C region	IGHG2	28	0.005	ND
Nuclear receptor coactivator 1	NCOA1	23	0.003	ND
cAMP-specific 3′,5′-cyclic phosphodiesterase 4B	PDE4B	25	0.003	ND
cAMP-specific 3′,5′-cyclic phosphodiesterase 4D	PDE4D	22	0.002	ND
cAMP-dependent protein kinase catalytic subunit alpha	PRKACA	24	0.004	ND
Trypsin-1	PRSS1	42	0.052	ND
Tyrosine-protein phosphatase non-receptor type 1	PTPN1	30	0.014	ND
Proto-oncogene serine/threonine-protein kinase Pim-1	PIM1	20	0.003	ND

ND: no related diseases in TTD database.

proteins, which play hub roles (have high dgree and/or high betweenness centrality) in the network, are mainly selected to illustrate the pharmacological activities of this formula. Especially, the compunds and targets with high binding affinity (SVM score >0.9 and RF score >0.9) in the drug-targeting model (Yu et al., 2012) are also included. Finally, a total of 92 highly-connected nodes include 35 candidate drugs (Table 2) and 57 potential proteins (Table 3) are used to construct a core Drug–Target Network (cD–T Network, as seen in Fig. 6 and Supporting information Table S2). Fig. 6 shows the global view of the cD–T Network with color-coded nodes: Eph (red), RC (magenta), SAA (purple), RG (cyan) and targets (yellow). It shows that each herb can connect with most of the targets, indicating the potential synergistic effects among them.

3.4.2. Target–Disease Network (T–D Network)

The T–D Network linking potential targets and diseases is constructed for exploring the protein interactions and the therapeutic targets for diseases. It has been proven that different diseases might share common symptom thus potentially be cured by the same formula. In other words, one formula might be used to treat multiple diseases (Jiang, 2005). As shown in Fig. 7, 44 target proteins (circle, jasper) are connected to various kinds of diseases (square, purple), 35 of which have at least one link to other diseases, such as Nervous System Diseases, Pathological Conditions, Signs and Symptoms, Respiratory Tract Diseases, Cardiovascular Diseases and so on.

It is shown that inducing sweating and reducing cough and asthma are the two main functions of MHD (Can and Cheng, 2010). The target analysis shows that neurotransmitters, such as adrenergic receptors (ADRs), dopamine receptor, Histamine H1 receptor, Muscarinic acetylcholine receptor, etc. (Hösli and Hösli, 1993), are responsible for regulating the process of sweating (Liu et al., 2006). In addition, many compounds such as 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin (RG.11) and neoglycyrol (RG.6) are found to hit heat shock protein HSP 90-alpha (HSP90AA1), a kind of stress protein which allows cells to adapt to gradual changes in their environment and to survive in lethal conditions (Garrido et al., 2001). Therefore, it is deduced that RG may activate and promote the expression of such proteins to release disease syndromes.

Fourteen of 44 target proteins (as shown in Table 3) have effects on Respiratory Tract Disease (RTD), including cough and asthma. *Herba Ephedra* is one of the most frequently used oral treatment for cold both in TCM and in conventional Western

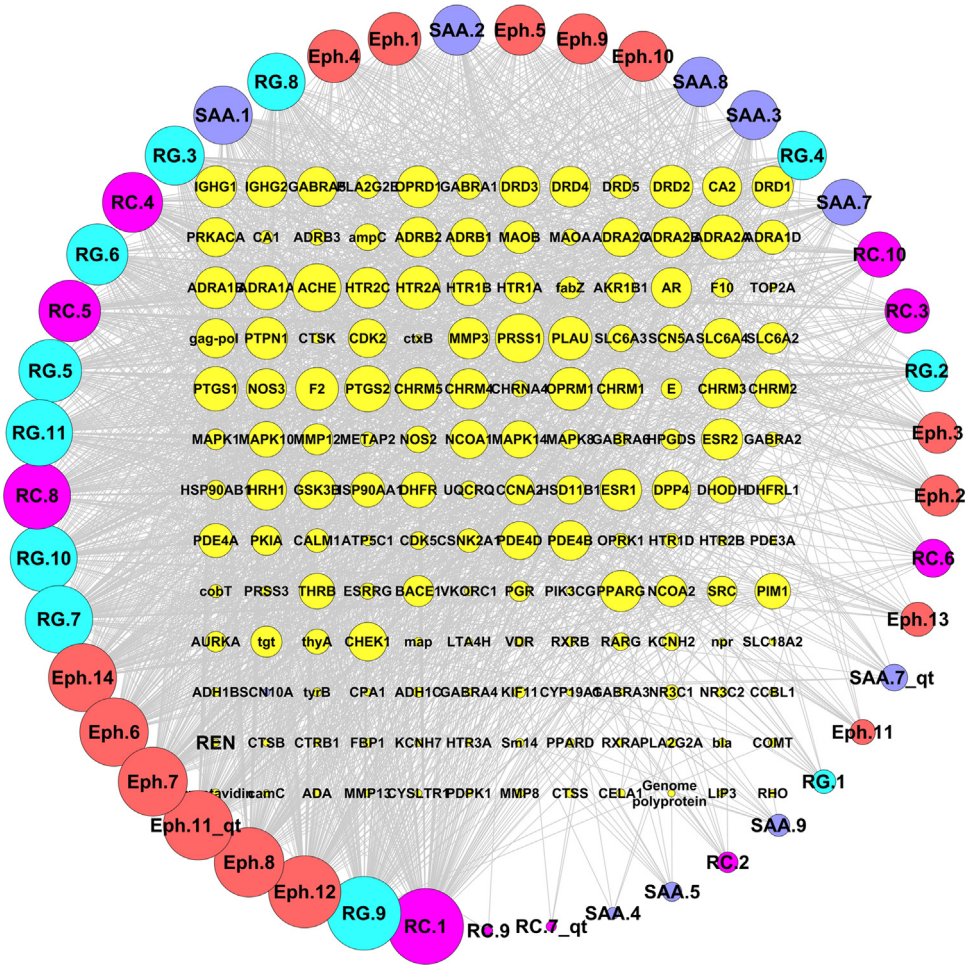


Fig. 6. core Drug–Target Network (cD–T Network). The cD–T Network is constructed by linking the most potential drugs and targets which have high degree and high betweenness centrality, and consists of 92 nodes and 1049 edges, with 36 candicate drugs and 56 potential targets. The area of the node is proportional to the degree. The yellow circles are the common targets of all four herbs, illustrating the synergistic effect in the mixture. (Eph, *Herba Ephedrae*; RC, *Ramulus Cinnamomi*; SAA, *Semen Armeniacae Amarum*; RG, *Radix Glycyrrhizae*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

medicine (Graham and Blaiss, 2000). Interestingly, we found that the mechanism of action of anti-asthma is closely related to sweating, i.e. beta-1 adrenergic receptor and beta-2 adrenergic receptor are targeted by ephedrine (Eph.1) and pseudoephedrine (Eph.4) (Vansal and Feller, 1999). In addition, isoliquiritigenin (RG.9) and 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin (RG.11) from RG are found to hit cAMP-specific 3′,5′-cyclic phosphodiesterase 4B (PDE4B) and peroxisome proliferator-activated receptor gamma (PPARG), which are respectively responsible for asthma. And estrone (SAA.1) from SAA can treat cough by hitting delta-type opioid receptor (OPRD1). In conclusion, we believe that Eph possibly plays the leading role in treating respiratory tract diseases, while RG and SAA (especially RG) play supplementary roles in this formula.

Quite apart from the above-mentioned main functions of MHD, we still find that this formula has many other important pharmacological effects (Ames et al., 1981; Jenkins et al., 2002; Andraws et al., 2005; Asl and Hosseinzadeh, 2008). For instance, 21 of the 44 target proteins are related with cardiovascular diseases which are mainly hit by ingredients from Eph, SAA and RG. For example, estrone (SAA.1) from SAA targets estrogen receptor beta (ESR2), the adrenergic receptor is hit by ephedrine (Eph.1) and pseudoephedrine (Eph.4) from Eph, isoliquiritigenin (RG.9), licochalcone B (RG.10), and 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin (RG.11) from RG. Nineteen drug targets are found relevant with inflammation, nausea and vomiting, and so forth (pathological conditions, signs and symptoms).

4. Discussion

Combination therapy is a fundamental principle of Traditional Chinese Medicine, which is developed for the purpose of maximizing the efficacy and minimizing the adverse effects or toxicity (Sucher, 2013). TCM prescription is produced on the assumption of *Jun–Chen–Zuo–Shi*, according to the different functions of ingredients in the formula (Lin and Li, 2009). An herbal formula is not a simple quantitative addition of different herbs (Jia et al., 2004), instead, all the herbs of a defined prescription should correspond exactly to the related diseases, showing a significantly better effect than the constituent herb used alone (Scholey and Kennedy, 2002). Thus, approach to investigate the possible occurrences and modes of synergistic actions of herbal ingredients is needed.

Recently, there is an increasing focus on validating and explaining the combination principle of TCM by different modern methods, such as the component combination (Liu and Su, 2009), *in silico* modeling (Wang et al., 2005) and so on. All these are important to deepen our knowledge of the combination nature

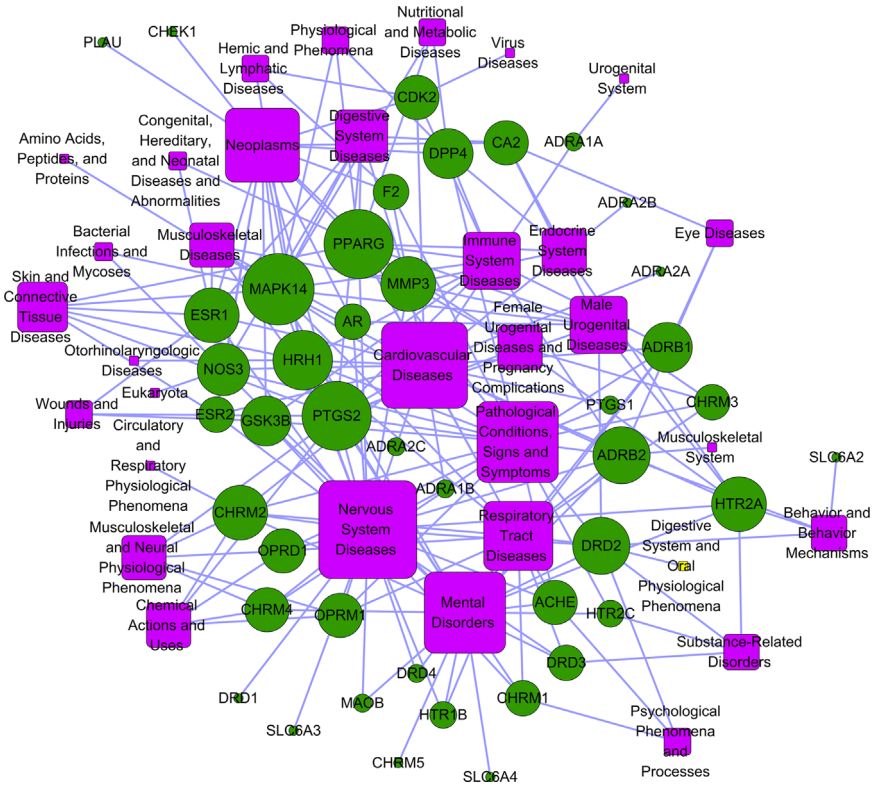


Fig. 7. Target–Disease Network (T–D Network). In T–D Network, potential drugs are connected with those related diseases. Forty-four target proteins (circle, jasper) are connected to various kinds of diseases (square, purple). (Eph, *Herba Ephedrae*; RC, *Ramulus Cinnamomi*; SAA, *Semen Armeniacae Amarum*; RG, *Radix Glycyrrhizae*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of TCM. In this work, the systems pharmacology method is introduced and applied to investigate TCM, which possibly allows us to understand the combination rule of herbal formulae from the active chemical, target proteins, disease networks perspectives. However, our study still has some limitations. The model used for the oral bioavailability screening is developed for single chemical entities which does not take into account any processing of the compounds in the gut that may be the result of other compounds in the formula. And the druglikeness evaluation approach, which based on known Western drugs, might also partial miss out on the chance that Chinese medicine might contain compounds with different properties which still have effects.

4.1. The reasonability of the combination rule of Jun–Chen–Zuo–Shi

Usually, a formula consists of several medicinal herbs, in which one represents the principal component, and the others serve as adjuvant to increase the therapeutic effectiveness or facilitate the delivery of the principal component in the TCM theory (Wang et al., 2008). It is known that nearly 100,000 formulae have been recorded in TCM books such as *Treatise on Cold Pathogenic Diseases*, *Puji Fang* and so on. An interesting and well clinical validated example is “Decoction of Four Noble Ingredients”. In this formula, *Radix ginseng* (Ren-shen) is used to strengthen the spleen, as the *Jun* drug; *Rhizoma Atractylodis Macrocephalae* (Bai-shu) serves as the *Chen* drug to invigorate the spleen and eliminate the “dampness”; *Poria* (Fu-ling) is used for eliminating the “dampness” and strengthening the spleen, and therefore, as the *Zuo* drug; and *Radix Glycyrrhizae* (Gan-cao) serves as the *Shi* drug to mediate the therapeutic actions of the other three components (Jia et al., 2004). However, the mechanism involved in this combination is still unclear from either molecular or systems levels.

In trying to address the possible curative effects of formulae with the current systems pharmacology approach, the MHD is selected as a model and the proposed principle of herbal combination is displayed in Fig. 8. Eph, as *Jun* herb in MHD, is mainly used to relive the common cold-related key syndromes, such as fever, headache, asthma and inflammation. RC is applied as a *Chen* herb, with SAA and RG as *Zuo*, *Shi* herbs in this formula respectively to modulate or augment the therapeutic effects of Eph through targeting the same receptors such as ADRs, PDE4B, PPARG and OPRD1. Interestingly, the target network analysis demonstrates an unexpected mechanism of MHD for cold therapy, i.e., multiple components, such as ephedrine (Eph.1), pseudoephedrine (Eph.4) and methylpseudoephedrine (Eph.10), coumarin (RC.1), cinnamaldehyde (RC.6), cinnamicacid (RC.10), estrone (SAA.1), liquiritin (RG.5), phaseol (RG.8) and isoliquiritigenin (RG.9), interact with the same key targets ADRs.

4.1.1. “Jun”, the sovereign drug targeting illness

In consideration of the high pertinence and remarkable efficacy, *Jun* herb plays a leading role in the formula for treating diseases. For example, Realgar-Indigo naturalis formula has been proven effective in treating human acute promyelocytic leukemia due to the primary efficacy of the *Jun* herb Realgar (Wang et al., 2008). Tetraarsenic tetrasulfide, which belongs to Realgar, serves as the principal component of the formula to enhance the promyelocytic leukemia-retinoic acid receptor degradation and therapeutic efficacy (Wang et al., 2008). In more than 10,000 traditional Chinese herbal formulae, it is found that *Jun* herb usually has two features: the highest dose and low toxicity in the mixture (Yi and Chang, 2004). However, this is only a clinic experience-derived theory which cannot uncover the molecular mechanism in building the herbal combination.

From a molecular level, the present work shows that the *Jun* herb possesses two extraordinary characteristics: many active molecules and powerful pharmacological effects. The OB screening

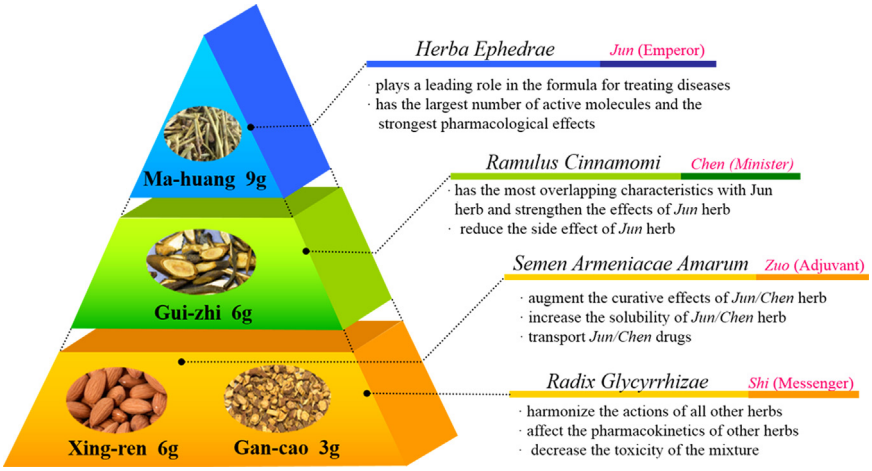


Fig. 8. The diagram of the combination principle of MHD. Eph, as *Jun* herb in MHD, is mainly used to relive the common cold-related key syndromes, such as fever, headache, asthma and inflammation. RC is applied as a *Chen* herb with SAA and RG as *Zuo*, *Shi* herbs respectively in this formula to modulate or augment the therapeutic effects.

Table 4
The number of molecules in each molecule database.

Herb	No. of ingredients	OB ≥ 30% (%)	DL ≥ 0.18 (%)	Dose ratio	Relative blood concentration (OB × dose ratio/8) (%)
Herba Ephedrae	237	63.3	8.4	3	23.7
Ramulus Cinnamomi	117	57.3	7.7	2	14.3
Semen Armeniacae Amarum	86	40.7	33.7	2	10.2
Radix Glycyrrhizae	288	52.8	76.0	1	6.6

shows that Eph has 150 potential active chemicals (Table 4), whose relative concentration of Eph active components in blood circulation is the maximum as up to 23.7%. In the network analysis, it is also found that Eph is most connected to those targets involved in the treatment of cold disease, as exemplified by the adrenergic receptors (Fig. 4 and Table S2). Moreover, many targets of the active compounds like leucopelargonidin (Eph.6), kaempferol (Eph.12), leucocyanidin (Eph.14), etc., play a central role (hubs) in the Drug–Target Network (as seen in Fig. 4), which are the main regulators controlling various singling pathways (Bai and Abernethy, 2013). All these provide strong evidence for explaining why Eph plays a *Jun* role for the potency of MHD.

4.1.2. "Chen", the minister drug boosting the Jun functions

For a *Chen* herb, the primary mission is to assist the *Jun* herb in the TCM theory. Take Dang-gui-bu-xue decoction as an example, it is demonstrated that *Chen* herb *Angelica* can increase the vitality of the active component Astragaloside contained in the *Jun* herb *Astraglus*, leading to the improvement pharmacological action of *Jun* herb (Wang et al., 2009). In our previous study, it is also proved that *Panax notoginseng* is used as the *Chen* drug to enhance the effectiveness of *Radix Salviae Miltiorrhizae* in the compound Dansen Formula by sharing most drug targets (X. Li et al., 2012).

In this work, by compound structural comparisons and target analysis, to certain extent, Eph and RC show similar physicochemical features in ingredients (Fig. 1) and have many overlapping drug targets. This indicates that Eph and RC could produce enhancing pharmacological synergism, due to that two drugs direct at a similar receptor target or physiological system (Spinella, 2002). It is known that, overdose of single Eph to treat cold disease is not clinically suggested, since it may cause severe side effects on nervous system (Abourashed et al., 2003). This might be the main reason why RC is added in this formula in hundreds of years' clinical practice, as it can enhance the pharmacological actions, and meanwhile reduce the side effects of Eph. In addition, we also notice that RC is rich in essential oils, which may serve as solvents to increase the Eph solubility in water, thus improving the overall bioavailability of the lipophilic ingredients in the mixture. All these might help us understand why RC can act as a minister herb to improve the pharmacological actions of Eph.

4.1.3. "Zuo", the auxiliary drug assisting Jun/Chen herb functions

The main role that *Zuo* herb plays in a formula is to increase the pharmacological effects of *Jun*/*Chen* herb. Normally, the classic method to investigate the *Zuo* herb is the recipe decomposition, which investigates whether *Zuo* herb affects the formula functions by dividing the receipt into different combinations (Xu and Pan, 2009). For example, Borneol, one of the commonly used adjuvant drug in TCM, has been demonstrated to increase the *Jun*/*Chen* functions in many TCM recipes (Yang et al., 2008). As this drug is a good cosolvent, it can increase the bioavailability for many hydrophobic compounds (Wei and Wu, 2010). In addition, this compound can inhibit the P-gp function and improve the permeability of drug through the blood–brain barrier (BBB) (Wei and Wu, 2010).

In MHD, SAA is considered as the *Zuo* herb, which has the lowest number of active ingredients (Table 2) and also target few proteins displayed in Table S2. However, results indicate that SAA might have pharmacokinetic synergy with *Jun*/*Chen* herb, which participates in the processes of drug absorption, distribution, biotransformation, or elimination (Spinella, 2002). And the main findings are (1) SAA contains about 50% lipid-like substances (Nanos et al., 2002), which increase the solubility and absorption of orally administered Eph, thus improving the pharmacological function of the mixture; and (2) through interaction with P-gp and/or CYP3A, this herb affects the ADME of certain chemicals of Eph and RC. For example, stigmasterol (SAA.3) and chlorogenic

acid (SAA.8) are known P-gp/CYP3A4 inhibitors, and cholesterol (SAA.2), eicosadienoic acid (SAA.5), squalene (SAA.6), amygdalin (SAA.7) and eicosenoic acid (SAA.9) are potential inhibitors. The entire results demonstrate that SAA might play an assistant role in promoting the combination effects through pharmacokinetic synergy with other herbs.

4.1.4. "Shi", the messenger drug guiding the drug effect

The two most important roles of *Shi* herb is to harmonize the action of all the other ingredients and to improve their pharmacokinetics. As we know *Herba Menthae* is one of the most commonly used messenger herbs in TCM. Its principal active ingredient, menthol can increase the bioavailability of other mixed compounds (Wang and Xu, 1996). In this work, RG is also applied as a messenger herb (Zhang, 2010), and its role mainly includes three aspects:

(1) RG has broad pharmacological actions. Druglikeness screening and T–D Network analysis reveal that it has broad pharmacological actions such as anti-respiratory disease, anti-inflammation and so on (Liu et al., 2013), thus RG can increase the function of the herb combination. (2) It can decrease the toxicity of the mixture. Chemically, the compound in RG, such as glycyrrhizic acid (RG.12), can react with alkaloids in herbal mixtures to produce precipitate, thus converting toxic to non-toxic compounds. Pharmacologically, compounds neoglycyrol (RG.6) and 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin (RG.11) can bind to HSP 90-alpha, increasing the adaptation ability of the body against the environmental stresses (Liu et al., 2013); and (3) it can affect the ADME of other herbs by pharmacokinetic synergy. 18β-glycyrrhetic acid (RG.1), glyasperin F (RG.2), glabridin (RG.3), shinpterocarpin (RG.4), liquiritin (RG.5), neoglycyrol (RG.6), licopyranocoumarin (RG.7), phaseol (RG.8), and glycyrrhizic acid (RG.12) can interact with P-gp/CYP3A4-mediated drug transport/metabolism, which might affect the pharmacological effects of other herbs due to the drug–drug interactions.

5. Conclusion

TCM is a comprehensive and abstruse subject, and the main therapeutic concept is herb formula which is based on the combination principle of "*Jun–Chen–Zuo–Shi*". Even though the principle has accumulated a mass of clinical experience, it has long since been discredited as a mystery due to the absence of systems-based analysis methods and scientific explanations (Liu et al., 2008). In this work, we successfully develop a systems pharmacology method integrating the pharmacokinetic analysis, Drug–Target Network, and target–disease network to dissect this combination principle.

Due to the fact that there exist thousands of TCM formulae, elucidation of the combinational principles for all formulae is quite difficult. Hence, in this manuscript, we choose Ma-huang Decoction, a widely-used and representative TCM formula, as an example to decipher the combination principles of TCM. The study of Ma-huang Decoction outlines most of the key problems involved in the formula combination rules, and can be regarded as a model for the study of other TCM formulae.

Based on the deep investigation of the function and the synergistic effect of each herb in the molecular/systems level, the combination principle of TCM formula can be explained as follows: *Jun* herb plays a leading role in a formula for treating diseases by acting on the main targets; *Chen* herb enhances the *Jun* pharmacological action by interacting with the common targets of *Jun*, thus leading to the reduction of *Jun* dose in a prescription; and *Zuo–Shi* herbs can improve the bioavailability and decrease the toxicity of *Jun–Chen* herbs. Our results suggest that the systems pharmacology method is an efficient way to study the molecular mechanisms for the combination rule of herbal formulae. It is reasonable to believe that the combination of multidisciplinary technologies in a systems level would be a main avenue for study of the TCM theory.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.09.018.

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