Journal of Ethnopharmacology 151 (2014) 452–460

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Investigation into the mechanism of *Eucommia ulmoides Oliv*. based on a systems pharmacology approach

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ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form
30 October 2013
Accepted 30 October 2013
Available online 13 November 2013

Keywords:
Traditional Chinese Medicine
Eucommia ulmoides Oliv.
Systems pharmacology
Network analysis

ABSTRACT

Ethnopharmacology relevance: Though Traditional Chinese Medicine (TCM) has long been playing a significant role in the maintenance of health for people in Asia as well as many other places, the mechanism of its action still remains ambiguous for most of the plants used in TCM, such as *Eucommia ulmoides Oliv.*, a kind of herb that is widely used to help regulate hypertension and the immune system nowadays. However, its functioning mechanism is still unknown. Thus it is necessary to exploit the mechanism of *Eucommia ulmoides Oliv.*

Methods: A systems pharmacology approach combining drug-likeness evaluation, oral bioavailability prediction, multiple drug targets prediction as well as network pharmacology techniques has been used.

Results: This comprehensive systematic approach helps successfully to identify 41 candidate compounds contained in *Eucommia ulmoides Oliv.* while 39 potential targets hit by these ingredients and helps to uncover the synergistic mechanism of action on a systematic level.

Conclusions: Our work successfully explains the mechanism of the efficiency of *Eucommia ulmoides Oliv.* for the treatment of hypertension and enhancing immune. These results not only provide a new insight for the understanding of the chemical and pharmacological basis of *Eucommia ulmoides Oliv.*, but also provide an efficient way for drug discovery from herbal medicine.

1. Introduction

With a history of more than 2500 years of empirical test and refinement, Traditional Chinese Medicine (TCM) is regarded as a valuable treasure for Asia (Cheung, 2011). It aims to make dysfunctioning living organisms to return back to their normal states with a holistic way that has already played a significant role in the maintenance of health among Asians (Cheung, 2011; Zhao et al., 2009). Elsewhere around the globe, it is common to use TCM as well. It is reported that the United States, who is the largest importer of TCM products from China, spent as much as US$7.6 billion alone in 2010 (Cheung, 2011). Given the importance of TCM, more and more attention has been drawn onto the relating study. However, the working mechanism of TCM which relates to the therapeutic effectiveness is still unknown in most circumstances due to the lack of scientific and technologic approaches (Zhao et al., 2009). Since multiple active components in the herbs may exert synergistic therapeutic efficacies, diversified ingredients in TCM as well as a wide range of related targets complicate the pharmacological research significantly, making it difficult to uncover the mysterious mechanisms using traditional experimental methods (Gu et al., 2009; Wang et al., 2013). Consequently, a comprehensive approach which is able to capture the holistic property of TCM is really needed in research.

As a newly emerged field of pharmacology, systems pharmacology utilizes network analysis to help to understand the mechanism of multiple actions of drugs across multiple scales ranging from molecular and cellular levels to tissue and organism levels (Berger and Iyengar, 2009). Based on genomics, proteomics, metabolomics and some other technological platforms, it is able to study the essence of TCM and the function of herbal ingredients in a holistic way (Tao et al., 2013). Furthermore, a significant area of integration between systems pharmacology and drug discovery is polypharmacology which describes a phenomenon that many effective drugs act on more than one target while these targets

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http://dx.doi.org/10.1016/j.jep.2013.10.067

involve multiple diseases (Boran and Iyengar, 2010; Hopkins, 2007). By mapping the polypharmacology network, both the explicit targets hit by active compounds and additional diseases related with important drug targets might be uncovered (Hopkins, 2007). Thus the application of systems pharmacology to TCM provides new possibilities to understand the interactions among active ingredients of herbs, relevant targets and various diseases which in turn highlights the mechanism of action. Presently, we will use a previously developed robust model based on systems pharmacology to shed light on the mystery of one of the oldest TCMs, namely, *Eucommia ulmoides Oliv.* (Li et al., 2012b).

Eucommia ulmoides Oliv. (also known as *Du-Zhong* or *Tu-chung*) is the dehydrated bark derived from a 15–20-year-old *Eucommia ulmoides Oliv.* tree (Lee et al., 2005). It is one of the oldest traditional Chinese medicinal herbs distributed mainly in Shanxi, Hunan, Hubei, Sichuan and Yunnan Provinces and it has been widely used in China, Japan, Korea and some other countries (Lee et al., 2005; Luo et al., 2010). According to the ancient records of Chinese medicinal herbs namely *Shennong Bencao Jing* and *Bencao Gangmu*, the pharmacological effects of *Eucommia ulmoides Oliv.* were used as a tonic for liver and kidney, preventing miscarriage and senescence, regulating blood pressure etc (Hu, 1996). Nowadays, *Eucommia ulmoides Oliv.* has been applied to treat diseases due to its amelioration of hypertension as well as its enhancing effects on the immune system. However, several fundamental questions still remain uncovered including: 1) what the active ingredients of the herb are; 2) what explicit targets the active ingredients hit; and 3) how these components exert effects on various diseases.

In the present work, an integrated model of systems pharmacology developed in our previous study that combines drug-likeness evaluation, oral bioavailability prediction, multiple drug targets prediction as well as network pharmacology techniques has been used to cope with the above questions (Li et al., 2012b). We firstly identified its candidate compounds (more likely to be the active ingredients) and potential targets, and then mapped these compounds and targets onto functional ontologies to generate several drug-target-disease networks which help to understand the holistic and synergic essence of the herbal medicine from a systematic point of view. The obtained results may not only provide new insights for a deeper understanding of the chemical and pharmacological basis of *Eucommia ulmoides Oliv.*, but also provide an efficient way for drug discovery from herbal medicine.

2. Materials and methods

2.1. Database building

After discarding those compounds with unknown structures, we finally obtained a total of 166 compounds as *Eucommia ulmoides Oliv.*'s ingredients from our own database: TcmSP: Traditional Chinese Medicines for Systems Pharmacology Database and Analysis Platform (http://tcmspnw.com). As a chemically oriented herbal encyclopedia, TcmSP is capable of providing detailed and up-to-date information about herbal components and structural data. These ingredients were saved as mol2 format for further analysis and were optimized by Sybyl 6.9 (Tripos Associates, St. Louis, MO) with the same parameters as described in our previous work (Zhang et al., 2011). Considering the process of oral administrated Chinese herb, the glycosyls of the ingredients will be deglycosylated by enteric bacteria in the intestine (Németh et al., 2003). As a result, 50 components of *Eucommia ulmoides Oliv.* with glycosyls were deglycosylated according to the rule of glycosidase hydrolysis reaction. The obtained products were further optimized by procedures as mentioned above. All detailed information about these ingredients is provided in Table S1.

2.2. Drug-likeness evaluation

Given the costs, length and complexity of the drug-discovery process, it is vital to recognize what a drug is like. In order to obtain drug-like compounds, a database-dependent model was applied to calculate the drug-likeness (see Eq. (2.1)) of each compound by using the Tanimoto coefficient (Willett et al., 1998).

$$DL(a,b)=\frac{ab}{\|a\|^2+\|b\|^2-ab} \tag{2.1}$$

where a is the molecular property of each compound while b represents the average molecular properties of the whole compounds in the Drugbank database (http://www.drugbank.ca/). The Drugbank database is a unique bioinformatics and cheminformatics resource that contains 6511 compound data. These compounds are either FDA approved drugs or chemicals under clinical trials which have a great possibility to become licensed drugs. When calculating their average drug-likeness index, a value of 0.18 was obtained (Liu et al., 2013). Thus we assume if one compound has drug-likeness larger than 0.18, it is more likely to be developed into a drug in the future. Therefore, $DL > 0.18$ is regarded as one threshold for screening possible candidate drugs presently.

2.3. Oral bioavailability (OB) prediction

Oral bioavailability, one of the most important pharmacokinetic parameters, represents the speed of a drug to become available to the body and the eventually absorbed extent of the oral dose (Turner et al., 2004). Since poor OB is indeed a main reason responsible for the unsuccessful development of compounds into therapeutic drugs in drug screening cascades, it is valuable to conduct oral bioavailability screening on the compounds (Ahmed and Ramakrishnan, 2012; Veber et al., 2002). However, the prediction of OB is very challenging because of the complex function of many biological and physicochemical factors affecting bioavailability (Wang et al., 2013). However, thanks to the rapid development of computational chemistry, we have developed a novel and robust in-house software OBioavail1.1 to predict the oral bioavailability for each compound (Xu et al., 2012). This software was built based upon 805 structurally different drugs and drug-like molecules and was integrated with the transport (P-glycoprotein) and metabolism (P450 3A4) information. According to previous standard Wang et al. developed (Wang et al., 2012), $OB > 40\%$ was selected as another screening threshold and the qualified molecules satisfying both DL and OB thresholds were regarded as candidate compounds. The OB criterion used here mainly focuses on two principles: (1) Using the least ingredients to extract information from *Eucommia ulmoides Oliv.* as much as possible; (2) The obtained model will be explained by the reported pharmacological data reasonably.

2.4. Target prediction

Considering the fact that most drugs act by binding to specific proteins and some Traditional Chinese Medicines might target multiple proteins due to the existence of its multiple active components, target identification is helpful to elucidate the mechanism of action of *Eucommia ulmoides Oliv.* through the analysis of drug-target and target-disease networks (Gu et al., 2009; Yıldırım et al., 2007). In the present work, based on a systematic model developed in our previous work, we predicted the candidate targets by using candidate ingredients of the herb (Yu et al., 2012). This model was built based upon 6511 drugs and 3999 targets with known compound-protein interactions in Drugbank database (http://www.drugbank.ca/). The chemical, genomic and pharmacological information for drug target was integrated on a large scale and two powerful

mathematical methods namely Random Forest (RF) and Support Vector Machine (SVM) were applied. As an effective tool in property prediction, RF is a classification and regression algorithm (Breiman, 2001). It can be developed as a classifier which consists of a number of decision trees while each tree casts a unit vote for the most popular class (Breiman, 2001). When dealing with the prediction of drug-target interactions, RF is more robust against the overfitting problem and runs more efficiently on large dimensional datasets (Yu et al., 2012). SVM is another promising classification and regression method (Sun et al., 2008), which maps the input vectors to a very high-dimensional feature space non-linearly to get a maximal margin hyperplane (Cortes andVapnik, 1995; Yu et al., 2012). Because of its good performance in dealing with linearly non-separable problems, SVM has also been applied to predict the interaction between drugs and proteins (Yu et al., 2012). Presently, the Random Forest soft package (http://www.stat.berkeley.edu/users/breiman/) and the LIBSVM suite of programs (http://www.csie.ntu.edu.tw/~cjlin/libsvm) were used to build the RF and SVM prediction models, respectively. The performance of the predicted models is impressive with a concordance of 85.83%, a sensitivity of 79.62%, a specificity of 84.12% for the RF optimal model and a concordance of 70.80%, a sensitivity of 41.78%, a specificity of 92.76% for the SVM optimal model. In this work, the overlap targets between the RF and SVM models located in top 50 ranking targets were treated as candidate targets. These targets were further validated by the Uniprot (http://www.uniprot.org/) (Bairoch et al., 2005), Drugbank (http://www.drugbank.ca/) (Wishart et al., 2008) and TTD (http://bidd.nus.edu.sg/group/ttd/) (Zhu et al., 2010). Taking the Uniprot as an example, one mission of Uniprot is to provide a comprehensive, high-quality and freely accessible resource of functional information about proteins. The name of each candidate target was inputted into the search box of Uniprot database to get its functional annotation. Then we would check if its functional information is relevant with hypertension or regulation of immune system.

2.5. Drug-target-disease network construction

To elucidate the multicomponent therapeutic mechanism of *Eucommia ulmoides Oliv.* in the treatment of hypertension and the enhancement of immune function from a network target perspective, we constructed two visualized networks: candidate compound-potential target network (C-T network) and potential target-disease network (T-D network), to understand the holistic and synergic essence of herbal medicine from a systematic point of view (Zhao et al., 2009). The C-T network was constructed by linking the candidate compounds to the corresponding validated potential targets while the T-D network was built by linking the potential targets to their relevant diseases. Information on the disease was mined by mapping the terminology of the potential targets to related disease in the TTD database. The obtained 132 diseases were then classified into 14 disease categories by projecting into the Medical Subject Headings (http://www.nlm.nih.gov).

Both networks were generated by Cytoscape 2.8.1, an open source of bioinformatic package for biological network analysis and visualization (Smoot et al., 2011). In this graphic network, a node represents a candidate compound, a target or a disease, while an edge encodes the interaction of candidate compound-potential target or potential target-disease. In order to disclose the importance of a node and how this node affects the signal transmission of related nodes, two statistical parameters namely degree and betweenness were applied to the analysis of the obtained network. The former is defined as the number of which a node connects to, while the latter is the ratio of the number of shortest paths passing through a node to the number of total paths passing through the nodes (Azuaje et al., 2011).

3. Results and discussion

Traditional Chinese Medicine, developed through thousands of years of observation and accumulated experience, poses a stable therapeutic effect in a more holistic way when compared with western medicine (Zhao et al., 2009). However, due to the great number of different biological components contained in each herbal medicine as well as the complex background theories of TCM, the therapeutic mechanism is still ambiguous for most of the medicinal plants in the TCM research (Tao et al., 2013). *Eucommia ulmoides Oliv.*, one of the traditionally used herbal medicines, has been widely used as an anti-hypertensive and enhancing immune medicine (Ha et al., 2003; Takeshi et al., 2001). In our work, a novel systems pharmacology approach integrated with chemogenomics, polypharmacology and network biology was applied to uncover the therapeutic mechanism of *Eucommia ulmoides Oliv.* from a systematic level.

3.1. Drug-likeness and oral bioavailability evaluation

Drug-likeness (DL), a qualitative property of chemicals, means that how pharmacokinetic and pharmaceutical properties of compounds like solubility and potency correspond to the majority of known drugs, in brief how “drug like” a prospective chemical is (Ursu et al., 2011; Walters and Murcko, 2002). Considering the value of drug-likeness on saving time and costs in the process of drug discovery, it was used to filter out compounds with undesirable pharmaceutical properties in the database (Ursu et al., 2011). In addition, apart from drug-likeness, oral bioavailability (OB) which represents the percentage of an oral dose that reaches the systemic circulation and produces a pharmacological effect, is also an important pharmacokinetic parameter to screen candidate compounds which have the potential to be further developed into drugs (Li et al., 2012b). It has been reported that most chemicals in the TCM fail to reach the active site and produce a pharmacological effect because of their poor pharmacokinetic properties, especially the OB (Li et al., 2012b; Yang et al., 2007). Consequently, predicting the OB of the known compounds of *Eucommia ulmoides Oliv.* is necessary in order to obtain candidate compounds that play key roles in the pharmacodynamic process.

The obtained result shows that 32 candidate compounds (Table S2) were screened from the 166 chemicals (19.3%) which possess not only satisfactory drug-likeness (≥ 0.18), but also OB ($\geq 40.0\%$). Interestingly, it has been proved that some of the candidate compounds possess good pharmacological effects on hypertension or the regulation of immune system. For instance, tabernaemontanine (M5, DL=0.61, *OB*=58.7%), pinoresinol O-β-D-glucopyranoside (M18, DL=0.52, OB=100%), rutin (M87, DL=0.28, OB=47.0%) and dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucopyranoside (M114, DL=0.39, OB=48.4%) were reported to exhibit anti-hypertension activity (Ji, 2011; Luo et al., 2006). Besides, for 1-Hydroxy-pinoresinol 4′,4′′-di-O-β-D-glucopyranoside (M6, DL=0.55, OB=100%), medioresinol di-O-β-D-glucopyranoside (M16, DL=0.62, OB=87.2%) and syringaresinol di-O-β-D-glucopyranoside (M74, DL=0.72, OB=66.9%), they present potently inhibitory effect on cAMP phosphodiesterase, that brings about the improvement of cAMP's concentration in the vascular smooth muscle, thus activates protein kinase A, which helps to expand the blood vessels and reduce the blood pressure by regulating the flow of calcium (Deyama et al., 1988; Luo et al., 2006; Wei et al., 1995). In addition, some other candidate compounds like epipinoresinol (M11, DL=0.52, OB=68.2%) and syringaresinol O-β-D-glucopyranoside (M20, DL=0.72, OB=56.4%) help to kill the pathogens by antibodies and thus improve the defensive ability of immune system (Wei et al., 1995; Yoshitebua et al., 1988) while kaempferol (M71, DL=0.24, OB=67.4%) has a regulatory effect on the

immune system by regulating the activation and proliferation of T cell and the phagocytic function of macrophage (Mu, 2010).

It is worth noting that chlorogenic acid (M37, DL=0.33), one of the most abundant ingredients in *Eucommia ulmoides Oliv.*, shows a relative poor OB value of 11.9% even though this compound enables to present effective anti-hypertension, antiviral, antioxidant activities (Cheng, 2006; Liu and Qiu, 2003; Li et al., 2010). This can be explained by the previous study that the oral absorption of chlorogenic acid is relatively low and most of the chlorogenic acid has been biotransformed into various metabolites by the microbe in the colon (Wu et al., 2006). In addition, despite that quercetin is able to flexibly regulate blood pressure according to the concentration of itself, the OB of quercetin (M86, DL=0.28) is as low as 11.4% (Luo et al., 2006). This is consistent with the fact that quercetin is able to be absorbed by human but the OB value is less than 30% because of its special compound structure (Wang, 2006). As a result, these two compounds should be taken into account in the following discussion.

After observing the obtained data and reading relative published papers carefully, we found that ajugoside (M28, DL=0.10, OB=78.5%), alkavervir (M29, DL=0.04, OB=51.7%), aucubin (M31, DL=0.06, OB=100%), coniferol (M43, DL=0.04, OB=53.7%), genipin (M59, DL=0.10, OB=69.5%), and asperuloside (M122, DL=0.17, OB=69.8%) present either potent anti-hypertension activity or the ability to promote the immune system, with relatively high OB but poor DL (Cheng, 2006; Luo et al., 2006; Tang et al., 1998; Yang et al., 1995). In addition, according to the report from University of Wisconsin, pinoresinol-di-O-β-D-glucoside (M17, DL=0.52, OB=4.2%) was tested on the anesthetized hypertensive rats and identified as the major antihypertensive principle of *Eucommia ulmoides Oliv.* (Sih et al., 1976). Thus, the seven ingredients of *Eucommia ulmoides Oliv.* were also regarded as candidate compounds. To sum it up, 41 ingredients of *Eucommia ulmoides Oliv.* are finally screened and regarded as candidate compounds.

3.2. Target identification

Since TCM contains a lot of pharmacologically effective compounds, a pivotal challenge lies in the identification of molecular targets which will help to shed light on the mechanism of TCM's action in a system-level view. Traditionally, the sequencing of expressed-sequence tags (ESTs), serial analysis of gene expression (SAGE) differential display, homology cloning and relevant approaches have been used to confirm the therapeutic targets of drugs through experiments (Fryer et al., 2002). However, given the time-consuming, expensive, challenging feature and a narrow application scope of this process, computational methods should be the first choice (Lomenick et al., 2010). Thanks to the application of our systematic model based on the RF and SVM methods, presently the binding of candidate components of *Eucommia ulmoides Oliv.* to those targets that are related with hypertension and/or the enhancement of immune system have been studied.

The 41 candidate compounds yield 103 candidate targets (Table S3) and the connections between them reach up to 263 (Table S4). The obtained targets were further subjected to the Uniprot, Drugbank and TTD databases to check if they are relevant with hypertension or the regulation of immune system. Finally, 39 potential targets (Table 1) are reserved and 3 candidate compounds (M109, M115, M117) without any relevant targets are removed, indicating that most of the candidate compounds we screened previously have effective therapeutic actions. Furthermore, the average target hit by one compound has been calculated and and ended up with a value of 1.03, showing that the action of some compounds may be promiscuous. For example, kaempferol not only acts as an inhibitor of inducible nitric oxide synthase (Hämäläinen et al., 2007), arachidonate 5-lipoxygenase (Deng et al., 2007) and aldose reductase (Yoo et al., 2008) but also as a weak agonist of estrogen receptor beta (Tang et al., 2008); Astragalin has an effective regulation effect on the expression of inducible nitric oxide synthase (Kim and Kim, 2011) and less selective estrogen receptor beta agonist activity (Yang et al., 2011); Epicatechin and helenalin interact with inducible nitric oxide synthase at the same time (Davis et al., 2005; Kim et al., 2004). These confirm that multiple active constituents in the *Eucommia ulmoides Oliv.* hit two or more targets and may treat diseases in a synergistic manner.

Table 1
The information of 39 potential targets.

No.	Potential target
T9	Aldose reductase
T10	Alpha-1A adrenergic receptor
T11	Alpha-2A adrenergic receptor
T12	Alpha-2B adrenergic receptor
T15	Androgen receptor
T16	Arachidonate 5-lipoxygenase
T17	ATP-binding cassette transporter sub-family C member 8
T19	Beta-1 adrenergic receptor
T20	Beta-2 adrenergic receptor
T24	Calmodulin
T25	cAMP and cAMP-inhibited cGMP 3′,5′-cyclic phosphodiesterase 10A
T27	cAMP-specific 3′,5′-cyclic phosphodiesterase 4D
T31	Cathepsin K
T33	cGMP-inhibited 3′,5′-cyclic phosphodiesterase A
T34	cGMP-specific 3′,5′-cyclic phosphodiesterase
T42	Dipeptidyl peptidase 4
T44	Epidermal growth factor receptor
T45	Estrogen receptor
T46	Estrogen receptor beta
T54	Glucocorticoid receptor
T57	Glycogen synthase kinase-3 beta
T59	Heat shock protein HSP 90-alpha
T60	Heat shock protein HSP 90-beta
T64	Integrin alpha-L
T68	Mineralocorticoid receptor
T69	Mitogen-activated protein kinase 14
T70	Muscarinic acetylcholine receptor M1
T71	Muscarinic acetylcholine receptor M2
T74	Nitric-oxide synthase, inducible
T76	Nitric-oxide synthase, endothelial
T78	Peroxisome proliferator-activated receptor gamma
T79	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma isoform
T81	Prostaglandin G/H synthase 1
T82	Prostaglandin G/H synthase 2
T84	Proto-oncogene tyrosine-protein kinase LCK
T85	Proto-oncogene tyrosine-protein kinase Src
T98	Trypsin-1
T101	Vascular endothelial growth factor receptor 2
T103	Voltage-dependent calcium channel subunit alpha-2/delta-1

3.3. Compound-target-disease network analysis

The well accepted “one drug, one target” theory has been found to be less effective than hoped because of the intrinsic robustness of living systems against various perturbations (Kitano, 2007). Consequently, the focus of drug discovery has shifted to the “multi-drug, multi-target” theory. Interestingly, given the multiple ingredients of TCM and its remarkable pharmacological efficacy in body, this theory may be able to help to uncover the synergistic effect between multi-components and multi-targets. However, due to the method used in the TCM research that focuses mainly on the path of partitioned reductive analysis, the characteristic of the scientific system of a herbal medicine is unable to be captured and the synergistic therapeutic mechanism is still poorly uncovered (Liu et al., 2013). Thanks to the rapid development of network pharmacology recently, the above problems can be solved from a

system point of view. Presently, the network method is applied to detect both the candidate ingredients of *Eucommia ulmoides Oliv.*, and the effective targets interacted with these ingredients as well as to elaborate the mechanism of action of this herbal medicine.

3.3.1. C-T network: detecting key players of Eucommia ulmoides Oliv. for treating diseases

After deleting 3 compounds with no targets, the remaining 38 candidate compounds and their relevant 39 potential targets were adopted to generate a bipartite graph of drug-target interaction network. Fig. 1 shows a global view of C-T network in which green diamonds and red circles represent the candidate compounds and potential targets of *Eucommia ulmoides Oliv.*, respectively. This network consists of 77 nodes (38 candidate compounds and 39 potential targets) and 263 edges. The centralization and heterogeneity of this net are 0.259 and 0.819 respectively, indicating that certain compounds or targets are more central and biased than the others in the network. For example, trypsin-1 (T98) has the largest number of drug interactions while muscarinic acetylcholine receptor M1 (T70) has only one drug interaction.

As a fundamental topological parameter of a network, degree may pinpoint and offer insights into highly influential compounds or targets (Azuaje et al., 2011). Interestingly, the degree of some nodes (Table S5) displays a rather big number of compound-target interactions while others are middle or small. This is consistent with the previous results of centralization and heterogeneity. Astragalin (M30) exhibits the largest number of target connections (16), followed by epipinoresinol (M11) with 15 potential targets and erythraline (M53), kaempferol (M71), isochlorogenic acid C (M144) with 14 potential targets. Similarly, the more central potential targets are trypsin-1 (T98), dipeptidyl peptidase 4 (T42) and heat shock protein HSP 90-alpha (T59) connected by 26, 25 and 18 candidate compounds, respectively. These high-degree nodes are referred to as hubs (Azuaje et al., 2011), suggesting their more important role in helping to treat the hypertension or enhance the immune. Interestingly, of all the 38 candidate compounds, 13 compounds possess degree larger than 10 under an average value of 6.9 in which 7 are reported to be active compounds. The excellent hit rate ($>50\%$) suggests the reliability and rationality of our network analysis to pinpoint the key players of *Eucommia ulmoides Oliv*.

Another basic parameter of network nodes is betweenness, a key measure to assess the relevance of the location of nodes within a network (Goňi et al., 2008). A node is considered central if it involves in lots of paths linking pairs of nodes. Interestingly, we find that values of degree and betweenness are correlated with each other strongly and the node with high betweenness (Table S5) tends to possess large degree. For example, the top ten targets with high betweenness values are trypsin-1 (T98), dipeptidyl peptidase 4 (T42), heat shock protein HSP 90-alpha (T59), estrogen receptor (T45), estrogen receptor beta (T46), androgen receptor (T15), prostaglandin G/H synthase 2 (T82), peroxisome proliferator-activated receptor gamma (T78), glycogen synthase kinase-3 beta

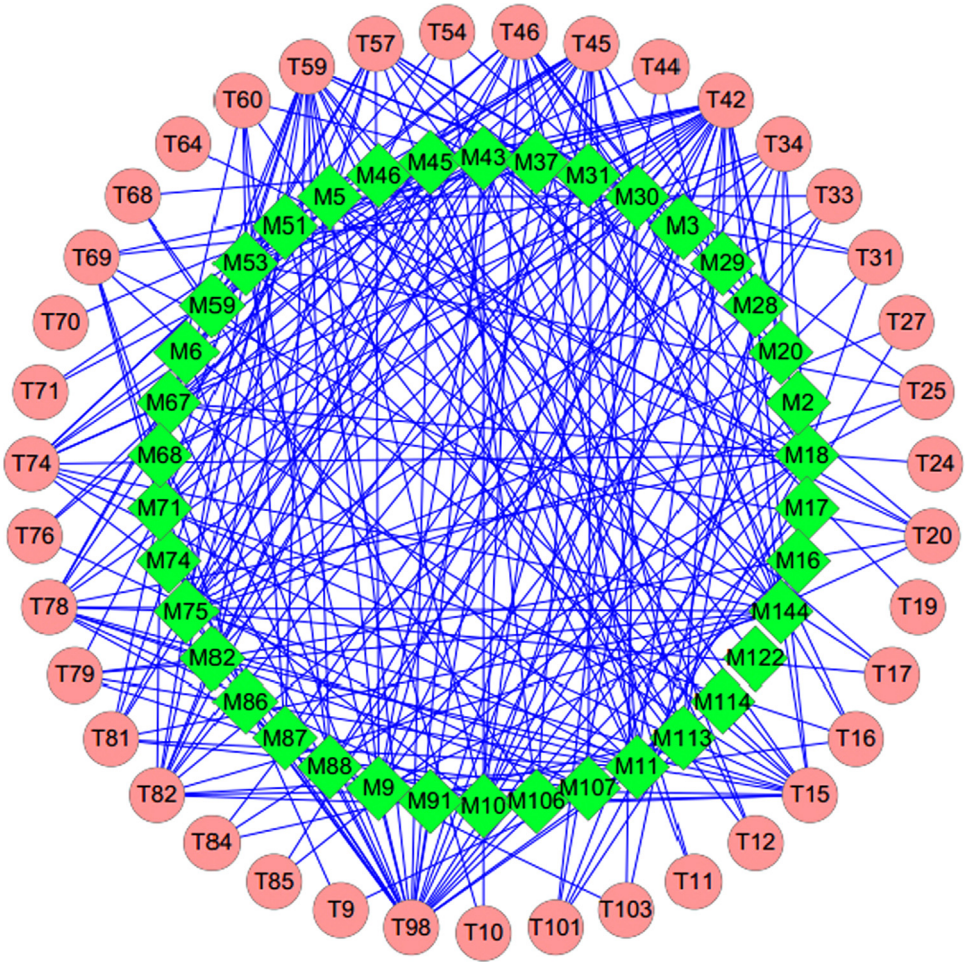

Fig. 1. The global view of C-T network. The green diamond and red circle nodes represent the candidate compounds and potential targets for *Eucommia ulmoides Oliv.*, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(T57) and inducible nitric oxide synthase (T74), that are exactly the same as the top ten targets with large degree except for the slight difference of target order. Consequently, compounds or targets with higher degree and betweenness would be key players of *Eucommia ulmoides Oliv*.

3.3.2. C-T Network: illustrating the mechanism of action of *Eucommia ulmoides Oliv.* on treating diseases based on the compound-target interactions

Based on the holism, a serial of potential targets may be modulated by multiple compounds from TCM in a synergistic manner and a new equilibrium of living system finally achieves with less harmful impact. This "multi-drug, multi-target" theory is probably the therapeutic mechanism of *Eucommia ulmoides Oliv*.

Hypertension, one of the most common causes for cardiovascular and cerebrovascular complications, is widespread in many countries and estimated to occupy 4.5% of current global disease burden (Luo et al., 2010; Whitworth, 2003). As a natural plant for the treatment of hypertension, *Eucommia ulmoides Oliv.* has been widely used in Asia for a long time (Luo et al., 2010). Among all the 39 potential targets, targets concerned with hypertension are estrogen receptor (T45), androgen receptor (T15), prostaglandin G/H synthase 2 (T82), estrogen receptor beta (T46), etc. Among these, estrogen receptor possesses the highest number of compound-target interactions, indicating its key role in the treatment of hypertension. It may increase the expression of endothelial nitric oxide synthase to produce NO by regulating its promoter activity which can spread around to adjacent smooth muscle cells (SMCs) and improve the level of cyclic guanosine monophosphate (cGMP) responsible for dilating SMCs and then lower the blood pressure (Li, 2012; Luo et al., 2010). Estrogen receptor beta, connected with 14 compounds, modulates the expression of numerous vasodilator and vasoconstrictor proteins in which multiple components belong to the renin-angiotensin system (Zhu et al., 2002). As a member of this system, renin hydrolyzes angiotensinogen into angiotensin I that converts into angiotensin II later under the angiotensin-converting enzyme and then angiotensin II affects the development of hypertension as well as its complication via various ways (Luo et al., 2010). Prostaglandin G/H synthase 2, one of the two known isoforms of Prostaglandin G/H synthase (PGHS), is an important enzyme leading to the production of prostaglandins and thromboxane which are critical in the regulation of vasoconstrictive with 13 compound-target interactions (Davidge, 2001). These hypertension relevant targets may synergistically mediate the living system from different part and make the system return back to a healthy state after hitting by a serial of compounds.

After a careful inspection of our network, we find that a number of targets related with large number of compounds are also involved in the enhancement of the immune reaction which is another critical function of *Eucommia ulmoides Oliv.*, including dipeptidyl peptidase 4 (T42), glycogen synthase kinase-3 beta (T57), heat shock protein HSP 90-alpha (T59), inducible nitric oxide synthase (T74), peroxisome proliferator-activated receptor gamma (T78), trypsin-1 (T98), etc. Dipeptidyl peptidase 4 acts as a positive regulator in the co-stimulation process of T cell that enlarges the extent of T cell activation, consequently leading to an enhanced immune response (Pacheco et al., 2005). Glycogen synthase kinase-3 beta and peroxisome proliferator-activated receptor gamma are closely involved with Nuclear factor-kappa B which plays a key role in the expression of numerous cytokines and adhesion molecules helping to improve the immune responses (Chen et al., 2001; Ghosh et al., 1998; Hoeflich et al., 2000). Heat shock protein HSP 90-alpha and trypsin-1 are somewhat related with macrophages that may help to produce potent cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-6 which in turn recruit neutrophils, T lymphocytes and other immune cells beneficial for the immune enhancement (Kim et al., 2012; Koshikawa et al., 1998; Li et al., 2012a). The NO generated by inducible nitric oxide synthase plays an essential role in the modulation of immune system response (Kim et al., 2012). Interestingly, there are a total of 35 compounds interacted with these targets and most of them belong to lignans that may promote the activity of macrophages and the production level of IL-2 by T lymphocytes, thus enhancing the immunologic function (Yu et al., 2008).

3.4. Target-disease network

Given the fact that most complex diseases are triggered by an unbalanced modulating network in which multiple genes or their products stay in a state of dysfunction and that proteins targeted by a drug tend to associate with various diseases, we have constructed T-D network to explore other potential therapeutic effects of *Eucommia ulmoides Oliv*. In this network, 4 potential targets have not been found to have relevant diseases and the left 35 targets achieved 132 diseases that were classified into 14 groups according to the Medical Subject Headings (http://www.nlm.nih.gov). For example, hypertension, angina, atherosclerosis belong to cardiovascular diseases; bone metastases, melanoma, breast cancer pertain to neoplasms. Fig. 2 shows a global view of T-D network in which red circle nodes, green circle nodes and yellow hexagon nodes represent the potential targets, relevant diseases and disease categories respectively.

Among the 132 diseases, we find that most of them belong to neoplasms (39/132), cardiovascular diseases (25/132), immune system diseases (14/132), musculoskeletal diseases (8/132), mental disorders (8/132), respiratory tract diseases (8/132), nervous system diseases (7/132), indicating the potential therapeutic role of *Eucommia ulmoides Oliv.* in the treatment of these diseases. For instance, estrogen receptor beta (T46) which is predicted to be the potential target of kaempferol (M71) may be a therapeutic target for the treatment of a series of diseases, including breast cancer (neoplasms), cardiovascular diseases and neurodegenerative diseases (nervous system diseases) (Rouayrenc et al., 2000). It has been reported that kaempferol could stimulate transcriptional activity of estrogen responsive element in the promoters of target genes through the modulation of estrogen receptor beta (Tang et al., 2008), indicating the possible therapeutic effect of this compound in curing the above mentioned diseases. These results confirm the good performance of our T-D network and provide a clear view of the relationships between candidate ingredients of the herbal and the diseases through the protein targets.

4. Conclusion

Presently, a systems pharmacology methodology integrating drug-likeness evaluation, oral bioavailability prediction, multiple drug targets prediction and network analysis has been applied to the investigation of the therapeutic mechanism of *Eucommia ulmoides Oliv*. Our main findings are as following:

1) 41 ingredients of *Eucommia ulmoides Oliv.* are screened and regarded as candidate compounds. Besides, 39 potential targets hit by these ingredients are identified.
2) The network analysis clearly elucidates the synergistic action mechanism of *Eucommia ulmoides Oliv.* on the treatment of hypertension and the enhancement of immune based on the "multi-drug, multi-target" theory about compound-target interactions.
3) The T-D network displays that *Eucommia ulmoides Oliv.* has certain therapeutic efficiency for the control of other diseases

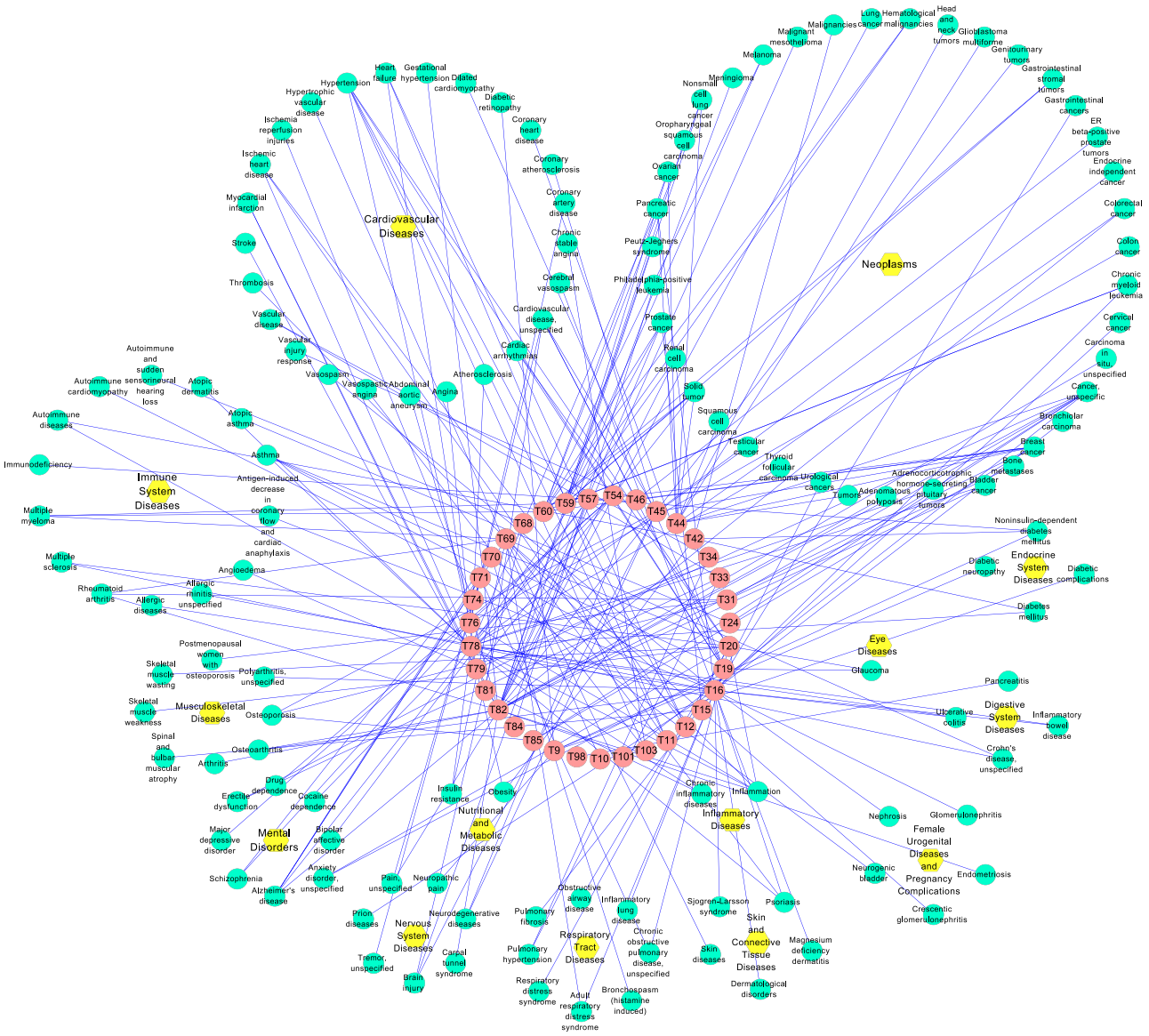

Fig. 2. The global view of T-D network. The red circle nodes, green circle nodes and yellow hexagon nodes represent the potential targets, relevant diseases and disease categories respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

such as neoplasms, cardiovascular diseases, immune system diseases, musculoskeletal diseases, mental disorders, respiratory tract diseases, nervous system diseases, indicating that the medicinal herb may also be applicable in multiple diseases.

4) The present work provides an alternative *in silico* strategy for the deep investigation and understanding of the chemical and pharmacological basis of TCM, which will promote drug discovery from herbal medicines.

Acknowledgments

Thanks for the support given by the high-performance computing platform of Northwest A&F University and the National Natural Science Foundation of China (Grant no. 11201049).

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.2013.10.067.

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