Article

Network pharmacology and component analysis of four herbs decoction molecular mechanism in hypertension treatment

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Received 25 March 2021; *Accepted 5 April 2021*; *Published 1 June 2021*

Abstract

Traditional Chinese Medicines (TCM) are known for their curative effects on hypertension through a holistic approach. The molecular mechanisms of the formulation comprising *Polygonum multiflorum*, *Rehmannia glutinosa*, *Senna obtusifolia* and *Crataegus*, used by Chinese practitioners in ameliorating hypertension, however remain a mystery. This initial study is thus aimed at unveiling the molecular mechanisms of this TCM formulation in treating hypertension. The methanolic extract compounds of the decoction were identified through Liquid chromatography mass spectrometry-mass spectrometry (LC-MS/MS). Oral bioavailability and drug likeness were then measured to filter out identified compounds. Several databases, such as the SwissTargetPrediction, STRING, OMIM and KEGG, were used to retrieve information on the predicted targets for the purpose of developing a network using Cytoscape Version 3.8. Enrichment analysis was then performed to elucidate the mechanisms of the decoction in hypertension mitigation. A total of 11 compounds identified were revealed to possess bioavailable and drug like characteristics, based on the Veber and Quantitative Estimation of Drug-likeness (QED) parameters. Pathway analysis showed enrichment of pathways such as cardiac muscle contraction, fluid shear stress and atherosclerosis, dilated cardiomyopathy, renin-angiotensin system and hypertrophic cardiomyopathy (HCM), which are all strongly associated with hypertension. The network pharmacology analysis clearly shows that this TCM decoction ameliorates hypertension through several indirect pathways where most of the targets are involved in HCM, which is caused by hypertension.

Keywords *Polygonum multiflorum*; *Rehmannia glutinosa*; *Senna obtusifolia; Crataegus;* hypertension; network pharmacological analysis; network medicine; ethnomedicine

Network Biology
ISSN 2220-8879
URL: http://www.iaees.org/publications/journals/nb/online-version.asp
RSS: http://www.iaees.org/publications/journals/nb/rss.xml
E-mail: networkbiology@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

In the category of noncommunicable diseases, cardiovascular disease is the foremost cause of mortality worldwide, accounting for approximately 17.7 million deaths in 2017, more than three quarters of which were from low-income and middle-income countries (GBD 2017 Causes of Death Collaborators, 2018). The severity of cardiovascular disease also causes economic burdens resulting in it being categorized as the most costly disease among noncommunicable diseases, with costs predicted to increase to $368 billion by 2035 (Dunbar et al., 2018). One of the leading causative factors of cardiovascular disease, with its strong association established by numerous studies, is hypertension (Torpy et al., 2003; Xiong et al., 2015). One previous report projected that 1.56 billion adults may develop hypertension by 2025 (Tabrizi et al., 2016). Due to substantial evidence of its role as a silent killer, significant efforts have expended in the identification of critical hypertension levels and ideas for treatments to prevent or halt atherosclerosis and further development of cardiovascular disease (Leening and Ikram, 2018). Western medicine is currently the most common treatment for hypertensive conditions but it is limited by availability, cost and adverse effects (Chobanian et al., 2003). The efficacy and limitation of western drugs in treating such conditions remain unsatisfactory thus it is essential that alternative approaches be considered and explored.

Due to the bottlenecks in drug discovery focus has been shifted from one target to network targets, or more commonly known as multitargets, to derive solutions (Li et al., 2014). In tackling the complexity of the multitarget mechanism, the rapid growth in bioinformatics provides a powerful platform for studying network-based drug discoveries, which is a more cost-effective drug development approach (Zhang et al., 2019). Bioinformatics can be used to mine and comment on the biological information of disease networks (Shi et al., 2014). Further, through utilization of one of its powerful tools, network pharmacology has been widely used to reveal the relationships between compound-targets and correlate it to disease gene targets and pathways, via a sea of data in databases (Liu et al., 2019). Data from network pharmacological analysis, including underlying pathways, genes and protein-protein interaction and integration of these data, can ultimately lead to the formulation of mathematical models to decipher the system biology of humans, in its response to drugs' efficacies (Zhang et al., 2019). The first concept or pioneer of network pharmacological analysis was developed for unraveling the effects of traditional Chinese medicine on system biology (Li et al., 2014).

Traditional Chinese Medicine (TCM) is well known for the use of formulations comprising multiple herbs to treat numerous diseases through a holistic view and multitarget approach (Xiong et al., 2011), concomitant with the pivotal concept of new modes of drug discovery. A wide variety of cases have been studied and randomized trials performed using TCM as treatment for hypertension (Li et al., 2010; Zhong et al., 2011) where some have shown curative effects (Xiong et al., 2011). However, the establishment of evidence for TCM's effects on hypertension is still in progress as the unravelling of the molecular mechanisms of TCM remains limited and vague. There are still a lot of uncertainties about the curative effects of TCM due to its complex multitarget properties in the human system biology. This hampers the development of novel TCM (Zhang et al., 2019) cures, particularly those involving prescriptions which have been passed down from generation to generation verbally by Chinese practitioners.

One such prescription recommended by Chinese practitioners in East Malaysia involves the use of a formulation comprising the combination of *Polygonum multiflorum,* (何首乌 Hé shǒu wū) *Rehmannia glutinosa* (熟地黄 Shú dì huáng), *Senna obtusifolia* (决明子 Jué míng zǐ) and *Crataegus* (山楂 Shān zhā), as medicine for reducing or preventing hypertension. These plants are known for their anti-hyperlipidaemia, hypodynamia and anti-ageing properties (Lin et al., 2015; Wang et al., 2013). They are also high in antioxidants and are antigenotoxic (Weng and Yen, 2015), besides having cardiotonic and cardioprotective

characteristics (Parikh et al., 2015). However, identification of the bioactive compounds and mechanisms of action of this decoction remains undetermined. To ascertain the efficacy of this decoction from a cost-effective perspective, we deciphered its molecular mechanisms in the human system biology through liquid chromatography mass spectrometry-mass spectrometry (LC-MS/MS) screening and the bioinformatic approach, via network pharmacological analysis.

2 Material and Methods

2.1 Preparation of plant materials

Dried samples of the *Polygonum multiflorum,* (何首乌 Hé shǒu wū; Locality: Henan Province, batch number: ECN000274), *Rehmannia glutinosa* (熟地黄 Shú dì huáng; Locality: Shandong Province, batch number: ZY001813), *Senna obtusifolia* (决明子 Jué míng zǐ; Locality: Shaanxi province, batch number: 20181230007) and *Crataegus* (山楂 Shān zhā; Locality: Shandong Province, batch number: 955616020137) were purchased from Traditional Chinese Medicine shop, Ming Kuong Company Sdn Bhd, Kota Kinabalu, Sabah, Malaysia. A combination of the selected plants was then boiled, using the specific amounts recommended by Chinese practitioners. Plant extracts were concentrated under vacuum at 45^0C using a rotary evaporator and subjected to Liquid chromatography mass spectrometry-mass spectrometry (LC/MS-MS) screening for compound identification.

2.2 Liquid chromatography mass spectrometry-mass spectrometry (LC-MS/MS) analysis

The extracts were dissolved in 2 ml of methanol and filtered using 0.22 µm hydrophobic PTFE prior to LC-MS/MS analysis using the Agilent 6520 Accurate Mass Q-TOF LC/MS system. Reverse-phase liquid chromatography was used, with a multistep gradient consisting of Solvent A (water HPLC grade + 0.1% formic acid HPLC grade) and Solvent B (acetonitrile HPLC grade + 0.1% formic acid HPLC grade) at a flow rate of 0.2 mL/min. Data acquisition was performed using positive and negative ionization modes (separate runs) for both profiling (MS1) and fragmentation (MS2), with a detection range of between 100 to 1500 m/z.

2.3 Identification of compounds from databases

The raw data obtained were processed using MZmine 2.53, and only the MS1 features (associated with their respective MS2 spectra) that appeared in at least 2 of the 3 biological replicates were retained and converted into MSP formatted files, using a custom PERL script. MSFinder 3.26 was used for both molecular formula prediction and compound annotation of the MSP (MSFinder) files.

2.4 Oral Bioavalability (OB) and Drug Likeness (DL) screening and establishment of a compound-target network

The identified compounds' oral bioavailability (OB) and drug likeness (DL) were analyzed and calculated using ADMETlab (Dong et al., 2018) and DruLiTo, based on Veber rule (Veber et al., 2002) and Quantitative Estimate of Drug likeness (QED). The bioactive compounds were filtered based on OB≥50% and DL=pass criteria. Predicted target interaction with compounds were obtained through the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) based on chemical similarities (Gfeller et al., 2014) and the relationships of a compound target's (CT) visual interaction network was produced using Cytoscape program Version 3.8 (Shannon et al., 2003). All of the genes' names were standardized using the UniProt database (http://www.uniprot.org/).

2.5 Establishment of a hypertension gene network

Genes related to hypertension or cardiovascular disease were downloaded and identified via the Online Mendelian Inheritance in Man, OMIM (https://omim.org/) database and the human gene database, GeneCards (http://www.genecards.org/), using the keywords 'hypertension' and 'cardiovascular disease related genes of *Homo sapiens'*, while other species were excluded (Amberger et al., 2019; Stelzer et al., 2016). The identified

hypertension or cardiovascular disease genes were then used to construct a protein-protein interaction (PPI) network with the compound target genes.

2.6 Construction of protein-protein interaction (PPI) network

Protein-protein interaction **(**PPI) analysis was performed using STRING 11.0 (https://string-db.org/) database and generated in accordance with systematic co-expression analysis, detection of shared selective signals across genomes and automated text-mining of the scientific literature (Szklarczyk et al., 2019). It was imported to Cytoscape Version 3.8 for network analysis and data visualization through STRINGApp (Doncheva et al., 2019), using the confidence score of 0.7 for higher confidence in the interaction.

2.7 Gene Ontology (GO) functional annotation and KEGG pathway analysis

The mechanisms of action of the decoction in hypertension were deciphered by analyzing the primary pharmacological units through Gene Ontology (GO) (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (https://www.genome.jp/kegg/pathway.html)(Kanehisa et al., 2019; The Gene Ontology Consortium, 2019) analysis. Only biological processes and pathways with p-values<0.05 were considered as significant in this enrichment analysis. The data were analyzed using R version 3.6.3 (RStudio Team, 2020).

3 Results

3.1 ADME profile of the screened components from *Polygonum multiflorum, Rehmannia glutinosa, Senna obtusifolia* and *Crataegus*

A total of 51 compounds, were identified from MSFinder after performing LC-MS/MS (Supplementary Table 1). The absorption, distribution, metabolism and excretion (ADME) properties of the metabolites of the selected plants' compounds were analyzed as ADME properties are pivotal keys in bioactivities. OB limits F (20%) and F (30%) indicators were used to filter the chemical constituents of the plant samples that displayed favourable pharmacokinetic properties. The ADME-related parameters of OB and DL were selected to screen for potential bioactive compounds in the TCM decoction. This is because only bioactive compounds can contribute to therapeutic effects (Pang et al., 2018). The values of OB ≥50% and DL=pass were considered as being indicative of good absorption after oral administration and thus, demonstrative of chemical suitability for drug development. The results indicated that only 11 compounds met the OB ≥50% and DL=pass criteria, as shown in Table 1.

Table 1 Oral Bioavailability (OB) and Drug Likeness (DL) profile of 11 bioactive compounds from the TCM formulation.

Compound	Chemical Formula	Existing in Medicinal Plants	OB		DL	
			F (20%)	F (30%)	Vebe r	QED
2-methoxy-N-[2-(methylthio)phenyl]benzamide	$C_{15}H_{15}NO_2S$	*Crataegus*	0.704	0.612	Pass	Pass
camazepam	$C_{19}H_{18}ClN_3O_3$	*Crataegus*	0.652	0.685	Pass	Pass
Purine	$C_5H_4N_4$	*Crataegus,Senna obtusifolia, Rehmannia glutinosa*	0.653	0.66	Pass	Pass
D-glucose	$C_6H_{12}O_6$	*Crataegus,Senna obtusifolia,Polygonum*	0.589	0.507	Pass	Pass

		multiflorum				
L-Valine	$C_5H_{11}NO_2$	*Senna obtusifolia*	0.803	0.717	Pass	Pass
1-(dimethylamino)propan-2-ol	$C_5H_{13}NO$	*Senna obtusifolia*	0.597	0.677	Pass	Pass
L-Isoleucine	$C_6H_{13}NO_2$	*Senna obtusifolia*	0.793	0.707	Pass	Pass
2-Aminobenzoic acid	$C_7H_7NO_2$	*Senna obtusifolia*	0.869	0.795	Pass	Pass
Gallic acid	$C_7H_6O_5$	*Senna obtusifolia,Polygonum multiflorum*	0.673	0.616	Pass	Pass
pyroglutamic acid	$C_5H_7NO_3$	*Senna obtusifolia,Polygonum multiflorum, Rehmannia glutinosa*	0.657	0.582	Pass	Pass
L-lactic acid	$C_3H_6O_3$	*Polygonum multiflorum, Rehmannia glutinosa*	0.519	0.577	Pass	Pass

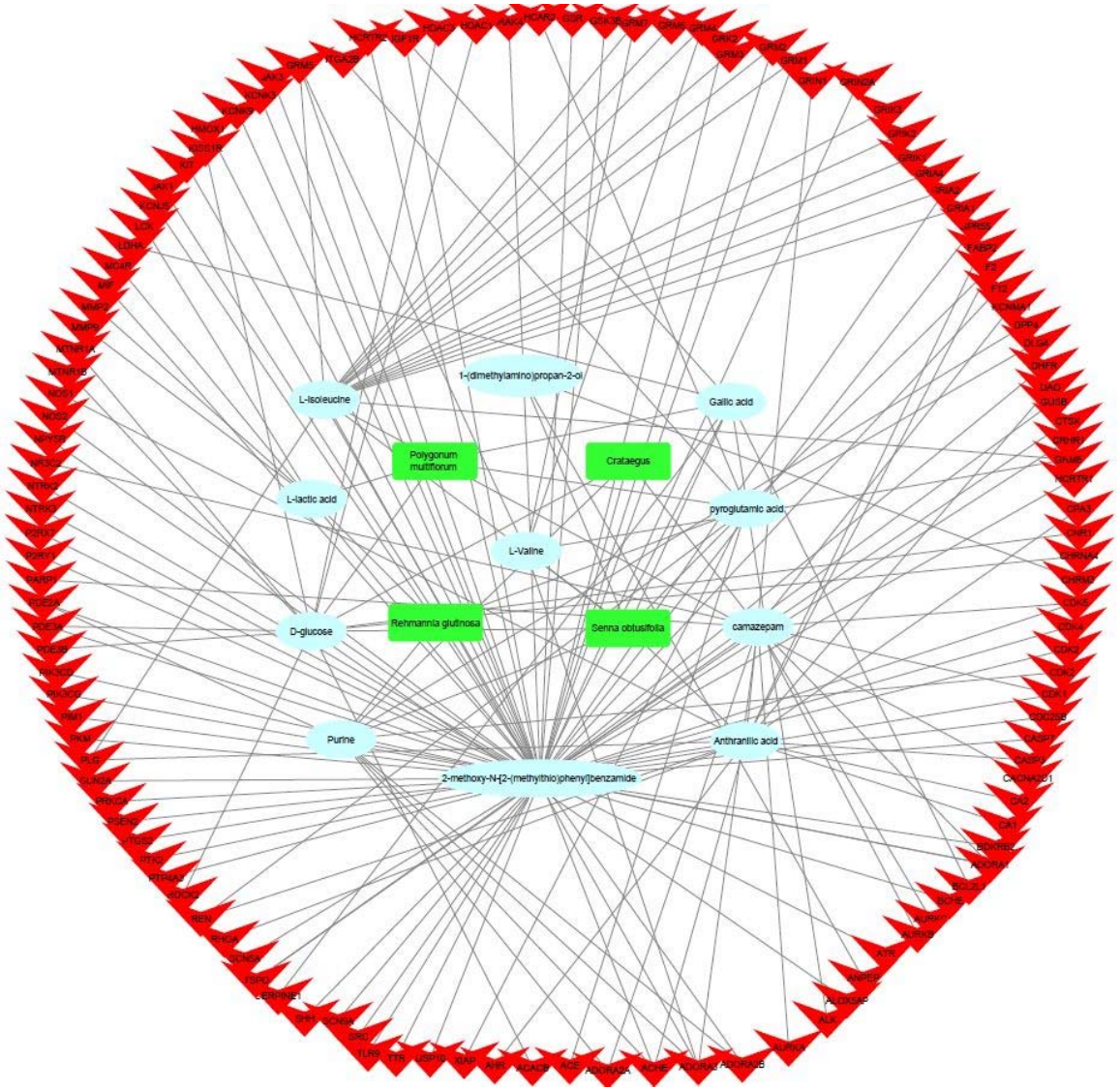

Fig. 1 Plant-Compound-Target Network analysis. Medicinal plants (green rectangle) were connected to its respective compound (blue oral) to link to predicted target gene (red arrow).

3.2 CT visual interaction network

To visualize the relationships of the medicinal plants' compounds and targets, the plant-compound-target network of the decoction was constructed using Cytoscape Version 3.8, as shown in Fig. 1. Only 11 compounds from the four medicinal plants were identified to be suitable as bioactive compounds, as attested by their OB and DL criteria.

There were also duplicate compounds detected in the different plants used for the decoction through network visualization, as illustrated in Fig. 2. For instance, D-glucose was found in *Crataegus, Senna obtusifolia* and *Polygonum multiflorum,* while pyroglutamic acid was found in all the plants except for *crataegus,* as shown in Table 1. The 11 selected compounds were found to exhibit activity on 124 targets, as illustrated in Fig. 1 and Fig. 2. Some of the compounds were also found to have the same targets, as shown in Fig. 2. For example, ADORA3 is the target gene for purine, camazepam, L-isoleucine and L-valine. In the meantime, only 1-(dimethylamino)propan-2-ol and L-lactic acid targets were found to be unique, which do not share an edge with other compounds. On other hand, PLG, IGF1R, REN, F2, PDE3A, TSPO, CDK1, CDK5 and ADORA2B were targets that were shared by two different compounds.

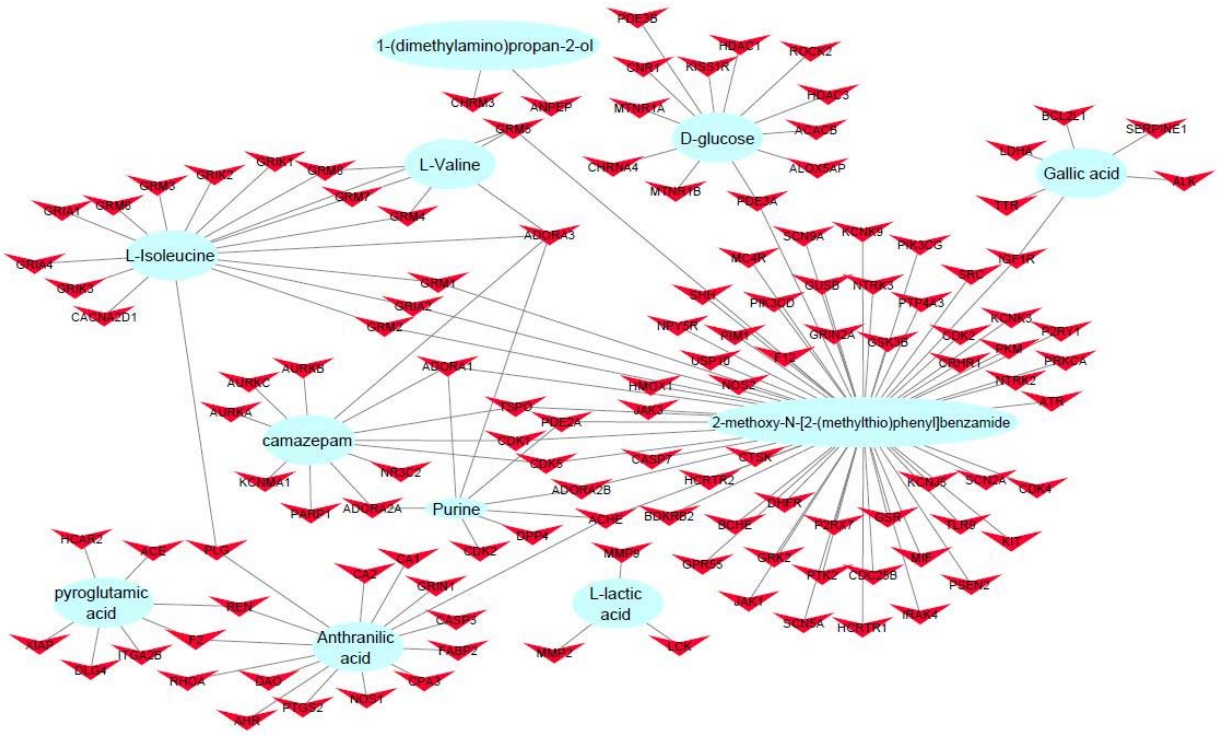

Fig. 2 Compound-Target Network. Visualization of connection of 11 bioactive compounds (blue arrow) with its target genes (red arrow) from the decoction of four medicinal plants.

3.3 PPI Network

The PPI analysis results from STRING, after input of all the targets, revealed diversified interactions in the PPI network, as depicted in Fig. 3. The illustrated figure shows that viable protein target nodes (n=164) were linked to edges (n=497) at an average node degree of 6.06 and average local clustering coefficient of 0.591. The confidence level of 0.7 was selected as the high evidence level from STRING to indicate strong interactions.

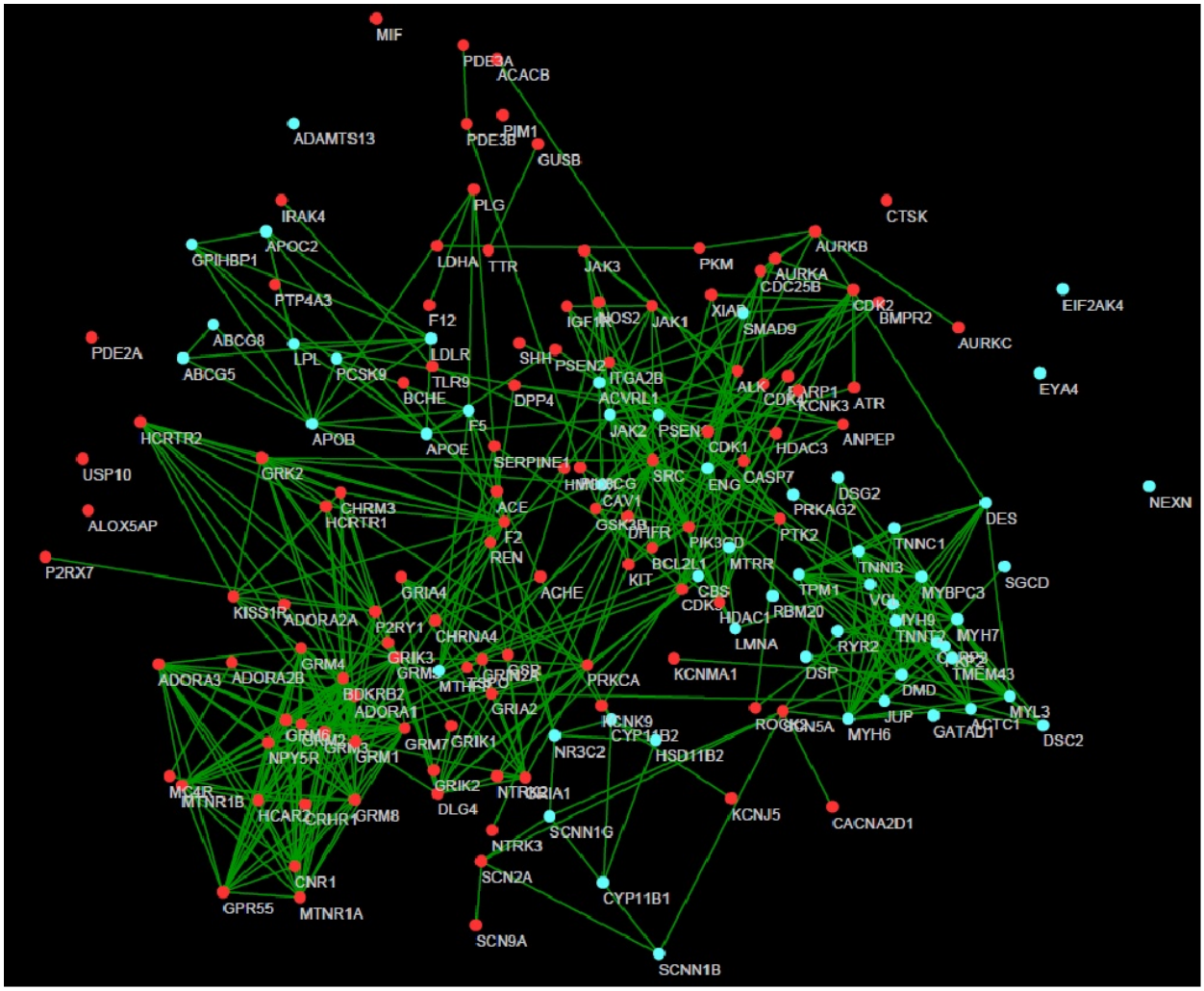

Fig. 3 Visualization of PPI interaction network analyzed by STRING using Cytoscape. Compound targets (red node) and hypertension-related targets (blue node) were linked together through edges (green) for its PPI interaction.

3.4 GO and KEGG pathway enrichment analysis

GO and KEGG pathway enrichment analysis were carried out to determine the characteristics of the compound-related and disease-related targets. GO analysis revealed that most of the existing potential targets were enriched in the regulation of biological processes, regulation of biological quality, signal transduction, response to chemicals, regulation of cell communications and response to stress (Fig. 4 (a)). According to the GO enrichment analysis, the top 30 enriched hypertension-related biological processes included regulation of heart contraction, regulation of blood circulation, heart process and cardiac muscle regulation. These findings suggest that rather than hypertension-related biological processes, other numerous biological processes were instead affected by the synergies of this decoction. As shown in Fig. 4 (b), KEGG enrichment analysis showed that 131 pathways (p value < 0.05) were affected by this TCM. Among the top 30 enriched pathways, neuroactive ligand-receptor interaction, pathways in cancer, hypertrophic cardiomyopathy (HCM), glutamatergic synapse and dilated cardiomyopathy (DCM) comprise the top 5 pathways, where HCM and DCM are closely related to the hypertension pathways.

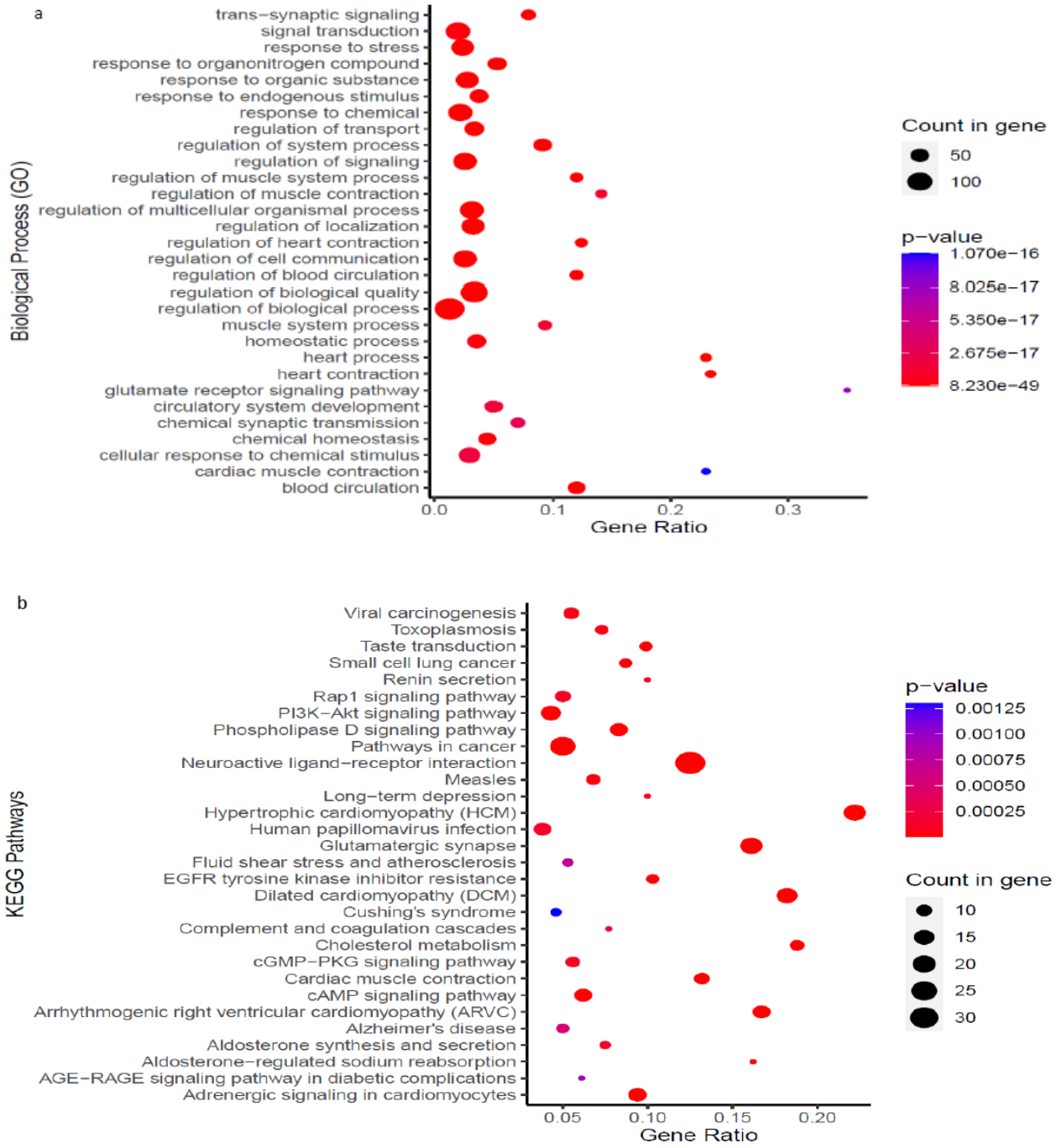

Fig. 4 The top 30 most significant biological processes (GO) and KEGG pathway enrichment analysis. The x-axis exhibit gene ratio involved in the y-axis (GO and KEGG pathways) with the bigger node size, more targets of genes are involved while a gradient of red to blue showed the significance of the enrichment analysis with red indicating the most significant value. (a) Biological process from GO enrichment analysis. (b) KEGG pathway from KEGG enrichment analysis.

3.5 Target-disease network

Based on the enrichment analysis shown in Fig. 4, the target-pathway network was constructed to visualize the mechanisms of this decoction in hypertension treatment, by associating the pathways and targets in the network (Fig. 5). Other pathways known to be associated with hypertension, such as Cushing's syndrome,

AGE-RAGE signaling pathway in diabetic complications and complement and coagulation cascades, were included. The network constructed depicts the synergy of the TCM in managing or preventing hypertension through pathways such as cardiac muscle contraction, fluid shear stress and atherosclerosis, DCM, renin-angiotensin system, HCM and arrhythmogenic right ventricular cardiomyopathy (ARVC). A total of 36 compound targets and 42 disease targets identified were mostly involved in HCM and DCM.

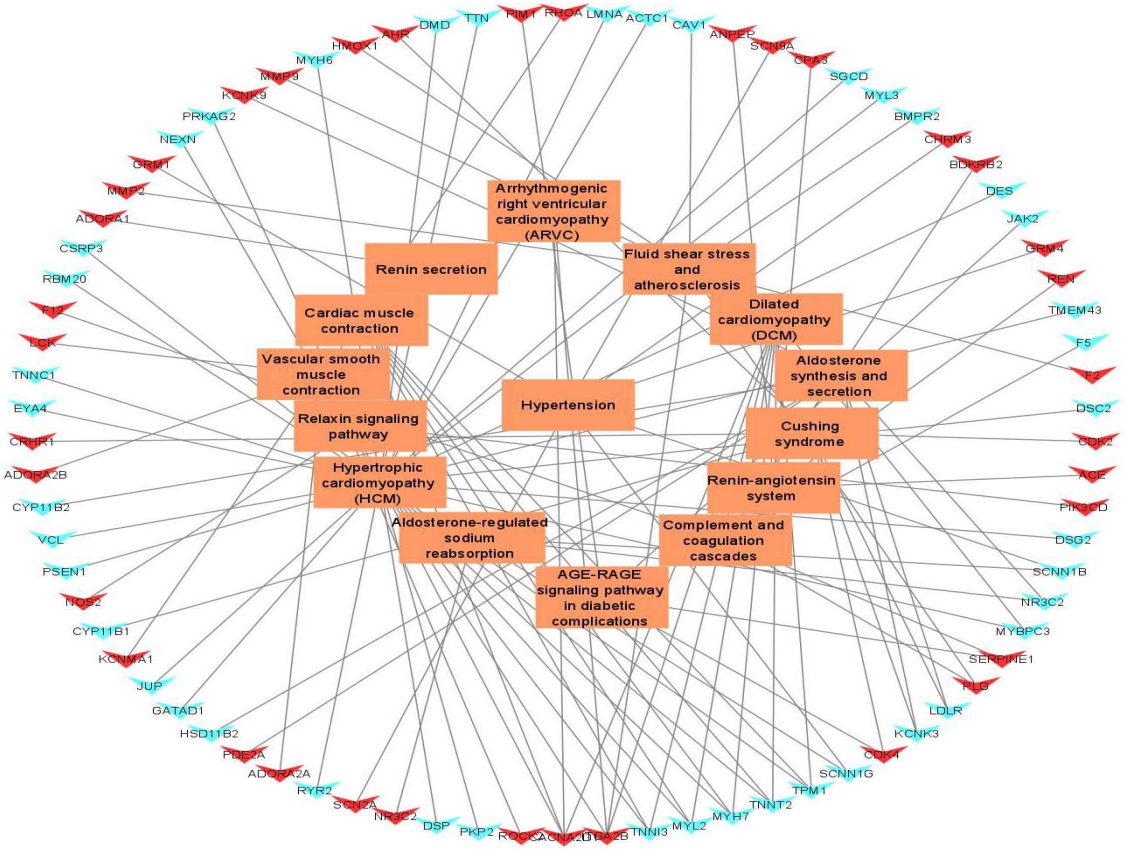

Fig. 5 Target–disease network constructed to visualize the interaction between targets to hypertension-related pathways. Fifteen hypertension-related pathways (orange rectangle) were connected to compound targets (red arrow) and disease targets (blue arrow) to establish the correlation produced by TCM decoction from the four selected medicinal plants.

4 Discussion

Substantial evidence from previous studies, either *in-vitro* or clinical trials, have revealed TCM's curative effects on hypertension and potential for drug discovery (Xiong et al., 2011; Zhong et al., 2011). In this study, we performed LC-MS/MS for identification of the chemical constituents existing in the TCM formulation consisting of *Polygonum multiflorum, Rehmannia glutinosa, Senna obtusifolia* and *Crataegus,* to determine the bioactive compounds responsible for mitigating hypertension. From the numerous compounds detected (Supplementary Table 1), only the bioactive compounds which can contribute therapeutic effects (Pang et al., 2018) were selected, based on their OB and DL values. The OB and DL values of constituents are pivotal keys for successful development of therapeutic drugs with good oral absorption and permeability across the blood-brain barrier (Bhowmik et al., 2015; Tao et al., 2013), in line with their molecular physicochemical properties (Jia et al., 2020). While most network pharmacological analysis selections are based on OB≥30%, in actual fact there are no universal criteria to define high and low bioavailable compounds (Bickerton et al., 2012). Thus, in this study, instead of selecting bioactive compounds with OB≥30%, we chose OB≥50% (as

shown in Table 1) as the arbitrary classification threshold, with the hope that the selected bioactive compounds with higher OB would result in higher absorption in the human system (Bhowmik et al., 2015). Our selection was based on the observation that a lot of drugs failed in clinical trials due to poor absorption in the human system.

The filtering process revealed that 11 compounds fulfilled the selection criteria (Table 1), with some found in duplicate within the four medicinal plants. A total of 124 genes were predicted as the target genes for these 11 compounds, as illustrated in Fig. 1. While the SwissTargetPrediction Database estimates predictions of protein targets for drug-like molecules using similarity principles efficiently, as has been experimentally observed previously, it does not however provide calculations for large peptides (Daina et al., 2019). From our results shown in Fig. 2, one prime example is 2-methoxy-N-[2-(methylthio)phenyl]benzamide, which was shown to possess most of the predicted targets, which might indicate that it has been experimentally observed to bind to or homologs thereof. It is an established fact that the targets for this bioactive compound, involving hypertension, is through regulation of blood circulation, such as ADORA1, ADORA2B, HMOX1, KCNJ5, NOS2 illustrated in Fig. 1 and Fig. 4(a) (Ayer et al., 2016; Chang et al., 2017; Chen et al., 2013; Förstermann and Sessa, 2012).

Apart from the target compounds, hypertension disease targets were also determined through the OMIM and GeneCards databases. In addition to the hypertension disease gene targets, we also searched the databases for hypertension-related diseases such as cardiovascular disease or categories of diseases possessing both keywords, which possess strong inter correlations, as shown in previous reports (Torpy et al., 2003; Xiong et al., 2015). Subsequently, the targets for the bioactive compounds were overlapped with the targets from diseases to construct the PPI network and visualized using Cytoscape Version 3.8 for better illustration, as depicted in Fig. 3, with confidence level of 0.7. Our findings revealed that two main clusters were formed through this network, which consisted only of the target compounds (red node) and target diseases (blue node), in the bottom left and bottom right of the network, respectively. It showed that the target compounds were involved in neuroactive ligand receptor interaction while the target disease clusters were involved in HCM pathways. This indicates that the TCM decoction might trigger the central neural pathways, one of the multiple converging pathways leading to hypertension (Cheng et al., 2019; Scheuer, 2008), through neuroactive ligand receptor interaction, to ameliorate hypertension.

The enrichment analysis for GO in biological processes and KEGG pathways demonstrated the involvement of this TCM decoction in numerous biological processes and pathways in the human system biology, as summarized in Fig. 4. As shown in Fig. 4 (a), the biological processes of high significance involve the regulation of biological quality, glutamate receptor signaling pathway, response to stress, regulation of localization and trans-synaptic signaling, among others. Among these biological processes, we found that regulation of heart contraction and regulation of blood circulation, which are strongly associated with hypertension (Chaudhry et al., 2020; Tzeng et al., 2014), are also biological processes with *p*-value of 6.58×10^{-19} and 2.84×10^{-21}, respectively. This is concomitant with our KEGG pathway enrichment analysis (Figure 3 (b)) which revealed the high significance of HCM, DCM and cardiac muscle contraction, with *p*-values of 2.12×10^{-17}, 1.89×10^{-14} and 5.93×10^{-08}, respectively. All of these pathways are inter-linked, either directly or indirectly (Chaudhry et al., 2020). Additionally, other top pathways found are neuroactive ligand-receptor interaction, glutamatergic synapse, pathways in cancer and adrenergic signaling in cardiomyocytes, among others.

Our PPI network associated with enrichment analysis also suggests that this TCM decoction resolves hypertension conditions through neuroactive ligand receptor interaction (Cheng et al., 2019). We thus constructed a network to visualize all the hypertension-related pathways by overlapping the compound targets

and disease targets, as shown in Fig. 5. No compound targets related to hypertension were however detected which indicates that the synergies of the decoction might instead be involved in pathways indirectly linked to hypertension, to improve hypertension conditions in the human system biology. For example, one of the compound targets, angiotensin converting enzyme (ACE), plays a role in elevating hypertension via constriction of the blood vessels in the pathway of the renin angiotensin system (Fountain and Lappin, 2020). This indicates that this TCM decoction might be contributing to hypertension amelioration through alternative pathways instead of affecting hypertension itself directly, as most of the targets involved neuroactive ligand receptor interaction, one of the pathways leading to hypertension (Cheng et al., 2019) and HCM, a disease caused by hypertension (Marian and Braunwald, 2017).

5 Conclusion

By and large, the TCM formulation comprising *Polygonum multiflorum, Rehmannia glutinosa, Senna obtusifolia* and *Crataegus* ameliorates hypertension through triggering biological processes and pathways such as the regulation of blood circulation and cardiac muscle contraction, instead of affecting hypertension itself directly, to improve conditions of HCM and DLM. Thus, even though this TCM formulation does not act directly on hypertension it works indirectly through regulation of the blood circulation and alternative pathways, to ameliorate hypertension and hypertension related diseases. This indicates the high potential of this TCM formulation for use in preventing hypertension and improving cardiovascular disease conditions. However, there are limitations in this study which warrants further experiments, through *in-vitro* or *in-vivo* mode, in the future, to validate the network pharmacological analysis.

List of Abbreviations

TCM	Traditional Chinese Medicine
LCMS/MS	Liquid chromatography-mass spectrometry-mass spectrometry
OB	Oral bioavailability
DL	Drug likeness
QED	Quantitative Estimate of Drug likeness
CT	Compound target
PPI	Protein-protein interaction
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ADME	Absorption, distribution, metabolism and excretion
HCM	Hypertrophic cardiomyopathy
DCM	Dilated cardiomyopathy
ARVC	Arrhythmogenic right ventricular cardiomyopathy
ACE	Angiotensin converting enzyme

Supplementary File

Supplementary Table 1 can be found in supplementary material.

Acknowledgment

The authors would like to express their sincere thanks for the funding support of Malaysia Toray Science Foundation (Ref: 18/G105) and thank the Director General of Health, Malaysia, for his permission to publish this article.

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