

Network pharmacology-based prediction of the active compounds and mechanism of Buyang Huanwu Decoction for ischemic stroke

KAI WANG^{1,2*}, LU LEI^{1*}, JINYI CAO^{1*}, YI QIAO^{1,3*}, RUIMIN LIANG², JIALIN DUAN¹, ZHIJUN FENG¹, YI DING¹, YANG MA¹, ZHIFU YANG¹ and ENHU ZHANG²

¹Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032;

²College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi 712046;

³Department of Pharmacology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, P.R. China

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Abstract. Buyang Huanwu Decoction (BYHWD) is used to promote blood circulation and is widely used in Chinese clinical practice for the treatment and prevention of ischemic cerebral vascular diseases. However, the mechanism and active compounds of BYHWD used to treat ischemic stroke are not well understood. The current study aimed to identify the potential active components of BYHWD and explore its mechanism using network pharmacology and bioinformatics analyses. The compounds of BYHWD were obtained from public databases. Oral bioavailability and drug-likeness were screened using the absorption, distribution, metabolism and excretion (ADME) criteria. Components of BYHWD, alongside the candidate targets of each component and the known therapeutic targets

of ischemic stroke were collected. A network of target gene compounds and cerebral ischemia compounds was established using network pharmacology data sources. The enrichment of key targets and pathways was analyzed using STRING and DAVID databases. Moreover, three of key targets [IL6, VEGFA and hypoxia-inducible-factor-1 α (HIF-1 α)] were verified using western blot analysis. Network analysis determined 102 compounds in seven herbal medicines that were subjected to ADME screening. A total of 42 compounds as well as 79 genes formed the principal pathways associated with ischemic stroke. The 16 key compounds identified were baicalein, beta-carotene, baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, bifendate, formononetin, calycosin, astragaloside IV, stigmaterol, sitosterol, Z-ligustilide, and dihydrocapsaicin. The core genes in this network were IL6, TNF, VEGFA, HIF-1 α , MAPK1, MAPK3, JUN, STAT3, IL1B and IL10. Furthermore, the TNF, IL17, apoptosis, PI3K-Akt, toll-like receptor, MAPK, NF- κ B and HIF-1 signaling pathways were identified to be associated with ischemic stroke. Compared with the control group (no treatment), BYHWD significantly inhibited the expression of IL6 and increase the expression of HIF-1 α and VEGFA. Network pharmacology analyses can help to reveal close interactions between multi-components and multi-targets and enhance understanding of the potential effects of BYHWD on ischemic stroke.

Correspondence to: Professor Zhifu Yang, Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, 127 Changle West Road, Xincheng, Xi'an, Shaanxi 710032, P.R. China

E-mail: yangtian_1973@163.com

Professor Enhu Zhang, College of Pharmacy, Shaanxi University of Chinese Medicine, 1 Middle Section, Chenyangzhai Century Avenue, Qindu, Xianyang, Shaanxi 712046, P.R. China

E-mail: 824887855@qq.com

*Contributed equally

Abbreviations: BYHWD, Buyang Huanwu Decoction; ADME, absorption, distribution, metabolism, and excretion; TCM, traditional Chinese medicine; OB, oral bioavailability; DL, drug-likeness; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PPI, protein-protein interaction; BMECs, brain microvascular endothelial cells; OGD, oxygen glucose deprivation; BP, biological process; MF, molecular function; CC, cellular component; HIF-1 α , hypoxia-inducible factor 1-alpha; TLRs, toll-like receptors

Key words: traditional Chinese medicine, Buyang Huanwu decoction, network pharmacology, cerebral ischemia, pharmacological mechanism

Introduction

Ischemic stroke, also known as cerebral ischemia, is an acute cerebrovascular disease that is associated with high disability and mortality rates (1,2). According to statistics compiled by the American Heart Association in collaboration with the National Institutes of Health and other government agencies, stroke has become the third most common cause of death worldwide after cancer and heart disease in recent years, placing a huge psychological and financial burden on many families (3).

Buyang Huanwu decoction (BYHWD) has been used to promote blood circulation and was proposed by Wang Qingren in the 'Correction on Errors in Medical Classic'. It is composed of Radix Astragali, the dried roots of

Astragalus membranaceus Bge; Angelicae Sinensis Radix, the dried roots of *Angelica sinensis* Diels; Paeoniae Radix Rubra, the dried roots of *Paeonia lactiflora* Pall; Chuanxiong Rhizoma, the dried rhizomes of *Ligusticum chuanxiong* Hort; Persicae Semen, the dried seeds of *Prunus persica* Batsch; Carthami Flos, the dried flowers of *Carthamus tinctorius* L; and Pheretima, the dried bodies of *Pheretima aspergillum* (E.Perrier), in the ratio of 120:6:4.5:3:3:3. This formula has been widely used in Chinese clinical practice for the treatment and prevention of ischemic cardio-cerebral vascular diseases (4). Previous studies have demonstrated that BYHWD is efficacious in treating ischemic stroke and other vascular diseases (5). However, the exact mechanism of BYHWD in improving ischemic stroke and its active components remain unclear. As such, there is a great interest in identifying the active compounds and molecular targets in BYHWD that act on ischemic stroke. The present study aimed to identify the potential active constituents of BYHWD and explore its mechanism of action in the amelioration of ischemic stroke.

BYHWD is a multi-component, multi-channel and multi-target agent that is a product under the guidance of the holistic view and dialectical theory (6). It has unique advantages for the treatment of complex diseases, but due to the complex composition of TCM compounds, the modernization process can be slow (7). Network pharmacology is a novel method that combines system network analysis and pharmacology that can be used to clarify the synergistic effects and underlying mechanisms of compound-compound, compound-target and target-disease networks at the molecular level. This helps to elucidate the interactions among compounds, genes, proteins and diseases (8,9). Due to its robust and systematic nature, which is consistent with the overall view of TCM and the principle of dialectical theory, it has been widely applied in TCM research (10). Therefore, the current study used network pharmacology to study the mechanism of BYHWD in the treatment of ischemic stroke, providing a reference and theoretical basis for its experimental research and clinical application.

Materials and methods

Chemical database collection of BYHWD-compound information. Information on the compounds included in BYHWD was collected from two phytochemical databases, the TCM systems pharmacology database (TCMSP, <http://ibts.hkbu.edu.hk/LSP/tcmsp.php>) and the TCM Database@Taiwan (<http://tcm.cmu.edu.tw/>). The bidimensional chemical structures were acquired from NCBI PubChem (<http://pubchem.ncbi.nlm.nih.gov/>).

Pharmacokinetic ADME evaluation. OB represents the ability of a compound to circulate in the body after oral administration. OB can indicate whether the active compounds in a formula can be delivered throughout the body and produce a physicochemical effect (11). DL is an indicator for determining the similarity or likeness of a compound and its physicochemical properties with conventional drugs. DL can help determine if a certain compound has a therapeutic effect (11). All compounds were selected using the *in silico* integrative ADME model provided by the TCMSP database. Chemicals without ADME information were removed from the final list.

Compounds were only retained if $OB \geq 30$ and $DL \geq 0.18$ to satisfy the criteria suggested by the TCMSP database (12).

Target genes related to the identified compounds. The TCMSP database is a unique pharmacological database of traditional Chinese medicines describing the relationships between drugs, targets and diseases. The name of the identified compound was input into the TCMSP database version 2.3 (<https://tcmspw.com/tcmsp.php>) to obtain its target name, after which the target name of each compound was input into the UniProt (<http://www.uniprot.org/>) database to convert to the target gene and confine the species to *Homo sapiens*. Subsequently, the various IDs of the targets were transformed into UniProt IDs (13).

Collection of the cerebral ischemia target genes. Therapeutic Target Database (TTD) (<https://db.idrblab.org/ttd/>) explores therapeutic protein and nucleic acid targets within a targeted disease (14). DisGeNET (<http://www.disgenet.org/web/DisGeNET/menu/home>) is a discovery platform containing one of the largest publicly available collections of genes and variants associated with human diseases (15). By searching the key word 'cerebral ischemia', the targets related to this disease were collected from each database.

Construction of the networks and pathway analyses. The constructed herbal-chemical-protein networks were visualized using Cytoscape version 3.6.1 (<http://www.cytoscape.org/>). The relationship between active ingredients and common targets of component diseases in BYHED were summarized into a table and imported into Cytoscape software to obtain a network of drug ingredient actions and disease targets. The nodes in the networks represent herbs and chemicals, and the edges indicated interactions between herbs and chemicals, and between chemicals and target genes (16). The common targets of components and diseases were imported into the ClueGo plug-in for Cytoscape software. The main functional annotation clusters were ranked using the Biocarta functional annotation cluster tool (<http://amp.pharm.mssm.edu/Harmonizome/dataset/Biocarta+Pathways>; updated on July 3, 2016) (17). The functional pathways of BYHWD associated with cerebral ischemia were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (<http://www.genome.jp/kegg/pathway.html>). Gene Ontology (GO) enrichment evaluation was determined using the DAVID database version 6.8 (<https://david.ncifcrf.gov/>). Data with $P < 0.05$ were screened from the enrichment analysis results.

Protein-protein interaction (PPI) analysis. Screened drug component-disease common targets were imported into the STRING (<https://string-db.org/>) database to build the PPI network model. The protein category was set as *Homo sapiens*. The minimum interaction threshold was set as the medium highest confidence (> 0.9) and the PPI network was obtained using the default setting of the other parameters. In the network, the size of the nodes represented the degree size. The higher the degree, the better the correlation between the protein and therapeutic mechanism (18). Fig. 1 illustrated network pharmacology analysis workflow.

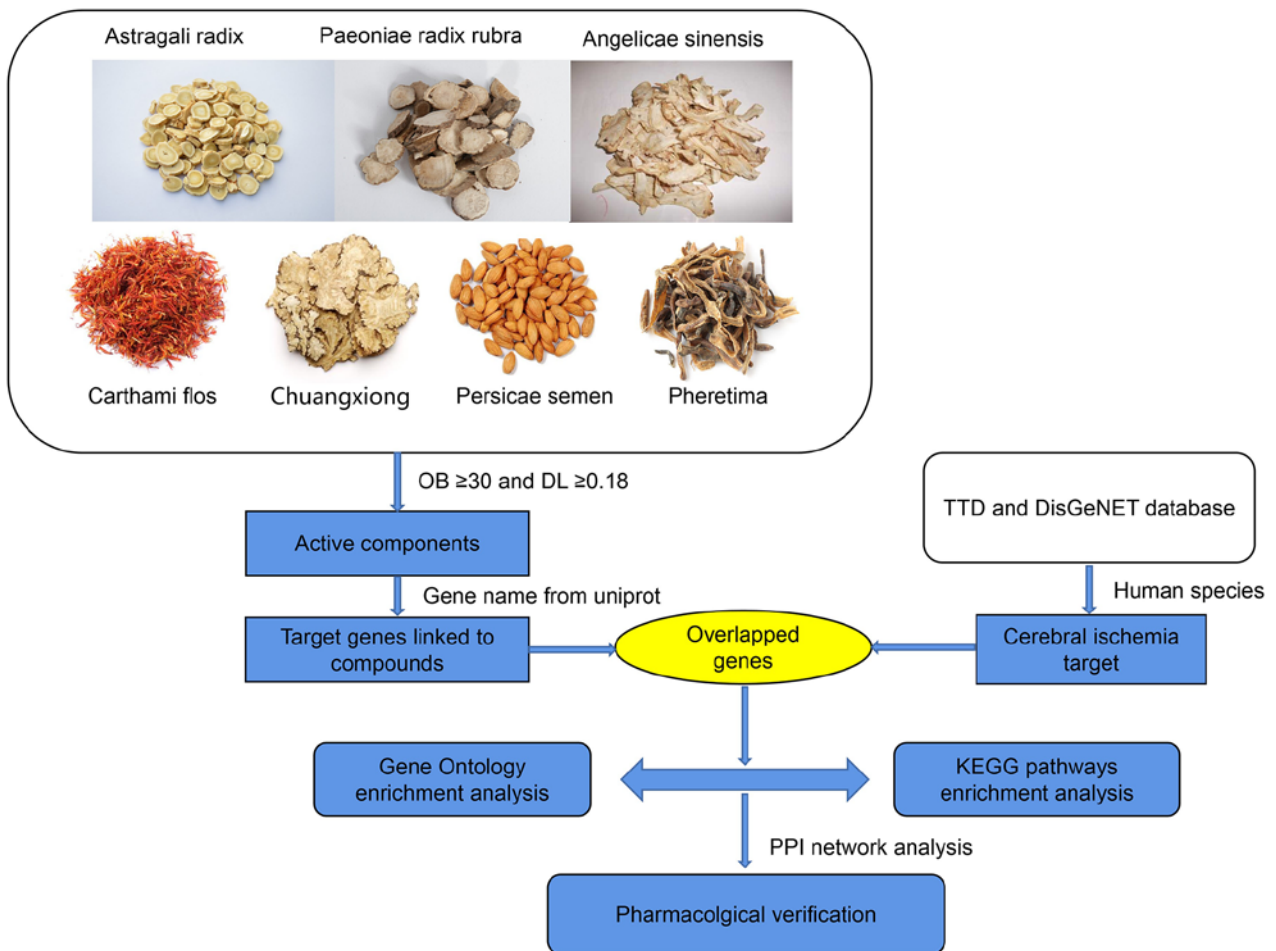


Figure 1. Workflow of network pharmacology analysis. OB, oral bioavailability; DL, drug-likeness; TTD, Therapeutic Target Database; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

Materials for pharmacological experimental verification. BYHWD is a granule made of Astragali Radix, Angelicae Sinensis Radix, Paeoniae Radix Rubra, Chuangxiong Rhizoma, Persicae Semen, Carthami Flos and Pheretima according to the ratio 120:6:4.5:3:3:3:3. BYHWD was obtained from Guangdong Yifang Pharmaceutical Co., Ltd. (batch no. 7070352). Rat brain microvascular endothelial cells (BMECs) were purchased from the Cell Biologics, Inc. (cat. no. C57-6023). Primary antibodies against β -actin (cat. no. ab227387), VEGFA (cat. no. ab46154), hypoxia-inducible-factor-1 α (HIF-1 α) (cat. no. ab179483) and IL6 (cat. no. ab9324) and secondary antibodies [HRP-conjugated goat anti-rabbit (cat. no. ab7090) and HRP-conjugated goat anti-mouse (cat. no. ab97040)] were purchased from Abcam.

Cell culture and treatments. BMECs were divided into three groups: Control, oxygen-glucose deprivation (OGD) and OGD group treated with BYHWD (80 μ g/ml) groups. OGD was established as follows: Cells were rinsed once with glucose-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) (19) and transferred to an anaerobic chamber (Thermo Fisher Scientific, Inc.) containing a gas mixture composed of 7% CO₂ and 93% N₂ for 6 h at 37°C. The cells were then returned to the normal culture conditions (37°C). Control BMECs were cultured in complete DMEM under normal conditions (37°C) and did not receive

BYHWD treatment. BYHWD was dissolved in complete DMEM and treated with a 0.22 μ m membrane filter, after which BYHWD group cells were treated (37°C) with 80 μ g/ml BYHWD for 12 h.

Western blotting. BMECs were lysed with cold radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) for 30 min and protein concentrations were analyzed by the BCA method. Whole-cell lysates (30 μ g) were fractionated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min and incubated overnight at 4°C with primary antibodies against VEGFA (1:1,000; anti-rabbit), HIF-1 α (1:1,000; anti-rabbit), IL6 (1:1,000; anti-mouse), β -actin (1:1,000; anti-rabbit). The membranes were subsequently incubated with a secondary antibody (1:10,000; goat anti-rabbit or goat anti-mouse antibody) at 37°C for 1 h. The blots were visualized using an ECL-Plus reagent (Santa Cruz Biotechnology, Inc.) and analyzed using Quantity One System image analysis software version 4.6.2 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data in the present study were expressed as the mean \pm SD. One-way ANOVA followed by

a Tukey's post hoc test was used for analyzing differences between groups. $P < 0.05$ was indicated as the statistical significance.

Results

Compounds in the herbal medicines. BYHWD consists of seven herbal medicines, namely Astragali Radix, Angelicae Sinensis Radix, Paeoniae Radix Rubra, Chuanxiong Rhizoma, Persicae Semen, Carthami Flos and Pheretima. All identified compounds were subjected to ADME screening. Of the 780 compounds assessed, 102 had an OB $\geq 30\%$ and DL ≥ 0.18 , which met the suggested drug screening criteria. Of these, 21, 23, 4, 29, 9, 23 and 5 were Astragali Radix, Carthami Flos, Angelicae Sinensis Radix, Paeoniae Radix Rubra, Chuanxiong Rhizoma, Persicae Semen and Pheretima, respectively. There were 6 compounds that did not meet the aforementioned requirements and were therefore considered to be bioactive compounds: Hydroxysafflor yellow A, astragaloside IV, ferulic acid, ligustrazine, Z-ligustilide and linoleic acid. These compounds are the major components of BYHWD and their effects on cerebral ischemia have been investigated previously (20-25). Duplicate components and those with ambiguous targets were removed. The compound information of BYHWD is presented in Table I, and the composition of each structure is presented in Fig. 2.

Combination of the compound-target and cerebral ischemia target genes. A total of 507 genes and compounds related to BYHWD were obtained from the TCMS database (Table SI). A total of 274 genes associated with cerebral ischemia were retrieved from the TTD and DisGeNET version 6.0 databases (Table SII). A total of 79 overlapping genes were pinpointed by matching the 518 compound genes with the disease-associated genes (Fig. 3). Therefore, these target genes were used as prediction targets of BYHWD in the treatment of stroke.

Potential target genes and network analysis. The constructed herbal-chemical-protein networks were visualized using Cytoscape, which included 42 compounds, with 79 target genes, 129 nodes and 490 edges (Fig. 4). In particular, 16 of the compounds (baicalein, beta-carotene, baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, bifendate, formononetin, calycosin, astragaloside IV, stigmasterol, sitosterol, Z-ligustilide and dihydrocapsaicin) were associated with >5 genes. Additionally, 33 genes (PTGS2, MMP9, NOS2, MAPK1, MAPK3, TNF, EGFR, APP, JUN, TP53, IL6, IL2, STAT3, NOS3, IL10, CYCS, VEGFA, HIF-1 α , BCL2, CASP9, CAT, PARP1, CASP3, CCL2, MPO, NFE2L2, CSF2, RELA, ALB, NFKBIA, PPARG, NR3C2 and ICAM1) were regulated by >4 compounds. The compound-target gene network demonstrated intimate communication between several components and multiple targets. This is helpful to better understand the potential pharmacodynamic substances and targets of BYHWD in the treatment of cerebral ischemia.

GO and KEGG enrichment analysis of potential target genes. GO enrichment and KEGG pathway enrichment analyses of the 79 potential targets were performed to determine the underlying molecular mechanism of BYHWD in cerebral ischemia.

GO enrichment analysis provided biological process (BP), molecular function (MF) and cellular component (CC) results. Using the DAVID database (with $P < 0.05$), the results revealed that the main BPs were 'positive regulation of transcription from RNA polymerase II promoter', 'inflammatory response', 'negative regulation of apoptosis', 'angiogenesis', 'positive regulation of NF- κ B transcription factor activity', 'apoptosis process', 'immune response' and 'response to oxidative stress'. The MFs were mainly associated with 'DNA binding', 'transcription factor activity', 'heparin-binding', 'heme-binding', 'sequence-specific DNA binding' and 'identical protein binding'. CC analysis revealed that there was a higher proportion of protein in the nucleus and extracellular space. Furthermore, GO analysis revealed the top six enriched conditions in the BP, CC and MF categories (Fig. 5). Furthermore, the 20 BPs are listed in Table II. To examine the signaling pathways and functions of these target genes, KEGG pathway functional enrichment analysis was performed. The signaling pathways were obtained by screening their statistical significance ($P < 0.05$), and the resulting target genes were found to primarily interact with the TNF, IL17, PI3K-Akt, toll-like receptor (TLR), MAPK, NF- κ B and HIF-1 signaling pathways. Therefore, these signaling pathways appear to be closely associated with the potential effects of BYHWD in cerebral ischemia. The identified target genes are listed in Table III. A KEGG bubble diagram was created using the top 20 signals (Fig. 6A). Additionally, the main functional annotation clusters were ranked by the Biocarta functional annotation cluster tool and were presented in Fig. 6B.

PPI network analysis of the target genes. The drug component-disease common target was imported into the STRING database to construct the PPI network. When using an interaction score of >0.9 as a cut-off, the PPI network contained 79 nodes and 327 edges, with an average node degree of 8.28 and PPI enrichment P-value of $<1.0 \times 10^{-16}$ (Fig. 7A). Cytoscape version 3.6.1 was then used to analyze the degree, PPI network topological eigenvalues, degree of node color, size reaction center, edge thickness and color depth to determine a combined score. The top 20 target genes with a high degree of connectivity were selected as the hub genes of BYHWD for cerebral ischemia (Fig. 7B). The core genes in this network were IL6, TNF, VEGFA, HIF-1 α , MAPK1, MAPK3, JUN, STAT3, IL1B and IL10.

Experimental validation. To confirm the results of the network analysis and verify the key targets of BYHWD, three key targets (IL6, VEGFA and HIF-1 α) were selected for pharmacological validation (Fig. 8). Western blotting results revealed that, when compared with the control group, BYHWD significantly inhibited the expression of IL6 and increased the expression of HIF-1 α and VEGFA ($P < 0.05$).

Discussion

Ischemic stroke is one of the leading causes of death and disability worldwide. Previous studies have indicated that the injury mechanism of ischemic stroke includes apoptosis, necrosis, inflammation, immune regulation and oxidative stress (26). However, no effective treatment has been found

Table I. Final selected compounds among the seven herbal medicines of Buyang Huanwu Decoction.

No.	Compound	OB (%)	DL	Herbal medicine
1	Kaempferol	41.88	0.24	Astragali Radix, Carthami Flos
2	Quercetin	46.43	0.28	Astragali Radix
3	Mairin	55.38	0.78	Astragali Radix
4	Jaranol	50.83	0.29	Astragali Radix
5	Hederagenin	36.91	0.75	Astragali Radix, Persicae Semen
6	Isorhamnetin	49.60	0.31	Astragali Radix
7	Bifendate	31.10	0.67	Astragali Radix
8	Formononetin	69.67	0.21	Astragali Radix
9	Isoflavanone	109.99	0.30	Astragali Radix
10	Calycosin	47.75	0.24	Astragali Radix
11	AstragalosideIV	17.74	0.15	Astragali Radix, Persicae Semen
12	Poriferast-5-en-3beta-ol	36.91	0.75	Carthami Flos
13	Lignan	43.32	0.65	Carthami Flos
14	Phytoene	39.56	0.50	Carthami Flos
15	Phytofluene	43.18	0.50	Carthami Flos
16	Pyrethrin II	48.36	0.35	Carthami Flos
17	6-Hydroxykaempferol	62.13	0.27	Carthami Flos
18	Baicalein	33.52	0.21	Carthami Flos, Paeoniae Radix Rubra
19	Quercetagetin	45.01	0.31	Carthami Flos, Paeoniae Radix Rubra
20	Beta-carotene	37.18	0.58	Carthami Flos
21	Baicalin	40.12	0.75	Carthami Flos, Paeoniae Radix Rubra
22	Beta-sitosterol	36.91	0.75	Carthami Flos, Paeoniae Radix Rubra, Angelicae sinensis Radix
23	Stigmasterol	43.83	0.76	Carthami Flos, Paeoniae Radix Rubra, Angelicae sinensis Radix
24	Luteolin	36.16	0.25	Carthami Flos
25	Ferulic acid	39.56	0.06	Angelicae sinensis Radix
26	Cis-ligustilide	36.91	0.75	Angelicae sinensis Radix
27	Ellagic acid	43.06	0.43	Paeoniae Radix Rubra
28	Paeoniflorin	53.87	13.88	Paeoniae Radix Rubra
29	Sitosterol	36.91	0.75	Paeoniae Radix Rubra, Chuangxiong Rhizoma
30	Spinasterol	42.98	0.76	Paeoniae Radix Rubra
31	(+)-catechin	54.83	0.24	Paeoniae Radix Rubra
32	Stigmast-7-en-3-ol	37.42	0.75	Paeoniae Radix Rubra
33	Ethyl oleate (NF)	32.40	0.19	Paeoniae Radix Rubra
34	Campest-5-en-3beta-ol	37.58	0.71	Paeoniae Radix Rubra
35	Hydroxysafflor yellow A	4.77	0.68	Carthami Flos
36	Perlolyrine	65.95	0.27	Chuangxiong Rhizoma
37	Wallichilide	42.31	0.71	Chuangxiong Rhizoma
38	Ligustrazine	20.01	0.03	Chuangxiong Rhizoma
39	Z-ligustilide	53.72	0.07	Chuangxiong Rhizoma
40	Sitosterol alpha1	36.91	0.75	Persicae Semen
41	Gibberellin A44	101.61	0.54	Persicae Semen
42	3-O-p-coumaroylquinic acid	37.63	0.29	Persicae Semen
43	Folinic acid	68.96	0.71	Persicae Semen
44	Linoleic acid	41.90	0.14	Pheretima
45	Arachidonic acid	45.57	0.20	Pheretima
46	Dihydrocapsaicin	47.07	0.19	Pheretima

OB, oral bioavailability; DL, drug-likeness.

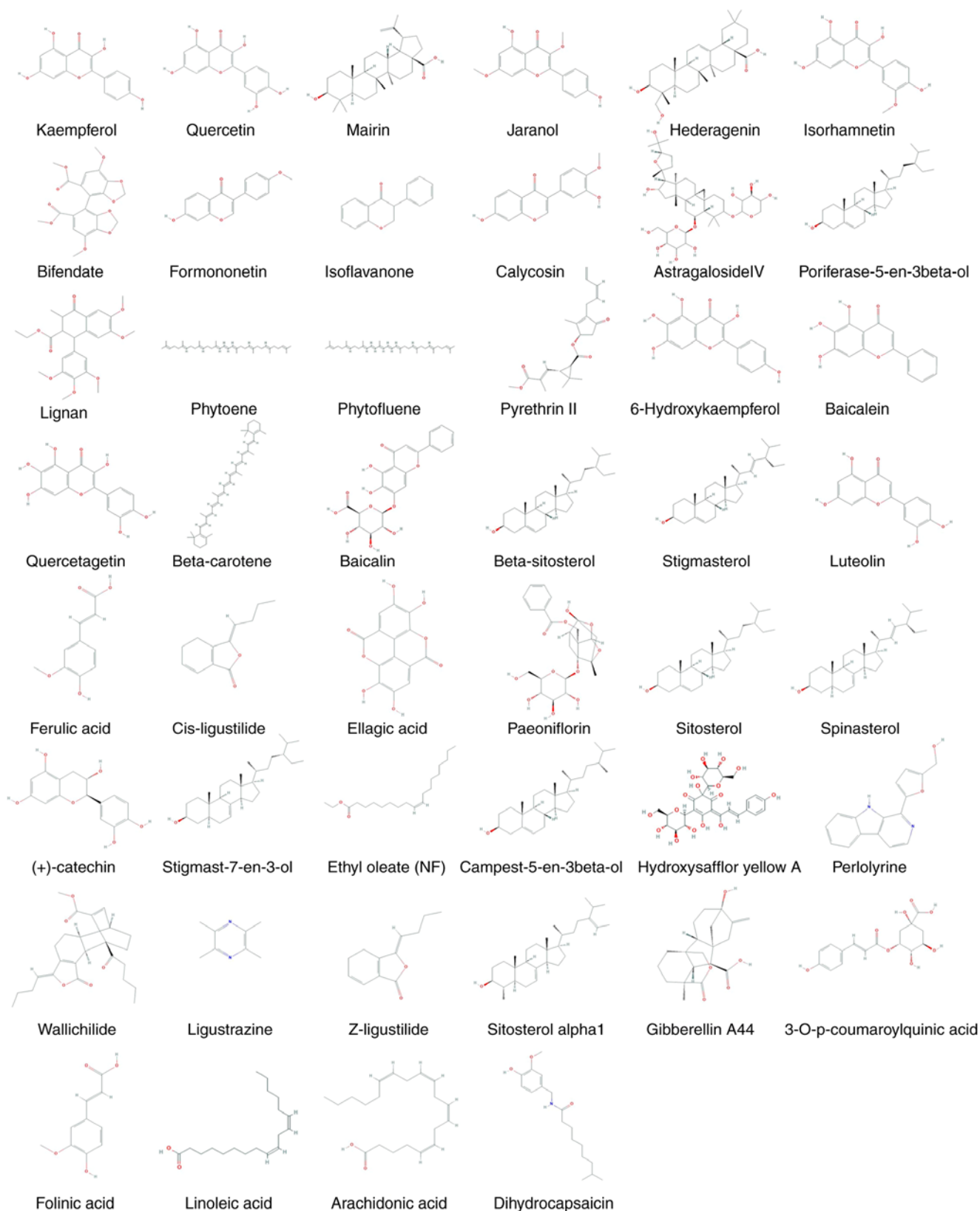


Figure 2. Final selected compound structures of Buyang Huanwu Decoction.

to prevent damage to the brain, except for tissue plasminogen activator; however, this single compound or single target drug has limited efficacy. Therefore, it is thought that a promising treatment approach for ischemic stroke should utilize multiple-component agents with multiple targets (27).

BYHWD activates blood circulation. It is a classic prescription that has been used by doctors for many generations. TCM cures disease using a multi-approach, multi-target and integer concept. It has unique advantages for the treatment of complex diseases, but due to the complex composition of TCM, its

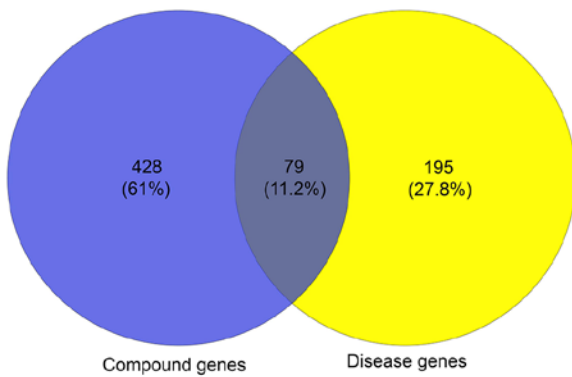


Figure 3. Venn diagram for compound genes and disease genes, 79 targets were common to Buyang Huanwu Decoction (compound genes) and cerebral ischemia (disease genes).

application in modern medicine has been slow. Furthermore, network pharmacology has provided a novel method of determining the pharmacological mechanisms of TCMs. Scholars have attempted to apply network pharmacology to evaluate the ingredients, targets and mechanisms of herbal formulas (8).

In the present study, network pharmacology was performed to investigate the pharmacological mechanisms of BYHWD in relation to cerebral ischemia. The network pharmacological analysis of BYHWD identified seven herbs, 42 compounds and 79 target gene-regulated pathways associated with cerebral ischemia. Additionally, 16 compounds (baicalein, beta-carotene, baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, bifendate, formononetin, calycosin, astragaloside IV, stigmaterol, sitosterol, Z-ligustilide and dihydrocapsaicin) were associated with >5 genes. Previous studies have reported that baicalein has potent neuroprotective properties under *in vitro* and *in vivo* systems (28). Beta-carotene serves as an antioxidant, inhibiting free radical production. It may also regulate cell growth and death (29). Baicalin inhibits microglial cell activation and reduces inflammation, oxidative damage and brain edema (30). In addition, kaempferol has strong anti-inflammatory and antioxidant effects. Numerous scientific reports have revealed that it serves a beneficial role in different inflammatory-related diseases, such as cardiovascular and neurodegenerative diseases. Luteolin suppresses inflammation in the brain tissue and regulates different cell signaling pathways (31). Quercetin has antioxidant stress and neuroprotective effects (32). Moreover, hydroxysafflor yellow A protects BMECs against OGD/reoxygenation-induced injury by inhibiting autophagy via the Class I PI3K/Akt/mTOR signaling pathway (33). Treatment of experimental stroke mice with isorhamnetin attenuated cerebral edema, improved blood-brain barrier function and upregulated the gene expression of certain tight junction proteins, including occludin, zonula occluden-1 and claudin-5 (34). Furthermore, calycosin protected the rat brain against ischemic injury by inhibiting calpain activation (35). Dihydrocapsaicin-treated cerebral ischemia-reperfusion rats demonstrated attenuated cerebral and blood-brain barrier damage by inhibiting oxidative stress and inflammatory pathways (36). These findings suggest that the main components of BYHWD are effective for treating cerebral ischemia. However, as the compounds in the database may be incomplete, the predicted active ingredients of

BYHWD may also be incomplete, which is a limitation of network pharmacology.

Genes with high degrees of differential articulation were acquired as a result of the PPI system analysis of the current study. Of these, IL6, TNF, VEGFA, HIF-1 α , MAPK1, MAPK3, JUN, STAT3, IL1B and IL10 were recognized as the center genes. IL6 is a multifunctional cytokine with a wide range of biological activities, including regulation of the immune system and generation of acute phase reactions (37). VEGF is a pleiotropic growth factor that is crucially involved in neurovascular remodeling in the ischemic brain. VEGF promotes angiogenesis, protects ischemic neurons from injury, has potent anti-inflammatory actions and promotes brain plasticity (38). HIF-1 α regulates the expression of gene encoding molecules that participate in erythropoiesis, cell proliferation and energy metabolism, and is closely associated with the regulation of neuronal survival in ischemia (39,40). HIF-1 α can upregulate the expression of proteins associated with the vascular system and can promote the angiogenesis of VEGF and its receptors to increase blood flow and reduce ischemic injury (41). TNF is a typical cytokine involved in the acute phase of systemic inflammation and is closely associated with the severity of cerebral ischemia (42). IL10 is a potent anti-inflammatory mediator and, if overexpressed, can suppress neuronal degeneration (43).

GO and KEGG pathway analyses were used to further understand the interaction and action pathways of target genes. GO analysis revealed that target genes were strongly associated with the following BPs: 'positive regulation of transcription including ribonucleic acid (RNA) polymerase II promoter', 'inflammatory response', 'transcription', 'DNA-templated', 'negative regulation of the apoptotic process', 'positive regulation of transcription', 'angiogenesis' and 'response to hypoxia'. The enriched MF ontologies were 'DNA binding transcription factor activity', 'sequence-specific DNA binding', 'identical protein binding', 'heparin-binding' and 'heme-binding'. The KEGG pathway analysis primarily pertained to TNF, IL17, apoptosis, PI3K-Akt, TLR, MAPK, NF- κ B and the HIF-1 signaling pathway. The results indicated that these pathways may interact to exert their combined effects against cerebral ischemia, which could explain the apparent effects of BYHWD.

Cerebral ischemia results in decreased cerebral blood flow and decreased oxygen supply, which leads to HIF-1 signaling pathway activation and upregulated HIF-1 α expression. This helps to recover blood circulation in the penumbra after cerebral ischemia, transport glucose and mediate hypoxia adaptation after hypoxia, serving a protective role in promoting cell survival and inhibiting brain tissue apoptosis (44). The MAPK and PI3K/Akt signaling pathways are the main pathways related to apoptosis after cerebral ischemia. Currently, it is hypothesized that MAPK signaling serves a dual role in the process of cell apoptosis, while PI3K/Akt signaling is important to the cell survival signaling pathway (45). Multiple neurotrophic factors inhibit apoptosis by activating the PI3K/Akt signaling pathway, thus playing a protective role in the brain (45). The PI3K/Akt signaling pathway is involved in the regulation of various intracellular signaling pathways and serves a key role in promoting cell survival and proliferation, anti-apoptosis, regulating glucose metabolism and protein synthesis (46). TLRs, as inflammatory signal receptors,

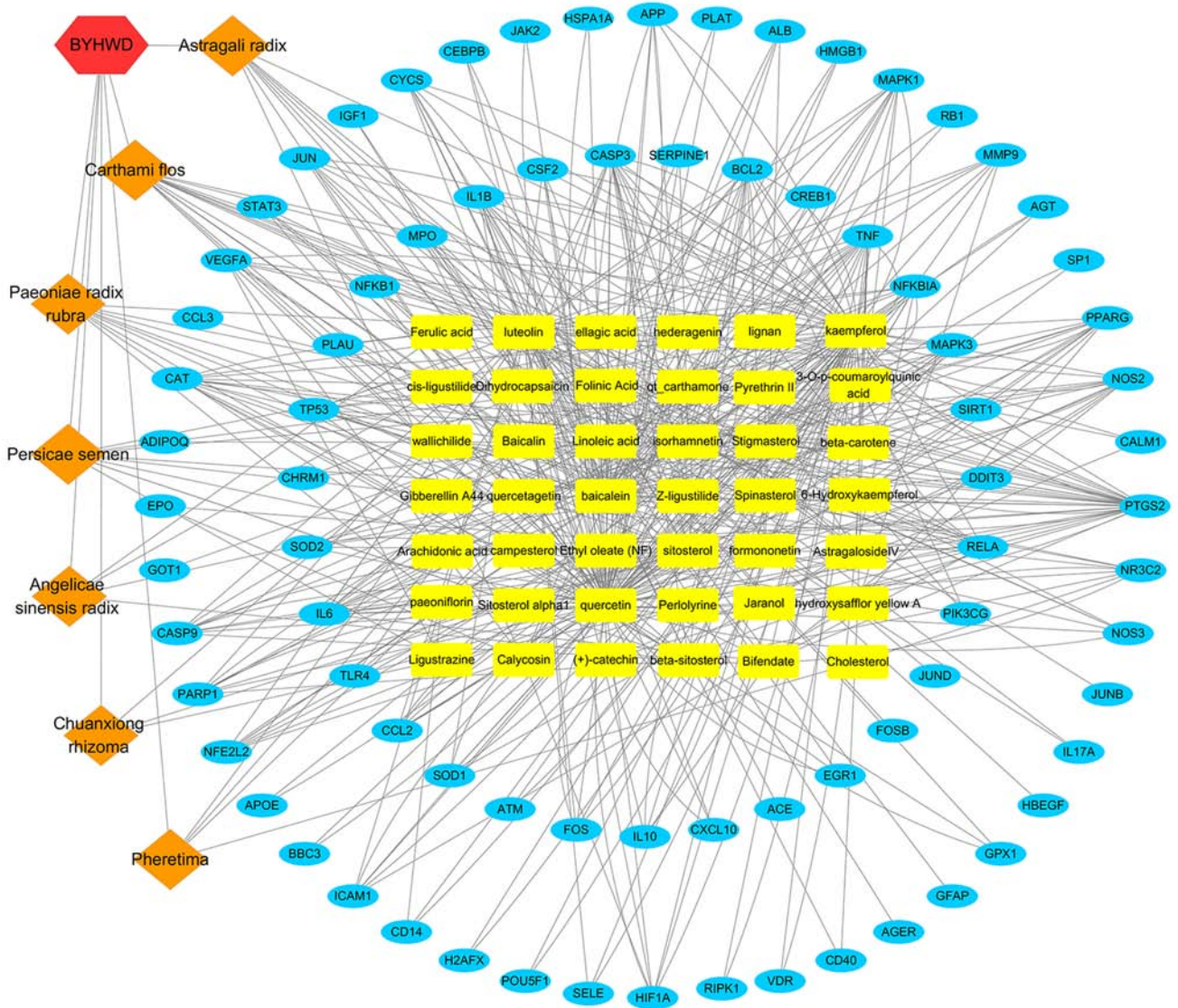


Figure 4. Network of BYHWD. Seven herbal medicines and 42 compounds were predicted to have 79 common potential protein targets. The common-target network included 129 nodes and 490 edges. Blue represents protein targets, orange represents the herbal medicines, yellow represents the compounds and red represents BYHWD. BYHWD, Buyang Huanwu Decoction.

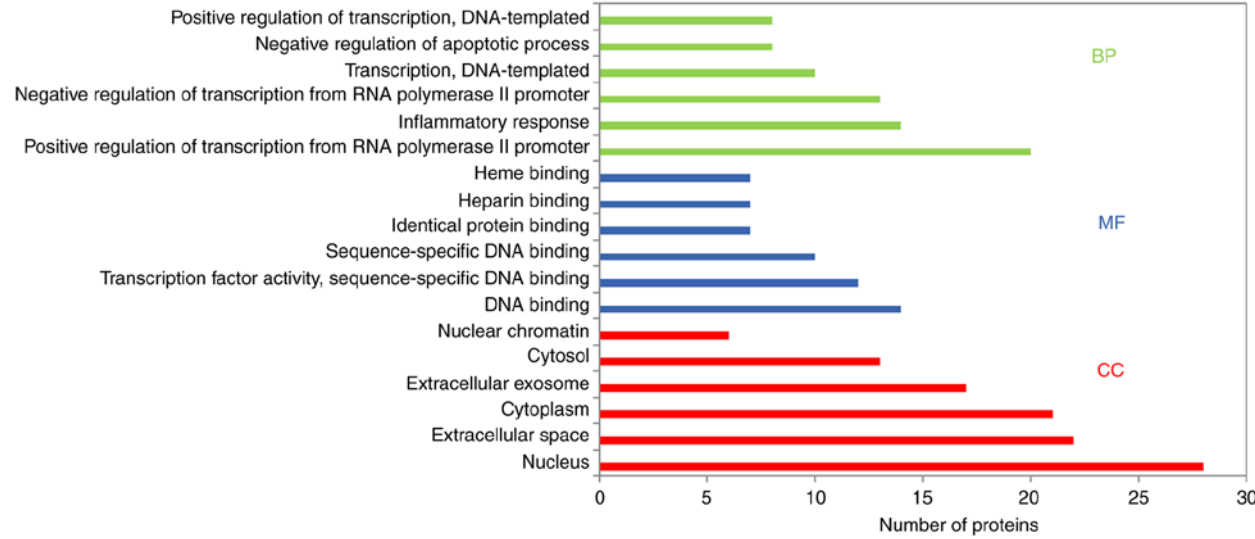


Figure 5. Gene Ontology analysis of candidate targets. The database revealed the top six enriched items in the BP, CC and MF categories. BP, biological process; CC, cell component; MF, molecular function.

Table II. Biological processes of potential target genes based on GO enrichment analysis.

GO ID	Biological process	Genes	P-value
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	HMGB1, TNF, RELA, PPARG, TP53, IGF1, NFKBIA, RB1, CD40, SIRT1, IL10, VDR, IL17A, APP, HIF-1 α , SP1, POU5F1, RIPK1, JUN and MAPK3	6.60x10 ⁻¹²
GO:0006954	Inflammatory response	PIK3CG, HMGB1, CCL2, PTGS2, RELA, TLR4, CD40, AGER, IL10, CXCL10, IL17A, JAK2, NFE2L2 and CD14	7.35x10 ⁻¹¹
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	EGR1, VDR, TNF, POU5F1, RELA, JUN, PPARG, TP53, NFKB1, RB1, SIRT1, DDIT3 and EPO	3.56x10 ⁻⁷
GO:0006351	Transcription, DNA-templated	EGR1, VDR, HIF-1 α , CEBPB, JUN, MAPK3, PPARG, TP53 and STAT3	5.98x10 ⁻⁵
GO:0043066	Negative regulation of apoptotic process	CASP3, ALB, BCL2, TP53, IGF1, CAT, SIRT1 and STAT3	1.78x10 ⁻⁴
GO:0045893	Positive regulation of transcription, DNA-templated	CEBPB, JUN, PPARG, NFKB1, NFE2L2, STAT3 and EPO	1.78x10 ⁻⁴
GO:0031663	Lipopolysaccharide-mediated signaling pathway	MAPK1, CCL2, TNF, MAPK3, NFKBIA, NOS3 and TLR4	7.02x10 ⁻¹⁰
GO:0001525	Angiogenesis	PIK3CG, HIF-1 α , JUN, VEGFA, SERPINE, NOS3 and SIRT1	2.14x10 ⁻⁵
GO:0042127	Regulation of cell proliferation	JUN, NFKBIA, JAK2, NOS2, CD40, PLAU and CXCL10	8.12x10 ⁻⁵
GO:0006357	Regulation of transcription from RNA polymerase II promoter	FOS, SP1, NFKB1, RB1, NFE2L2, STAT3 and SOD2	1.22x10 ⁻³
GO:0001666	Response to hypoxia	PLAT, VEGFA, NOS2, AGER, PLAU and EPO	1.96x10 ⁻⁵
GO:0051092	Positive regulation of NF-kappaB transcription factor activity	TNF, RELA, RIPK1, CAT, CD40 and AGER	7.26x10 ⁻⁵
GO:0006915	Apoptotic process	APP, RIPK1, CYCS, TP53, RB1 and EPO	2.98x10 ⁻⁴
GO:0055114	Oxidation-reduction process	GPX1, NOS3, CAT, NOS2, SOD1 and SOD2	1.43x10 ⁻³
GO:0006955	Immune response	CSF2, IL6, JUN, CD40, IL10 and CXCL10	6.95x10 ⁻³
GO:0043491	Protein kinase B signaling	CSF2, CCL2, TNF, IGF1 and CD40	1.21x10 ⁻⁵
GO:0071356	Cellular response to lipopolysaccharide	JUN, SERPINE1, NOS2, IL10 and CXCL10	1.82x10 ⁻⁴
GO:0006979	Response to oxidative stress	GPX1, APP, PTGS2, APOE and MPO	2.91x10 ⁻⁴
GO:0045766	Positive regulation of angiogenesis	HIF-1 α , VEGFA, SERPINE1, NOS3 and SIRT1	6.75x10 ⁻⁴
GO:0043524	Negative regulation of neuron apoptotic process	CCL2, APOE, JUN, BCL2 and SOD1	7.56x10 ⁻⁴

GO, Gene Ontology.

serve an important role in the inflammatory cascade reaction triggered by cerebral ischemia and are closely related to the expression of various inflammatory mediators (47). Therefore, it is of great significance to intervene in the TLR signaling pathway during the initial stage of the inflammatory response to effectively reduce inflammatory injury in the acute stage of ischemic stroke (47).

The current study verified three key targets (IL6, VEGFA and HIF-1 α) using western blotting, which were predicted in the network. Therefore, the pharmacological mechanism of BYHWD in the treatment of ischemic stroke can be more clearly verified. The results revealed that when compared with the control group, BYHWD significantly inhibited the expression of IL6 and increased the expression of HIF-1 α and VEGFA.

In conclusion, the network pharmacology analysis of BYHWD identified seven herbs, 42 compounds and 79 target genes that were associated with ischemic stroke. The current study did not validate the identified active components, which was a limitation of the study. As the results of network pharmacology analysis revealed that baicalein, beta-carotene, baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, bifendate, formononetin, calycosin, astragaloside IV, stigmaterol, sitosterol, Z-ligustilide and dihydrocapsaicin were the main effective components of BYHWD in the treatment of ischemic stroke, subsequent studies should first verify these results. Based on the pathway enrichment results of the present study, it was determined that the effects of BYHWD against ischemic stroke may be due to some of the ingredients that can simultaneously target multiple

Table III. Potential target gene function based on Kyoto Encyclopedia of Genes and Genomes pathway analysis.

KEGG ID	Pathway	Pathway genes	P-value
hsa05167	Kaposi sarcoma-associated herpesvirus infection	PTGS2/CALM1/PIK3CG/RELA/RB1/MAPK1/MAPK3/NFKBIA/CASP3/CSF2/CYCS/JUN/NFKB1/STAT3/TP53/VEGFA/IL6/CASP9/ICAM1/FOS/HIF-1 α /CREB1/JAK2	1.55x10 ⁻¹⁹
hsa04668	TNF signaling pathway	PTGS2/RELA/MMP9/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CEBPB/CSF2/IL1B/JUN/NFKB1/IL6/ICAM1/CCL2/FOS/SELE/RIPK1/CXCL10/JUNB/CREB1	2.80x10 ⁻²³
hsa05163	Human cytomegalovirus infection	PTGS2/CALM1/RELA/RB1/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CYCS/IL1B/NFKB1/STAT3/TP53/VEGFA/IL6/CASP9/CCL2/RIPK1/SP1/CREB1/CCL3	1.83x10 ⁻¹⁶
hsa04933	AGE-RAGE signaling pathway in diabetic complications	RELA/MAPK1/MAPK3/TNF/BCL2/CASP3/IL1B/JUN/NFKB1/STAT3/VEGFA/IL6/ICAM1/CCL2/SELE/AGER/EGR1/NOS3/AGT/SERPINE1/JAK2	7.02x10 ⁻²³
hsa04657	IL17 signaling pathway	PTGS2/RELA/MMP9/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CEBPB/CSF2/IL1B/JUN/NFKB1/IL6/CCL2/FOS/CXCL10/FOSB/IL17A/JUND	5.04x10 ⁻²²
hsa05161	Hepatitis B	RELA/MMP9/RB1/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CYCS/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/FOS/CREB1/JAK2	6.08x10 ⁻¹⁷
hsa05152	Tuberculosis	CALM1/NOS2/RELA/MAPK1/MAPK3/TNF/BCL2/CASP3/CEBPB/CYCS/IL1B/NFKB1/IL6/TLR4/CASP9/CD14/VDR/IL10/CREB1/JAK2	3.94x10 ⁻¹⁶
hsa05145	Toxoplasmosis	NOS2/PIK3CG/RELA/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CYCS/NFKB1/STAT3/TLR4/CASP9/IL10/CD40/HSPA1A/JAK2	2.25x10 ⁻¹⁷
hsa04210	Apoptosis	RELA/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CYCS/JUN/NFKB1/TP53/CASP9/PARP1/BBC3/ATM/FOS/RIPK1/DDIT3	6.85x10 ⁻¹⁶
hsa05142	Chagas disease (American trypanosomiasis)	NOS2/RELA/MAPK1/MAPK3/NFKBIA/TNF/IL1B/JUN/NFKB1/IL6/TLR4/CCL2/FOS/IL10/ACE/SERPINE1/CCL3	1.03x10 ⁻¹⁶
hsa05418	Fluid shear stress and atherosclerosis	CALM1/RELA/MMP9/TNF/BCL2/IL1B/JUN/NFKB1/TP53/VEGFA/NFE2L2/ICAM1/CCL2/FOS/SELE/NOS3/PLAT	1.87x10 ⁻¹⁴
hsa05202	Transcriptional misregulation in cancer	PPARG/RELA/MMP9/CEBPB/CSF2/IGF1/MPO/NFKB1/PLAU/TP53/IL6/ATM/CD14/CD40/SP1/DDIT3/PLAT	2.41x10 ⁻¹²
hsa05169	Epstein-Barr virus infection	RELA/RB1/NFKBIA/TNF/BCL2/CASP3/CYCS/JUN/NFKB1/STAT3/TP53/IL6/CASP9/ICAM1/RIPK1/CD40/CXCL10	8.51x10 ⁻¹²
hsa05166	Human T-cell leukemia virus 1 infection	RELA/RB1/MAPK1/MAPK3/NFKBIA/TNF/CSF2/JUN/NFKB1/TP53/IL6/ICAM1/ATM/FOS/CD40/EGR1/CREB1	3.38x10 ⁻¹¹
hsa04151	PI3K-Akt signaling pathway	PIK3CG/RELA/MAPK1/MAPK3/BCL2/IGF1/NFKB1/TP53/VEGFA/IL6/TLR4/CASP9/NOS3/CREB1/JAK2/EPO/CHRM1	5.39x10 ⁻⁸
hsa04620	Toll-like receptor signaling pathway	RELA/MAPK1/MAPK3/NFKBIA/TNF/IL1B/JUN/NFKB1/IL6/TLR4/CD14/FOS/RIPK1/CD40/CXCL10/CCL3	2.89x10 ⁻¹⁵
hsa05162	Measles	RELA/NFKBIA/BCL2/CASP3/CYCS/IL1B/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/BBC3/FOS/HSPA1A	2.81x10 ⁻¹³
hsa05164	Influenza A	RELA/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CYCS/IL1B/NFKB1/IL6/TLR4/CASP9/ICAM1/CCL2/CXCL10/JAK2	5.60x10 ⁻¹²
hsa05170	Human immunodeficiency virus 1 infection	CALM1/RELA/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CYCS/JUN/NFKB1/TLR4/CASP9/ATM/FOS/RIPK1	2.12x10 ⁻¹⁰
hsa04010	MAPK signaling pathway	RELA/MAPK1/MAPK3/TNF/CASP3/IGF1/IL1B/JUN/NFKB1/TP53/VEGFA/CD14/FOS/JUND/DDIT3/HSPA1A	2.60x10 ⁻⁸
hsa05133	Pertussis	CALM1/NOS2/RELA/MAPK1/MAPK3/TNF/CASP3/IL1B/JUN/NFKB1/IL6/TLR4/CD14/FOS/IL10	1.55x10 ⁻¹⁹

Table III. Continued.

KEGG ID	Pathway	Pathway genes	P-value
hsa04064	NF-kappa B signaling pathway	PTGS2/RELA/NFKBIA/TNF/BCL2/IL1B/NFKB1/PLAU/TLR4/PARP1/ICAM1/ATM/CD14/RIPK1/CD40	2.80x10 ⁻²³
hsa04066	HIF-1 signaling pathway	NOS2/RELA/MAPK1/MAPK3/BCL2/IGF1/NFKB1/STAT3/VEGFA/IL6/TLR4/HIF-1 α /NOS3/SERPINE1/EPO	1.83x10 ⁻¹⁶

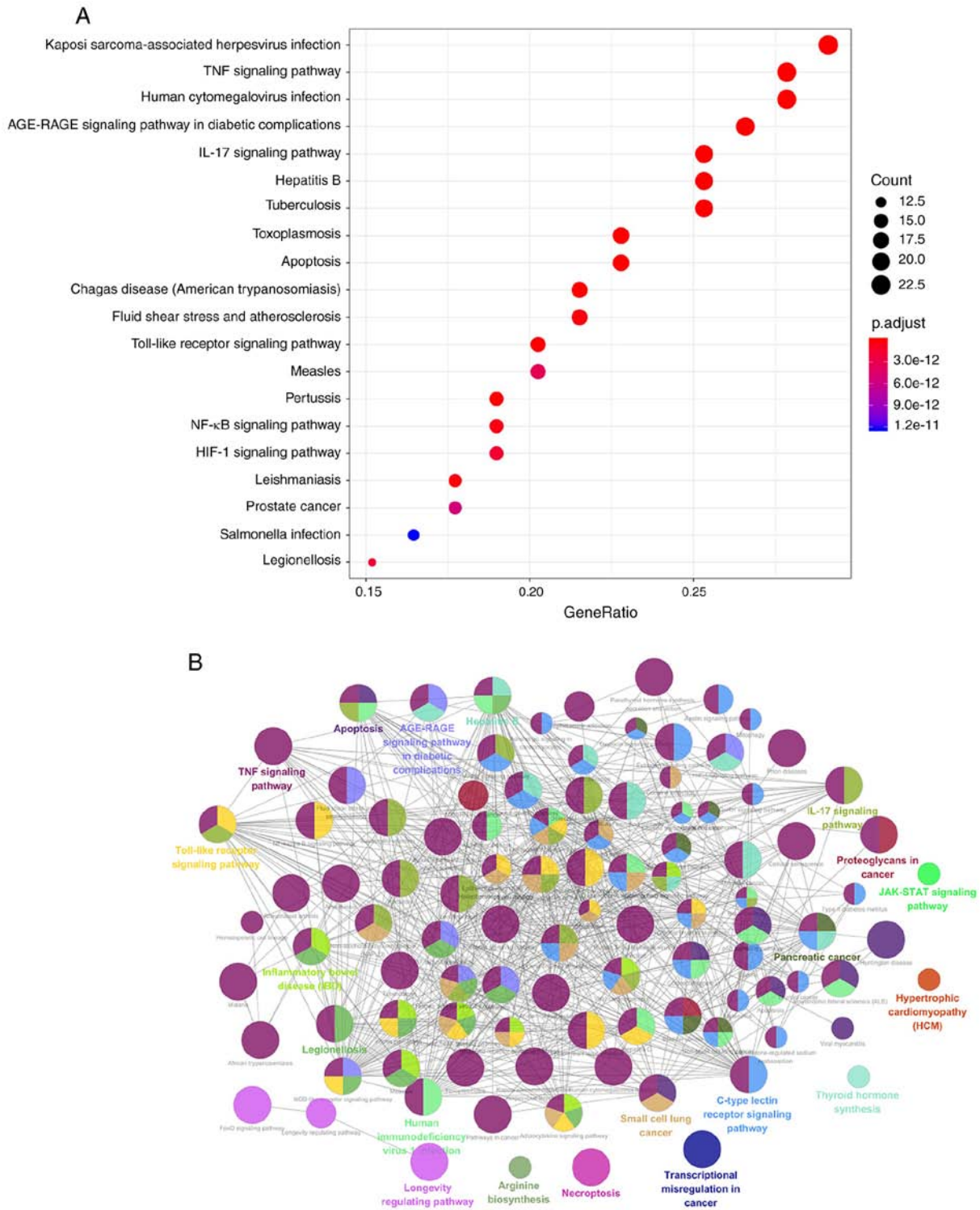


Figure 6. Buyang Huanwu Decoction in the treatment of cerebral ischemia signaling pathway. (A) KEGG pathways of target genes and (B) main functional annotation clusters determined through Biocarta analysis.

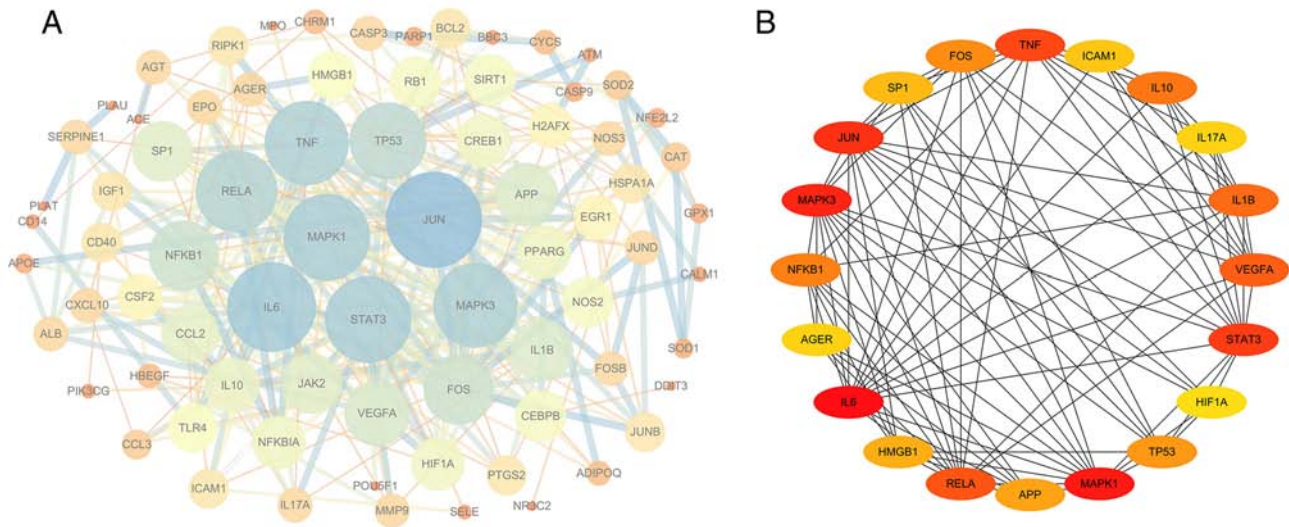


Figure 7. PPI networks of the active ingredients of Buyang Huanwu Decoction for the treatment of cerebral ischemia. (A) The network of PPI relationships had 79 nodes and 327 edges, with an average node degree of 8.28. Each node represented the relevant gene. The edge and line thickness indicated the strength of supporting data. (B) The top 20 hub genes in the PPI network are presented. The darker the color, the higher the score. PPI, protein-protein interaction.

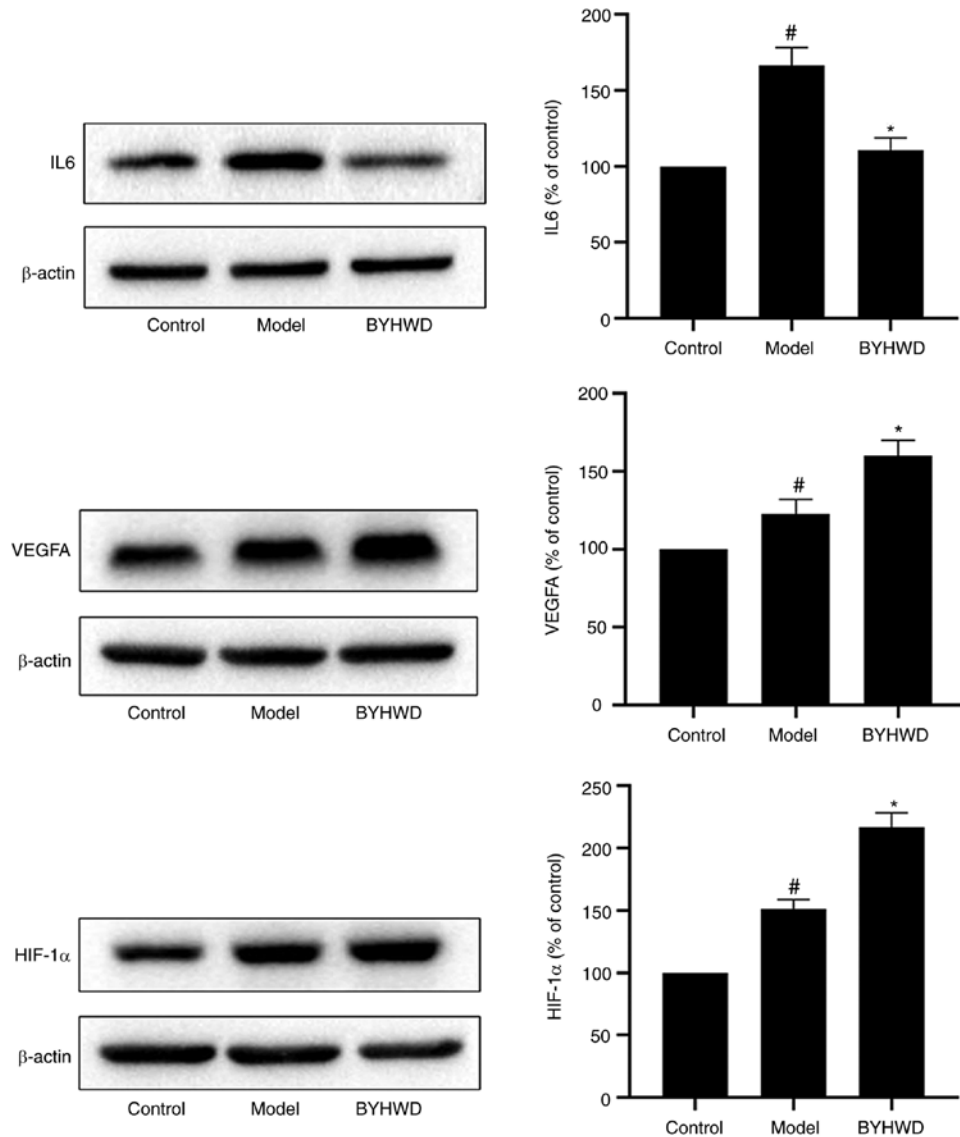


Figure 8. Effects of BYHWD on the levels of IL6, VEGFA and HIF-1α in brain microvascular endothelial cells. Data were expressed as the mean ± SD. [#]P<0.05 vs. the control group. ^{*}P<0.05 vs. the model group. BYHWD, Buyang Huanwu Decoction; HIF-1α, hypoxia-inducible-factor-1α.

pathways, such as the TNF, IL17, apoptosis, PI3K-Akt, TLR, MAPK, NF- κ B and HIF-1 signaling pathways. Furthermore, genes with high degrees of differential articulation were identified from PPI system analysis. IL6, TNF, VEGFA, HIF-1 α , MAPK1, MAPK3, JUN, STAT3, IL1B and IL10 were recognized as center genes. The results indicated that compound-target gene networks can reveal close interactions between multi-components and multi-targets, enhancing understanding of the potential effects of BYHWD in ischemic stroke.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KW, LL, JC and YQ designed the experiments. KW and LL conducted the experiments and researched the literature. KW, JC, YQ and RL collected and analyzed the data. KW wrote the manuscript. KW, JC and ZY revised the manuscript. JD, ZF, YD and YM interpreted the data. ZY and EZ obtained funding and designed the study. KW and LL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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