Study on the Mechanism of Buyang Huanwu Decoction for the treatment of Ischemic Stroke Based on Network Pharmacology

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Abstract

Background: Buyang Huanwu Decoction (BYHWD) is one of the representative prescriptions for tonifying qi and promoting blood circulation. This formula has been widely used in Chinese clinical practice for treatment and prevention of ischemic cerebral vascular diseases. However, the mechanism and active compounds of BYHWD used in clinical practice for ischemic stroke are not well understood. The purpose of this study was to understand the potential active components of BYHWD and further explore its mechanism of improving ischemic stroke.

Methods: This study was based on network pharmacology and bioinformatics analysis. The compounds of BYHWD were obtained from public databases. Oral bioavailability as well as drug-likeness were screened by using absorption, distribution, metabolism, and excretion (ADME) criteria. Then, components of BYHWD, candidate targets of each component and known therapeutic targets of ischemic cerebral were collected. A network of compound-target genes and compound-ischemic cerebral was established by means of network pharmacology data sources. The enrichment of key targets and pathways was analyzed by using string database and DAVID database. In addition, we verified three key targets predicted by western blot analysis (IL6, VEGFA and HIF1A).

Results: Network pharmacology analysis results of BYHWD identified 7 herbs, 42 compounds and 79 target genes associated to cerebral ischemia. The 10 key compounds were baicalein, beta-carotene, Baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, Bifendate, formononetin, Calycosin, AstragalosideIV, Stigmasterol, sitosterol, Z-ligustilide, Dihydrocapsaicin. Core genes in this network were IL6, TNF, VEGFA, HIF1A, MAPK1, MAPK3, JUN, STAT3, IL1B and IL10. And pathways TNF, IL-17, Apoptosis, PI3K-Akt, Toll-like receptor, MAPK, NF-kappa B and HIF-1 signaling pathway, etc. related to ischemic stroke were identified. In vitro experiments, The results showed that compared with the control group (no treatment), BYHWD could significantly inhibit the expression of IL6 and increased the expression of HIF1A and VEGFA.

Conclusions: Network pharmacology analysis can reveal close interactions between multi-components and multi-targets, and enhance our understanding of the potential effects of BYHWD in cerebral ischemia.
1. Background
Ischemic stroke is an acute cerebrovascular disease, also known as cerebral ischemia. It has the characteristics of high disability and high mortality[1, 2]. According to statistics compiled by the American heart association (AHA) 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) is one of the representative prescriptions for tonifying qi and promoting blood circulation in the "Correction on Errors in Medical Classic" by Wang Qingren, a famous doctor in the Qing Dynasty. It is composed of: Radix Astragali, the dried roots of *Astragalus membranaceus* (Fisch.) Bge; Angelicae sinensis Radix, the dried roots of *Angelica sinensis*(Oliv.)Diels; Paeonieae Radix Rubra, the dried roots of *Paeonia lactiflora* Pall; Chuangxiong Rhizoma, the dried rhizomes of *Ligusticum chuanxiong* Hort; Persicae Semen, the dried seeds of *Prunus persica*L. Batsch; Carthami Flos, the dried flowers of *Carthamus tinctorius* L; Pheretima, the dried bodies of *Pheretima aspergillum*E.Perrier[], in the ratio of 120 : 6 : 4.5 : 3 : 3 : 3 : 3. According to the traditional Chinese medicine literature, BYHWD was used to promote blood circulation, and activate energy (qi) flow through energy meridians. This formula has been widely used in Chinese clinical practice for treatment and prevention of ischemic cardio-cerebral vascular diseases[4]. Many researches show that BYHWD has notable curative effectiveness in 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 acting on ischemic stroke. The present study aimed to identify the effective constituents of BYHWD and to further explore its action mechanisms in the amelioration of ischaemic stroke.

Traditional Chinese medicine compound has the characteristics of “multi-component, multi-channel, multi-target”. It is a product of traditional Chinese medicine under the guidance of holistic view and dialectical theory. It has unique advantages for the treatment of complex diseases, but because of the
complex composition of traditional Chinese medicine compound to make its modernization process slow. Network pharmacology, a novel method which combined the system network analysis and the pharmacology, could clarify the synergistic effects and underlying mechanisms among the networks of compound-compound, compound-target and target-disease in the molecular level, which let us know the interactions among the compounds, genes, proteins and diseases. Due to its integrity and systematicness, which are consistent with the overall view of TCM and the principle of dialectical theory, it has been widely applied in TCM research[6]. Therefore, this study applied network pharmacology method to study and discuss the mechanism of BYHWD in the treatment of ischemic stroke, providing reference and theoretical basis for its experimental research and clinical application.

In this study, information on compounds in herbal medicines of BYHWD was acquired coming from public databases, and oral bioavailability (OB) and drug-likeness (DL) were evaluated utilizing ADME standards. Then, components of BYHWD, candidate targets of each component and known therapeutic targets of ischemic stroke were collected. By utilizing these data, compounds of BYHWD-com pound targets and compounds – ischemic stroke target networks were established via network pharmacology databases. Key targets and pathway enrichment were analyzed by STRING database and DAVID database. In addition, we verified three of the key targets predicted in the network by western blot analysis. The workflow of this study on BYHWD against ischemic stroke based on network pharmacology was drawn (Fig. 1).

2. Materials And Methods
2.1. Chemical databases collection of BYHWD
The compound of buyang huanwu decoction was collected from two phytochemical databases:

Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, http://lirts.hkbu.edu.hk/LSP/tcmsp.php) and TCM Database@Taiwan (http://tcm.cmu.edu.tw/).

Bidimensional chemical structures were also gotten from NCBI PubChem (http://pubchem.ncbi.nlm.nih.gov/).

2.2. Pharmacokinetic ADME evaluation
OB represents the ability of a compound to circulate in the body after oral administration. OB can show whether the active compounds in a formula can be delivered through out the body and produce
a physicochemical effect. 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[7]. All compounds were selected using the in silico integrative ADME model administered by the TCMSP Database. Chemicals without ADME information were removed from the final list. The ADME system used in this study includes predict OB and DL, and compounds were retained only if $\text{OB} \geq 30$ and $\text{DL} \geq 0.18$ to satisfy criteria suggested by the TCMSP database[8].

2.3. Target genes related to the identified compounds
Collected all the genes related to the compound from the database, The official names of gene were taken from UniProt (http://www.uniprot.org/) by confining the species to ‘Homo sapiens’.
Subsequently, various ID forms of the targets were transformed into UniProt IDs.

2.4. Target genes collection of cerebral ischemia
Information on cerebral ischemia associated target genes was collected from the therapeutic targets database (TTD; http://bidd.Nus.edu.sg/group/cjttd/TTD_HOME.asp) and DisGeNETv6.0(http://www.disgenet.org/web/DisGeNET/menu/home), and simply “Homo sapiens” proteins connected to cerebral ischemia were selected.

2.5. Construction of networks and pathway analyses
The constructed herbal-chemical-protein networks were visualized using Cytoscape ver.3.6.1 (http://www.cytoscape.org/), Nodes in networks represent herbs and chemicals, and edges indicate interactions between herbs and chemicals, and between chemicals and target genes[9]. The functional pathways of BYHWD related to cerebral ischemia diseases were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/pathway.html) and gene ontology (GO) enrichment evaluation based upon the database for annotation, Visualization and Integrated Discovery(DAVID) version 6.8 (https://david.ncifcrf.gov/). The value of $P < 0.05$ was screened from the enrichment analysis results.

2.6. Protein-Protein interaction (PPI) analyses
The screened drug component-disease common target was imported into the STRING (https://string-db.org/) database to build the protein-protein interaction (PPI) network model, and 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 with the default setting of other parameters. In the network, the size of the nodes represents the size of the degree. The higher the degree, the better the correlation between protein and therapeutic mechanism[10].

2.7. Pharmacological experimental verification

2.7.1. Materials

Buyang Huanwu Decoction is a granule made up of Astragali Radix, Angelicae sinensis Radix, Paeoniae Radix Rubra, Chuangxiong Rhizoma, Persicae Semen, Carthami Flos and Pheretima according to the ratio of 120 : 6 : 4.5 : 3 : 3 : 3 : 3 (Manufacturer: Guangdong Yifang pharmaceutical co. LTD. Product batch: 7070352). The rat Brain Microvascular Endothelial cells (BMECs) were purchased from the Cell Biologics Company (#C57-6023, Chicago, IL). Primary antibody against β-actin, VEGFA, HIF1A, IL6 and Secondary antibody were all purchased from Abcam technology (Cambridge, UK). All other reagents are analytical reagents and commercially available.

2.7.2. Cell culture and treatments

Cells were divided into three groups: control group, OGD group and OGD group treated with HH (100 µg/mL). Oxygen Glucose Deprivation (OGD) was established as follow: cells were rinsed once with glucose-free DMEM (Gibco, Rockville, MD) and transferred to an anaerobic chamber (Forma Scientific, Waltham, MA) containing a gas mixture composed of 7% CO2 and 93% N2 for 6 h at 37 °C. Then, the cells were returned to the normal culture condition. Control BMECs were cultured in complete DMEM under normal conditions. BYHWD groups were treated with 80 µg/mL BYHWD (dissolved in complete DMEM and filtered with a 0.22 µm membrane filter) for 12 h. The control group was treated without BYHWD.

2.7.3 Western blot assay

BMECs were lysed with cold RIPA buffer (Rockford, IL, USA) for 30 min. The whole cell lysates were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore, USA). The membranes were blocked with 5% bovine serum albumin (BSA) and incubated overnight with primary antibodies against VEGFA (1:1000, rabbit), HIF1A (1:1000, rabbit), IL6 (1:1000, rabbit) at 4°C. Subsequently, membranes were incubated with
secondary antibody at a 1:10000 dilution at 37°C for 1 h. The blots were visualized with ECL-Plus reagent (Santa Cruz, USA) and analyzed with Quantity One System image analysis software (Bio-Rad, USA).

2.7.4 Statistical analysis
The results were expressed as mean ± standard deviation (S.D.). Differences between multiple groups were analyzed by One-way analysis of variance (ANOVA) and differences between two groups were analyzed using the t-test. Significant difference was considered when P < 0.05.

3. Results
3.1. Compounds in herbal medicines
BYHWD consists of seven herbal medicines, namely Astragali Radix, Angelicae sinensis Radix, Paeonie Radix Rubra, Chuangxiong Rhizoma, Persicae Semen, Carthami Flos and Pheretima. A total of 102 compounds were identified in BYHWD, among which 21 were Astragali Radix, 23 were Carthami Flos, 4 were Angelicae sinensis Radix, 29 were Paeonie Radix Rubra, 9 were Chuangxiong Rhizoma, 23 were Persicae Semen and 5 were Pheretima. All identified compounds were subjected to ADME screening, among compounds below these requirements, six (Hydroxysafflor yellow A, AstragalosideIV, Ferulic acid, Ligustrazine, Z-ligustilide, Linoleic acid) were considered bioactive compounds. These compounds are the major components of BYHWD, and their effects on cerebral ischemia is have been investigated previously[11–16]. Remove duplicate components and remove components with ambiguous targets. The composition table of BYHWD was obtained, as shown in Table 1.

3.2. Combination of compound target genes and cerebral ischemia target genes
A total of 518 genes and compounds related to BYHWD were obtained from the database. A total of 274 genes related to cerebral ischemia were retrieved from the TTD and DisGeNETv6.0 database, and 79 overlapping genes were pinpointed through matching the mentioned 518 compounds genes with disease-associated genes (Fig. 2).

3.3. Potential target genes and network analysis
The constructed herbal-chemical-protein networks were visualized using Cytoscape, this network of 7 herbs 42 compounds and 79 target genes has 129 nodes and 490 edges (Fig. 3). Particularly, among the 16 compounds (baicalein, beta-carotene, Baicalin, kaempferol, luteolin, quercetin, hydroxysafflor...
yellow A, isorhamnetin, Bifendate, formononetin, Calycosin, AstragalosideIV, Stigmasterol, sitosterol, Z-ligustilide, Dihydrocapsaicin) were linked to more than five genes. Additionally, 33 genes (PTGS2, MMP9, NOS2, MAPK1, MAPK3, TNF, EGFR, APP, JUN, TP53, IL6, IL2, STAT3, NOS3, IL10, CYCS, VEGFA, HIFA, BCL2, CASP9, CAT, PARP1, CASP3, CCL2, MPO, NFE2L2, CSF2, RELA, ALB, NFKBIA, PPARG, NR3C2, ICAM1) were regulated by more than four compounds. The compound-target gene network showed intimate communications between several components and multiple targets, and facilitated a better understanding of the possible curative results of cerebral ischemia.

3.4. PPI network analysis of target genes

The drug component-disease common target was imported into the STRING database to construct the PPI network model of protein-protein interaction. With an interaction score of > 0.9 was selected, the network of PPI relationships has 79 nodes and 327 edges, the average node degree was 8.28, and PPI enrichment p-value was < 1.0e-16 (Fig. 4A). Cytoscape3.6.1 was used to analyze the degree and combined score of PPI network topological eigenvalues, the degree of node color and size reaction center, the edge thickness and colordepth reflect the combined score. The highest twenty target genes with a high degree of connectivity were selected as the hub genes for cerebral ischemia (Fig. 4B).

3.5. GO and KEGG enrichment analysis of potential target genes

To explore the molecular mechanism of BYHWD on cerebral ischemia, GO enrichment analysis and KEGG pathway enrichment of the 79 potential targets were executed. GO enrichment analysis results included biological process (BP), molecular function (MF) and cellular component (CC). Analyze in the DAVID database, take a value of P < 0.05, the results showed that the main biological processes have inflammatory response, negative regulation of apoptosis, angiogenesis, positive regulation of NF-kB transcription factor activity, apoptosis process, immuneresponse, and response to oxidative stress. The molecular function is mainly related to DNA binding, transcription factor activity, heparin binding, heme binding, cytokine activity and growth factor activity. Cell component analysis showed that nucleus and extracellular space for the higher proportion. An introduction of the GO analysis was discovered with the top 6 enriched conditions in the BP, CC and MF categories (Fig. 5) and the
remarkable 20 biological processes in which they are involved were presented in Table 2. Subsequently, to examine the signaling pathways and functions of these target genes, we performed functional enrichment analysis using KEGG pathways. Obtained the signal pathway with statistical significance (p < 0.05), and target genes were found to interact mainly with the TNF signaling pathway, IL-17 signaling pathway, PI3K-Akt signaling pathway, Toll-like receptor signaling pathway, MAPK signaling pathway, NF-kB signaling pathway, and HIF-1 signaling pathway. Therefore, these signaling pathways appear to be closely associated with the potential effects of BYHWD against cerebral ischemia. Identified target genes were listed in Table 3 and take the top 20 signal pathways and draw KEGG bubble diagram (Fig. 6A). Additionally, main functional annotation clusters ranked by the Biocarta functional annotation cluster tool are presented in Fig. 6B.

3.6. Experimental validation
To confirm the results of the network analysis and verify the key targets of BYHWD brain protection, we selected three key targets (IL6, VEGFA and HIF1A) for pharmacological validation (Fig. 7). Western blot results showed that compared with the control group (no treatment), BYHWD could significantly inhibit the expression of IL6 and increased the expression of HIF1A and VEGFA (p < 0.05).

4. Discussion
Ischemic stroke is one of the leading causes of death and disability worldwide. Studies have shown that the injury mechanism of ischemic stroke includes apoptosis, necrosis, inflammation, immune regulation, oxidative stress, etc[17]. However, no effective treatment has been found to prevent damage to the brain in such cases except tissue plasminogen activator. This single compound or single target drug has limited effect on ischemic stroke. Therefore, a promising treatment approach for ischemic stroke is utilization of multiple-component agents with multiple targets[18]. Buyang huanwu decoction has the functions of supplementing qi, activating blood circulation and clearing collaterals. As a classic prescription for treating qi deficiency and blood stasis, it has been attached great importance by doctors of all generations. Traditional Chinese medicine cures disease through multi-approach, multi-target and integerconcept. It has unique advantages for the treatment of complex diseases, but because of the complex composition of traditional Chinese medicine
compound to make its modernization process slow. Furthermore, network pharmacology has provided a new way for exploring pharmacological mechanisms of TCMs. Many scholars have been made an effort to apply network pharmacology to explore the complex of ingredients, targets, and mechanisms of herbal formulas[19].

In this study, network pharmacology was used to investigate the pharmacological mechanism of BYHWD related to cerebral ischemia, which improved the accuracy of targeted prediction to some extent. Network pharmacological analysis of BYHWD identified seven herbs, 42 compounds, and 79 target gene-regulated major pathways related to cerebral ischemia. In particular, the 16 compounds (baicalein, beta-carotene, Baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, Bifendate, formononetin, Calycosin, AstragalosideIV, Stigmasterol, sitosterol, Z-ligustilide, Dihydrocapsaicin) were linked to more than five genes. A number of studies have reported that baicalein has potent neuroprotective properties under in vitro as well as in vivo systems[20]. Beta-carotene serve as an antioxidant, inhibiting free radical production. It may regulate cell growth and death[21]. Baicalin inhibited microglial cell activation and reduced inflammation, oxidative damage, and brain edema[22]. Additionally, Kaempferol has strong anti-inflammatory and antioxidant effects. Numerous scientific reports showed that it has beneficial role on different inflammatory-related diseases such as cardiovascular, and neurodegenerative diseases. Luteolin suppresses inflammation in the brain tissues and regulates different cell signaling pathways[23]. Quercetin has antioxidant stress and neuroprotective effects[24]. Moreover, Hydroxysafflor yellow A protects brain microvascular endothelial cells (BMECs) against oxygen glucose deprivation/reoxygenation(OGD/R) induced injury by inhibiting autophagy via the Class IPI3K/Akt/mTOR signaling pathway[25].

Treatment of experimental stroke mice with isorhamnetin attenuated cerebral edema, improved blood-brain barrier function, and upregulated gene expression of tight junction proteins including occludin, ZO-1, and claudin-5[26]. Furthermore, calycosin protected the brain against ischemic injury through inhibiting calpain activation[27]. Dihydrocapsaicin treated cerebral I/R rats attenuate cerebral and BBB damage through inhibition of oxidative stress and inflammatory pathways[28]. In a word, these findings suggest that the main components of BYHWD are effective for treating cerebral
ischemia.

Genes with high degrees of differential articulation were actually acquired depending on PPI system analysis, IL6, TNF, VEGFA, HIF1A, MAPK1, MAPK3, JUN, STAT3, IL1B and IL10 were recognized as center genes. Interleukin-6 (IL-6) is a multi-functional cytokine with a wide range of biological activities such as regulation of the immune system, generation of acute phase reactions[29]. Vascular endothelial growth factor (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[30]. HIF1A regulates the expression of genes encoding molecules that participate in erythropoiesis, cell proliferation, and energy metabolism, and is closely associated with the regulation of neuronal survival in ischemia[31, 32]. HIF1A can up-regulate the gene expression of proteins related to the vascular system and promote the angiogenesis of VEGF and its receptors to increase blood flow and reduce ischemic injury[33]. TNF is a typical cytokine involved in the acute phase of systemic inflammation and is closely related to the severity of cerebral ischemia[34]. IL-10 is a potent anti-inflammatory mediator, and, if overexpressed, can suppress neuronal degeneration[35].

GO and KEGG pathway analysis to better comprehend the interaction and action pathway of target genes. In results, GO analyses revealed that target genes were majorly associated with the biological processes of positive regulation of transcription from RNA polymerase II promoter, inflammatory response, transcription, DNA-templated, negative regulation of apoptotic process, positive regulation of transcription, DNA-templated, angiogenesis, response to hypoxia, response to hypoxia. The enriched molecular function ontologies were dominated through DNA binding transcription factor activity, sequence-specific DNA binding, sequence-specific DNA binding, identical protein binding, heparin binding and heme binding so on. KEGG pathway analysis were primarily pertaining to TNF, IL-17, Apoptosis, PI3K-Akt, Toll-like receptor, MAPK, NF-kappa B and HIF-1 signaling pathway. Our results suggest that these pathways might 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, decreased oxygen supply, induced
activation of the HIF-1 signaling pathway, and upregulated HIF1A expression, which contributes to the recovery of blood circulation in the penumbra after cerebral ischemia and the transport of glucose, and mediates hypoxia adaptation after hypoxia, and plays a protective role in promoting cell survival and inhibiting apoptosis in brain tissues[36]. MAPK and PI3K/AKT signaling pathways are the main signaling pathways related to apoptosis after cerebral ischemia. Currently, it is believed that MAPK signaling plays a dual role in the process of cell apoptosis, while PI3K/Akt signaling is an important cell survival signaling pathway. Multiple neurotrophic factors inhibit apoptosis by activating the PI3K/Akt signaling pathway, thus playing a protective role in the brain[37]. The PI3K/Akt signaling pathway is involved in the regulation of various intracellular signaling pathways and plays a key role in promoting cell survival and proliferation, anti-apoptosis, regulating glucose metabolism and protein synthesis[38]. Toll-like receptors (TLRS), as inflammatory signal receptors, play an important role in the inflammatory cascade reaction triggered by cerebral ischemia and are closely related to the expression of various inflammatory mediators. Therefore, it is of great significance to intervene the TLRs signaling pathway in the initial stage of inflammatory response to effectively reduce the inflammatory injury in the acute stage of ischemic stroke[39].

In addition, we verified three of the key targets (IL6, VEGFA and HIF1A) that predicted in the network by using western blot analysis. Therefore, the pharmacological mechanism of BYHWD in the treatment of cerebral ischemia can be more clearly verified. The results showed that compared with the control group (no treatment), BYHWD could significantly inhibit the expression of IL6 and increased the expression of HIF1A and VEGFA.

5. Conclusion
In summary, network pharmacology analysis of BYHWD identified 7 herbs, 42 compounds and 79 target genes associated to cerebral ischemia. The 10 key compounds were baicalein, beta-carotene, Baicalin, kaempferol, luteolin, quercetin, hydroxysafflor yellow A, isorhamnetin, Bifendate, formononetin, Calycosin, AstragalosideIV, Stigmasterol, sitosterol, Z-ligustilide, Dihydrocapsaicin. Based on the results of pathwayenrichment, we determined that effects of BYHWD against cerebral ischemia may be due to that ingredients of BYHWD can simultaneously target multiple pathways like
TNF, IL-17, Apoptosis, PI3K-Akt, Toll-like receptor, MAPK, NF-kappa B and HIF-1 signaling pathway and so on. Also, genes with high degrees of differential articulation were actually acquired depending on PPI system analysis, IL6, TNF, VEGFA, HIF1A, MAPK1, MAPK3, JUN, STAT3, IL1B and IL10 were recognized as center genes. Our results indicate that compound-target gene networks can reveal close interactions between multi-components and multi-targets, and enhance our understanding of the potential effects of BYHWD in cerebral ischemia.

**Abbreviations**
BYHWD, Buyang Huanwu Decoction; ADME, Absorption, Distribution, Metabolism, and Excretion; AHA, American heart association; 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; IL-6, Interleukin-6; VEGFA, Vascular endothelial growth factor-A; HIF1A, Hypoxia-inducible factor 1-alpha; TLRS, Toll-like receptors.

**Declarations**

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**Author contributions:**
WK, LL, CJ, QY designed the experiments; WK, LL conducted experiments and researched literature; WK, CJ, QY, LR collected and analyzed the data; WK wrote the manuscript; WK, CJ, YZ revised the manuscript. DJ, FZ, DY, MY conducted data interpretation; YZ, ZE conducted Funds Collection. All authors commented on the results and approved the final manuscript.

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**Availability of data and materials**

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

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no conflict of interest.

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Tables
Due to technical limitations, Table 1 is provided in the Supplementary Files.

Table 2
Biological processes of potential target genes based on GO enrichment analysis.
| 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,HIF1A,SP1,POU5F1,RIPK1, JUN, MAPK3 | 6.60E-12  |
| GO:0006954 | inflammatory response                                                              | PIK3CG, HMGB1, CCL2, PTGS2,RELA, TLR4, CD40, AGER, IL10, CXCL10, IL17A, JAK2, NFE2L2, CD14     | 7.35E-11  |
| GO:0000122 | negative regulation of transcription from RNA polymerase II promoter                | EGR1,VDR,TNF,POU5F1,RELA,JUN, PPARG, TP53, NFKB1, RB1, SIRT1, DDIT3, EPO                         | 3.56E-07  |
| GO:0006351 | transcription, DNA-templated                                                      | EGR1,VDR,HIF1A,CEBPB,JUN,MAPK3, PPARG, TP53, STAT3                                            | 5.98E-05  |
| GO:0043066 | negative regulation of apoptotic process                                           | CASP3, ALB, BCL2, TP53, IGF1, CAT, SIRT1, STAT3                                              | 1.78E-04  |
| GO:0045893 | positive regulation of transcription, DNA-templated                                | CEBPB,JUN, PPARG,NFKB1, NFE2L2, STAT3, EPO                                                    | 1.78E-04  |
| GO:0031663 | lipopolysaccharide-mediated signaling pathway                                       | MAPK1, CCL2, TNF, MAPK3, NFKBIA, NOS3, TLR4                                                  | 7.02E-10  |
| GO:0001525 | angiogenesis                                                                        | PIK3CG,HIF1A,JUN,VEGFA,SERPINE, NOS3, SIRT1                                                  | 2.14E-05  |
| GO:0042127 | regulation of cell proliferation                                                    | JUN,NFKBIA,JAK2,NOS2,CD40,PLAU, CXCL10                                                        | 8.12E-05  |
| GO:0006357 | regulation of transcription from RNA polymerase II promoter                         | FOS,SP1,NFKB1, RB1, NFE2L2,STAT3, SOD2                                                         | 0.001215038 |
| GO:0001666 | response to hypoxia                                                                | PLAT,VEGFA,NOS2,AGER, PLAU,EPO                                                                | 1.96E-05  |
| GO:0051092 | positive regulation of NF-kappaB transcription factor activity                     | TNF, RELA, RIPK1, CAT, CD40, AGER                                                             | 7.26E-05  |
| GO:0006915 | apoptotic process                                                                  | APP, RIPK1, CYCS, TP53, RB1, EPO                                                               | 2.98E-04  |
| GO:0055114 | oxidation-reduction process                                                        | GPX1, NOS3, CAT, NOS2, SOD1, SOD2                                                              | 0.001431211 |
| GO:0006955 | immune response                                                                     | CSF2, IL6, JUN, CD40, IL10, CXCL10                                                             | 0.006954104 |
| GO:0043491 | protein kinase B signaling                                                         | CSF2, CCL2, TNF, IGF1, CD40                                                                   | 1.21E-05  |
| GO:0071356 | cellular response to lipopolysaccharide                                             | JUN, SERPINE1, NOS2, IL10, CXCL10                                                              | 1.82E-04  |
| GO:0006979 | response to oxidative stress                                                       | GPX1, APP, PTGS2, APOE, MPO                                                                    | 2.91E-04  |
| GO:0045766 | positive regulation of angiogenesis                                                | HIF1A, VEGFA, SERPINE1, NOS3, SIRT1                                                            | 6.75E-04  |
| GO:0043524 | negative regulation of neuron apoptotic process                                    | CCL2, APOE, JUN, BCL2, SOD1                                                                    | 7.56E-04  |

Table 3

Functions of potential target genes based on KEGG pathway analysis.
hsa05167 Kaposi sarcoma-associated herpesvirus infection PTGS2/CALM1/PIK3CG/REL/RA/B1/MAPK1/MAPK3/NFKBIA/CASP3/CSF2/CYCS/JUN/NFKB1/STAT3/TP53/VEGFA/IL6/CASP9/ICAM1/FOS/HIF1A/CREB1/JAK2 1.55E-19

hsa04668 TNF signaling pathway PTGS2/REL/MMP9/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CEBPB/CSF2/IL1B/JUN/NFKB1/IL6/ICAM1/CCL2/SELE/RIPK1/CXCL10/JUNB/CREB1 2.80E-23

hsa05163 Human cytomegalovirus infection PTGS2/CALM1/REL/RA/B1/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CSF2/IL1B/NFKB1/STAT3/TP53/VEGFA/IL6/CASP9/CCL2/RIPK1/SPl/CREB1/CCL3 1.83E-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 7.02E-23

hsa04657 IL-17 signaling pathway PTGS2/REL/MMP9/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CEBPB/CSF2/IL1B/JUN/NFKB1/IL6/TLR4/CASP9/FOS/JUND 5.04E-22

hsa05161 Hepatitis B RELA/MMP9/RB1/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CSF2/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/FOS/CREB1/JAK2 6.08E-17

hsa05152 Tuberculosis CALM1/NOS2/REL/A/PAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CEBPB/CSF2/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/CD40/HSPA1A/JAK2 3.94E-16

hsa05145 Toxoplasmosis NOS2/PIK3CG/REL/A/PAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CSF2/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/FOS/CREB1/JAK2 2.25E-17

hsa04210 Apoptosis RELA/MAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/JUN/NFKB1/TP53/CASP9/PARP1/BBC3/ATM/FO/S/DETT3 6.85E-16

hsa05142 Chagas disease (American trypanosomiasis) NOS2/REL/A/PAPK1/MAPK3/NFKBIA/TNF/BCL2/JUN/NFKB1/IL6/TLR4/CCL2/FO/S/ACE/SERPINE1/CCL3 1.03E-16

hsa05418 Fluid shear stress and atherosclerosis CALM1/REL/A/PAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CSF2/JUN/NFKB1/TP53/IL6/TLR4/CASP9/FOS/CREB1/JAK2 1.87E-14

hsa05202 Transcriptional misregulation in cancer PPARG/REA/PAPK1/MAPK3/CEBPB/CSF2/IGF1/MPO/NFKB1/PLAU/TP53/IL6/ATM/CD40/SP1/DDIT3 2.41E-12

hsa05169 Epstein-Barr virus infection RELA/RB1/NFKBIA/TNF/BCL2/CASP3/CSF2/JUN/NFKB1/STAT3/TP53/IL6/CASP9/ICAM1/RIPK1/CD40/CXCL10 8.51E-12

hsa05166 Human T-cell leukemia virus 1 infection RELA/RB1/MAPK1/MAPK3/NFKBIA/TNF/CSF2/JUN/NFKB1/TP53/IL6/ICAM1/ATM/FO/S/CD40/EGR1/CREB1 3.38E-11

hsa04151 PI3K-Akt signaling pathway PIK3CG/REL/A/PAPK1/MAPK3/BCL2/IGF1/NFKB1/TP53/VEGFA/IL6/TLR4/CASP9/NOS3/CREB1/JAK2/EPO/CHRM1 5.39E-08

hsa04620 Toll-like receptor signaling pathway RELA/MAPK1/MAPK3/NFKBIA/TNF/IL1B/JUN/NFKB1/IL6/TLR4/CD14/FOS/RIPK1/CD40/CXCL10/CCL3 2.89E-15

hsa05162 Measles RELA/NFKBIA/BCL2/CASP3/CSF2/JUN/NFKB1/STAT3/TP53/IL6/TLR4/CASP9/BC3/FO/S/HSPA1A 2.81E-13

hsa05164 Influenza A RELA/MAPK1/MAPK3/NFKBIA/TNF/CASP3/CSF2/JUN/NFKB1/IL6/TLR4/CASP9/ICAM1/CXCL10/JAK2 5.60E-12

hsa05170 Human immunodeficiency virus 1 infection CALM1/REL/A/PAPK1/MAPK3/NFKBIA/TNF/BCL2/CASP3/CSF2/JUN/NFKB1/TLR4/CASP9/ATM/FO/S/RIPK1 2.12E-10

hsa04010 MAPK signaling pathway RELA/MAPK1/MAPK3/TNF/CASP3/IGF1/IL1B/JUN/NFKB1/TP53/VEGFA/CD14/FO/S/JUND/DDIT3/HSPA1A 2.60E-08

hsa05133 Pertussis CALM1/NOS2/REL/A/PAPK1/MAPK3/TNF/CASP3/IL1B/JUN/NFKB1/IL6/TLR4/CD14/FO/S/IL10 4.64E-16

hsa04064 NF-kappa B signaling pathway PTGS2/REL/NFKBIA/TNF/BCL2/IL1B/NFKB1/PLAU/TLR4/PARP1/ICAM1/ATM/CD14/RIPK1/CD40 3.44E-14
Figures

Figure 1

Workflow of network pharmacology analysis.
Figure 1

Workflow of network pharmacology analysis.
Figure 2

Venn diagram for compound genes and disease genes.
Figure 2

Venn diagram for compound genes and disease genes.
Figure 3

Network of BYHWD, seven herbal medicines and 42 compounds predicted to have 79 common potential protein targets. The blue ellipses represent the protein targets, orange diamonds delineate the herbal medicines, yellow ellipses represent the compounds and the red hexagon delineates the BYHWD.
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Protein–protein interaction (PPI) networks of active ingredients of BYHWD for the treatment of cerebral ischemia. (A) Each node represents the relevant gene, the edge means line thickness indicates the strength of data support. (B) Hub top 20 genes in PPI network, the darker the color, the higher the score.
Protein–protein interaction (PPI) networks of active ingredients of BYHWD for the treatment of cerebral ischemia. (A) Each node represents the relevant gene, the edge means line
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Figure 5
GO analysis of candidate targets. Database showed the six remarkably enriched items in the biological processes (BP), cell component (CC) and molecular function (MF).
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GO analysis of candidate targets. Database showed the six remarkably enriched items in the biological processes (BP), cell component (CC) and molecular function (MF).

A

B
Figure 6

(A) KEGG pathways of target genes. (B) Main functional annotation clusters by Biocarta analysis.
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(A) KEGG pathways of target genes. (B) Main functional annotation clusters by Biocarta analysis.
Figure 7

Effects of BYHWD on the levels of IL6, VEGFA and HIF-1α in BMECs. (#p<0.05 compared with control group; *p<0.05 compared with model group).
Figure 7

Effects of BYHWD on the levels of IL6, VEGFA and HIF-1α in BMECs. (#p<0.05 compared with control group; *p<0.05 compared with model group).

Supplementary Files
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