Journal of Experimental and Clinical Application of Chinese Medicine

ORIGINAL RESEARCH

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Leech-Derived Hirudin in Yangyin Tongnao Granules: Network Pharmacology Peels Back the Layers of Its Ischemic Stroke Relief Action

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DOI: https://doi.org/10.62767/jecacm604.3725

Keywords

Yangyin Tongnao Granules

Hirudin

Ischemic stroke

Cerebral ischemia-reperfusion injury

Network pharmacology

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Received: 28 October 2025

Revised: 12 November 2025

Accepted: 5 December 2025

Published: 26 December 2025

Journal of Experimental and Clinical Application of Chinese Medicine **2025; 6(4): 17-28.**

Abstract

Background: This study applied network pharmacology to explore the mechanism of Yangyin Tongnao Granules (YYTN) and its key component hirudin in improving cerebral ischemia-reperfusion injury (CIRI). **Methods:** From Traditional Chinese Medicine (TCM) Systems Pharmacology and the literature, the active components of 6 herbs of YYTN were obtained; the targets were obtained based on their sources (the target of leech came from GeneCards, and the other 5 TCM came from HERB, totaling 2879); the CIRI targets were obtained from DisGeNet (300) and GeneCards (144, with a score > 1) (a total of 420). The "TCM - component - target" network and protein-protein interaction (PPI) network (Search Tool for the Retrieval of Interaction Gene/Proteins, confidence = 0.4) were constructed. The core targets were screened using the Matthews Correlation Coefficient method; Kyoto Encyclopedia of Genes and Genomes (KEGG)/Gene Ontology (GO) enrichment analysis was performed on the core targets and 13 targets of hirudin. **Results:** The "TCM - Components - Targets" network contained 6 TCM, 251 components, and 198 targets; the PPI network had 196 nodes and 4760 edges. The top 5 core targets were tumor protein P53 (TP53), Jun proto-oncogene (JUN), Fos proto-oncogene (FOS), signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa B P65 subunit (RELA); the core targets were enriched in 152 pathways in KEGG, while the hirudin target was enriched in 31 pathways and in 487 GO entries. **Conclusions:** YYTN can improve CIRI through multiple components, multiple targets and multiple pathways. Hirudin may exert its effect through pathways such as complement and coagulation cascades.

1 Introduction

Ischemic stroke accounts for over 85% of all types of stroke. Its pathogenesis is complex and involves multiple pathological processes such as neuronal apoptosis, neuroinflammation, and oxidative stress, posing a serious threat to human health [1,2]. Traditional Chinese medicine has demonstrated advantages of multi-target and multi-pathway in the treatment of ischemic stroke, and holds promising prospects for research and application [3,4].

Yangyin Tongnao Granules (YYTN) is an innovative national traditional Chinese medicine (TCM). It is composed of six herbs: *Rehmannia glutinosa*, *Astragalus membranaceus*, *Pueraria lobata*, *Ligusticum chuanxiong* hort, *Whitmania pigra* Whitman, and *Dendrobium nobile* Lindl. Clinical trials at phases II and III have been completed (approval number: 2003L00206). Previous studies have confirmed that YYTN has antioxidant, anti-inflammatory, antithrombotic, increased cerebral blood flow, improved microcirculation, and inhibited apoptosis of nerve cells effects [5,6]. In the MCAO rat model, the active components of YYTN reduced the levels of tumor necrosis factor-α and cytochrome c, and increased the content of total superoxide dismutase, exerting anti-inflammatory, anti-apoptotic and antioxidant effects [7]. Studies have shown that YYTN exert therapeutic effects on ischemic stroke by regulating the expression level of HIF-1α, VEGFA and PAI-1 and altering the activity of the HIF-1 signaling pathway [8]. It can also up-regulate the expression of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) in brain ischemic rats [9]. Among them, as a key medicinal insect of YYTN, leech has the functions of unblocking meridians and removing blood stasis, and improving blood circulation, and has a clear improvement effect on symptoms such as limb weakness after stroke [10]. Hirudin, as the main active component of leeches, has been proven to alleviate neural damage and inflammatory responses in acute cerebral stroke [11]. Moreover, it can promote cerebral vascular generation by activating the Wnt/β-catenin pathway in the regulation of angiogenesis, thereby exerting neuroprotective effects on middle cerebral artery occlusion/reperfusion (MCAO/R) rats [12]. However, the specific target and signaling pathway of hirudin's role in regulating angiogenesis in ischemic cerebral stroke remain unclear at present, and the "TCM - components - target" network relationship of the overall function of YYTN also lacks systematic analysis.

Network pharmacology is based on the research concept of "multiple components - multiple targets - multiple pathways", which can systematically explore the correlations between the active components of TCM and disease targets, providing an efficient research method for clarifying the mechanism of action of TCM compound and single component ingredients [13]. In this study, through the network pharmacology method, the active components and action targets of YYTN were systematically screened, the relevant action network was constructed and enrichment analysis was performed, with a focus on analyzing the potential mechanism of hirudin in improving ischemic stroke, aiming to provide a theoretical basis for subsequent *in vitro* and *in vivo* experimental verification, and promoting in-depth research and application of YYTN and hirudin in the treatment of ischemic stroke.

2 Materials and methods

2.1 Database source

This study employed multiple types of databases and software to support the analysis process. Specifically, the TCM Systems Pharmacology (TCMSP) database (https://old.tcmsp-e.com/tcmsp.php) was used to obtain the active ingredients of YYTN [14]. The HERB database (http://herb.ac.cn/) was used to directly search for the action targets of five types of TCM

(*Rehmannia glutinosa Libosch*, *Astragalus membranaceus*, *Pueraria lobata*, *Ligusticum chuanxiong* hort, and *Dendrobium nobile* Lindl) [15]. The GeneCards database (https://www.genecards.org/) was used to retrieve the action targets of leech [16]. At the same time, using the keyword "cerebral ischemia reperfusion", the software screened for disease targets related to cerebral ischemia reperfusion injury (CIRI) with a score greater than 1. The DisGeNet database (https://www.disgenet.org/home) was used to supplement the acquisition of CIRI-related disease targets with the keyword "Reperfusion Injury" [17]. The Search Tool for the Retrieval of Interaction Gene/Proteins (STRING) database (https://string-db.org) was used to construct the protein-protein interaction (PPI) network [18]. And the Cytoscape 3.7.1 software (Institute of Systems Biology, Seattle, WA, USA) was used, which not only was used to construct the "TCM - component - target" network and the "hirudin - disease target - pathway" network, but also the built-in CytoHubba plugin was used to screen the core targets.

2.2 YYTN active components and compound target screening

This study employed a stepwise screening strategy for the active components of YYTN and the compound targets: In the active component screening stage, first, the active components of the six TCM in YYTN were retrieved from the TCMSP database, and then the component information was supplemented and verified based on the published relevant literature. Finally, a complete list of the active components of YYTN was compiled, with a particular focus on extracting the active component information from the leech. In the compound target screening stage, a source-based search method was adopted - the target of the leech was retrieved from the GeneCards database, while the targets of the other five TCM were directly retrieved from the HERB database. Subsequently, the target information of the six TCM was combined and duplicate targets were eliminated, ultimately obtaining the compound action targets of YYTN.

2.3 CIRI disease target acquisition

Search for CIRI-related targets using the keyword "Reperfusion Injury" in the DisGeNet database; search for disease-related targets using the keyword "cerebral ischemia reperfusion" in the GeneCards database (with a Score > 1). Merge the search results from the two databases and remove duplicate targets. Finally, obtain the CIRI disease-related targets.

2.4 Construction of the "TCM - component - target" network

Using the Venny 2.1.0 online tool, the intersection of the YYTN compound target (2879) and the CIRI disease target (420) was obtained to identify the common action targets for improving CIRI by YYTN. Based on the re-generated plotting data, the 6 types of TCM of YYTN, the selected active ingredients, and 198 common action targets were imported into the Cytoscape 3.7.1 software. The node attributes were set - the TCM were green squares, the components were red triangles, and the targets were purple/blue cross shape. "TCM - component - target" network and "hirudin - disease target - pathway" network were constructed.

2.5 Construction of PPI network and screening of core targets

Import 198 common interaction targets into the STRING database, set the species as "Homo sapiens", use the default threshold of the database (confidence = 0.4), hide the isolated nodes, and obtain the original data of the PPI network. Import the original PPI network data into the Cytoscape 3.7.1 software, use the built-in CytoHubba plugin to calculate the association scores of each target (the darker the node

color, the higher the Matthews Correlation Coefficient (MCC) score), and determine the top 30 core targets.

2.6 Enrichment analysis

A Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted for 30 core targets, with a significance threshold of $p < 0.05$ set; The KEGG enrichment bubble plot for the top 10 pathways was generated using R software (v4.3.1, https://www.r-project.org/) with the ggplot2 package (v3.4.4). For the 13 targets directly associated with hirudin, a KEGG and Gene Ontology (GO) enrichment analysis was re-performed. In the KEGG enrichment analysis, a significance threshold of $p < 0.05$ was set, and the top 10 pathway enrichment graph was drawn using R software (v4.3.1) with the ggplot2 package (v3.4.4). In the GO enrichment analysis, a significance threshold of $p < 0.05$ was set, and significant entries were obtained, covering three dimensions: biological process (BP), cellular component (CC), and molecular function (MF), and the GO enrichment analysis graph was drawn.

3 Results

3.1 Analysis of the active components of YYTN and construction of the "TCM - Components - Targets" network

A total of 696 compounds from 6 herbs in YYTN were obtained from the TCMSP database and literature. There are 349 compounds in CHUAN XIONG, with the most active ingredients (Figure 1A). 35 compounds were isolated from leech (SHUI ZHI), as shown in Figure 1A. In terms of CIRI disease targets, 300 targets were retrieved from the DisGeNet database using the keyword "Reperfusion Injury", and 144 targets were obtained from the GeneCards database (with a score > 1) using the keyword "cerebral ischemia reperfusion". After merging and removing duplicates, a total of 420 disease targets were obtained. Using the Venny 2.1.0 online tool to take the intersection of compound targets and disease targets, a total of 198 common target points for improving CIRI by YYTN were finally obtained. Subsequently, the "TCM - components - targets" network was successfully constructed by Cytoscape 3.7.1, which includes 6 TCM nodes, 251 component nodes and 198 target nodes; among them, leech corresponds to 12 component nodes and 46 target nodes, and hirudin is independently associated with 13 target nodes (Figure 1B).

3.2 Core target KEGG enrichment analysis

The 198 common interaction targets were imported into the STRING database (with confidence level of 0.4), and the constructed PPI network consisted of 196 nodes and 4760 edges. The average node degree was 48.6, and the average local clustering coefficient was 0.664, indicating strong interactions among the targets. Using the MCC method of the CytoHubba plugin and the node color depth in Figure 2A, the top 30 core targets were selected. Among them, the top 5 core targets were tumor protein 53 (TP53), Jun proto-oncogene (JUN), Fos proto-oncogene (FOS), signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa B P65 subunit (RELA) (Figure 2A). These targets are all involved in the key pathological processes of ischemic stroke. Subsequently, KEGG enrichment analysis was conducted on 30 core targets, resulting in a total of 152 significant pathways ($p < 0.05$). Figure 2B showed the top 10 pathways, which were the lipid and atherosclerosis pathway, the Kaposi's sarcoma-associated herpesvirus infection pathway, the hepatitis B pathway, the EB virus infection pathway, the measles pathway, the human cytomegalovirus infection pathway, the AGE-RAGE signaling pathway (diabetic complications), the TNF signaling pathway, the endocrine resistance pathway, and the prolactin signaling pathway.

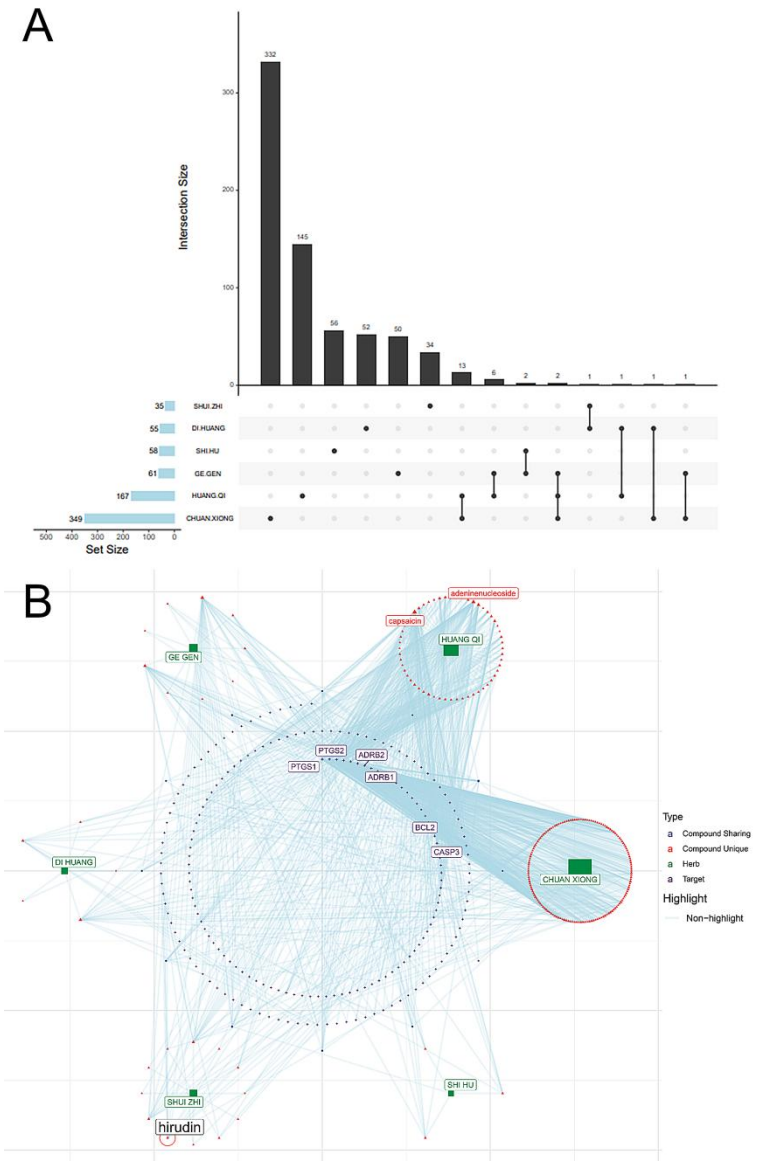

Figure 1 The "Traditional Chinese Medicine (TCM) - Components - Targets" network of Yangyin Tongnao Granules (YYTN). (A) Upset diagram showing intersections of active components among six YYTN TCMs (CHUAN XIONG, HUANG QI, GE GEN, SHI HU, DI HUANG, SHUI ZHI). Displays unique/shared components, with emphasis on 35 components in leech (SHUI ZHI). Components are from TCMSP database and literature. Upper columns: number of intersection combinations; left horizontal columns: total active components per TCM. (B) "TCM - Components - Targets" network. Shows associations between TCMs, their active components, and predicted targets (from GeneCards database), visualized via Cytoscape 3.7.1. Nodes: green squares (TCM), red triangles (active components), purple crosses (targets); connections indicate "TCM-Component" or "Component-Target" relationships.

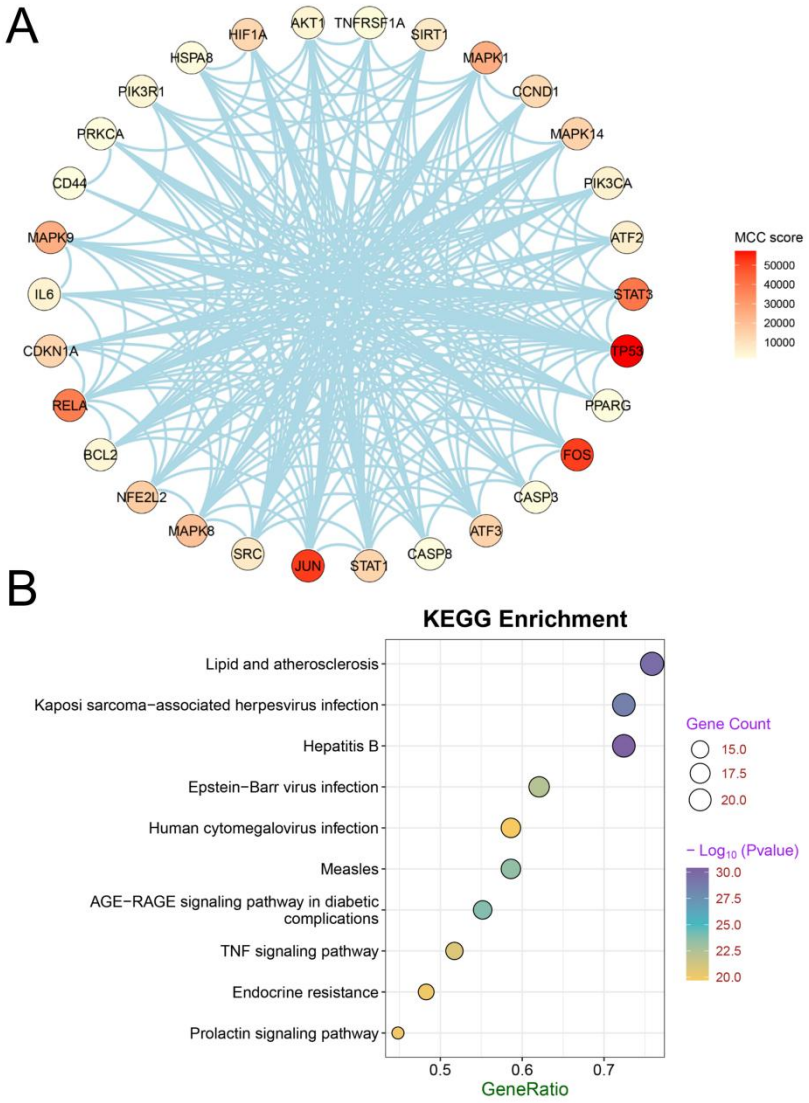

Figure 2 Core target screening and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. (A) Core target screening. 198 common drug-disease targets were imported into Search Tool for the Retrieval of Interaction Gene/Proteins (STRING) database (with confidence level of 0.4) to construct a PPI network. Top 30 core targets were selected via Cytoscape 3.7.1's CytoHubba plugin using the Matthews Correlation Coefficient (MCC) method. Nodes represent targets; node color darkness positively correlates with MCC score (darker = higher score, stronger gene correlation). (B) KEGG enrichment analysis bubble chart of core targets. 30 core targets underwent KEGG analysis (significant threshold: adjusted $p < 0.05$), with the top 10 pathways plotted via R software with the ggplot2 package (v3.4.4), showing key YYTN pathways for improving CIRI. X-axis: gene ratio (core targets in pathway/total core targets); y-axis: KEGG pathway names. Node size = number of enriched core targets (Count); color intensity = $-\log_{10}$ (adjusted p) (darker = more significant enrichment).

3.3 "Hirudin - disease target - pathway" network construction

From the YYTN "TCM - Components - Targets" network, 13 direct target points related to hirudin (such as coagulation factor II (F2), tissue-type plasminogen activator (PLAT), Plasminogen Activator, Urokinase (PLAU), Cluster of Differentiation 40 Ligand (CD40LG), etc.) were extracted. Combined with the disease targets of CIRI and the significantly enriched pathways, a "hirudin - disease target - pathway" network was constructed (Figure 3A). This network

clearly showed the association between hirudin (red nodes) and 13 associated target points (blue nodes), and the significantly enriched pathways (purple nodes) (Figure 3A). Among them, target points such as PLAU and F2 were directly connected to key pathways such as the complement and coagulation cascades, and the NF-κB signaling pathway (Figure 3A).

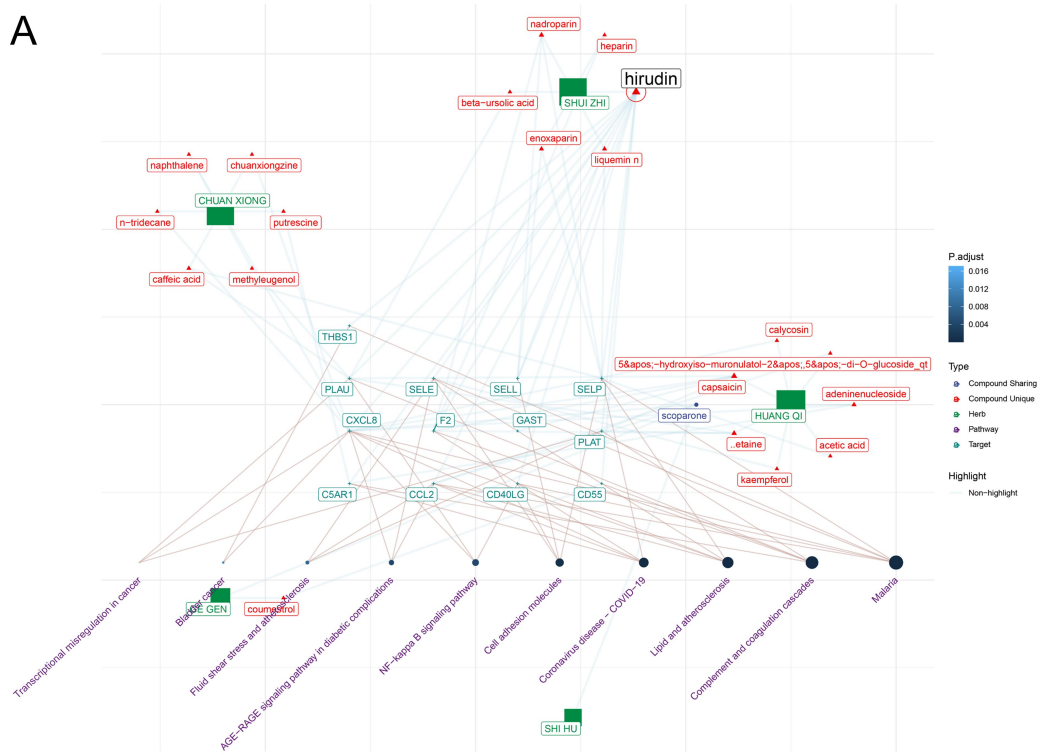

Figure 3 "Hirudin - disease target - pathway" network construction. (A) "Hirudin - disease target - pathway" network. Visualizes hirudin's potential mode in regulating CIRI via an association network of hirudin targets (from YYTN active components), CIRI disease targets (DisGeNet/GeneCards), and pathways (KEGG enrichment results), generated with Cytoscape 3.7.1. Nodes: red (hirudin), blue (13 associated targets), purple (pathways); connections indicate interrelationships.

3.4 Hirudin target enrichment analysis

KEGG enrichment analysis was conducted on the 13 target points directly associated with hirudin, resulting in 31 significant pathways ($p < 0.05$). Figure 4A presented the top 10 pathways, including the malaria pathway, the lipid and atherosclerosis pathway, the coronavirus disease-COVID-19 pathway, the complement and coagulation cascades pathway, and the cell adhesion molecule pathway, etc. Among them, the complement and coagulation cascades pathway, the NF- κ B signaling pathway, and the CIRI pathological mechanism are highly correlated.

GO enrichment analysis yielded a total of 487 significant entries ($p < 0.05$). Figure 4B presented the core enrichment results from three dimensions (BP, CC, and MF). BP was mainly enriched in processes such as leukocyte migration, regulation of leukocyte migration, and coagulation, CC was mainly enriched in regions such as the external side of plasma membrane and secretory granule membrane, and MF was mainly enriched in functions such as sulfur compound binding and receptor ligand activity (Figure 4B). This further validates the protective mechanism of hirudin against CIRI at the molecular level.

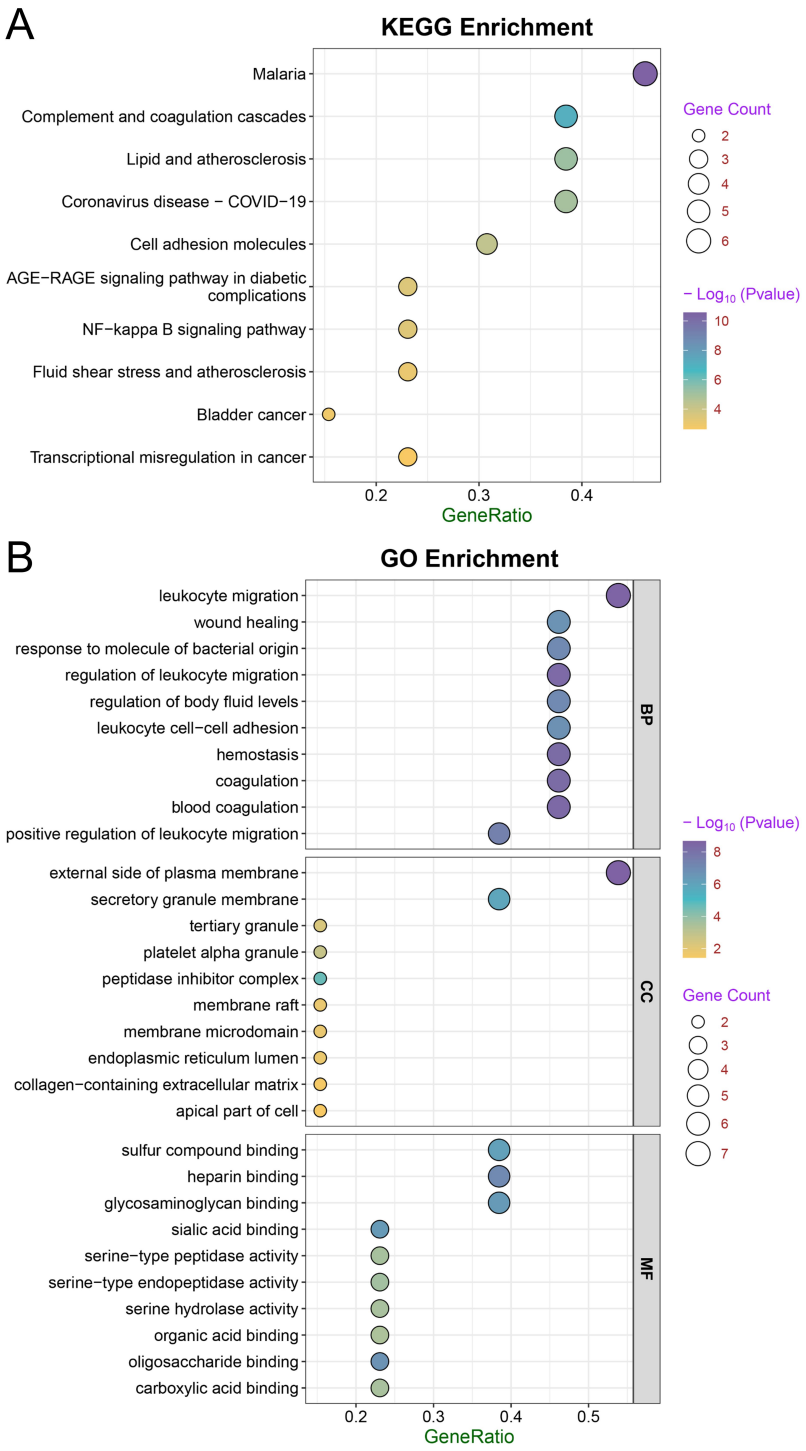

Figure 4 KEGG and Gene Ontology (GO) enrichment analysis bubble plot for the hirudin target. (A) KEGG enrichment analysis bubble plot of hirudin targets. Hirudin targets underwent KEGG analysis (significant threshold: adjusted $p < 0.05$), with the top 10 pathways plotted via R software with the ggplot2 package (v3.4.4). X-axis: gene ratio; y-axis: KEGG pathway names. Node size = number of enriched targets (Count); color shade = -$\log_{10}$ (adjusted p) (darker = more significant enrichment). (B) GO enrichment analysis bubble plot of hirudin targets. Hirudin targets underwent GO analysis (significant threshold: adjusted $p < 0.05$), with the top 30 GO entries plotted via R software with the ggplot2 package (v3.4.4). X-axis: gene ratio; y-axis: GO entry names. Node size = number of enriched targets (Count); color intensity = -$\log_{10}$ (adjusted p) (darker = more significant enrichment). "BP" = biological process, "CC" = cellular component, "MF" = molecular function.

4 Discussion

CIRI is a crucial pathological process in the treatment of ischemic stroke. In recent years, with the increasing incidence of ischemic stroke, the neurological functional impairment and poor prognosis caused by CIRI have become increasingly prominent, seriously affecting the quality of life of patients [19,20]. TCM has demonstrated unique value in the treatment of CIRI due to its advantages of "overall regulation and multi-target intervention". YYTN, as a TCM compound with anti-thrombotic and improving microcirculation effects [21], the regulatory mechanism of the active component of the medicinal herb leech, which is the core component of YYTN, in CIRI still lacks systematic analysis. This study, based on the network pharmacology method, conducts in-depth exploration of the mechanism of action of YYTN and hirudin in improving CIRI.

In the target retrieval stage, this study identified a total of 2879 YYTN compound targets. The constructed "TCM - components - targets" network contains 6 TCM nodes, 251 component nodes, and 198 common targets. Among them, leech corresponds to 12 component nodes and 46 target nodes. In the latest research, it was confirmed that the leech extract enhanced the pro-angiogenic effect of endothelial cell-derived exosomes in a mouse model of ischemic stroke [22]. Hirudin, as a key component of leech, can alleviate acute ischemic stroke both *in vitro* and *in vivo* by inhibiting nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) inflammation bodies-mediated neural inflammation [23]. In this study, leech was associated with 13 targets alone (such as PLAU, CD40LG and C-C Motif Chemokine Ligand 2 (CCL2)), and PLAU was directly related to core pathological links such as CIRI coagulation abnormalities and inflammatory activation. Targeting PLAU can protect CIRI by inhibiting the NF- κ B signaling pathway [24]. In addition, a study have shown that hirudin exerts neuroprotective effects against CIRI by inhibiting CCL2-mediated ferrotosis and inflammatory pathways [25]. This discovery strongly supports the hypothesis that "hirudin is the key active component of YYTN for improving CIRI".

The PPI network analysis further highlights the collaborative value of the core targets. The PPI network constructed by 198 common targets contains 196 nodes and 4760 edges, with an average node degree of 48.6, suggesting a close interaction among the targets. The top 5 core targets (TP53, JUN, FOS, STAT3, RELA) selected by the MCC method are all key regulatory factors for ischemic brain injury. TP53 mediates neuronal apoptosis [26], and it has been reported that methylation in the promoter region of the TP53 gene is associated with ischemic stroke [27]. JUN and FOS are involved in stress inflammation [28], STAT3 and RELA regulate immunity and vascular repair [29]. Studies have shown that RELA and STAT3 can mediate autophagy, and serve as potential biomarkers for the diagnosis and treatment of ischemic stroke [30]. Their high correlation indicates that YYTN can improve CIRI from multiple dimensions, which is consistent with the previous pharmacological conclusion of "YYTN's antioxidant and antithrombotic effects".

The enrichment analysis results clearly identified the pathways of action for YYTN and hirudin. Among the 152 significantly enriched KEGG pathways of the core targets, the lipid and atherosclerosis pathway was highly enriched. The lipid and atherosclerosis pathway was closely related to the onset of ischemic stroke [31]. This abnormality in the lipid pathway leads to cerebral vascular stenosis, which is an important trigger for ischemic stroke [32], suggesting that YYTN can alleviate cerebral vascular damage by regulating lipid metabolism. The 13 targets of hirudin had 31 significantly enriched KEGG pathways, among which

the complement and coagulation signaling pathway was in line with its "anti-thrombosis" classic function, and could improve blood flow in the cerebral ischemic area [33,34]. The NF-κB signaling pathway indicated that it can inhibit the release of inflammatory factors and alleviate neuroinflammation [35,36]. The entries such as "white blood cell migration" and "extracellular membrane lateral binding" in the GO analysis further verified the mechanism of action of hirudin at the molecular functional level.

Firstly, network pharmacology relies on database predictions and lacks *in vivo* and *in vitro* experiments to verify the functions of core targets (such as PLAU) and pathways. Secondly, the synergistic effects among the components of YYTN have not been analyzed. For instance, it is not clear whether there is complementarity in target sites between the components of *Ligusticum chuanxiong hort* and hirudin.

5 Conclusion

In conclusion, this study has identified the core targets and pathways through which YYTN and hirudin improve CIRI, providing a direction for future research. This study is a preliminary predictive investigation, and it is important to emphasize that all findings and conclusions require further experimental validation. Future work should focus on verifying the roles of key targets (such as PLAU) and related pathways through methods such as gene knockout and pathway inhibition experiments. Additionally, the multi-component synergistic mechanisms should be further explored, and patient-specific target networks constructed in combination with clinical samples, so as to facilitate the individualized application of YYTN and hirudin.

Acknowledgements

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

All authors contributed to the study conception and design. M.S. designed, performed experiments and wrote original draft; F.S. and Y.L. performed experiments and wrote original draft; W.L. performed experiments and revised the manuscript; F.S., Y.L. and W.L. performed experiments and analyzed the data; M.Z. and W.L. designed experiments and revised the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

No ethical approval was required for this article.

Funding

This work was supported by the Zhejiang Rehabilitation Medical Center College level project (Grant No. ZKJC2503) and by Hangzhou Health Science and Technology Project (Grant No.B20252055).

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Supplementary

Not applicable.

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