Exploring the therapeutic potential of Qi Teng Mai Ning recipe in ischemic stroke and vascular cognitive impairment

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Author contributions
Yao JY conceptualized and developed the methodology, prepared the original draft, and conducted the investigation. Yang YL curated the data, reviewed and edited the writing, and was responsible for the visualization. Chen WI carried out the analysis, operated the software, and performed validation. Fan HY supervised and administered the project, reviewed and edited the writing, and handled correspondence.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
QTMRD, Qi Teng Mai Ning; IS, ischemic stroke; VCI, vascular cognitive impairment; HADb, Human Autophagy Database; ATG9B, autophagy related 9B; FOS, Fos proto-oncogene, AP-1 transcription factor subunit; BAX1, BCL2 Antagonist/Killer 1; CC2L2, C-C Motif Chemokine Ligand 2; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PDB, Protein Data Bank; PPI, protein-protein interaction; BP, biological process; CC, cellular component; MF, molecular function; FC, fold change; OGD/R, oxygen-glucose deprivation/reperfusion; DEGs, differentially expressed genes; DMEM, Dulbecco’s modified eagle medium; TCM, traditional Chinese medicine.

Citation

Abstract
Background: This study aims to explore the therapeutic effects of the Qi Teng Mai Ning recipe on ischemic stroke and vascular cognitive impairment through its potential to modulate cellular autophagy, with a focus on identifying its active ingredients and their target proteins. Methods: The study began with the identification of active ingredients in the Qi Teng Mai Ning recipe. It proceeded to screen the gene expression omnibus database for ischemic stroke and vascular cognitive impairment-associated differentially expressed mRNAs and to identify cellular autophagy-related proteins via the Human Autophagy Database. These proteins were annotated with Gene Ontology and Kyoto Encyclopedia of Genes and Genomes functions and subjected to molecular docking with the recipe’s core active ingredients. In vitro cell experiments were conducted on hypoxic HT22 cells, involving CCK8 assay, lentiviral transfection to silence autophagy related 9B (ATG9B), immunofluorescence staining, and qPCR validation to investigate the effects of the recipe on autophagy. Results: The analysis identified 104 active ingredients targeting 408 proteins and forming a complex ingredient-target network. Intersecting 55 ischemic stroke-related and 909 vascular cognitive impairment-related differentially expressed mRNAs revealed 14 co-expressed mRNAs. Molecular docking showed quercetin, kaempferol, myrcene, and conferone as key ingredients targeting autophagy-related proteins. Cellular experiments indicated that the recipe significantly enhanced cell viability under hypoxic conditions, reduced apoptosis, and modulated the expression of autophagy-related factors, thereby decreasing apoptosis rates in HT22 cells. Conclusion: The Qi Teng Mai Ning recipe offers protective effects against ischemic stroke and vascular cognitive impairment by modulating autophagy-related proteins. Its efficacy highlights the potential of traditional Chinese medicine in treating these conditions, though further research is needed to fully understand its mechanisms and clinical applications.

Keywords: Qi Teng Mai Ning recipe; autophagy; ischemic stroke; vascular cognitive impairment; traditional Chinese medicine
These approaches suggest the possibility of repairing or regenerating damaged brain tissue, thereby offering a hopeful perspective beyond the limitations of current symptom-focused treatments [11]. Concurrently, TCM has been revisited for its wealth of natural compounds, some of which have shown promising therapeutic effects in chronic diseases outside their traditional use cases [12-14]. The increasing recognition of natural products in medical research underscores their potential role in developing new therapeutic strategies for diseases such as IS and VCI [15]. The QTMND, composed of a blend of aromatic and vine herbs, embodies the TCM philosophy of harnessing natural compounds for health benefits. These herbs, such as Ephedrae Herba, shallot, Semen Brassicae, Piperis Fructus, and Ferulea asafoetida, alongside vine herbs like Spatholobus suberectus Dunn and Sargent gloryvine, have been traditionally used for their purported effects on blood flow and vascular health [16]. Despite these traditional uses, the precise mechanisms by which the QTMND affects cerebrovascular diseases remain largely unexplored.

Autophagy is a fundamental catabolic pathway in eukaryotic organisms and can be induced by a variety of triggers, such as hypoxia, infection, and oxidative stress [17]. According to previous studies, autophagy has a dual role: it can have a protective mechanism against pathological processes; however, excessive autophagy can disrupt cell homeostasis [18]. The dual role of autophagy in disease pathogenesis underscores the complexity of targeting this pathway for therapeutic interventions [19, 20]. By mediating apoptosis and maintaining the integrity of the blood-brain barrier, autophagy plays a vital role in the pathophysiology of IS and VCI [21, 22].

Recent studies have emphasized the importance of modulating autophagy to improve the pathological progression of neurological disorders, suggesting that interventions that can influence this pathway may offer new hope for treating conditions like IS and VCI [23]. Recent studies have shown a significant interest in the modulation of autophagy as a therapeutic target for IS and VCI. Notably, non-invasive interventions like vagus nerve stimulation have demonstrated the potential to reduce neuroinflammation, which is a critical aspect of the pathophysiology of ischemic brain damage [24]. Through a combination of bioinformatics analysis and in vitro cell experiments, this research seeks to elucidate the mechanisms by which this TCM formula may exert its effects, thereby providing a theoretical basis and experimental evidence for its application in treating VCI and IS. By bridging traditional knowledge with modern scientific inquiry, this study endeavors to contribute valuable insights to the field, potentially informing future clinical practices and policy decisions related to the management of cerebrovascular diseases and cognitive impairments [25]. To uncover the pharmacological components of the QTMND, comprehensive searches were conducted within the TC MSP and Herb databases, leveraging the TC MSP and Batman-TCM platforms to pinpoint its principal active constituents. This research endeavors to shed light on the QTMND’s influence on autophagy, aiming to demystify its operational mechanisms and potential applications in the contexts of IS and VCI. Employing a combination of bioinformatics analysis and in vitro cell experiments, this study probes into how this TCM formula might target autophagy to ameliorate cognitive deficits post-IS. The goal is to establish both a theoretical framework and molecular experimental support for integrating TCM into the therapeutic landscape for IS and VCI, thereby enhancing its clinical utility.

Materials and methods

Active pharmaceutical ingredients and targets screening

The active ingredients of the 12 drugs contained in the QTMND, Ephedrae Herba, shallot, Semen Brassicae, Piperis Fructus, Ferula assafoetida, Spatholobus Caulis, Piperis Kadiurae Caulis, Polygoni Multiflori Caulis, Trachelomeri Caulis et Folium, Sagarodontoxae Caulis, Tetrapanacis Medulla and Uncariae Ramulus Cum Uncis, were searched through the TC MSP (https://tcmsp-e.com/) and the Herb database (http://herb.ac.cn/), with the following screening criteria: oral bioavailability ≥ 30%; drug-likeness ≥ 0.18; half-life ≥ 4 [26, 27].

The Qi Teng Mai Ning recipe (QTMND) highlights the effectiveness of traditional Chinese medicine in addressing ischemic stroke and cognitive impairment through cellular autophagy. Key ingredients like quercetin and kaempferol target proteins that enhance cell survival and regulate autophagy, showcasing QTMND’s potential as a treatment for neurovascular disorders.

Medical history of objective

The QTMND is a classical traditional Chinese medicine (TCM) formulation, derived from principles laid down in ancient texts and practiced over centuries. While the specific origins of QTMND are not detailed as some other formulas might be, its composition – featuring aromatic and vine herbs such as Ephedrae Herba, Semen Brassicae, and Sargent gloryvine – is rooted in the longstanding TCM philosophy of using natural compounds to maintain balance and health. The herbs in QTMND have been traditionally used to influence blood flow and vascular health, embodying the holistic approach of TCM in treating complex diseases. This historical usage is complemented by contemporary research, which probes into the molecular dynamics of these ingredients, bridging ancient wisdom with modern scientific inquiry.

Background

In the rapidly evolving landscape of global health, cerebrovascular diseases, particularly stroke, have emerged as a forefront concern, fueled by an aging population, accelerated urbanization, unhealthy diets, and an increase in risk factors [1]. As the second leading cause of death worldwide and the third leading cause of combined disability and death, stroke represents a significant public health challenge. In 2019 alone, stroke accounted for approximately 12.22 million new cases and 6.55 million deaths globally, underscoring its substantial impact on global mortality and morbidity [1]. Stroke prevalence is largely driven by modifiable risk factors, including hypertension, hyperlipidemia, diabetes, smoking, poor diet, and excessive alcohol consumption, suggesting potential avenues for prevention and management [2].

Stroke is broadly categorized into hemorrhagic and ischemic types, with ischemic stroke (IS) accounting for about 85% of cases. IS occurs when a blood vessel supplying the brain is obstructed, often due to thrombosis or embolism, leading to the infarction of brain tissue [3]. This condition is closely associated with atherosclerosis, cardiogenic embolism, vascular occlusion, and hypercoagulable states [4]. IS is one of the most common types of strokes worldwide, accounting for approximately 85% of all stroke cases. According to data from the World Health Organization, over 130,000 people die from IS each year globally [5]. Despite the high incidence of IS and its contribution to the global disease burden, current treatments, which focus on revascularization through thrombolysis, mechanical thrombectomy, and anti-platelet therapy, have limited efficacy [4, 6, 7]. Moreover, the post-stroke phase often involves cognitive and emotional disorders, for which specific and effective treatments are scarce.

Vascular cognitive impairment (VCI) further complicates the landscape of cerebrovascular diseases. As a syndrome of cognitive decline secondary to cerebrovascular damage, VCI encompasses a spectrum from mild cognitive impairments to dementia [8]. Epidemiologically, VCI is a major contributor to dementia, accounting for 15% to 30% of cases, and significantly deteriorates the quality of life of affected individuals [9, 10]. Epidemiologically, VCI is a major contributor to dementia, accounting for 15% to 30% of cases, and significantly deteriorates the quality of life of affected individuals. The exploration of regenerative medicine and the potential role of stem cells in providing neuroprotection against ischemic brain injury has opened new avenues in the quest for effective treatments for VCI.

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For drugs that could not be found in the TCMSP database, the Batman-TCM platform (http://bionet.ncpb.org.cn/Batman-TCM/) was used to obtain the active ingredients and the corresponding potential targets, with the following screening criteria: score cut-off set to 100; adjusted P-value cut-off set to 0.05. The gene names were corrected according to the matching targets in the Uniprot database (https://www.uniprot.org/).

### Disease target acquisition

Gene chip data related to “IS” and “VCI” were downloaded from the gene expression omnibus database (https://www.ncbi.nlm.nih.gov/geo/), filtered by (i) Search terms “Ischemic Stroke” and “Vascular Cognitive Impairment”; (ii) Human. For background correction, normalization, and expression value calculation, the Bioconductor R package in R software was used, and the limma package determined the differentially expressed mRNAs between the two groups. P-value < 0.05 and expression fold changes of ≥ 1.5 (log2(fold change (FC)) ≥ 0.58) were the criteria for screening differential genes, where log2FC ≥ 0.58 represented up-regulation of mRNA expression and log2FC ≤ –0.58 represented down-regulation of mRNA expression. The final “IS” and “VCI” differentially expressed genes (DEGs) were collected. The heat map package was used to construct a heat map and cluster the filtered DEGs, and the P-value was converted to $-\log_{10}(P$-value) was grouped according to log2FC (up-regulated DEGs group, down-regulated DEGs group, and non-statistically significant DEGs group). Lastly, the processed data were imported into R for volcano plotting.

### Disease-autophagy-related target screening and protein-protein interaction (PPI) network construction

Genes associated with autophagy were retrieved by searching the Human Autophagy Database (HADB) (http://www.autophagy.lu/) to analyze the interactions between IS-related and VCI-related genes and autophagy-related genes. The R language (http://www.r-project.org/) software and Perl language program were employed to obtain the intersection of the disease-related genes and the autophagy-related genes, which was subsequently input into the Venny 2.1 software (http://bioinfogp.cnb.csc.es/tools/venny/index.html) to construct the Venn diagram. The STRING database (https://string-db.org/) was utilized to construct the PPI network, setting the protein species to “Homo sapiens”, fixing the Settings to Set Settings to “medium confidence: 0.4” and hiding unrelated genes, leaving all other parameters at their default settings to construct the PPI network. Further topological analysis of the PPI network was performed using the Cytoscape 3.7.2 software (https://cytoscape.org/) to screen key targets.

### Gene Ontology (GO) biofunctional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

GO analysis of the shared targets of the QTMD in the treatment of IS and VCI was performed using the clusterProfilerGO. R package in the R programming language (http://www.r-project.org/). GO analysis is primarily used to describe the functions of gene products, including the cellular component (CC), molecular function (MF), and biological process (BP) [29–31]. The clusterProfilerKEGG. R package was used to perform KEGG pathway enrichment analysis, and the path view package was used to map the corresponding signaling pathways. The degree of core pathway enrichment was determined using the enrichment factor values in order to investigate the possible biological functions and signaling mechanisms of the active ingredients of the QTMD in treating cognitive impairment in IS.

### Gene screening for active ingredients, diseases, and autophagy-related targets

We utilized programs written in R (http://www.r-project.org/) and Perl to identify the overlap between genes related to diseases and those associated with drug ingredients. Specifically, we examined the interactions between autophagy-related genes in IS-VCI and the target genes of the active ingredients in the Qi Teng Mai Ning formula. These overlapping gene sets were further analyzed using Venny 2.1 software (http://bioinfogp.cnb.csc.es/tools/venny/index.html) to create Venn diagrams illustrating the intersections.

### Molecular docking validation

To verify the interactions between the top four primary active compounds (excluding any known toxic compounds) and the key proteins identified in preliminary network pharmacology screenings, we conducted molecular docking validations. We acquired the structural formulas of these active compounds from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and generated their 3D structures using Chem3D software, converting them into the mol2 format. The core proteins’ structural domains, in PDB format, were sourced from the Protein Data Bank (PDB) database (http://www.rcsb.org/). Using PyMOL software, we processed the proteins to remove water and phosphate groups. The PDB files for the active compounds and core protein genes were converted to pdbqt format using AutoDockTools 1.5.6 to identify active pockets. Molecular binding energy calculations were executed using a Vina script, and the molecular docking results were visualized. Additionally, Discovery Studio 2019 was employed to identify docking sites and calculate the LibDockScore, facilitating flexible binding analysis. These results were further visualized using PyMOL software. A binding energy below 0 indicates a spontaneous binding potential between the ligand and receptor. A Vina binding energy of $\leq 5.0$ kcal/mol and a LibDockScore greater than 100 denote stable docking [32].

### Cell culture

The HT22 cell line, derived from mouse hippocampal neurons, exhibits significant sensitivity to variations in oxygen and glucose levels, making it a highly suitable model for our study. The HT22 cells were acquired from Millipore, located in Boston, MA, USA, and authenticated using short tandem repeat profiling. Following authentication, the cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Tianhang, Huzhou, China) and 1% penicillin-streptomycin solution (Solarbio, Beijing, China). To ensure the integrity of our experiments, the cells were tested for mycoplasma contamination, confirming their contamination-free status.

### Preparation of the oxygen-glucose deprivation and reoxygenation injury model

For the experiments, $2 \times 10^{5}$ cells were seeded per well in 6-well culture plates. Once the cell confluence reached approximately 70%, the culture medium, comprising DMEM enriched with 1% penicillin-streptomycin solution and 10% fetal bovine serum, was replaced with a specialized DMEM-D-glucose medium from Gibco, based in Waltham, MA, USA. The cells were then incubated in a nitrogen atmosphere (comprising 1% O$_{2}$ and 5% CO$_{2}$) to induce hypoxia. This incubation took place for 12 h, after which the cells were transferred to a CO$_{2}$ incubator to undergo a 12-h period of reoxygenation. At the beginning of the reoxygenation phase, the original culture medium was replaced with a sugar-free medium (Gibco, Waltham, MA, USA). Conferon (10 μM) was added immediately to the medium to initiate cell recovery [33, 34].

### Autophagy related 9B (ATG9B) knockdown

Referencing previous studies, the knockdown of ATG9B in HT22 cells was achieved using two targeted shRNAs (named shATG9B) and a non-targeting scrambled RNA (named EV) constructed into lentiviral vectors [35]. The shRNA sequences were as follows: shATG9B-1: CCGCGCGCTGGTCGCTCCCAGGTCGCGCGTTTT; shATG9B-2: GCGGCCTTGCAGCCTGGCCACCTTCA. Stably transfected cells were selected using a FACSARia II cell sorter (BD Biosciences, San Jose, CA, USA), with all lentiviral particles packaged by Hanbio Biotechnology Co., Ltd. (Shanghai, China).
Cell proliferation assays
Following transfection, HT22 cells were seeded at a density of 5 × 10^5 cells per well in a 96-well plate. After 48 h of incubation, 10 μl of CCK8 solution was added to each well, followed by an additional 2 h of incubation. Finally, the absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader. The experiment was repeated three times.

Immunofluorescence staining
Cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. After fixation, cells underwent a blocking and permeabilization step using PBS containing 0.3% Triton X-100 and 8% goat serum (to prevent non-specific staining) at room temperature. For immunostaining, cells were incubated overnight at 4 °C with primary antibodies against LC3 (Cat#: 14600-1-AP, 1:100, Proteintech, Rosemont, IL, USA) and ATG9B (Cat#: 24050-1-AP, 1:100, Proteintech, Rosemont, IL, USA). Subsequent to three washes with PBST, the cells were incubated for 2 h at room temperature with Cy3-conjugated goat anti-mouse IgG (Cat#: bs-0296G-Cy3, 1:200, Bioss, Woburn, MA, USA) and FITC-conjugated goat anti-rabbit IgG (Cat#: bs-0295G-FITC, 1:200, Bioss, Woburn, MA, USA), followed by three additional washes with PBST. Then, the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Beyotime Institute of Biotechnology, Shanghai, China) for 30 min. Stained images were captured using a laser confocal microscope (FV3000, Olympus, Tokyo, Japan) or a fluorescence microscope (BX53, Olympus, Tokyo, Japan) and analyzed with ImageJ software.

Real-time PCR
Total RNA was extracted using the TRizol Reagent kit (Takara, Mountain View, CA, USA) and its purity and concentration were determined by spectrophotometry. The cDNA was synthesized by reverse transcription under the following conditions: 25 °C for 5 min, 50 °C for 15 min, 85 °C for 5 min, and 4 °C for 10 min. The cDNA was then diluted 10-fold and subjected to real-time fluorescence quantitative PCR amplification using the specific reaction system. The amplification procedure consisted of the following steps: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, and annealing at 60 °C for 30 s. Gapdh gene was used as an endogenous control, and the relative expression was calculated using the 2^(-ΔΔCt) method.

Statistical analysis
The data were analyzed using GraphPad Prism 8. Measurement data are presented as mean ± standard deviation, with one-way ANOVA employed for comparisons among multiple groups and Tukey’s Honestly Significant Difference test for post-hoc analysis between two groups. A P-value of < 0.05 was considered statistically significant.

Results
Active pharmaceutical ingredients and corresponding gene targets of Qi Teng Mai Ning
Our comprehensive analysis of the QTMDN using the Batman-TCM platform and the TCMS platform database, followed by gene name correction according to the Uniprot database, revealed 104 active ingredients. These ingredients correspond to a network of 408 potential effect genes (Figure 1). Utilizing Cytoscape 3.7.2 software, we constructed the QTMDN-Active Ingredient-Activity Related Gene Network, offering a visual representation of the intricate relationships between the recipe’s active ingredients and their gene targets.

The network analysis highlighted eight active ingredients with the highest degree of connectivity, suggesting their pivotal role in the recipe’s therapeutic effects. These are quercetin, alpha-pinene, kaempferol, limonene, delta-3-carene, 3-carene, myrcene, and gamma-terpinene. Notably, quercetin emerged as a central node, indicating its significant influence on the gene network. The results elucidate the significant correlation between the active ingredients of the QTMDN and the gene targets, potentially contributing to the recipe’s therapeutic efficacy.

Figure 1 QTMDN active ingredient-target network. This figure provides a visual representation of the complex interplay between the active ingredients of the QTMDN and their corresponding gene targets. The network is composed of nodes and edges, with each node representing either a formula, herb, ingredient, or target, and each edge depicting the biologically relevant interactions between them. QTMDN, Qi Teng Mai Ning recipe.
Differential gene expression analysis in IS and VCI

Our investigation into IS and VCI revealed significant gene expression alterations, as evidenced by the data obtained from the GSE201482 and GSE201482 datasets. Filtering criteria were meticulously applied, with a focus on the human species and the disease states of IS and VCI. The study incorporated 20 healthy adults and 20 patients with IS, alongside 10 individuals with normal cognitive function and 10 patients with VCI. DEGs were identified through a rigorous selection process, considering a \( P \)-value < 0.05 and a fold change in expression of \( \geq 1.5 \) (\( \log_{2}(\text{FC}) \geq 0.58 \)). From the GSE22255 dataset, 55 DEGs were discerned, with 50 being up-regulated and 5 down-regulated. The GSE201482 dataset unveiled 909 DEGs, consisting of 548 up-regulated and 361 down-regulated mRNAs. In Figure 2A, we present a heat map of the top 55 DEGs from the GSE22255 dataset, illustrating a predominant upregulation of gene expression in IS patients, signified by intense red clusters. Conversely, down-regulated genes are represented in blue. The heatmap is ordered by the magnitude of differential expression and annotated to denote age and clinical group status. This pattern of gene expression demarcation was corroborated by the volcano plot (Figure 2B), where a distinct dispersal of DEGs is noticeable, with up-regulated genes in red, down-regulated in blue, and non-significant genes in gray. The volcano plot’s axes depict \( \log_{2}(\text{fold change}) \) against the negative \( \log_{10}(\text{P-value}) \), highlighting key genes that surpassed the significance threshold. Similarly, Figure 2C exhibits the heat map for the top 100 DEGs from the GSE201482 dataset, focusing on VCI. The visualization follows the same color coding, with the clinical status denoted at the top. The corresponding volcano plot (Figure 2D) aligns with the previous dataset’s findings, underscoring significant genes with a stark contrast between up- and down-regulated genes within the context of VCI. Our results, articulated through methodically constructed graphical representations, elucidate the gene expression trends in IS and VCI. The heat maps and volcano plots together furnish a comprehensive view of the genomic landscape altered in these conditions.

Network and interaction analysis of autophagy-related genes in IS-VCI

Our investigation into the molecular underpinnings of IS and VCI led to the identification of 14 autophagy-related genes pivotal in the disease pathophysiology. Using the HADb, we cross-referenced 257 autophagy-related genes with datasets GSE22255 and GSE201482 to...
discern the genetic landscape specific to IS-VCI (Figure 3). The resulting gene interaction networks were meticulously constructed using Cytoscape 3.7.2 software, delineating the dynamic regulatory interactions among these genes (Figure 4A, 4B). The 14 IS-VCI autophagy-related genes revealed through our Venn diagram analysis underpin a complex network of PPIs. Subsequent analysis conducted on the STRING database, with a minimum interaction score set to medium confidence, further elucidated the PPIs, emphasizing the centrality of autophagy within this biological web (Figure 4C). The Degree algorithm, applied via the CytoHubba plugin, allowed us to pinpoint the top 13 core target genes, establishing a hierarchy of influence within the network (Figure 4D). Figure 3 demonstrates the intersection of autophagy-related genes in IS-VCI, while Figure 4 provides a comprehensive view of gene and protein interactions related to autophagy in IS and VCI. Figure 4A exhibits the DEG interaction network within the GSE22255 dataset for IS, with the intricate interplay of upregulated and downregulated genes represented as red and green triangles, respectively. The central node, indicative of the highest degree of interaction, is aptly highlighted, underscoring its significance in the network. Conversely, Figure 4B extends this analysis to the GSE201482 dataset for VCI, incorporating additional genes signified by cyan circles that remained unaltered in expression but may still play a regulatory role. The PPI network depicted in Figure 4C visualizes the relationship between the proteins of the identified autophagy-related genes, with the central node of autophagy serving as the cornerstone of the network. Lastly, Figure 4D graphically represents the core genes, emphasizing the strength of their interactions within the network and thereby providing a basis for targeted therapeutic strategies.

Results of GO and KEGG enrichment analysis of IS-VCI autophagy-related genes

![Figure 3 Intersection of autophagy-related genes in IS and VCI](image)

![Figure 4 Network analysis of gene and protein interactions in IS and VCI related to autophagy](image)
Utilizing the Bioconductor and clusterProfiler packages in R, we delineated the central BPs, CCs, and MFs associated with these genes. The analysis illuminated predominant BPs, such as cellular responses to external and extracellular stimuli and macroautophagy (Figure 5A).

On the cellular level, CCs were primarily enriched in autophagosomes, phagophore assembly sites, and autophagosome membranes (Figure 5B), while MFs were largely concentrated in BH domain binding and protein kinase regulator activities (Figure 5C). The GO functional enrichment, as illustrated in Figure 5D, presents a clear histogram of enrichment scores, providing an unequivocal visual representation of the data. Similarly, KEGG pathway enrichment analysis elucidated functions primarily involved in pathways such as Kaposi sarcoma-associated herpes virus infection, colorectal cancer, and human cytomegalovirus infection, along with other autophagy-related signaling pathways (Figure 6A). The signaling pathway diagram with IS-VCI autophagy-related genes is meticulously depicted in Figure 6B, offering a schematic representation of the molecular interactions and components involved in autophagy, as identified by our research.

Intersection of QTMND targets with IS-VCI autophagy-related genes
In the quest to identify potential therapeutic targets for IS-VCI, we investigated the intersection of QTMND active ingredient-related genes with autophagy-related genes implicated in IS-VCI. This intersection was elucidated through a meticulous analysis using an online Venn diagram tool, revealing a significant overlap comprising four genes: Fox proto-oncogene, AP-1 transcription factor subunit (FOS), ATG9B, BCL2 Antagonist/killer 1 (BAK1), and C-C Motif Chemokine Ligand 2 (CCL2) (Figure 7). These genes emerged as potential targets for the active ingredients in the QTMND, which may modulate the activity of autophagy-related proteins and offer a novel intervention strategy for cardiovascular IS and VCI. Figure 7 is an illustrative representation of the gene overlap, depicted as a Venn diagram. The shared genes between the QTMND recipe and autophagy-related genes in IS-VCI are clearly demarcated, with the pink circle delineating the autophagy-related genes and the orange circle representing the QTMND recipe genes. This graphical representation not only simplifies the complex interaction between the QTMND recipe and autophagy in IS-VCI but also serves as an aesthetically pleasing and easily interpretable illustration of our findings.

Molecular docking studies of bioactive compounds with IS-VCI autophagy-related proteins
In our recent molecular docking studies, we have identified significant interactions between bioactive compounds and proteins implicated in IS-VCI autophagy. Using Chem3D software, we rendered 3D structures...
of the active ingredients quercetin, kaempferol, myrcene, and conferone and procured 3D structures of the proteins FOS, ATG9B, BAK1, and CCL2 from the PDB database. Subsequent conversion to PDBQT format and docking studies using AutoDockTools 1.5.6 and Vina script revealed that except for myrcene’s interaction with CCL2, which demonstrated a binding energy above −5.0 kcal/mol, all other compounds formed stable dockings with binding energies below this threshold. Discovery Studio 2019 software facilitated the semi-flexible docking of these compounds with their corresponding proteins, with LibDockScore validating the stability of these dockings. Particularly, conferone showed the highest stability in dimer formation with the core proteins FOS and BAK1, as assessed by root mean square deviation, chemical energy, and docking fraction parameters. Figure 8 and Figure 9 depict the 3D and 2D molecular docking models generated by PyMOL and Discovery Studio 2019 software, respectively. These figures illustrate the intricate interactions between the bioactive compounds and the target proteins, highlighting key binding sites and interaction points. For instance, Figure 8A, 9A show the interactions between FOS and quercetin, indicating potential hydrogen bonds and hydrophobic interactions at the binding site. Similarly, the docking models of ATG9B with kaempferol (Figures 8F, 9F) and BAK1 with conferone (Figures 8L, 9L) demonstrate the ligand’s orientation and the molecular contacts essential for stability and specificity of binding. The details of these molecular interactions are crucial, as they lay the groundwork for the potential development of pharmacological interventions targeting autophagy processes in IS-VCI. The data provide a comprehensive view of the chemical affinities and stabilities of these compound-protein complexes, which is vital for the progression of these compounds through the drug development pipeline. Our findings are a testament to the utility of molecular docking studies in the preliminary assessment of drug-target interactions, offering a valuable tool for the prioritization of compounds for further therapeutic development.

**Cellular responses to oxygen-glucose deprivation/reperfusion (OGD/R) and conferone treatment with shATG9B-1 modulation**

Our study evaluated the molecular and cellular mechanisms underlying the response to OGD/R and the effects of conferone treatment with shATG9B-1 modulation. Through a series of bar graphs and immunofluorescence imaging, we elucidated the roles of ATG9B in cell survival, apoptosis, autophagy, and inflammation. Figure 10A presents a bar graph of ATG9B mRNA levels post-OGD/R treatment.

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**Figure 6 KEGG pathway enrichment and autophagy pathway analysis.** (A) Bubble diagram for KEGG enrichment analysis. This diagram provides a visual representation of pathway enrichment based on the genes studied. Each bubble represents a KEGG pathway, with the size indicating the number of genes involved, and the color gradient representing the P-value from high (yellow) to low (red), indicating the level of statistical significance. The y-axis shows various biological pathways, while the x-axis indicates the enrichment factor. (B) Schematic representation of the autophagy signaling pathway. This detailed pathway diagram shows the molecular interactions and components involved in autophagy as identified in the research. The diagram includes main pathway components, with arrows indicating the direction of action or influence. KEGG, Kyoto Encyclopedia of Genes and Genomes.

**Figure 7 Gene overlap between QTMND and autophagy-related genes in IS and VCI.** This figure displays a Venn diagram that identifies the shared and unique genes between the TCM formula Qi Teng Mai Ning and the genes associated with autophagy in IS and VCI. The pink circle represents the set of genes related to autophagy in IS-VCI, and the orange circle represents the genes associated with the QTMND recipe. QTMND, Qi Teng Mai Ning recipe; IS, ischemic stroke; VCI, vascular cognitive impairment.
We observed that control cells displayed baseline ATG9B expression, while shATG9B-1 transfection led to a significant knockdown of ATG9B mRNA. Post-OGD/R, we noted a marked decrease in ATG9B mRNA levels upon conferone treatment, which was partially reversed by shATG9B-1, indicating a modulatory role of ATG9B in response to stress and pharmacological intervention. In Figure 10B, cell viability, assessed via the CCK8 assay, showed high viability in control cells. Cells subjected to OGD/R exhibited reduced viability, while conferone treatment improved cell survival post-OGD/R. However, this improvement was negated by shATG9B-1 transfection, highlighting ATG9B’s potential protective role against OGD/R-induced cytotoxicity. The immunofluorescence images in Figure 10C demonstrate BCL2 expression in neuronal cells, with control cells showing baseline levels. OGD/R treatment upregulated BCL2 expression, which was downregulated with conferone treatment. However, shATG9B-1 transfection restored BCL2 expression to levels similar to those seen in OGD/R alone, suggesting a regulatory mechanism involving ATG9B in the apoptosis pathway. Figure 10D shows Beclin1 expression, which increased following OGD/R treatment in control cells. Conferone treatment reduced Beclin1 levels, but this reduction was counteracted by shATG9B-1, indicating an interaction between ATG9B and autophagic processes. Analysis of IL-1β expression through immunofluorescence in Figure 10E revealed an increase after OGD/R, indicative of an inflammatory response. Conferone reduced IL-1β expression, but shATG9B-1 reversed this effect, implying ATG9B's involvement in inflammatory pathway modulation. Finally, Figure 10F illustrates the expression of IL-6. Similar to IL-1β, IL-6 levels were elevated post-OGD/R treatment, reduced by conferone, and restored upon shATG9B-1 treatment, supporting ATG9B's regulatory role in inflammatory cytokine expression. These findings provide a comprehensive understanding of the role of ATG9B in neuronal survival and death mechanisms following ischemic conditions and therapeutic interventions.

Figure 8 Molecular docking 3D models of target proteins with bioactive compounds. (A) Molecular docking model showing the interaction of the protein FOS with the flavonoid quercetin. The model highlights the binding sites and the conformational fit of quercetin within the active site of FOS. (B) 3D representation of the FOS protein in complex with kaempferol, detailing the interaction points and the stability of the ligand within the protein’s binding pocket. (C) Docking model of FOS with the terpene Myrcene, depicting the molecular interactions and binding affinity between the small molecule and the protein. (D) The complex of FOS with the compound Conferone, illustrating the binding efficiency and spatial orientation of the ligand when docked with the protein. (E) ATG9B protein-quercetin interaction model, showing the docking position and potential hydrogen bonds and hydrophobic interactions between the protein and quercetin. (F) 3D docking model of ATG9B with kaempferol, highlighting the binding dynamics and the molecular fit within the active site. (G) Visualization of the ATG9B protein in complex with Myrcene, demonstrating the interaction sites and binding conformation. (H) The binding model of ATG9B with Conferone, showing the ligand’s orientation and interaction within the protein’s active region. (I) BAK1-quercetin complex model, highlighting the docking position, interaction sites, and binding stability. (J) Molecular docking model of BAK1 with kaempferol, indicating the ligand binding sites and the conformational placement within the protein. (K) Docking representation of BAK1 with Myrcene, depicting the ligand's binding conformation and interaction points. (L) BAK1-Conferone interaction model, showing the docking orientation and binding sites of the ligand within the protein's active pocket. (M) Molecular model of the CCL2 protein in complex with quercetin, illustrating the binding interactions and ligand conformation. (N) Docking model of CCL2 with kaempferol, showing the molecular interaction and binding stability within the protein's binding site. (O) CCL2 protein complexed with Myrcene, detailing the binding interactions and orientation of the ligand. (P) Interaction model of CCL2 with Conferone, highlighting the docking position and interaction points within the protein’s active site.

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Figure 9 Molecular docking 2D models of target proteins with bioactive compounds. (A) FOS-quercetin interaction depicted in a 2D model, highlighting the key hydrogen bonds and hydrophobic interactions between the protein and the flavonoid. (B) 2D representation of FOS-kaempferol binding, showing the specific atomic interactions and bond distances that contribute to the ligand’s affinity for the protein. (C) FOS-Myrcene docking model in 2D, illustrating the molecular contacts that define the interaction profile between the terpene and the protein. (D) The interaction of FOS with Conferone in a 2D model, detailing the binding pocket and the essential contacts. (E) ATG9B-quercetin 2D docking model, outlining the ligand’s binding orientation and the points of interaction within the protein’s active site. (F) 2D diagram of ATG9B-kaempferol interactions, depicting the stabilizing hydrogen bonds and the complementary fit of the ligand within the protein. (G) ATG9B-Myrcene interaction 2D model, displaying the interaction landscape and the binding conformation of the terpene. (H) 2D docking model of ATG9B with Conferone, highlighting the ligand’s key interactions and fit within the binding site. (I) BAK1-quercetin 2D interaction model, detailing the docking position and molecular interactions within the binding domain. (J) BAK1-kaempferol 2D model showing the interaction network and bond lengths critical to the binding affinity. (K) 2D interaction model of BAK1 with myrcene, illustrating the ligand’s docking conformation and interaction points. (L) BAK1-conferone 2D docking model, highlighting the compound’s orientation and key contacts within the protein’s active site. (M) CCL2-quercetin 2D interaction model, illustrating the binding interactions and molecular orientation of quercetin. (N) 2D docking representation of CCL2 with kaempferol, showing the interaction points and binding conformation. (O) CCL2-Myrcene 2D interaction model, detailing the docking orientation and molecular contacts. (P) 2D model of CCL2-Conferone interactions, depicting the ligand’s binding site and the critical points of interaction.
Discussion

Recent research underscores the pivotal role of autophagy, a crucial intracellular degradation process, in neuroprotection. The etiology of IS is multifaceted, incorporating oxidative stress, intracellular calcium imbalance, immune-inflammatory responses, inflammation-induced blood-brain barrier compromise, excessive reactive oxygen species production, apoptosis, and autophagy, among other mechanisms [36, 37]. The role of autophagy in IS is complex. It has been shown that the autophagy inhibitor 3-MA markedly reduces apoptosis, indicating that autophagy may contribute to ischemia-induced neuronal death [38]. The impact of autophagy on neurons seems to depend on the duration of ischemia and subsequent reperfusion [39]. Intriguingly, while autophagy can exacerbate cerebral ischemic damage through mechanisms such as microglia and astrocyte apoptosis and endothelial cell inflammatory secretion, it also offers protection by facilitating the differentiation of microglia to the anti-inflammatory M2 type and reducing cerebrovascular endothelial cell apoptosis [40-44].

VCI is an acquired mental retardation syndrome characterized by neurodegeneration, cognitive impairment, and memory difficulties for which there is no effective treatment [45]. The pathogenesis of VCI encompasses neuronal damage, immune-inflammatory response, oxidative stress, β-amyloid deposition resulting in blood-brain barrier disruption, and alterations in cerebrovascular reactivity [46-51]. Crucially, dysregulation of autophagy is implicated in VCI’s progression, contributing to neuronal damage and intracellular β-amyloid accumulation. This dysregulation is also associated with VCI caused by chronic cerebral hypoperfusion [22]. There is evidence that modulating autophagy could mitigate some VCI symptoms [52].

In our investigation, four genes of interest – FOS, ATG9B, BAK1, and CCL2 – were pinpointed through differential analysis using the HADB database alongside datasets GSE201482 and GSE22255. Among these, FOS contributes to the AP-1 transcription factor complex, playing a pivotal role in cellular processes such as proliferation, differentiation, apoptosis, and transformation [53]. Prior studies underscore FOS’s role in managing oxidative stress and neuronal apoptosis within IS models, emphasizing its significance [51]. Similarly, ATG9B, crucial for autophagosome formation and the initiation of autophagy, has been linked with autophagy dysregulation through gene mutations [54, 55]. This gene’s involvement extends to cancer progression, metastasis, and resistance to therapy, as well as susceptibility to

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coronary artery diseases [56–58]. BAK1, a member of the BCL2 protein family, regulates apoptotic processes and promotes neuronal survival by modulating apoptosis [59]. Conversely, CCL2, a pro-inflammatory chemokine that targets the CCR2 receptor, is integral to the inflammatory response mechanism in stroke, Alzheimer’s disease, and other neurological conditions [60, 61]. Emerging research suggests CCL2’s role in neurological recovery post-stroke, potentially offering protection through mechanisms like angiogenesis, neurogenesis, and inflammation modulation [62, 63]. Our findings corroborate these studies, underlining the involvement of autophagy-related genes in the pathogenesis and progression of IS and VCI, thus enriching the literature on these subjects.

Further, by examining the QTMND’s influence on gene and protein expression profiles in IS and comparing these effects with those of conventional treatments, we can deepen our understanding of its underlying mechanisms. Existing research has shed light on specific peripheral immune responses following cerebral hemorrhages and proteomic alterations in rat brains post-stroke [64, 65]. These findings pave the way for investigating how TCM recipes like Qi Teng Mai Ning might influence similar molecular pathways, potentially offering a novel perspective on stroke and cognitive impairment therapy through autophagy modulation. Furthermore, considering the utilization of hydrogen sulfide and its donors in the treatment of IS, particularly their role in neuroprotection through the modulation of autophagy, research on the Qi Teng Mai Ning formula may further substantiate the therapeutic potential of TCM components for IS [66]. This perspective aligns with current research on the application of Chinese medicinal components in the treatment of IS [67]. When discussing the therapeutic potential for IS and VCI, it is imperative not to focus on direct neuroprotective strategies but also to consider the role of inflammation in the pathogenesis of IS, where inflammatory markers could serve as diagnostic indicators for early neurological deterioration [68].

The principal bioactive constituents of the Qi Teng Mai Ning formula were acquired via the TCMSP and Batman-TCM platforms, with the top four core bioactive constituents identified as quercetin, kaempferol, myrcene, and conferone. Previous investigations have proposed the potential involvement of these compounds in IS and VCI. For instance, quercetin has demonstrated enhancements in cerebral blood flow, inhibition of thrombosis and oxidative stress, and interference with ischemia-induced apoptosis and necrosis. Moreover, it has exhibited protective attributes against IS and showcased clinical significance in various cardiovascular ailments such as atherosclerosis and myocardial ischemia [69, 70]. Quercetin’s engagement in antioxidant stress, neuronal safeguarding, and mitigation of ischemic/hypoxic injury through autophagy regulation has been postulated [71–74]. Kaempferol, on the other hand, has been noted for its ability to prevent inflammation and oxidative stress while enhancing mitochondrial function [75]. In instances of IS and traumatic brain injury, kaempferol has fortified the blood-brain barrier, shielded neurons, and minimized neuronal harm [76–78]. Through the AMPK/mTOR signaling pathway, kaempferol regulates autophagy and inhibits apoptosis, consequently reducing neurological injuries caused by ischemic brain events [79]. Myrcene has exhibited efficacy in scavenging free radicals and mitigating neuronal damage stemming from cerebral ischemia-reperfusion [80, 81]. Conferone primarily exerts anti-angiogenic effects and manifests cytotoxicity against various tumor cells, including breast cancer cells. Nevertheless, its role in cerebrovascular diseases like stroke remains inadequately elucidated [33, 82]. This study has revealed the pivotal role of the potential bioactive constituents of the Qi Teng Mai Ning formula in neurological disorders, corroborating prior literature findings. Therefore, we posit that the identified core bioactive ingredients may modulate and regulate autophagy, thus conferring a protective effect against IS and VCI.

Moreover, molecular docking models were established to validate the interactions between the top 4 core active ingredients (excluding known toxic constituents) and the core proteins identified through network pharmacology screening. The outcomes revealed that quercetin, kaempferol, myrcene, and conferone could all successfully dock with their respective core proteins (FOS, ATG9B, BAK1, and CCL2) in a semi-flexible manner, with conferone forming the most stable bond with FOS. Thus, the active constituents of the QTMND may partake in the protection against IS and VCI by modulating autophagy-related proteins.

Presently, there exists a dearth of research on the interaction between the TCM component conferone and autophagy-related proteins FOS, ATG9B, BAK1, and CCL2. Nonetheless, alterations in the expression levels of FOS, ATG9B, BAK1, and CCL2 have been demonstrated to impact the activation of the autophagy pathway. Furthermore, ATG9B serves as a pivotal protein in the autophagic process, contributing to the formation and fusion of autophagosomes. Additionally, BAK1 assumes a critical role within the autophagy mechanism, participating in the genesis of autophagosomes and the dynamic alterations of membrane structures. Lastly, CCL2, a chemotactic factor, engages in inflammatory responses and immune regulation, with associations with the autophagic process. Thus, it is postulated that the active ingredient conferone in the QTMND may interact with the autophagy genes FOS, ATG9B, BAK1, and CCL2, thereby regulating the activation of the autophagy pathway and the formation of autophagosomes. This forms a theoretical foundation for further exploration into the treatment of autophagy-related diseases with TCM [30, 81]. Furthermore, the adoption of sophisticated computational methodologies, such as machine learning, can explore the utility of machine learning in the identification of cancer biomarkers, showcasing the potential of these technologies in elucidating and comprehending the mechanisms of action of natural products [83, 84]. The amalgamation of such technologies could foster a more comprehensive understanding of the autophagy modulation effects of the QTMND, potentially culminating in more efficacious treatments for IS and its associated conditions.

Conclusions

In this investigation, the bioactive constituents of the Qi Teng Mai Ning formula manifested protective properties against IS and VCI by modulating autophagy-related proteins, offering a potential therapeutic avenue for the clinical management of IS complicated by VCI and laying a theoretical groundwork for the clinical application of the Qi Teng Mai Ning formulation. Nevertheless, the study encountered certain limitations. Specifically, we exclusively scrutinized the bioactive elements of the Qi Teng Mai Ning formulation via a TCM ingredient database without experimental verification of these constituents. Moreover, the exploration of IS and VCI disease targets relied on bioinformatics analysis, potentially introducing biases in dataset selection. Consequently, further validation through in vitro and in vivo experiments is imperative to validate the findings. Additionally, the study was confined to in vitro cell experiments, lacking pertinent in vivo animal studies, thereby constraining our capacity to fully elucidate the precise mechanism of action of the Qi Teng Mai Ning formulation. Thus, future endeavors will encompass additional in vivo animal investigations alongside pertinent clinical trials to comprehensively delineate the underlying mechanism of the Qi Teng Mai Ning formulation in IS and VCI treatment, with the aim of furnishing valuable insights for clinical practice.

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