Exploration of the Potential Mechanisms of Lingqihuangban Granule for Treating Diabetic Retinopathy Based on Network Pharmacology

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Research

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Abstract

**Background:** The Lingqihuangban Granule (LQHBG), a remarkable Chinese herbal compound, has been used for decades to treat diabetic retinopathy (DR) in Department of Ophthalmology, Shanghai General Hospital (National Clinical Research Center for Eye Diseases) with obvious effects. Through the method of network pharmacology, the present study constructed bioactive component-relative targets and protein-protein interaction network of the LQHBG and implemented gene function analysis and pathway enrichment of targets, discussing the mechanisms of traditional Chinese medicine LQHBG in treating DR.

**Materials and methods:** The bioactive ingredients of LQHBG were screened and obtained using TCMSP and ETCM databases, while the potential targets of bioactive ingredients were predicted by SwissTargetPrediction and ETCM databases. Compared with the disease target databases of TTD, Drugbank, OMIM and DisGeNET, the therapeutic targets of LQHBG for DR were extracted. Based on DAVID platform, GO annotation and KEGG pathway analyses of key targets were explored, combined with the screening of core pathways on Omicshare database and pathway annotation on Reactome database.

**Results:** A total of 357 bioactive components were screened from LQHBG, involving 86 possible targets of LQHBG treating DR. In PPI network, INS and ALB were identified as key genes. The effective targets were enriched in multiple signaling pathways, such as PI3K/Akt and MAPK pathways.

**Conclusion:** This study revealed the possible targets and pathways of LQHBG treating DR, reflecting the characteristics of multicomponent, multitarget and multipathway treatment of a Chinese herbal compound, and provided new ideas for further discussion.

**Background**

Diabetic retinopathy (DR) is a multifactorial neuromicrovascular complication of diabetes mellitus, serving as a main cause of blindness among working-aged people [1]. According to recent epidemiological studies, one-third of diabetic patients worldwide suffer from variant periods of DR, greatly increasing the economic and psychological burdens to individuals and society [2]. Currently, the treatment of DR is limited to laser photocoagulation of the retina and anti-vascular endothelial growth factor (VEGF) medications, accompanied by endocrine therapy and surgical methods, both of which have serious side effects and poor long-term prognosis, such as increased intraocular pressure, reverse aggravation of neovascularization and severe retinal hemorrhage [3, 4]. Therefore, it is urgent to seek potential targets and to explore new treatment methods and strategies.

Traditional Chinese Medicine (TCM) holds the opinion that diabetic retinopathy is associated with bad mood, tiredness and unhealthy diet [5], causing internal heat accumulation and damage to the liver, spleen, and kidney in the five internal organs. Then, deficiency of the Qi and imbalance of Yin and Yang bring about impaired blood circulation, such as congestion and hypoperfusion of the retinal microvasculature [6]; thus, the therapies of balancing Yin and Yang, promoting blood circulation and removing blood stasis are adopted in clinical practice. Traditional Chinese Medicine is a comprehensive
drug therapy that is widely used in Asian countries and that is gaining increasing amounts of attention and wide acceptance for its synergistic effect of multiple ingredients and minor side effects [7, 8]. Thus far, traditional Chinese medicine has shown its unique superiority and remarkable effects on the prevention and treatment of DR and has broad development prospects [9].

The LQHBG is a remarkable, prevalent Chinese herbal compound, consisting of Cistanche (Roucongrong), Lucid ganoderma (Lingzhi), Lycium barbarum (Gouqizi), Angelica sinensis (Danggui), Semen Cuscutae (Tusizi), Rhizoma atractylodis (Cangzhu), Ligusticum wallichii (Chuanxiong), Salvia miltiorrhiza (Danshen) and Codonopsis pilosula (Dangshen). It has been used for decades in Department of Ophthalmology, Shanghai General Hospital (National Clinical Research Center for Eye Diseases), with obvious effects, and many research articles have been published. According to the theory of traditional Chinese medicine, the spleen is the biochemical source of Qi and blood, with the prescription of Codonopsis pilosula possessing the functions of nourishing the spleen and stomach, benefitting Qi and blood, and Rhizoma atractylodis tonifying the spleen and reducing dampness. Cistanche, Semen Cuscutae and Lycium barbarum all benefit the kidney and essence, regulating the balance of Yin and Yang in the kidney. Among them, Lycium barbarum nourishes the liver and improves eyesight. In addition, Ligusticum wallichii and Salvia miltiorrhiza promote blood circulation and regulate Qi and blood transportation, while Lucid ganoderma serves as a treatment for fatigue syndrome and nourishes Qi and blood circulation. The LQHBG is a prescription mainly for tonifying the kidney and strengthening the spleen, treating DR and relieving the associated symptoms in many ways. Modern pharmacological studies show that some herbs of the LQHBG are capable of therapeutic effects on DR, such as Salvia miltiorrhiza [10, 11], Lycium barbarum [12, 13] and Rhizoma atractylodis, and the ingredients of single herbs also possess various functions [14, 15]. For example, by virtue of its remarkable antioxidant properties, Salvia miltiorrhiza elicits neuroprotective functions and prevents retinal neuronal apoptosis [16]. Its pharmacological effect of promoting blood circulation is applied to treat many blood circulation disorders, such as thrombosis, hypoperfusion, congestion and stasis [17]. It has been reported that Lycium barbarum prevents epithelial cell and neuronal apoptosis by regulating the Bcl-2/Bax and caspase3-mediated apoptotic pathway [18–20]. Kimura and Tsuneki’s studies manifest that β-eudesmol isolated from Rhizoma atractylodis has a definite blocking effect in anti-angiogenic action through the inhibition of the ERK signaling pathway [14, 15]. However, the therapeutic effects of the remaining herbs on DR, such as Codonopsis pilosula, Semen Cuscutae and Cistanche, have not been reported in the literature, and the 9 herbs of the LQHBG have not been studied as a whole for their synergistic and complementary effects on DR. Moreover, the relationship between these potential targets and molecular pathways has not been studied; thus, the pharmacodynamic substance basis of the prescription and its pharmacological mechanism are not yet clear. Therefore, the systematic and overall study of the biological active components and molecular mechanisms of the LQHBG in the treatment of DR is conducive to providing new ideas for the innovation of clinical medication.

With the rapid development of modern biological information technology, network pharmacology comes into existence with the characteristics of multitarget, multicomponent and multipathway, providing effective screening and prediction of targets and potential mechanisms [21]. Based on the systems
biology theory, the network pharmacology approach establishes relational prediction models between drugs and related targets, diseases and therapeutic targets and integrates the interactive networks [22]. Through the analysis of components in various network modules and specific interactions between the nodes, the relationships between drugs and potential targets and the mechanisms therein are explored systemically and comprehensively [23, 24]. Using the network pharmacology as a method, this study explores the effective bioactive ingredients and relative targets of the LQHBG in the treatment of DR and reveals its molecular mechanism and interactions between herbs, which are of great significance for the clinical promotion of the LQHBG and the innovation of drug therapy.

Materials And Methods

1.1 Obtaining the single-herb chemical components of the LQHBG

The major chemical components of the single herb of LQHBG were searched on the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCSMP) [25] to construct a database of its pharmaceutical ingredients. According to the study results, the obtained bioactive components were screened by the absorption, distribution, metabolism and excretion parameters (ADME) with oral bioavailability (OB) \( \geq 30 \) and drug-likeness (DL) \( \geq 0.18 \).

Similarly, the chemical components of each herb were searched on another database called the Encyclopedia of Traditional Chinese Medicine (ETCM) [26] to acquire the corresponding supplementary bioactive compounds.

1.2 Prediction of targets of the chemical components and acquisition of disease-related targets

Based on the results produced by TCSMP database, the smile chemical structures of the compounds for each single herb were searched on the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database [27], and then based on the structural characteristics of ligands, the predictive targets of the chemical components were gained by submitting them to the SwissTargetPrediction (http://www.swisstargetprediction.ch/) database [28]. The targets without detailed information were then filtered out. Regarding the bioactive compounds generated on ETCM database, the corresponding candidate target genes for each effective ingredient molecule were searched on the database. On the basis of the results, those with both a drug likeness grading higher than weak (i.e., such as moderate and good) and a prediction confidence index more than 0.80 were selected. Taking the above results from each database into consideration, the repetitive targets were filtered out, and the intersection was taken to construct a predictive targets database of the chemical components.
The index term DR was set to search on the Therapeutic Target Database (https://db.idrblab.org/ttd/) [29], the Drugbank database (https://www.drugbank.ca/) [30], the OMIM (https://omim.org/) [31] database and the DisGeNET database (http://www.disgenet.org/web/DisGeNET/menu/home) [32]. The results were summarized and the intersection was taken, and the known therapeutic targets of DR were then built.

1.3 Construction of the bioactive compound-targets network of LQHBG

Venn diagrams were utilized by importing gene names of the predictive targets of the chemical components and disease-related targets to analyze the same targets. With the filtered targets of the bioactive compounds of the LQHBG imported into Cytoscape (Version 3.6.0) [33], the bioactive compound-targets network was constructed. In the network, “node” represented the bioactive components of the LQHBG and relevant targets, while “edge” symbolized the interaction between bioactive compounds and targets. The relative network analysis of the characteristics of bioactive compounds and targets was employed to identify the significant nodes with the topology parameters of degree appearing dominant.

1.4 PPI network analysis and hub target screening

The target protein-protein interactions (PPIs) can be analyzed using the STRING (https://string-db.org/cgi/input.pl) online database [34]. The target information was imported into the STRING database with Homo sapiens being selected as the species and the comprehensive score of interactions by each pair of protein set at >0.4 as the screening condition to obtain the PPI information of the targets. Then, the PPI information was imported into Cytoscape software to visualize the PPI network of the interactive targets. The topological properties were analyzed through the Network analyzer function of the software with the two main network topology parameters of degree (degree) and close to the central (closeness centrality) on the key targets of the PPI network diagram. In addition, with regard to the target genes, the cytoHubba plug-in was adopted to calculate the top ten nodes ranked by Maximal Clique Centrality (MCC) scores that were the same as hub genes serving as supplementary. Meanwhile, the MCODE analysis tool in Cytoscape software was used to screen the modules in the PPI network diagram that may have significant interaction relationships. Moreover, the interactions among the crucial nodes were analyzed by consulting related literature.

1.5 GO biofunctional process and KEGG enrichment analysis of metabolic pathways
As Gene ontology (GO) biofunctional process and pathway analysis may reveal the degree of importance for variant gene targets and signal pathways among the protein-protein interaction network, this study made the use of DAVID (Version 6.8) platform [35] to conduct GO pathway analysis of the significant targets. Then, upon combining the Omicshare database screening core targets and pathways, the outcome was manifested in the form of a bubble diagram to illustrate the dynamic GO and KEGG analyses results. According to the enrichment factor value, the enrichment degree of core pathways was analyzed, aiming at exploring the probable mechanisms of the LQHGB in the treatment of DR. Furthermore, the annotation was added to the filtered core pathways using the Reactome database [36], and related graphics were created.

1.6 Molecular docking

We used the RCSB PDB platform (http://www.rcsb.org/pdb/home/home.do) [37] and downloaded the 3D structure documents of the key target proteins and used the PubChem database and downloaded the 3D structures of the bioactive compounds. The docking calculation was performed using the Yinfo Cloud Computing Platform for molecular docking verification, a friendly and versatile web server for biomedicinal, material, and statistical studies (https://cloud.yinfotek.com).

Results

2.1 Screening the components of the LQHGB

This study retrieved the reported nine herbs of the LQHGB and the screening conditions of the ADME parameters through the TCSMP database. Setting the indicators of OB $\geq 30\%$ and DL $\geq 0.18$ to the active ingredients of each herb, we were able to obtain the results for 6 effective molecules for Cistanche, 61 for Lucid ganoderma, 45 for Lycium barbarum, 2 for Angelica sinensis, 11 for Semen Cuscutae, 9 for Rhizoma atractylodis, 7 for Ligusticum wallichii, 65 for Salvia miltiorrhiza and 21 for Codonopsis pilosula, for a total of 227 bioactive components. On the ETCM database, the drug likeness grading higher than weak and the prediction confidence index more than 0.80 were chosen. After removing the unrelated chemical components and complementing the bioactive compounds through searching the literature, chemical components with high content and clear pharmacological effects were also used as candidate active components, though they did not meet the ADME parameters. As a result, another 1 effective molecule for Cistanche, 46 for Lucid ganoderma, 8 for Lycium barbarum, 23 for Angelica sinensis, 2 for Semen Cuscutae, 5 for Rhizoma atractylodis, 15 for Ligusticum wallichii, 17 for Salvia miltiorrhiza and 13 for Codonopsis pilosula, for a total of 130 bioactive components, were added. Ultimately, a sum of 357 corresponding bioactive compounds was collected. The specific information of the partial herbs of Cistanche Angelica sinensis, Semen Cuscutae and Ligusticum wallichii through TCSMP is shown in table 1. The complete data set is provided in the attachment.
| Mol ID     | Molecule Name  | OB(%) | DL   | Single Herb        |
|------------|----------------|-------|------|--------------------|
| MOL000358  | beta-sitosterol| 36.91 | 0.75 | Cistanche          |
| MOL005320  | arachidonate   | 45.57 | 0.2  | Cistanche          |
| MOL005384  | suchilactone   | 57.52 | 0.56 | Cistanche          |
| MOL007563  | Yangambin      | 57.53 | 0.81 | Cistanche          |
| MOL000098  | quercetin      | 46.43 | 0.28 | Cistanche          |
| MOL008871  | Marckine       | 37.05 | 0.69 | Cistanche          |
| MOL000358  | beta-sitosterol| 36.91 | 0.75 | Angelica sinensis  |
| MOL000449  | Stigmasterol   | 43.83 | 0.76 | Angelica sinensis  |
| MOL001558  | sesamin        | 56.55 | 0.83 | Semen Cuscutae     |
| MOL000184  | NSC63551       | 39.25 | 0.76 | Semen Cuscutae     |
| MOL000354  | isorhamnetin   | 49.6  | 0.31 | Semen Cuscutae     |
| MOL000358  | beta-sitosterol| 36.91 | 0.75 | Semen Cuscutae     |
| MOL000422  | kaempferol     | 41.88 | 0.24 | Semen Cuscutae     |
| MOL005043  | Campesterol    | 37.58 | 0.71 | Semen Cuscutae     |
| MOL005440  | Isofucosterol  | 43.78 | 0.76 | Semen Cuscutae     |
| MOL005944  | matrine        | 63.77 | 0.25 | Semen Cuscutae     |
| MOL006649  | sophranol      | 55.42 | 0.28 | Semen Cuscutae     |
| MOL000953  | CLR            | 37.87 | 0.68 | Semen Cuscutae     |
| MOL000098  | quercetin      | 46.43 | 0.28 | Semen Cuscutae     |
| MOL001494  | Mandenol       | 42    | 0.19 | Ligusticum wallichii |
| MOL002135  | Myricanone     | 40.6  | 0.51 | Ligusticum wallichii |
| MOL002140  | Perlolyrine    | 65.95 | 0.27 | Ligusticum wallichii |
| MOL002151  | senkyunone     | 47.66 | 0.24 | Ligusticum wallichii |
| MOL002157  | wallichilide   | 42.31 | 0.71 | Ligusticum wallichii |
| MOL000359  | sitosterol     | 36.91 | 0.75 | Ligusticum wallichii |
| MOL000433  | FA             | 68.96 | 0.71 | Ligusticum wallichii |
2.2 Selected targets

From 357 candidate compounds in the 9 herbs of LQHBG, a total of 3067 targets were predicted by the PubChem and SwissTargetPrediction databases, with 343, 532, 398, 140, 190, 150, 348, 550, and 416, respectively, for Cistanche, Lucid ganoderma, Lycium barbarum, Angelica sinensis, Semen Cuscutae, Rhizoma atractylodis, Ligusticum wallichii, Salvia miltiorrhiza, and Codonopsis pilosula, and a total of 1322 targets were acquired by the ETCM database, with 37, 320, 122, 47, 76, 238, 115, 134, and 233, respectively, for the above 9 herbs. Taking the intersection, we obtained 379 predictive targets without repetition for Cistanche, 763 for Lucid ganoderma, 489 for Lycium barbarum, 179 for Angelica sinensis, 233 for Semen Cuscutae, 353 for Rhizoma atractylodis, 436 for Ligusticum wallichii, 649 for Salvia miltiorrhiza and 593 for Codonopsis pilosula, for a total of 4073 targets. By searching the TTD, Drugbank, OMIM and Disgenet databases, the relevant targets of DR were obtained, with 8, 6, 59 and 246 targets being retrieved, respectively. After the repeated targets were deleted, 295 DR-related targets remained.

After drawing Venn diagrams of component-related targets and disease-related targets, 17 overlapping targets were obtained for Cistanche, 51 for Lucid ganoderma, 58 for Lycium barbarum, 39 for Angelica sinensis, 36 for Semen Cuscutae, 27 for Rhizoma atractylodis, 31 for Ligusticum wallichii, 46 for Salvia miltiorrhiza and 53 for Codonopsis pilosula, i.e., the targets related to the therapeutic effect of the active components of the LQHBG on DR. After the overlapping targets were deleted, 86 DR-related targets of the LQHBG action remained. The relevant information from the targets is shown in table 2, ranked randomly. The complete data set is provided in the attachment.
| Num | Gene      | Protein Name                          | Num | Gene      | Protein Name                          |
|-----|-----------|---------------------------------------|-----|-----------|---------------------------------------|
| 1   | VDR       | vitamin D receptor                    | 22  | FAAH      | fatty acid amide hydrolase            |
| 2   | ALOX15    | arachidonate 15-lipoxygenase          | 23  | HIF1A     | hypoxia inducible factor 1 subunit alpha |
| 3   | MMP2      | matrix metallopeptidase 2             | 24  | CNR1      | cannabinoid receptor 1                |
| 4   | PLG       | plasminogen                           | 25  | MMP9      | matrix metallopeptidase 9             |
| 5   | KDR       | kinase insert domain receptor         | 26  | ACE       | angiotensin I converting enzyme       |
| 6   | GLO1      | glyoxalase I                          | 27  | FLT1      | fms related tyrosine kinase 1         |
| 7   | PTPN1     | protein tyrosine phosphatase, non-receptor type 1 | 28  | SDHB      | succinate dehydrogenase complex iron sulfur subunit B |
| 8   | NOS2      | nitric oxide synthase 2               | 29  | RBP4      | retinol binding protein 4             |
| 9   | MMP3      | matrix metallopeptidase 3             | 30  | NOS1      | nitric oxide synthase 1               |
| 10  | NOS3      | nitric oxide synthase 3               | 31  | ICAM1     | intercellular adhesion molecule 1     |
| 11  | AKR1B10   | aldo-keto reductase family 1 member B10 | 32  | PPARA     | peroxisome proliferator activated receptor alpha |
| 12  | CYP2C19   | cytochrome P450 family 2 subfamily C member 19 | 33  | RAF1      | Raf-1 proto-oncogene, serine/threonine kinase |
| 13  | KEAP1     | kelch like ECH associated protein 1    | 34  | AGTR1     | angiotensin II receptor type 1         |
| 14  | ALB       | albumin                               | 35  | TGFB1     | transforming growth factor beta 1      |
| 15  | SERPINE1  | serpin family E member 1              | 36  | PTGS2     | prostaglandin-endoperoxide synthase 2 |
| 16  | CTSD      | cathepsin D                           | 37  | FLT4      | fms related tyrosine kinase 4         |
| 17  | MME       | membrane metalloendopeptidase         | 38  | EP300     | E1A binding protein p300               |
| 18  | IGF1R     | insulin like growth factor 1 receptor | 39  | C5AR1     | complement C5a receptor 1             |
| 19  | AKR1A1    | aldo-keto reductase family 1 member A1 | 40  | GLUL      | glutamate-ammonia ligase              |
| 20  | AKR1B1    | aldo-keto reductase family 1          | 41  | MAPK3     | mitogen-activated protein             |
2.3 Bioactive compound-target internet analysis of the LQHBG

To analyze the bioactive components screened from the LQHBG and the relevant targets for DR, Cytoscape software was used to construct the component-target network, as shown in figure 1. In the figure, the purple circle represented the active components, with 357 in total, the pink circle represented the relevant targets, with 86 in total, and the vermilion circle represented the 9 single herbs of LQHBG. There were 7 active components in Cistanche, which acted on 17 targets in the network diagram; Lucid ganoderma contained 107 active components, which acted on 51 targets; Lycium barbarum contained 53 active components, which acted on 58 targets; Angelica sinensis contained 24 active components, which acted on 39 targets; Semen Cuscutae contained 13 active components, which acted on 36 targets; Rhizoma atractylodis contained 14 active components, which acted on 27 targets; Ligusticum wallichii contained 22 active components, which acted on 31 targets; Salvia miltiorrhiza contained 82 active components, which acted on 46 targets; and Codonopsis pilosula contained 34 active components, which acted on 53 targets. In the network diagram, sitosterol (degree=36), quercetin (degree=34), Mandenol (degree=28) of Lycium barbarum and luteolin (degree=32) of Codonopsis pilosula had the highest degrees among the active ingredients in the LQHBG, followed by Lucidumol A (degree=22), Ganodermanondiol (degree=20) of Lucid ganoderma and NSC63551(degree=22) of Rhizoma atractylodis. However, the targets with high correlations with active components were CYP2C19 (degree=93), SERPINA6 (degree=89), PTPN1 (degree=83), VDR (degree=74), ALB (degree=71), and PPARA (degree=58). As seen from the active ingredient–target network diagram of the LQHBG, a single component can act on multiple targets at the same time, and correspondingly, a target can also be associated with multiple components at the same time, with the characteristics of multiple components and multiple targets.

2.4 PPI network analysis and hub target screening

The PPI network of the target is shown in figure 2 and contains 86 target proteins and 851 interaction edges, indicating close relationships between the targets. Among the red circles, the depth of the color represents the degree of the relationship between the edges, with the topological parameters of the degree algorithm being used, and the targets with degree > 50 were INS (degree = 63, closeness centrality = 0.812), ALB (degree = 60, closeness centrality = 0.788), GAPDH (degree = 58, closeness centrality = 0.774), TNF (degree = 54, closeness centrality = 0.745), IL6 (degree = 52, closeness centrality = 0.726), and MAPK3 (degree = 51, closeness centrality = 0.726), suggesting that the six targets, INS, ALB, GAPDH, TNF, IL6 and MAPK3, are the core targets of the LQHBG treatment of DR in the PPI network and mediating
important roles in the network. The top ten hub genes containing the above 6 targets with the addition of MMP9, PTGS2, CASP3 and SERPINE1 were extracted by the use of the cytoHubba plug-in. In addition, through the MCODE analysis tool in Cytoscape software, two significant modules were selected from the PPI network diagram. The first module contained 31 target proteins and 381 protein interaction edges, with an MCODE score of 25.4, while the second module contained 8 target proteins and 13 interacting edges, with an MCODE score of 3.714.

2.5 Annotation analysis of gene functions and pathways

Annotation analysis of the gene functions and pathways of 84 important targets was carried out on the DAVID platform (Version 6.8), and the results were imported into the Omicshare database and Reactome database. To determine the screening and annotation of the core genes and pathways of the LQHBG components used for the treatment of DR, three diagrams (figures 3, 4 and table 3) were constructed with the use of GraphPad Prism 8.0 and R studio.
Table 3
Enrichment results of key targets (Top 20)

| number   | Pathway                                             | count | Pvalue         |
|----------|-----------------------------------------------------|-------|----------------|
| ko04933  | AGE-RAGE signaling pathway in diabetic complications | 23    | 3.48E-24       |
| ko04668  | TNF signaling pathway                               | 20    | 1.37E-18       |
| ko04657  | IL-17 signaling pathway                             | 16    | 7.21E-15       |
| ko04071  | Sphingolipid signaling pathway                      | 17    | 1.47E-14       |
| ko04066  | HIF-1 signaling pathway                             | 15    | 8.08E-14       |
| ko04660  | T cell receptor signaling pathway                   | 15    | 4.38E-13       |
| ko04664  | Fc epsilon RI signaling pathway                     | 13    | 9.36E-13       |
| ko04010  | MAPK signaling pathway                              | 21    | 1.27E-11       |
| ko04064  | NF-kappa B signaling pathway                        | 14    | 3.31E-11       |
| ko04150  | mTOR signaling pathway                              | 15    | 1.82E-10       |
| ko04620  | Toll-like receptor signaling pathway                | 13    | 2.73E-10       |
| ko04920  | Adipocytokine signaling pathway                     | 11    | 1.12E-09       |
| ko04622  | RIG-I-like receptor signaling pathway               | 10    | 6.65E-09       |
| ko04621  | NOD-like receptor signaling pathway                 | 14    | 8.73E-09       |
| ko04350  | TGF-beta signaling pathway                          | 10    | 2.98E-08       |
| ko04068  | FoxO signaling pathway                              | 11    | 1.62E-07       |
| ko04370  | VEGF signaling pathway                              | 8     | 1.78E-07       |
| ko04015  | Rap1 signaling pathway                              | 12    | 1.54E-06       |
| ko04151  | PI3K-Akt signaling pathway                          | 15    | 5.58E-06       |

GO contains 3 subclasses, biological processes, cell components and molecular functions; 26 entries were enriched in biological processes, 10 entries were enriched in cell components and 16 entries were enriched in molecular functions, with the p-value being used as the significance standard of the enrichment degree. The top 10 items of the three subclasses of GO enriched by the targets are shown in figure 3. Among it, the red bar represented the biological processes, the green bar represented the cell components, and the blue bar represented the molecular functions.

There were 227 enriched KEGG pathways in total as the result of pathway analysis of the effective targets of the LQHXB. Figure 4 shows the effects of the related targets of the LQHXB mainly involved in many biological processes, such as cell cycle, the signal transduction, immune system, gene expression,
metabolism, and developmental biology, which reflected the LQHBG treatment of DR through regulating multiple complex biological processes, with the yellow to brown lines representing the importance of targets for enrichment of pathways and the p-value gradually increasing from yellow to brown. The bubble diagram of the top 20 pathways was significantly enriched by KEGG analysis and is shown in figure 5. After excluding extensive pathways, the top 20 signal transduction pathways are listed in table 4. After analysis, the 86 important targets were mainly distributed in the multiple pathways of advanced glycation end products (AGEs), phosphoinositol 3 kinase/protein kinase B (PI3K/Akt), mitogen-activated protein kinase (MAPK), transcriptional factor nuclear factor-κB (NF-κB), and transforming growth factor beta (TGF-β), indicating that the LQHBG treatment of DR acts on multiple pathways and that there is a complex interaction between these pathways.

| Target Protein | PDB ID | Name                          | Grid Score | Grid vdw energy | Grid es energy | Internal energy repulsive | Cluster Size |
|----------------|--------|-------------------------------|------------|-----------------|----------------|---------------------------|--------------|
| ALB            | 1N5U   | Ergosterol                    | -53.1529   | -51.5407        | -1.61218       | 12.05874                  | 8            |
| ALB            | 1N5U   | 5,6-Dihydroergosterol         | -54.6588   | -54.3065        | -0.35225       | 27.25711                  | 3            |
| ALB            | 1N5U   | Ganoderic Acid Am1            | -65.4211   | -50.3048        | -15.1163       | 14.35782                  | 8            |
| ALB            | 1N5U   | Ganoderic Acid B8             | -67.3951   | -57.6326        | -9.76248       | 37.90099                  | 8            |
| ALB            | 1N5U   | Ganoderic Acid C1             | -56.4813   | -46.0803        | -10.401        | 33.76432                  | 5            |
| ALB            | 1N5U   | Ganoderiol                    | -51.1042   | -47.1751        | -3.92914       | 17.13079                  | 8            |
| ALB            | 1N5U   | Lucidenic                     | -54.9702   | -46.4386        | -8.53164       | 18.73997                  | 8            |
| ALB            | 1N5U   | Lucidone                      | -47.5413   | -43.3316        | -4.2097        | 16.78487                  | 4            |

2.6 Molecular docking

Based on the screening results of PPI, the molecular docking method was adopted in this study to verify the key target protein, ALB, as a representative. Its PDB ID was imported into the Yinfo Cloud Computing Platform, docking with the 8 corresponding bioactive components of the LQHBG. The docking results are shown in table 4. Among them, Grid Score resembles docking score, meaning that the smaller the value is, the stronger the binding force is. Grid vdw and Grid es contribute to van der Waals force (which can be understood as a nonpolar effect) and electrostatic force (which can be understood as a polar effect)
respectively. InternalEnergy refers to the repulsive force between the acceptor and ligand. After analyzing the docking results, it was noted that 2 were less than -60 kcal/mol, accounting for 25 percent, 5 were between -60 kcal/mol and -50 kcal/mol, taking up 62.5 percent, and 1 was higher than -40 kcal/mol, for 12.5 percent. In general, a grid score less than -40 kcal/mol indicates that the drug molecule and target have good binding activity, while a grid score greater than -40 kcal/mol indicates that the drug molecule has poor binding activity with the target. These findings indicate that the bioactive components of LQHBG have good binding activity with the key target. Moreover, Ganoderic Acid B8 of Lucid Ganoderma has the strongest bonding force according to the pattern diagram of interaction analysis shown in figure 6. Among it, the ball with stick represented the ligand, as with the bioactive components of LQHBG, while the slender rod represented the residues of the receptor, as with the key target protein ALB, with the dotted line standing for the interaction force.

Discussion

The Lingqihuangban Granule (LQHBG) is a classic prescription for treating diabetic retinopathy disease with blood stasis syndrome, consisting of 9 herbs of Cistanche, Lucid ganoderma, Lycium barbarum, Angelica sinensis, Semen Cuscutae, Rhizoma atractylodis, Ligusticum wallichii, Salvia miltiorrhiza and Codonopsis pilosula, altogether playing roles in treating DR. To determine the relationship between the bioactive ingredients of LQHBG and DR and the potential mechanism, this study constructed a biological network of interactions between bioactive components and relative targets. In this study, 357 bioactive components in the Lingqihuangban Granule were screened, acting on a total of 86 DR targets and involving 851 interaction relationships. As seen from the active components–targets network diagram, for single herbs, active ingredients such as sitosterol, quercetin, and fucosterol of Lycium barbarum can both regulate CYP2C19 and PPARA targets, while ALB, TNF, and MAPK3 can be influenced by more than one active ingredient, showing that the multiple targets can be mediated by multiple bioactive ingredients. Among the 9 diverse herbs, sitosterol is the common bioactive ingredient of Lycium barbarum, Lucid ganoderma, Angelica sinensis and Ligusticum wallichii with the same targets, and quercetin is the common bioactive ingredient of Lycium barbarum, Lucid ganoderma, Cistanche and Semen Cuscutae, indicating that there are synergistic effects between drugs. All of the above findings reflected the mechanisms of coordination and joint regulation of LQHBG in the treatment of DR and show that there were a variety of interactions among targets, which together constituted a complex biological network system.

In the PPI network diagram, the higher the degree of connectivity was, the greater the possibility of LQHBG treating DR through these targets was. The results showed that INS, ALB, GAPDH, TNF, IL6 and MAPK3 were the six core targets selected according to the degree ranking. Some of the effective genes have been identified by animal experiments and clinical tests. For example, INS is a gene that encodes insulin and plays a vital role in the regulation of carbohydrate and lipid metabolism and stimulates glucose uptake with the binding of the insulin receptor (INSR). Previous studies illustrated that INS<sup>C94Y</sup> transgenic pigs developed a persistent diabetic phenotype, such as abnormalities of intraretinal
microvasculature similar to human beings, confirmed by regulating the functional proteins for Müller glial cell metabolism (e.g., GS) and the vital retinal water channel protein AQP4 [38]. Moore et al. reported that retinal microvascular endothelial cells (RMECs) treated with glycated albumin (AGE-Alb) gave rise to increases in the regulation of the NF-κB signaling pathway and leukocyte adhesion, accompanied by a dysfunctional blood–retinal barrier, while Treins et al. stated that AGE-Alb stimulated VEGF expression through an ERK-dependent pathway, playing an important role in the pathogenesis of proliferative diabetic retinopathy [39, 40]. Literature studies and network pharmacology results suggested that INS and ALB may be the key targets for the LQHBG treatment of DR, directly indicating the accuracy of network pharmacology in target prediction.

The results of DAVID (Version 6.8) Pathway and Reactome Pathway annotation analyses showed that the potential mechanisms of the LQHBG in the treatment of DR was mainly focused on the signaling pathways of biological metabolism, circulatory transport and anti-inflammatory and antioxidant functions. The diagram of the main effective pathways revealing the potential mechanisms of LQHBG for the treatment of DR is shown in Fig. 6. Based on the integration of pathway analysis and the theory of compatibility in traditional Chinese medicine, the Chinese herbal compound LQHBG played a hypoglycemic role of improving insulin resistance, reducing fat load and promoting the synthesis of liver glycogen. In addition, the components possess anti-inflammatory and antioxidant properties that regulate the synthesis of inflammatory factors and further prevent cell apoptosis and functional damage. At the same time, the herbs can effectively regulate blood circulation through reducing the oxidative stress response and preventing the proliferation of vascular endothelial cells, strengthening the adhesion of cells and the blood–retinal barrier (BRB). The above corresponded respectively with the theories of traditional Chinese medicine of invigorating spleen and benefiting digestion, tonifying kidney and warming Yang, nourishing blood and generating essence, with the result of clearing collaterals and clarifying eyes. To be specific, the pathways related to microvascular circulation include the AGE-RAGE pathway, the HIF-1α pathway and the VEGF pathway, which correspond to herbs containing the functions of strengthening Qi and blood and regulating the balance of Yin and Yang in the kidney, such as Lucid ganoderma, Salvia miltiorrhiza, Semen Cuscutae and Rhizoma atractylodis. All of the herbs acted on the AGE-RAGE signaling pathway, especially Lucid ganoderma and Salvia miltiorrhiza, reflecting the particularity and commonness of drug actions. The accumulation of AGEs plays a significant role in the progression of DR through exerting an inflammatory reaction and oxidative stress and increasing the expression of pro-apoptotic cytokines, which induce neurodegeneration, apoptosis of retinal pericytes and retinal microvascular dysfunction [41]. Brunelle et al. illustrated that AGEs activated the mitochondria-dependent apoptosis pathway by upregulating Bax expression and inhibiting Bcl-2 expression, participating in the release of pro-apoptotic cytochrome c from the mitochondria through the pores in the outer mitochondrial membrane, thus leading to the activation of caspase 9 and caspase 3 [42]. Moreover, the AGE-RAGE axis was linked to the blood-retinal barrier (BRB) breakdown through the mediation of leukocyte adherence barrier dysfunction, leading to the production of VEGF and angiogenic/vasopermeability growth factor in the retinal Müller glia [43] and to the activation of transcriptional factor nuclear factor-κB (NF-κB) and p38 MAPK expression, regulating pro-inflammatory
cytokine release [41, 44]. The quercetin of Lucid ganoderma, Lycium barbarum, and Semen Cuscutae and the luteolin of Salvia miltiorrhiza and Codonopsis pilosula mainly enriched the AGE-RAGE pathway and induced the apoptosis of retinal pericytes and neurons and mediated microvasculature dysfunction. Under hypoxia conditions, the production of hypoxia-inducible factor (HIF)-1α induces body glycolysis and erythrocytosis, giving rise to blood thickening and mitochondrial metabolism dysfunction [45]. Various clinical studies have confirmed that HIF-1α upregulates the transcription of the VEGF gene, stimulating angiogenesis and thus facilitating disease progression [46, 47]. The Ergosta-7,22-dien-3beta-yl palmitate in Lucid ganoderma, the sesamin in Semen Cuscutae and the epidanshenspiroketallactone in Salvia miltiorrhiza are mainly enriched in the HIF-1α pathway and prevent the occurrence of proliferative diabetic retinopathy (PDR) by reducing VEGF expression and suppressing angiogenesis.

The pathways related to biological metabolism include the sphingolipid signaling pathway and the PI3K-Akt signaling pathway, which corresponded to herbs containing the function of spleen's hypoglycemic effect through improving insulin resistance, reducing fat load and promoting the synthesis of liver glycogen, such as Codonopsis pilosula, Rhizoma atracylodos, Ligusticum wallichii and Lucid ganoderma. Evidence exists that the sphingolipid pathway is associated with inflammation and apoptosis through metabolic regulation [48]. A variety of sphingolipid metabolites, such as ceramide (Cer), ceramide-1-phosphate (C1P), sphingosine-1-phosphate (S1P), and lactosylceramide (LacCer), serve as the biologically active lipids, mediating important functions in biological metabolism and signal transduction [49]. Yaribeygi et al. illustrated that the upregulation of ceramides increases DR risk through inducing β-cell apoptosis and pancreatic inflammation [50], while Arai et al. reported that the accumulation of LacCer and S1P accelerate the proliferation of retinal endothelial cells and the eventual production of new blood vessels [51, 52]. The stigmasterone of Codonopsis pilosula, the NSC63551 of Rhizoma atraclylodis, and the myricanone of Ligusticum wallichii mainly enrich the sphingolipid pathway and inhibit the synthesis and secretion of adipokines and proinflammatory cytokines. Recent studies have shown that the PI3K/Akt/VEGF signaling pathway gives rise to enhanced viability of retinal vascular endothelial cells (RVECs) and inhibition of angiogenesis and apoptosis, stimulated by miR-21 overexpression and phosphatase and tensin homolog (PTEN) suppression [53]. In addition, glycogen synthase kinase-3b (GSK3b) is a critical enzyme that reduces the synthesis of liver glycogen and increases the blood sugar concentration. Evidence has shown that the PI3K/Akt/GSK3 signaling pathway is involved in the metabolism of glycogen, playing a crucial role in the glucose output responsible for insulin resistance [54]. The stimulation of PI3K is essential for the insulin-stimulated glucose uptake, while phosphorylated Akt induces the prevention of GSK3 expression, which promotes glucose intake and inhibits the inflammatory response [55, 56]. The myricanone of Ligusticum wallichii and the lauric acid of Codonopsis pilosula mainly act on the PI3K/Akt pathway and regulate glucose metabolism and fat metabolism to resist the progression of insulin resistance. The pathways related to anti-inflammatory and antioxidant functions include the MAPK signaling pathway, the NF-kappa B signaling pathway and the NOD-like receptor signaling pathway, which correspond to herbs containing the functions of strengthening Qi and blood and regulating the balance of Yin and Yang in the kidney, such as Lycium barbarum, Angelica sinensis and Cistanche. The mitogen-activated protein kinase (MAPK) signaling
pathway is an important signal transduction pathway, consisting of p38 MAPK, JNK, and ERK and correlating to certain pathological processes, such as cell proliferation, apoptosis, inflammation, and necrosis [57]. Under the condition of injury, MAPK aggravates the inflammation in a vicious circle, with the downstream P-p38 MAPK entering the nucleus to release inflammatory factors, such as IL-1 and IL-18, and regulating the expression levels of Bax and Bcl-2 and the apoptosis effector protein Caspase-3, which is closely associated with apoptosis [58, 59]. The mandenol of Lycium barbarum and the quercetin of Cistanche may inhibit the MAPK signaling pathway and alleviate the inflammatory reaction and the progression of apoptosis. Nod-like receptor family pyrin domain-containing protein (NLRP) is closely related to the chronic inflammatory reaction and innate immune response, accompanied by high expression levels of proinflammatory molecules such as IL-1β and TNF-α in DR [60]. It has been reported that increased levels of NLRP3 inflammasomes exert downstream inflammatory cytokines and signal transduction of the NF-κB pathway, which induce a cascade of inflammatory response in the aggravation of DR [61]. The inosine of Lycium barbarum and the suchilactone of Roucourong mainly act on the NF-kappa B signaling pathway and the NOD-like receptor signaling pathway, which block the activation of the inflammatory response and the oxidation reaction in preventing abnormal biological processes.

In short, DR is characterized by structural and functional changes that are affected by multiple factors. The current clinical studies identified new targets for the treatment of DR and confirmed that the traditional Chinese medicine components acting on these potential targets play important roles in the prevention and treatment of DR. In this study, the bioactive components and targets of the LQHBG in treating DR were analyzed and predicted through a network pharmacology method, while the mechanisms of action of key genes and pathways were analyzed through bioinformatics methods. It can be seen from the network diagram of bioactive components–targets and pathway analysis that quercetin is most the common component of the four single drugs in the LQHBG, which act together on the core gene of ALB, reflecting the synergistic effects of compound Chinese medicine components. Each herb consists of different bioactive ingredients, acting on diverse drug targets, referred to as the AGE-RAGE, HIF-1α, PI3K Akt, MAPK, and NF-kappa B signaling pathways, treating DR from different therapeutic aspects of the circulation, metabolism, inflammation, and aging, embodying the complementary effects of compound Chinese medicine in the treatment of DR with multiple components, multiple targets and multiple approaches, providing a new idea for the treatment of DR with complex pathogenesis. Because the results of this study were only obtained from available databases and the obtained pathways were relatively wide, failing to fully elucidate the molecular regulation and interaction relationships, the next step will be undertaking in vitro experiments and performing analysis of the biological technology, further verifying the accuracy of the targets of the Chinese herbal compound LQHBG and will be focusing on each single pathway to study the molecular regulation and interaction relationships, further exploring the specific mechanisms of action of this Chinese herbal compound in treating DR.

**Conclusion**

This study obtained the bioactive ingredients of the LQHBG and the predictive targets of DR and then used network pharmacology to manifest the correlations between the Chinese herbal compound LQHBG
and DR and the potential synergistic mechanisms of the LQHBG for the treatment of DR, providing new ideas for drug treatment.

**Abbreviations**

Lingqihuangban Granule  LQHBG

diabetic retinopathy  DR

Traditional Chinese Medicine  TCM

Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform  TCMSP

oral bioavailability  OB

drug-likeness  DL

Encyclopedia of Traditional Chinese Medicine  ETCM

Therapeutic Target Database  TTD

Online Mendelian Inheritance in Man  OMIM

protein-protein interaction  PPI

Maximal Clique Centrality  MCC

Database for Annotation, Visualization and Integrated Discovery  DAVID

Gene Ontology  GO

Kyoto Encyclopedia of Genes and Genomes  KEGG

Albumin  ALB

Insulin  INS

insulin receptor  INSR

advanced glycation end products  AGEs

phosphatidylinositol-3-kinase  PI3K

mitogen-activated protein kinase  MAPK

transcriptional factor nuclear factor-κB  NF-κB
transforming growth factor beta  TGF – β
Retinal microvascular endothelial cells  RMECs
glycated albumin  AGE-Alb
blood–retinal barrier  BRB
hypoxia-inducible factor  HIF
proliferative diabetic retinopathy  PDR
ceramide  Cer
ceramide-1-phosphate  C1P
sphingosine-1-phosphate  S1P
and lactosylceramide  LacCer
retinal vascular endothelial cells  RVECs
phosphatase and tensin homolog  PTEN
glycogen synthase kinase-3b  GSK3b
Nod-like receptor family pyrin domain-containing protein  NLRP
vascular endothelial growth factor  VEGF

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The manuscript is approved by all authors for publication.

Available of data and materials
All data generated or analyzed in this study are included in this article and its supplementary files.
Competing interests

The authors declare that they have no competing interests.

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Authors’ Contribution

All authors contributed significantly to this work. SH, CG and TS were participated in all aspects of this research involving data analysis, experimental performance and paper writing. CZ, TL and DD retrieved the literature and analyzed the data. QQ and LY contributed to the research design, supervision of the research, data analyses and revised the manuscript. All authors read and approved the final manuscript.

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**Figures**

![Figure 1](image_url)

**Figure 1**

Ingredients-related targets network of the Lingqihuangban Granule. The purple circle represented the active components, the pink circle represented the relevant targets, and the vermilion circle represented the 9 single herbs of LQHBG.
Figure 2

Protein-protein interaction network of the Lingqihuangban Granule a. Network diagram of PPI targets of the Lingqihuangban Granule. The depth of the color represented the degree of the relation between the edges b. Screening of significance modules in PPI network diagram. c. Screening of top 10 hub genes in PPI network diagram.
Figure 3

Functional annotation analysis of the targets. The red bar represented the biological processes, the green bar represented the cell components, and the blue bar represented the molecular functions.
Biological function analysis of key targets for active ingredients of the LQHBG for treatment of DR performed on Reactome platform. The effect of relative targets of the LQHBG mainly involved in many biological processes such as cell cycle, the signal transduction, immune system, gene expression, metabolism, developmental biology and so on, with the yellow to brown lines representing the importance of targets for enrichment of pathways and the P value being gradually increasing from yellow to brown.
Figure 5

Bubble diagram of KEGG enrichment of top 20 key targets for the LQHBG for treatment of DR.
Figure 6

Molecular docking interaction model of Ganoderic Acid B8 of Lucid Ganoderma. The left part described the receptor of target protein ALB docking with the ligand of bioactive component of LQHBG, with the purple foam representing the surface of the receptor protein and the framed area showing the docking point. The right framed part manifested the the enlarged docking point. The ball with stick represented the ligand the same as bioactive components of LQHBG, the slender rod represented the residues of receptor the same as the key target protein ALB, the dotted line represented the interaction force and the name showed the serial number of the residues of receptor ALB.
Figure 7

Potential mechanisms of LQHBG for treatment of DR

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