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Mechanism exploration of Gouqi-wentang formula against type 2 diabetes mellitus by phytochemistry and network pharmacology-based analysis and biological validation

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Abstract

Background: The Gouqi-wentang formula (GQWTF) is a herbal formula used by Academician Xiao-lin Tong for the clinical treatment of T2DM. GQWTF is beneficial to qi, nourishes Yin, clears heat, and promotes fluid production, but the effective components and their mechanism of action remain unclear.

Methods: The main components of GQWTF were detected by LC–MS, and the multi-target mechanisms of GQWTF in T2DM were elucidated using network pharmacology analysis, including target prediction, protein–protein interaction network construction and analysis, Gene Ontology (GO) terms, Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway annotation, and other network construction. Finally, the efficacy of the GQWTF was verified using biological experiments.

Results: First, the “herb-channel tropism” network suggested that GQWTF focuses more on treating diseases by recuperating the liver, which is considered as an important insulin-sensitive organ. Subsequently, a total of 16 active ingredients in GQWTF were detected and screened, and their biological targets were predicted. Then, “compound-target” network was constructed, where enrichment analysis of GQWTF targets reflected its potential pharmacological activities. After T2DM-related target identification, 39 cross targets of GQWTF and T2DM were obtained, and 30 key targets highly responsible for the beneficial effect of GQWTF on T2DM were identified by PPI analysis. GO analysis of these key targets showed that many biological processes of GQWTF in treating T2DM are key in the occurrence and development of T2DM, including components related to inflammatory/immune response, insulin, and metabolism. KEGG analysis revealed the regulation of multiple signalling pathways, such as insulin resistance, PPAR signalling pathway, FoxO signalling pathway, Fc epsilon RI signalling pathway, and pathways that influence diabetes primarily by regulating metabolism as well as other T2DM directly related pathways. Furthermore, a “formula-compound-pathway-symptom” network was constructed to represent a global view of GQWTF in the treatment of T2DM.

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Conclusions: This study explored the mechanism of action of GQWTF in T2DM by multi-component and multi-target multi pathways, which could provide a theoretical basis for the development and clinical application of GQWTF.

Keywords: T2DM, Gouqi-wentang formula (GQWTF), LC-MS, Network pharmacology, Biological validation

Background

With about 463 million adults suffering from diabetes mellitus worldwide, the disease has become a serious global public health concern. Type 2 diabetes mellitus (T2DM) accounts for up to 90% of the total number of these patients [1]. Epidemiological studies have shown that obesity, a high-calorie diet, and lack of physical activity are high risk factors for T2DM [2], suggesting a close correlation between high-calorie diet and T2DM [3]. With the improvement of people's living standards, the phenomenon of a high-calorie diet and inadequate physical activity will persist, and the number of people with T2DM worldwide is estimated to rise to 630 million by 2045 [4], prevention and control of T2DM is required.

Modern medical treatment of T2DM mainly focuses on the control of blood sugar by oral hypoglycemic drugs and injection of insulin, whereas improper use of insulin can lead to hypoglycemia [5]. Traditional Chinese medicine (TCM) has been in use for treating T2DM for 2000 years, which can improve the clinical symptoms, delay islet β -cell failure, improve insulin resistance, and avoid or reduce the use of glucose drugs. It improves the quality of the life of patients, and TCM offers a safe and effective alternative for the treatment of diabetes [6–8]. The Gouqi-wentang formula (GQWTF) was derived from Academician Tong Xiaolin's experience in the prevention and treatment of T2DM for many years. It consists of Gou-Qi-Zi (GQZ, *Lych fructus*), Sang-Ye (SY, *Mori folium*), Zhi-Mu (ZM, *Anemarrhenae rhizome*), Chi-Shao (CS, *Radix paeoniae rubra*), and Xi-Yang-Shen (XYS, *Panax quinquefolium*). The five herbs in the formula are combined to benefit the qi and nourish yin, which clear the heat and promote fluid production. Pharmacological studies have shown that the herbs in GQWTF could reduce blood sugar and cut off the progress of the disease, for example, polysaccharides extracted from GQZ have hypoglycemic and hypolipidemic effects [9]. Water extracts of SY can reduce the blood glucose of diabetic mice and improve glucose tolerance [10]. ZM extract significantly reduces fasting blood glucose and markedly increase the size and the number of insulin-producing β -cells in mice by mediating activation of AMPK [11]. A double-blind, randomized, cross-over clinical trial indicates that YYS can attenuate fasting

blood glucose, reduce blood glucose, and ameliorate glucose metabolism [12]; whereas CS ethanol extract plays a role in multiple hypoglycemic bioactivities in vivo by transcriptional inhibition of gluconeogenesis [13]. Due to the multiple components and multiple targeting characteristics of Chinese herbal medicine, studies aimed at single herbs are not enough to clarify the underlying mechanism of GQWTF. Therefore, it is necessary to resort to a new form of the TCM research system.

Network pharmacology provides an opportunity for research on TCM mechanisms, which are based on systems biology and network biology theory [14]. It analyses the relationship between drugs and diseases by constructing more reliable molecular networks associated with drug action targets and molecular networks of related biological entities. Thus, it can reflect specific disease states more realistically, integrate drug action target networks with disease biological networks, analyse drug-target-disease networks, and predict key targets. Therefore, network pharmacology can comprehensively analyse the effects of drugs on the human body from a holistic perspective, which is consistent with the "holistic" concept of TCM [15].

In this study, we implemented a systematic approach to explore the pharmacological mechanisms of GQWTF in the treatment of T2DM. The study was divided into two parts. The first part consisted of network pharmacology analysis. The ingredients of GQWTF were identified by liquid chromatography coupled with mass spectrometry (LC-MS). The final list of bioactive ingredients of GQWTF was selected based on existing research. Network pharmacology analysis predicted the bioactive ingredients' targeted genes/signalling pathways involved in T2DM. The second part consisted of animal experiments. The hypoglycemic effect of GQWTF was first validated in T2DM C57BL/6J mice fed with a high-fat diet. The predicted targets/pathways were further validated using samples from T2DM mice treated with GQWTF. In summary, we investigated the therapeutic mechanisms of GQWTF against T2DM for the first time by network pharmacology and experimental validation. Figure 1 shows the flow chart of this whole analysis.

(See figure on next page.)

Fig. 1 Workflow of this study

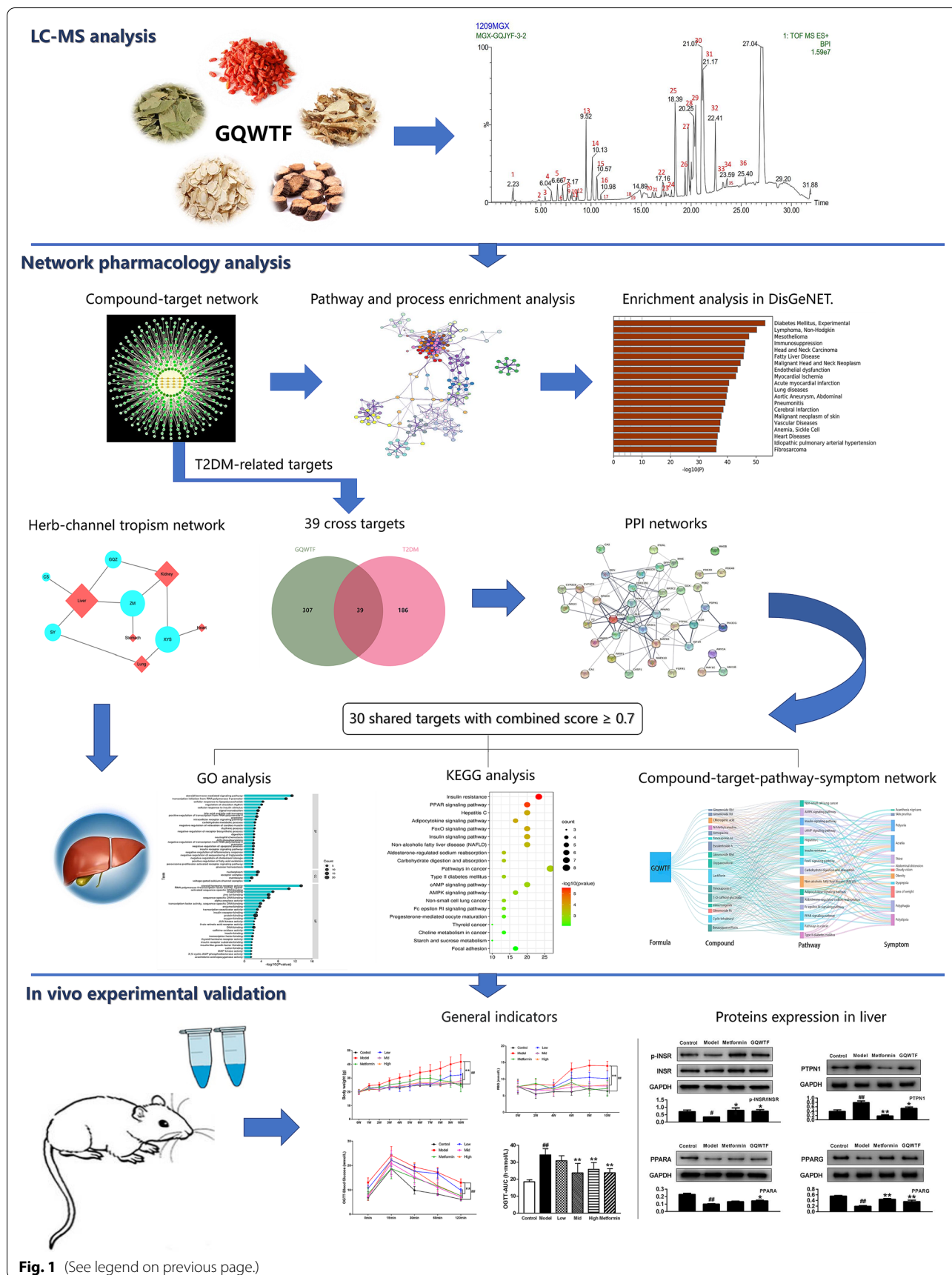


Fig. 1 (See legend on previous page.)

Methods

Collection of GQWTF meridian

According to TCM theory, meridian tropism can reflect the organ in which TCM can exert its curative effect. By searching Chinese Pharmacopoeia 2020 edition (National Medical Products Administration, China), we confirmed the TCM meridians of the herbs in GQWTF, and we imported the information into Cytoscape3.7.0 to construct the herb-meridian network.

Detection and screening of the active components of GQWTF

The prepared liquid medicine was filtered through a 0.22 μm filter membrane, and the liquid medicine was filled into a clean vial and half-stoppered before freezing to solid ice at an ultra-low temperature. The vial filled with liquid medicine was placed in a vacuum freeze dryer at an ultra-low temperature. Then, it is placed in a vacuum so that the water in the solid ice sublimation is directly excluded, thus forming the freeze-dried powder. The precisely weighed freeze-dried powder of GQWTF was placed in a 10 mL volumetric flask and dissolved in 8 mL of 50% methanol. The flask was then sealed and sonicated in a KQ-300 ultrasonic water bath (Kunshan Ultrasonic Instrument Co. Ltd., Jiangsu, China) operating at 40 kHz with an output power of 300 W for 30 min at room temperature. After standing and cooling, 10 mL methanol/water (50:50, v/v) was added and mixed well. The extract was filtered through a 0.22 μm microporous membrane and stored at 4 °C in the dark until analysis. Samples were analysed on a Waters Synapt High-Definition MS System (Waters Corporation, Milford, MA, USA) equipped with a Waters BEH Phenyl column (1.7 μm , 2.1 \times 100 mm). The analytical column was maintained at 35 °C and the mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The gradient running procedure was programmed as follows: 0–4 min, 95% A; 4–5 min, 95–90% A; 5–10 min, 90–88% A; 10–14 min, 88–75% A; 14–20 min, 75–63% A; 20–23 min, 63–50% A; 23–25 min, 50–10% A; 25–30 min, 10%–0 A; and 30–35 min, 0–95% A. The injection volume was 5 μL and the flow rate was 0.2 mL/min.

For mass spectrometry conditions, the cone voltage was set at 40 V in the negative ion mode, and the capillary voltage was set at 2500 V. We used a desolvation temperature of 400 °C, source temperature of 100 °C, and extraction cone of 3 V. The desolvation gas flow rate was set at 800 L/h, the cone gas flow rate was set at 50 L/h, and the mass range was from m/z 100 to 1000. The data were collected and acquired using MassLynx V4.1 software (Waters Co., Milford, MA, USA). The components

obtained by LC–MS analysis were screened by absorption, distribution, metabolism, and excretion (ADME) against related references. The inactive compounds were removed, and the remaining compounds were the potential active ingredients of GQWTF.

Collection and screening of targets of compounds and disease

We retrieved the structures of the active ingredients of GQWTF on PubChem and downloaded their SDF files. Then, the SDF files were imported into PharmMapper [16] (<http://lilab-ecust.cn/pharmmapper/>) to predict the potential targets of these compounds. The parameters were set as follows: generate conformers: Yes; maximum generated conformations: 300; select targets set: human protein targets only (v2010, 2241); and number of reserved matched targets (Max 1000): 300. All predicted targets with “fit scores” ≥ 3 were retained. The UniProt IDs of the targets were introduced into the UniProt database (<https://www.uniprot.org/uniprot/>) with the properties set at “reviewed” and “human” to search for the official symbol of each target.

Targets related to T2DM were searched using the keywords “Type 2 diabetes mellitus” in DrugBank [17] (<https://go.drugbank.com/>) and Therapeutic Target Database (TTD) [18] (<http://db.idrblab.net/ttd/>). After combining the targets of the two databases and removing repetitive targets, we identified disease targets and sent them to the UniProt database for normalisation.

Construction and analysis of compound-target network

The compound-target network was constructed for the 16 potential active ingredients of GQWTF and related targets using Cytoscape to interpret the pharmacological mechanisms of GQWTF in total, such as further observation of the biological functions of key components and targets.

For candidate targets of active ingredients in the GQWTF, pathway and process enrichment analyses were carried out using Metascape [19] with the following ontology sources: Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway, Gene Ontology (GO), biological processes (BP), Reactome Gene Sets, Canonical Pathways, CORUM, TRRUST, DisGeNET [20] (<https://www.disgenet.org/>), PaGenBase, transcription factor targets, WikiPathways, PANTHER Pathway, and COVID. Terms with a p -value < 0.01 , a minimum count of 3, and an enrichment factor > 1.5 were collected and used for filtering. The remaining significant terms were then hierarchically clustered into a tree based on the kappa statistical similarities among their gene memberships. A 0.3 kappa score was applied as the threshold to cast the tree

into term clusters, and the most statistically significant term within a cluster was chosen to represent the cluster.

Furthermore, gene list enrichment was identified in the ontology categories of DisGeNET, and all genes in the genome were used as the enrichment background. Terms with a p -value < 0.01 , a minimum count of 3, and an enrichment factor > 1.5 were collected and grouped into clusters based on their membership similarities.

Protein–protein interactions (PPI)

The overlapping targets of GQWTF and T2DM were taken by Venn diagram, and they were uploaded to the STRING database (<http://string-db.org/>) to obtain the PPI. With the species limited to “Homo sapiens”, we selected targets with “confidence score” > 0.7 for further enrichment analysis.

Enrichment analysis

Enrichment analysis of key targets obtained from the PPI network analysis of candidate targets of GQWTF for the treatment of T2DM, including GO and KEGG pathway analysis, was performed using the DAVID 6.8 database (<https://david.ncifcrf.gov/>), with a threshold value of $p < 0.05$. GO analysis was performed to reveal the function of gene targets, including BP, molecular functions (MF), and cellular components (CC). Key signalling pathways of gene targets were obtained by KEGG analysis.

Furthermore, a compound-target-pathway-symptom network was constructed by connecting the active compounds, overlapping targets, pathways, and symptoms. Pathway-symptom relationships were obtained from the literature.

Experimental design

Sixty healthy male C57BL/6J mice (7 weeks old) were purchased from the Model Animal Research Center of Nanjing University. Ten mice were housed in cages under specific pathogen-free grade (SPF grade) conditions. All mice were maintained under controlled conditions with a temperature of 22–25 °C, relative humidity of $55 \pm 5\%$, a regular 12 h light/dark cycle (light time 7:00–19:00), and free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee, Guang'an men Hospital, China Academy of Chinese Medical Sciences (IACUC-GAMH-2021-004).

After 1 week of adaptive raising, mice were randomly divided into six groups with 10 mice per group: control group (sterile water), model group (sterile water), metformin group (metformin 250 mg/kg/d), low-dose group (1/2 human equivalent dose (HED) of GQWTF converted by “body specific surface area method”), medium-dose group (HED of GQWTF), and high-dose group (double HED of GQWTFD). During the 10-week experiment,

except for the mice in the control group which were given normal feed, the other five groups received high-fat diet (60%) (D12492, Research Diets, New Brunswick, NJ, USA). Pharmacological intervention was started at the same time after grouping, and all pharmaceutical interventions were administered via gavage once a day.

During the experiment, body weight was measured once a week and fasting blood glucose (FBG) was measured every 2 weeks. After 10 weeks of treatment, an oral glucose tolerance test (OGTT) was performed at 0, 15, 30, 60, and 120 min after administration of 20% glucose at a dose of 2 g/kg. At the end of the experiment, all the mice were euthanized by cervical dislocation after a 12-h fasting period, and liver tissues were harvested and stored at -80 °C for western blot analyses. The protein was isolated from the hepatic tissue by phenylmethylsulfonyl fluoride, and the protein concentration was measured using a bicinchoninic acid assay kit. These protein samples were separated by 10% SDS–polyacrylamide gel electrophoresis, and the proteins were electroblotted onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA for 1 h and incubated with the following primary antibodies at 4 °C overnight: rabbit anti-p-INSR (1:1000 dilution, ab60946, Abcam plc, UK), rabbit anti-INSR (1:1000 dilution, ab137747, Abcam plc, UK), rabbit anti-PTPN1 (1:1000 dilution, ab252928, Abcam plc, UK), rabbit anti-PPARA (1:800 dilution, ab24509, Abcam plc, UK), rabbit anti-PPARG (1:500 dilution, ab209350, Abcam plc, UK), and rabbit anti-GAPDH (10B8) (1:10,000 dilution, KGAA002, KeyGEN BioTECH, China). After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody on a shaker at room temperature for 2 h. The antigen–antibody complexes were visualised using an enhanced chemiluminescence system. Relative protein expression levels of p-INSR, INSR, PTPN1, PPARA, and PPARG were normalised to that of GAPDH.

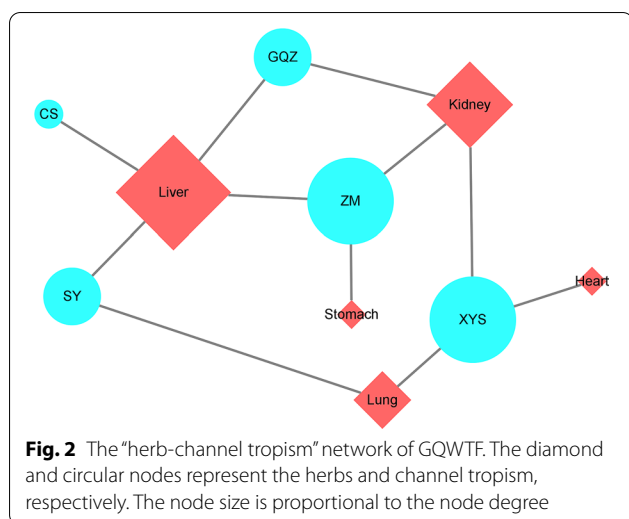
Statistical analyses

Data are expressed as the mean \pm standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using the SPSS software (SPSS 20.0, SPSS Inc., Chicago, IL, USA). Two-tailed Student's t -test, one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests, and Games-Howell tests were used to compare the groups. $p < 0.05$ was considered statistically significant.

Results

Channel tropism information of herbs in GQWTF

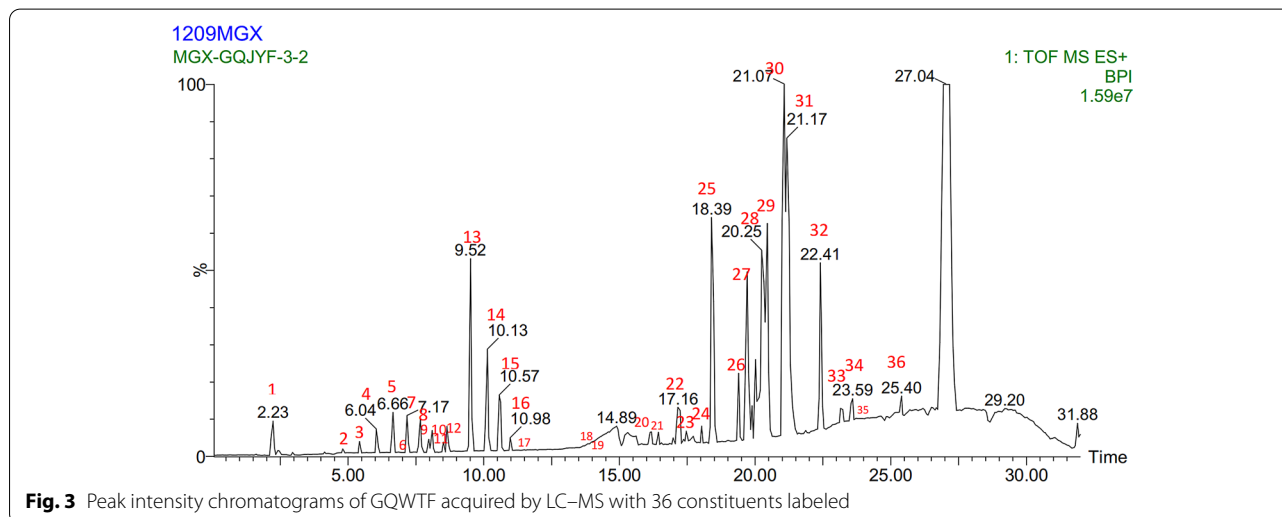
We used the channel tropism of each herb to construct a herb-channel tropism network, as shown in Fig. 2. The



Compound-target network construction of GQWTF

A total of 346 candidate targets of active ingredients in GQWTF were identified (Additional file 2: Table S2). We evaluated the relationships between these potential targets with active ingredients, and the herbs in the GQWTF with a constructed compound-target network (Fig. 4A), and the number of nodes and edges in this network were 362 and 1780, respectively.

The network indicated the potential relationship between compounds and targets, which implied the potential pharmacological mechanisms of GQWTF or the compounds. The nodes with the highest degree of connection to other compounds or targets represent hubs in the entire network, which are potential drugs or targets. For example, oxypaeoniflorin (degree = 214), benzoylpaeoniflorin (degree = 202), lactiflorin (degree = 192), timosaponin C (degree = 140), ginsenoside Rh4 (degree = 122), and 1-O-caffeoyl gluco-



organ affinity of the GQWTF herbs was highest for the liver, with the largest degree value of 4, followed by the kidney, lung, heart, and stomach in the network, with degree values of 3, 2, 1, and 1, respectively.

LC-MS analysis of GQWTF

Thirty-six compounds in GQWTF were identified by LC-MS (Additional file 1: Table S1), and Fig. 3 shows the peak intensity chromatograms. Compounds 1, 3, 4, 8, 9, 22, 23, 24, and 27 were unknown compounds, and compounds 28 and 29 were sulfur fumigation products of the herbs, which were discarded. After screening by ADME and related references [21–33], a total of 16 active ingredients were retained (Table 1).

side (degree = 121) were predicted to be important active compounds according to degree by topological analysis, indicating their critical roles in GQWTF. The targets of CRABP2 were linked to 14 compounds: oxypaeoniflorin, armapavine, benzoylpaeoniflorin, esculentoside A, ginsenoside Rb1, ginsenoside Rc, ginsenoside Rd, ginsenoside Rh4, lactiflorin, timosaponin C, timosaponin AI, *N*-methylcanadine, cyclotetraleucyl, and ristectorigenin. ZAP70 was linked to 14 compounds: oxypaeoniflorin, 1-O-caffeoyl glucoside, Benzoylpaeoniflorin, Chlorogenic acid, Esculentoside A, Ginsenoside Rb1, Ginsenoside Rc, Ginsenoside Rd, Ginsenoside Rh4, Lactiflorin, Timosaponin C, Timosaponin AI, Cyclo tetraleucyl, and Iristectorigenin. NR1I2, THRA, GSTA1, RARA, ABO, and PDPK1 were separately connected to 13 compounds,

Table 1 Active ingredients of GQWTF

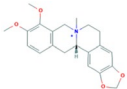

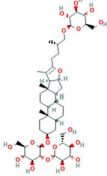
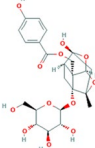
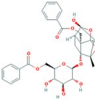
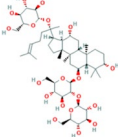
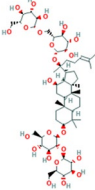
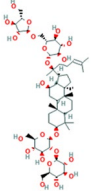
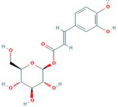

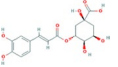
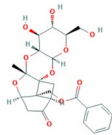
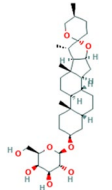
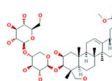
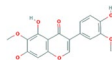
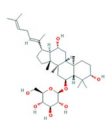
No	Peak No. ^a	RT (min)	M+H	Product ion	Identification result	Chemical structure
1	2	4.804	354.2853	321.1312;305.1577;300.2020;283.1756	<i>N</i> -Methylcanadine	
2	5	6.632	453.2111	437.2361;432.2805;415.2533	Cyclo tetraeleucyl	
3	6	7.134	903.4887; 957.5079	925.4880;741.4484	Timosaponin C	
4	7	7.173	497.2362	481.2635;476.3075	Oxypaeoniflorin	
5	10	8.125	585.2885	569.3146;564.3591;547.3326	Benzoylpaeoniflorin	
6	17	11.522	969.5364	789.4799;461.3982;443.3825	Ginsenoside Rd	
7	18	13.864	1109.6099	1131.5977	Ginsenoside Rb1	
8	19	14.044	1101.5818	947.5421; 785.4781; 461.3992	Ginsenoside Rc	
9	20	16.13	343.2959	365.2782; 250.1779;240.2325	1- <i>O</i> -caffeoyl glucoside	
10	21	16.54	314.1746	286.1446; 272.1521	Artemepavine	
11	25	18.39	355.1047	259.1665;237.1842;124.0868	Chlorogenic acid	

Table 1 (continued)

No	Peak No. ^a	RT (min)	M+H	Product ion	Identification result	Chemical structure
12	26	19.707	463.2819	259.1099;243.1355	Lactiflorin	
13	30	21.097	579.2934	383.2043;301.1412	Timosaponin A1	
14	32	22.408	827.4404	684.2042;425.2144;301.1410	Esculentoside A	
15	33	23.258	331.2245	309.2423; 301.13	Iristectorigenin	
16	35	23.953	637.306	659.2884	Ginsenoside Rh4	

^a Peak No. is consistent with the label of Fig. 3

indicating that different compounds could target synergistically a single gene.

Subsequently, we performed pathway and process enrichment analysis for candidate targets of active ingredients in the GQWT. Statistically enriched terms were identified, and the top 20 clusters were visualised. To capture the relationships between the resulting terms, a subset of enriched terms was selected and converted into a network plot, where terms with a similarity > 0.3 are connected by edges. We selected the terms with the best *p*-values from each of the 20 clusters. The network is visualised using Cytoscape with “force-directed” layout, where each circle node represents an enriched term and is sized proportional to the number of input genes that fall into that term and coloured by its cluster identity. The selected terms from each cluster were shown as labels to have their term description (Fig. 4B), including Pathways in cancer, Nuclear Receptor transcription pathway, myeloid leukocyte mediated immunity, cellular response to hormone stimulus, Ras Signaling, nucleotide metabolic process, positive regulation of kinase activity, monocarboxylic acid metabolic process, small molecule catabolic process, Hemostasis, Metabolism of lipids, VEGFA-VEGFR2 Signaling Pathway, Nuclear Receptors Meta-Pathway, response to xenobiotic stimulus, response to steroid hormone, regulation of lipid metabolic process,

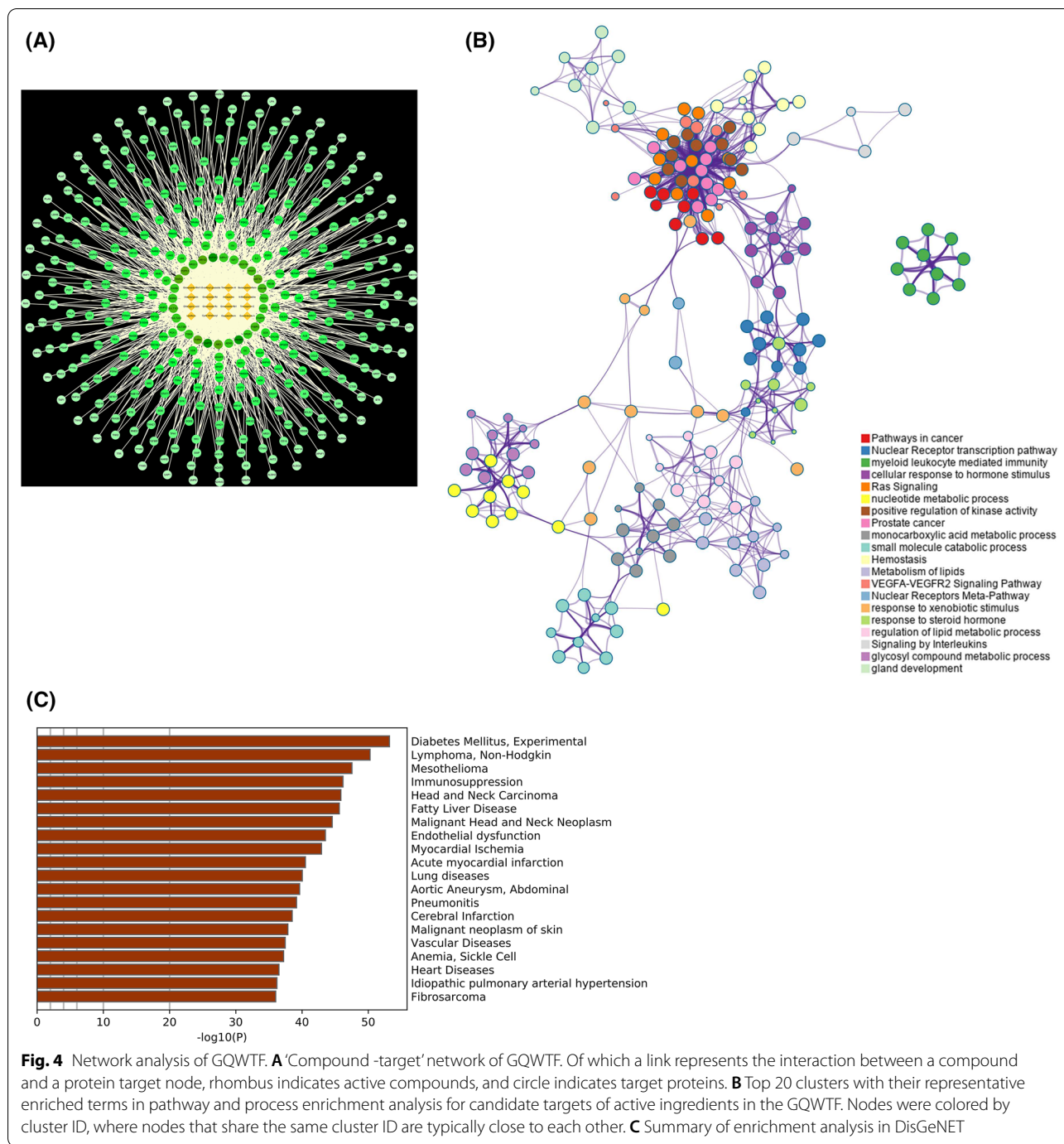
Signaling by Interleukins, glycosyl compound metabolic process, etc.

Furthermore, using the DisGeNet database, the gene information related to the disease in candidate targets of active ingredients in the GQWTF were obtained, and the top 20 enriched clusters (one term per cluster) are shown in Fig. 4C. The most significant of which were Diabetes Mellitus, Experimental; while the other involved diseases were Lymphoma, Non-Hodgkin; Immunosuppression; Fatty Liver Disease; Endothelial dysfunction Vascular Diseases; Vascular Diseases; etc.

GQWTF potential target-T2DM target network

For disease target identification, 153 and 92 target genes of “Type 2 Diabetes Mellitus” were identified from the Drugbank and TTD databases, respectively. Thus, we obtained 225 key nodes of target genes for disease targets after eliminating duplicates (Additional file 3: Table S3). Finally, a total of 39 targets were shared between 16 active ingredients of GQWTF-related targets and 225 T2DM-related targets (Fig. 5A, Table 2).

The PPI relationships among these 39 target genes were generated from the STRING database, The PPI relationship network has 39 nodes and 98 edges, with an average of 5.03 node degree (Fig. 5B). Next, 30 shared targets with high confidence scores (combined score ≥ 0.7) (Fig. 5C),



suggesting their strong interactions, were selected for further enrichment analysis.

GO and pathway enrichment analysis

For GO analysis, we identified 58 biological processes, 4 cellular components, and 37 molecular functions with an adjusted $p < 0.05$. Results show that many biological processes are related to diabetes, such as cellular response to

lipopolysaccharide, cellular response to insulin stimulus, bile acid and bile salt transport, carbohydrate metabolic process, digestion, neutrophil chemotaxis, insulin receptor signaling pathway, negative regulation of inflammatory response, negative regulation of sequestering of triglyceride, positive regulation of fatty acid oxidation, negative regulation of cholesterol storage, glucose homeostasis, and so on. GO analysis of cellular components

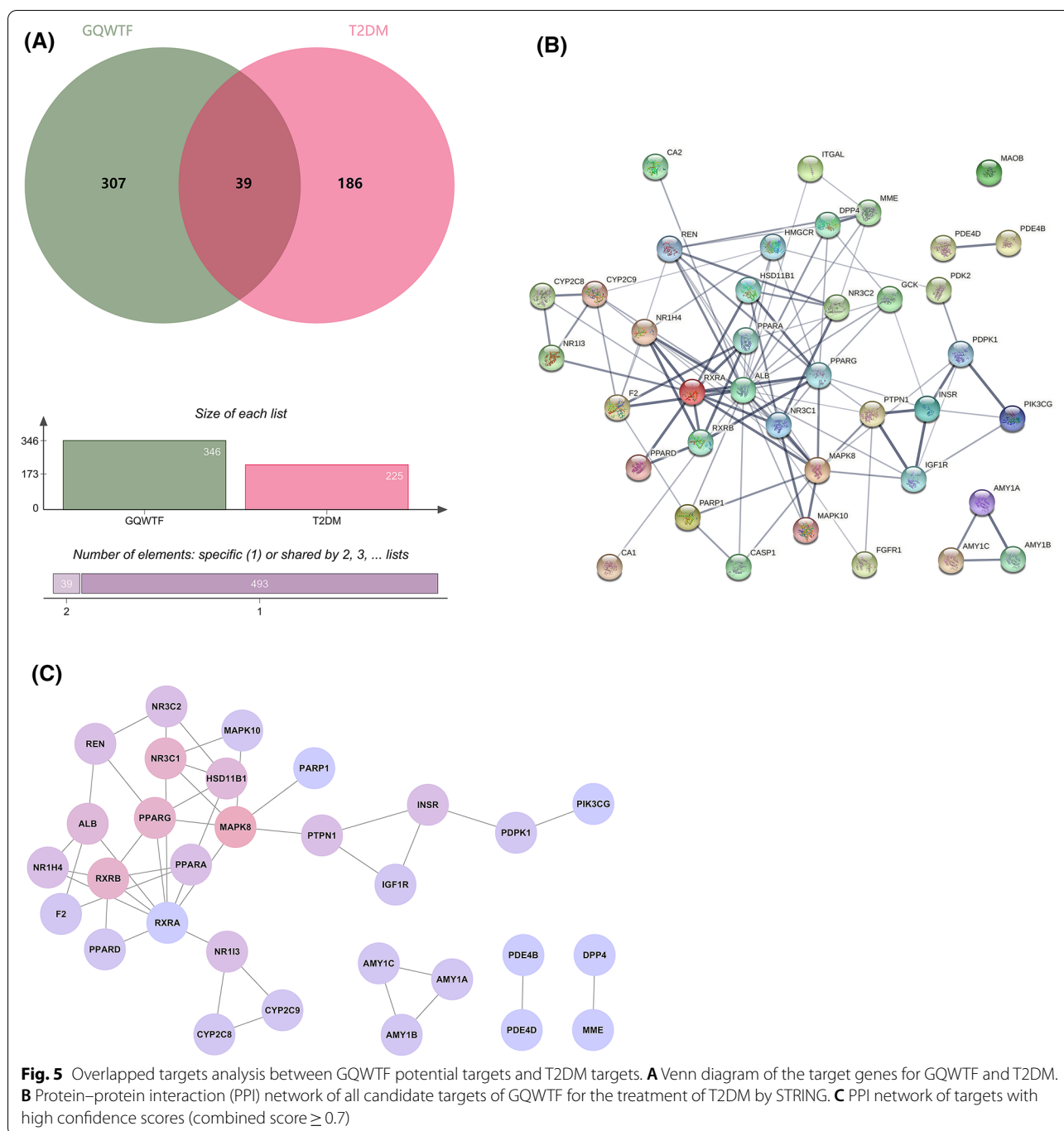


Fig. 5 Overlapped targets analysis between GQWTF potential targets and T2DM targets. **A** Venn diagram of the target genes for GQWTF and T2DM. **B** Protein-protein interaction (PPI) network of all candidate targets of GQWTF for the treatment of T2DM by STRING. **C** PPI network of targets with high confidence scores (combined score ≥ 0.7)

showed that these processes occur mainly in nucleoplasm, receptor complex, membrane, and voltage-gated calcium channel complex.

In addition to molecular functions, the enriched items were related to insulin receptor binding, protein binding, insulin binding, insulin receptor substrate binding, insulin-like growth factor I binding, MAP kinase activity, retinoid X receptor binding, insulin-like growth factor

receptor binding, fatty acid binding, and so on. The top 25 GO items with the lowest *p*-value were shown in Fig. 6A, and details of all items are provided in the Additional file 4: Table S4.

KEGG pathway analysis was conducted to explore these targets further. These targets were highly enriched in Insulin resistance, PPAR signaling pathway, Adipocytokine signaling pathway, FoxO signaling pathway, Insulin

Table 2 Potential targets of GQWTF against T2DM

No	Protein name	Gene symbol	Uniprot ID
1	Bile acid receptor	NR1H4	Q96R11
2	Caspase-1	CASP1	P29466
3	Cytochrome P450 2C8	CYP2C8	P10632
4	Cytochrome P450 2C9	CYP2C9	P11712
5	Hexokinase-4	GCK	P35557
6	cAMP-specific 3',5'-cyclic phosphodiesterase 4B	PDE4B	Q07343
7	Glucocorticoid receptor	NR3C1	P04150
8	Neprilysin	MME	P08473
9	Albumin	ALB	P02768
10	Peroxisome proliferator-activated receptor alpha	PPARA	Q07869
11	Renin	REN	P00797
12	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 2, mitochondrial	PDK2	Q15119
13	Nuclear receptor subfamily 1 group I member 3	NR1I3	Q14994
14	Peroxisome proliferator-activated receptor delta	PPARD	Q03181
15	Mitogen-activated protein kinase 8	MAPK8	P45983
16	Tyrosine-protein phosphatase non-receptor type 1	PTPN1	P18031
17	Integrin alpha-L	ITGAL	P20701
18	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	P04035
19	cAMP-specific 3',5'-cyclic phosphodiesterase 4D	PDE4D	Q08499
20	Dipeptidyl peptidase 4	DPP4	P27487
21	Poly [ADP-ribose] polymerase 1	PARP1	P09874
22	Mineralocorticoid receptor	NR3C2	P08235
23	Insulin-like growth factor 1 receptor	IGF1R	P08069
24	3-phosphoinositide-dependent protein kinase 1	PDPK1	O15530
25	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	P28845
26	Fibroblast growth factor receptor 1	FGFR1	P11362
27	Retinoic acid receptor RXR-alpha	RXRA	P19793
28	Amine oxidase [flavin-containing] B	MAOB	P27338
29	Insulin receptor	INSR	P06213
30	Mitogen-activated protein kinase 10	MAPK10	P53779
31	Peroxisome proliferator-activated receptor gamma	PPARG	P37231
32	Carbonic anhydrase 2	CA2	P00918
33	Retinoic acid receptor RXR-beta	RXRB	P28702
34	Prothrombin	F2	P00734
35	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform	PIK3CG	P48736
36	Carbonic anhydrase 1	CA1	P00915
37	Alpha-amylase 1C	AMY1C	P0DTE8
38	Alpha-amylase 1A	AMY1A	P0DUB6
39	Alpha-amylase 1B	AMY1B	P0DTE7

signaling pathway, Aldosterone-regulated sodium reabsorption, Carbohydrate digestion and absorption, Type II diabetes mellitus, cAMP signaling pathway, AMPK signaling pathway, Fc epsilon RI signaling pathway, Starch and sucrose metabolism, and so on. The top 20 KEGG items with the lowest *p*-value were shown in Fig. 6B, and

detailed information of all 35 pathways is provided in the Additional file 5: Table S5.

Furthermore, we searched the literature for the possible symptomatic improvement of these enriched pathways, such as thirst, polydipsia, acratia, loss of weight, polyphagia, polyuria, acanthosis nigricans, obesity, abdominal

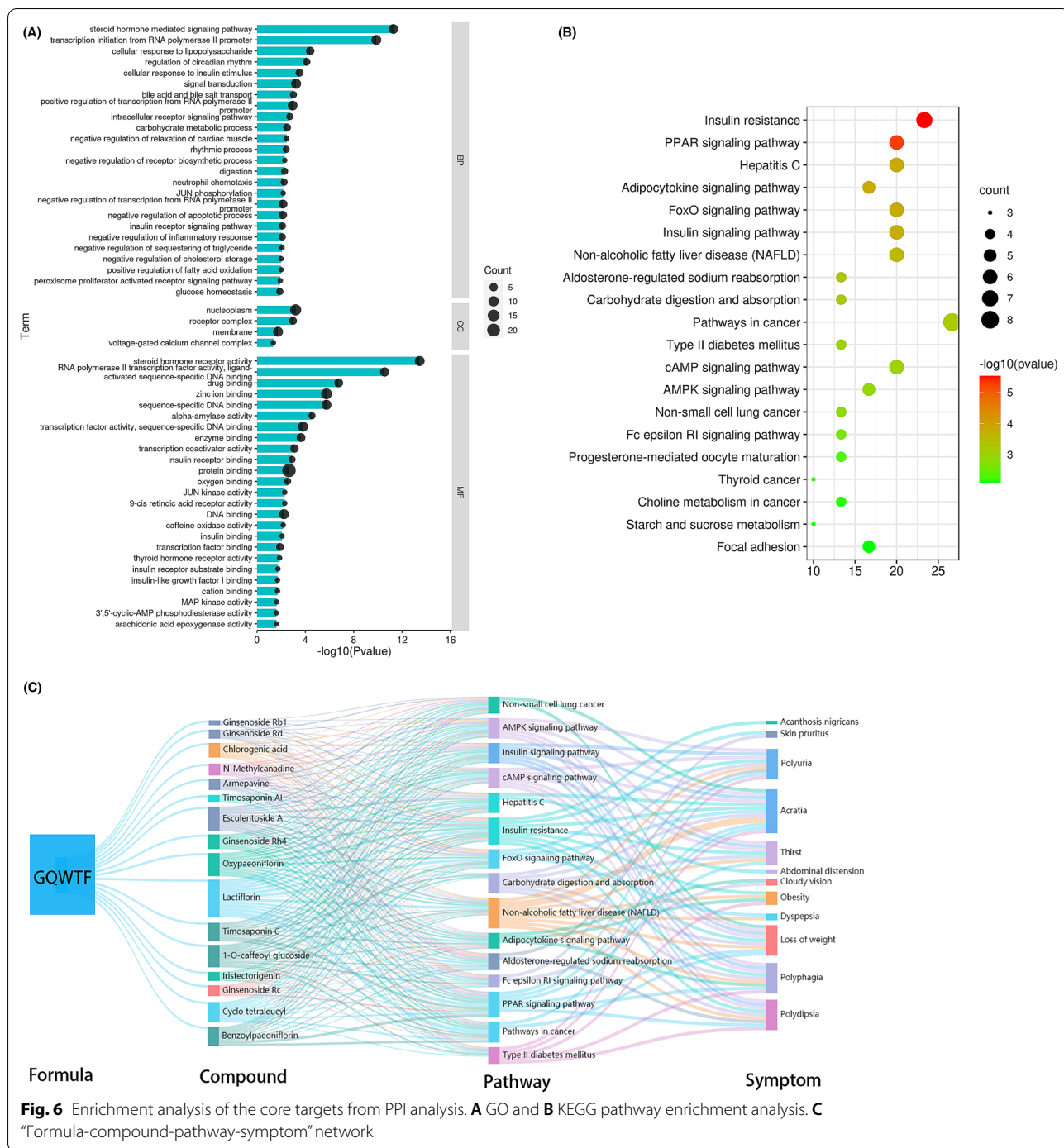


Fig. 6 Enrichment analysis of the core targets from PPI analysis. **A** GO and **B** KEGG pathway enrichment analysis. **C** "Formula-compound-pathway-symptom" network

distension, cloudy vision, dyspepsia, and skin pruritus. We then created a network, which is shown in Fig. 6C.

In vivo experimental validation

During the 10-week treatment period, the weight of mice in the HFD group continued to increase and was significantly higher than that of the ND group at

each time point. However, the weight of the mice significantly decreased in the drug-intervention groups (Fig. 7A). Similarly, the FBG levels were significantly higher in the HFD group than in the ND group. After the 10-week treatment period, GQWTF and metformin intervention achieved a significant glucose-lowering effect (Fig. 7B). The effects of GQWTF on

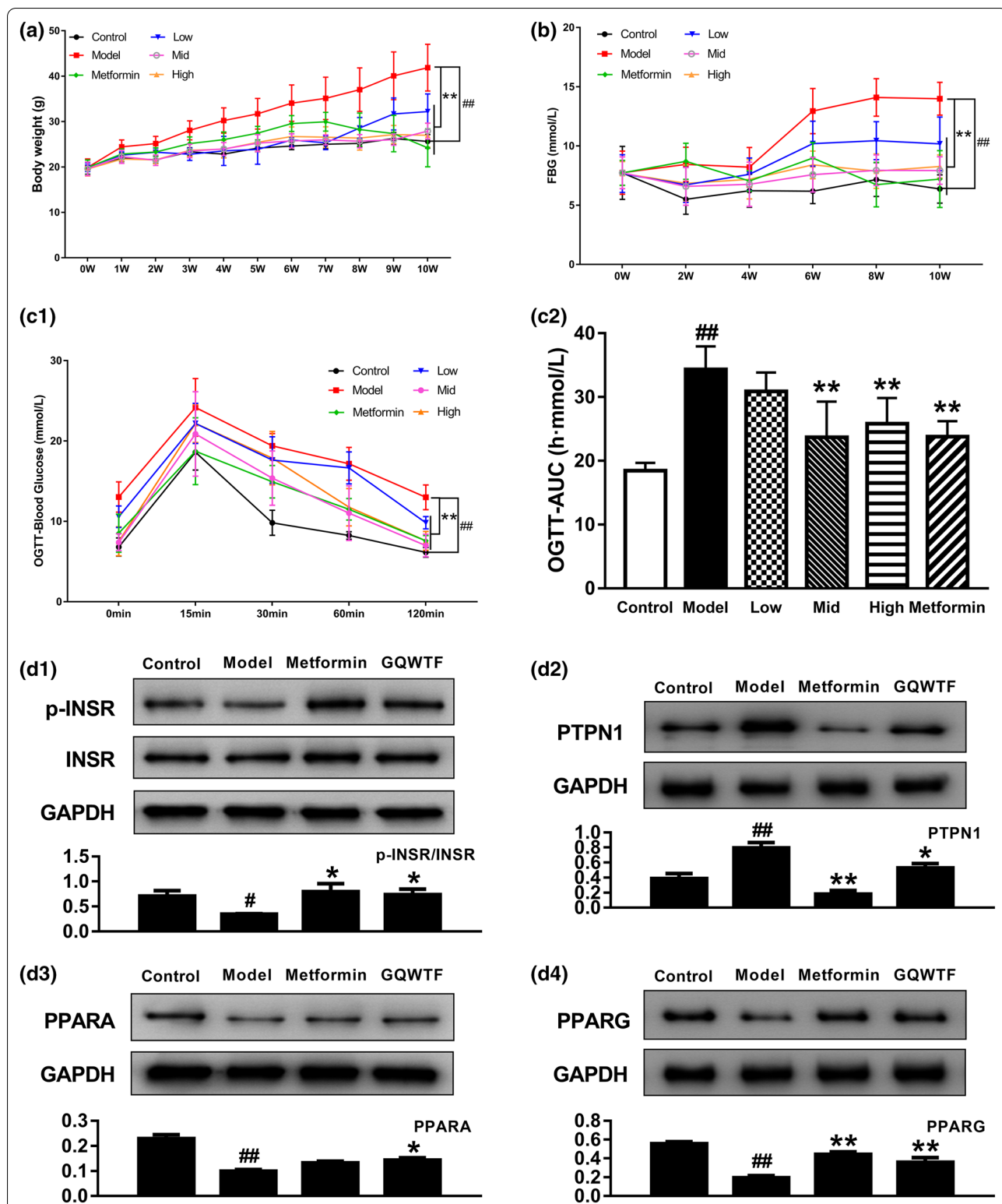


Fig. 7 Effects of GQWTF on 10-week high-fat diet induced mice. Body weight **A** was monitored once a week, and fasting blood-glucose (FBG) **(B)** every 2 weeks during 10-week trial. **C** Dynamic changes of blood glucose during oral glucose tolerance test (OGTT), and AUC is area under the blood glucose-time curves of OGTT. **D** The relative protein expression of PTPN1, PPARA, PPARG and phosphorylation of INSR in liver using western blot analysis. Data are shown as means \pm SEM of ten **(A, B)** or six **(C)** or three **(D)** independent experiments. # $p < 0.05$, ## $p < 0.01$, compared with control group; * $p < 0.05$, ** $p < 0.01$, compared with model group

reducing body weight and FBG levels showed good dose dependence.

OGTT is widely performed to evaluate whether the body is abnormally glucose tolerant and diabetic. The results showed that compared to the control group, the HFD-fed T2DM group had higher blood glucose levels during the 120 min period. GQWTF and metformin drug intervention also significantly reduced blood glucose during the test. Significant improvement in glucose clearance by drug treatment was also reflected by the lower peaks of blood glucose. Moreover, the AUC was calculated to further determine the improvement of glucose tolerance following long-term GQWTF treatment. The AUC in T2DM mice was much higher than that in control mice; meanwhile, the increased AUC in DM mice was reduced by GQWTF treatment (Fig. 7C).

Based on the pathway enrichment analysis, targets (INSR, PTPN1, PPARA, PPARG) from two of the most significant pathways, Insulin resistance, and PPAR signaling pathway, were selected for further experimental validation. As shown in Fig. 7D, western blot results showed that compared with the control group (normal diet), PTPN1 was upregulated in HFD-fed mice ($p < 0.05$), while treatment with GQWTF or metformin significantly inhibited the expression of PTPN1 ($p < 0.01$, $p < 0.05$). Treatment with drugs significantly blocked the inhibitory effect of HFD on INSR phosphorylation ($p < 0.05$). Furthermore, the overexpression of PPARA and PPARG induced by HFD was also abolished by treatment with GQWTF ($p < 0.01$, $p < 0.05$).

Discussion

GQWTF is a proposed prescription for the prevention and treatment of T2DM, which is composed of GQZ, SY, ZM, CS, and XYS. As a holistic medicine, TCM is mainly based on the observation of the curative effect of empirical clinical practice, and its effectiveness depends on a variety of compounds, targets, and approaches. Thus, it is difficult to explore its mechanism of action. This study draws lessons from the research ideas of network pharmacology, combined with the theory of TCM, analytical chemistry, and molecular biology technology through the analysis of complex and multi-level interactions of various networks to identify multi-components and multi-targets involved in the prevention and treatment of T2DM by GQWTF.

The theory of channel tropism is an important part of the theory of TCM, which can reflect the attribution of TCM and prefers certain viscera, meridians, or specific parts. In the “herb-channel tropism” network of GQWTF, more herbs showed affinities for the liver, which indicates that GQWTF focuses more on treating diseases by recuperating the liver. As an important organ

of systemic metabolism, the liver plays an important role in the development of insulin resistance and T2DM, related to hepatic fat accumulation and alterations in energy metabolism [34–36].

In this study, 16 potential active ingredients in GQWTF were obtained through qualitative analysis using LC–MS and ADME screening. Candidate targets of these active compounds in the GQWTF were identified by searching related database, thus “compound-target” network of GQWTF was built. The network topology parameter analysis results showed that Oxypaeoniflorin, Benzoylpaeoniflorin, Lactiflorin, Timosaponin C, Ginsenoside Rh4, and 1-O-caffeoyl glucoside, which had high degrees, were more important compounds in GQWTF. It also suggested that a single compound affects multiple targets, and some of the active ingredients in GQWTF could exert biological effects via multiple targets. Additionally, modern studies have also suggested the pharmacological activities of these compounds, such as hypoglycemic effect, anti-inflammation, anti-oxidation, and anti-tumour effects, [37–40] etc. The target linked to multiple compounds in the network also reflects the synergistic effect between different compounds. In brief, it can be seen that the multilevel and multi-target effects were jointly determined by multiple active compounds of GQWTF.

Furthermore, enrichment analysis of candidate targets of GQWTF revealed that it could exert a variety of pharmacological activities through multiple pathways, such as Pathways in cancer, VEGFA-VEGFR2 Signaling Pathway, regulation of lipid metabolic process, glycosyl compound metabolic process, Signaling by Interleukins, which reflects pharmacological activities such as anti-tumor, vascular regulation, glucose and lipid metabolism, and immune regulation, etc. As for the enrichment of these targets in the disease-related database, the most significant finding turned out to be Diabetes Mellitus and Experimental; thus, pharmacological prediction from the perspective of drugs alone has shown the potential of this prescription in the treatment of diabetes, which coincides with the theme of GQWTF in the treatment of T2DM in this study.

After retrieving T2DM related targets, 39 cross targets of GQWTF and T2DM were obtained, which were presumed to be putative targets of GQWTF for the treatment of T2DM. Moreover, we screened 30 core targets with stronger interactions through the PPI network for enrichment analysis. GO biological process enrichment analysis results showed that many biological processes of GQWTF in treating T2DM were closely involved in the key parts of the occurrence and development of diabetes mellitus, especially the top 25 items. For example, items related to inflammatory/immune response including

cellular response to lipopolysaccharide, neutrophil chemotaxis, negative regulation of inflammatory response, and T2DM have been proven to be associated with chronic low-grade inflammation in a large number of studies [41–43]. Items related to insulin including cellular response to insulin stimulus, and insulin receptor signaling pathway were also involved. Insulin is the only hormone that can reduce blood glucose in the body. It cannot play its biological activity directly, as it needs to combine with insulin receptor on target organ cells to play its physiological role, and abnormalities in these physiological processes may induce the occurrence of T2DM [44, 45]. Items related to metabolism including bile acid and bile salt transport, carbohydrate metabolic process, digestion, negative regulation of sequestering of triglyceride, positive regulation of fatty acid oxidation, negative regulation of cholesterol storage, and glucose homeostasis were also involved. These biological processes also influence each other, especially the regulation of glucose and lipid metabolism, along with the whole process of the occurrence and development of T2DM [46–48]. Furthermore, the enriched items in molecular function analysis focused more on insulin, such as insulin receptor binding, insulin binding, insulin receptor substrate binding, insulin-like growth factor I binding, insulin-like growth factor receptor binding, etc.

Finally, KEGG pathway enrichment analysis was used to investigate the effect of GQWTF in the treatment of diabetes by regulating multiple signalling pathways. Of which Insulin resistance, a condition where cells become resistant to the effects of insulin and is often found in obesity, T2DM, non-alcoholic fatty liver disease, etc. [49–51]; for PPAR signaling pathway, which plays significantly in inflammation, insulin sensitivity, lipid metabolism, as well as the expression of hepatic enzymes regulating glucose homeostasis [52, 53]; for FoxO signaling pathway, which controls important physiological events, including apoptosis, glucose metabolism, oxidative stress resistance, by converting the external stimuli of insulin, growth factors, cytokines, and oxidative stress into cell-specific biological responses by regulating the transcriptional activity of target genes [54, 55]; for Fc epsilon RI signaling pathway, which contributes to inflammatory responses by regulating mediators and cytokines [56, 57]; there are also pathways that influence diabetes primarily by regulating metabolism, such as cAMP signaling pathway [58], AMPK signaling pathway [59], Starch and sucrose metabolism [60], Carbohydrate digestion and absorption [61], Aldosterone-regulated sodium reabsorption [62], Adipocytokine signaling pathway [63]; and other T2DM directly related pathways like Insulin signaling pathway, and Type II diabetes mellitus, are involved. In addition, combined with literature search, the corresponding

symptoms of these pathways were synthesized to construct the "herbs-targets-pathways-symptoms" network, which represents a global view of GQWTF in treating T2DM, with their pathways that functions and related possible symptomatic improvement.

Finally, the efficacy of GQWTF was confirmed, and the key pathways were verified through animal experiments *in vivo*. The results showed that GQWTF reduced the body weight and blood glucose, which were abnormally elevated due to a high-fat diet. The abnormal glucose tolerance was also improved. In the validation of pathways, key targets (INSR, PTPN1, PPARA, and PPARG) from two of the most significant pathways in pathway enrichment analysis, Insulin resistance, and PPAR signaling pathway, were selected. The liver was selected according to the meridian network, which is also the key target tissue for insulin resistance in T2DM. INSR on hepatocyte surface would activate insulin receptor substrate (IRS) after binding with insulin, and then functionally mediate downstream like glucose/lipid metabolism, hepatocyte proliferation, DNA synthesis, and cell cycle regulation [64]. While PTPN1 dephosphorylates INSR and attenuates insulin signal transduction cascade, and human studies also demonstrated that PTPN1 expression increases in obese individuals and those with T2DM [65, 66]. For downstream PPARA and PPARG, which are important regulatory genes in cell differentiation and various metabolic processes, especially lipid and glucose metabolism [67, 68]. The regulatory effect of GQWTF on these key targets of these two pathways was verified by western blotting, the abnormal expression of proteins induced by high fat and the phosphorylation level of INSR were adjusted.

Conclusions

In summary, we analysed the main components of GQWTF by LC–MS, and thus had a further understanding of its pharmacodynamic basis. By using the method of network pharmacology, the mechanism of GQWTF in the treatment of T2DM was analysed systematically at the molecular level, and the reliability of network pharmacology prediction was verified by animal experiments *in vivo*. Therefore, the potential mechanism of the synergistic action of GQWTF in the treatment of T2DM through multiple components, targets, and pathways was elaborated.

Abbreviations

T2DM: Type 2 diabetes mellitus; TCM: Traditional Chinese medicine; GQWTF: Gouqi-wentang formula; GQZ: Gou-Qi-Zi; SY: Sang-Ye; ZM: Zhi-Mu; CS: Chi-Shao; XYS: Xi-Yang-Shen; LC–MS: Liquid chromatography coupled with mass spectrometry; ADME: Absorption, distribution, metabolism, and excretion; TTD: Therapeutic Target Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; BP: Biological processes; PPI: Protein–protein

interaction; MF: Molecular functions; CC: Cellular components; HFD: High-fat diet; HED: Human equivalent dose; FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; AUC: Area under curve.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13020-021-00479-2>.

Additional file 1: Table S1. Main compounds of GQWTF identified through LC-MS.

Additional file 2: Table S2. Candidate targets of active ingredients in GQWTF.

Additional file 3: Table S3. Candidate targets of T2DM.

Additional file 4: Table S4-1. The results of GO analysis-biological process (BP). **Table S4-2.** The results of GO analysis-cellular component (CC). **Table S4-3.** The results of GO analysis-molecular function (MF).

Additional file 5: Table S5. The results of KEGG analysis.

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Not applicable.

Authors' contributions

X-LT, L-HZ, and LH conceived and designed the research methods. LH, H-YY, X-XW, W-CD, L-LZ and XM collected the data. LH, and Y-JZ did the animal experiment. X-MW, and Q-YD helped with the animal experiment. LH, and H-YY analysed the data. LH and H-YY wrote the original draft. X-LT and L-HZ reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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

Declarations

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee, Guang'an men Hospital, China Academy of Chinese Medical Sciences (IACUC-GAMH-2021-004).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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