A systematic study of Erzhu Erchen decoction against damp-heat internalized type 2 diabetes based on data mining and experimental verification

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Author contributions
Wang PY conducted the data and wrote the first draft and revised the manuscripts. Shen JF and contributed the cell experiments. Lan Q, Ma GD, Zhang S and Wang T reviewed and edited the manuscripts. Zhang YZ designed and administratively supported the entire project. All the authors participated in the final approval of the manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
TCM, traditional Chinese medicine; EZECD, Erzhu Erchen decoction; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; qPCR, quantitative polymerase chain reaction; WGCNA, weighted gene co-expression network analysis; GSEA, gene set enrichment analysis; TCMSP, Traditional Chinese Medicine Systems Pharmacology; OD, optical density; HS, high glucose.

Results

Keywords: data mining; damp-heat internalized type 2 diabetes; Erzhu Erchen decoction; network pharmacology; bioinformatics

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Background

Type 2 diabetes (T2D) is a widespread lifelong illness that poses a major hazard to human health. According to its clinical characteristics of “polydipsia, polyphagia, polyuria, and weight loss” it is classified as “Xiaoke” (The concept of Type 2 diabetes in ancient Chinese medicine) in traditional Chinese medicine (TCM) [1]. Scholars have confirmed that the incidence of damp-heat internalized T2D is on the rise in clinical practice [2, 3]. Moreover, in epidemiological studies, about 50% of diabetic patients exhibited damp-heat symptoms [4, 5]. Therefore, the purpose of our study is to identify a medicine that does not just treat hyperglycemia but which also ameliorates damp-heat symptoms.

Erchen decoction, a traditional Chinese medicine, as a treatment for phlegm and fluid retention or as a standard treatment for phlegm [6], Erchen decoction is compatible with Atractylodes lancea and Atractylodes macrocephala, termed Erzhu Erchen decoction (EZECD). In clinical studies, EZECD is extensively used for the treatment of a variety of diseases caused by retention of phlegm dampness, such as obesity, fatty liver, diabetes, and hypertension [7, 8]. Pharmacological studies showed that Erchen decoction decreases insulin resistance in Zucker Diabetic Fatty rats [9]. Of note, Atractylodes lancea decreases blood glucose levels by decreasing glycogen uptake in muscles and the liver, blocking glycogen production, and reducing glycogen storage [10]. Atractylodes macrocephala may control blood glucose and inflammation by regulating insulin resistance and the MAPK signaling pathway [11]. Therefore, we believe that EZECD can treat both damp-heat syndrome and type 2 diabetes, while its mechanism remains unknown.

Network pharmacology, initially proposed by Li et al., as a vital part of bioinformatics, is increasingly recognized for its significance in compound acquisition [12]. In addition, TCM is characterized by multiple active compounds and complex targets, and is proposed as a multilevel and multi-system complex mode of action. Thus, the scope of TCM network pharmacology has increasingly evolved from a “one-target, one-drug mode” to a “network-target, multiple-compound-therapeutics mode” [13, 14]. However, much of the research indicated that the targets and pathways are mostly repeated and biological meaning limited, which may be due to the use of the same binary algorithm to screen the targets of diseases.

Weighted gene co-expression network analysis (WGCNA) was first developed by Zhang and Horvath in 2005 according to the scale-free topology criterion, more closely to the biologically motivated criterion [15]. WGCNA can cluster tens of thousands of disease genes into co-expression modules and verify the associated gene modules with clinical traits of samples according to the coefficient value of gene expression pattern, which can screen genes, using more complicated and closer to biological features algorithm.

Therefore, this paper was divided into four sections based on data mining and experimental verification. First, network pharmacology methodology was used to obtain the hub compound and related genes. Then, the data mining including GEO database and WGCNA were used to mine hub genes that EZECD treated damp-heat internalized T2D, and the affinity of hub compound and genes was verified using molecular docking. Further, the related pathways with hub genes were screened in violin plot and gene set enrichment analysis (GSEA). Finally, the pharmacological interaction between hub compound and genes will be further validated according to pharmacological database (Comparative Toxicogenomics Database) and quantitative polymerase chain reaction, aiming to provide a certain theoretical basis for more in-depth research. The overall design flowchart of this study was shown in Figure 1.

Materials and methods

The construction of herb-compound-genes-disease network and analysis

In the first place, the systematic pharmacology database and analysis
platform Traditional Chinese Medicine Systems Pharmacology (TCMSP) (https://old.tcmsp-e.com/tcmsp.php) of traditional Chinese medicine was applied to obtain the main compounds and corresponding targets of six herbs in Erzhu Erchen decoction [16]. Firstly, the search was gradually conducted using the names of the herbal medicines (Pinellia ternata, Atractylodis macrocephalae rhizoma, Poria, Glycyrrhizae radix et rhizoma, Atractylodis lanceae rhizoma and Cibotii retardatum (musciporum)) as the keywords, and ADMET values of OB ≥ 30% and DL ≥ 0.18 were screened in TCMSP and replenished with literature. Then, the information on the compounds of EZECD was obtained [17-19]. Ultimately, the corresponding gene names were matched with all targets by the UniProt protein database (https://www.uniprot.org/)[20].

For another, we searched for targets of damp-heat internalized T2D in the GeneCards database (https://www.genecards.org) with "type 2 diabetes" as the keyword, and then screened it by the relevance score > 25.5 (the data was taken twice as the value greater than the average) [21]. At the same time, the keyword "type 2 diabetes", was also searched in the OMIM (https://www.omim.org), DisGeNET (https://www.disgenet.org/), Pharm GKB (https://www.pharmgkb.org/) and TTD (http://db.idrblab.net/tdt)[22, 23]. Furthermore, "damp-heat internalization" as the keyword was used to get the diseased genes in the GeneCards and OMIM database. At last, the genes related to damp-heat internalized T2D were collected after merging and deleting the duplicate genes. Finally, the intersection targets of EZECD and damp-heat internalized T2D were performed by the Venny visionary tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html). The data was imported into Cytoscape 3.9.1 software to draw herb-compound-genes-disease network diagrams [24].

Gene Ontology (GO) enrichment analysis of the intersection targets

Human (Homo sapiens) is used as the screening criterion on the Metascape platform, and by selecting customized analysis, GO enrichment includes biological process (BP), cellular component (CC), and molecular function (MF) [25]. Then, the data from the GO analyses was plotted by SRplot (http://www.bioinformatics.com.cn/), that is a free online platform for analyze and visualization.

Construction of compound-disease targets-pathway network and the hub compound

Firstly, KEGG enrichment analysis was carried out to identify the most significant pathways in biological processes. P < 0.05 was deemed to be substantial [26]. Subsequently, the data from the KEGG enrichment analysis was also imported into the free online bubble chart platform SRplot (http://www.bioinformatics.com.cn/). Then, the top 10 pathways were selected by gene ratio, and the compound-disease targets-pathway network was constructed utilizing Cytoscape 3.9.1. In accordance with the results of “Analyze network” (degree value), we extracted the hub compound and main pathways eventually.

WGCNA for the hub module

According to the compound-disease target-pathway network, it is difficult to find additional genes and pathways for damp-heat T2D. With the R software package “WGCNA”, a gene co-expression network was established to explore gene-phenotype co-expression. (1) Pancreatic islet beta cells apoptosis could inhibit insulin secretion, thereby disturbing glucose metabolism, which has become a major cause of type 2 diabetes. However, GSE76894 and GSE118139 are mainly devoted to the islet tissue of diabetic and normal people, and to study diabetes-related genes. Therefore, the datasets of GSE76894 and GSE118139 were selected to data mining. (2) Construct a scale-free gene co-expression network using 15113 genes with no missing values and correlation coefficients to create a similarity matrix in GSE76894. The soft threshold was employed to transform the similarity matrix into an adjacency matrix. (3) Identify modules: The topological overlap matrix measured the average network connectivity for each gene. According to “minModuleSize” (50) and “mergeCutHeight” (0.25), each module has a separate color, with the grey module holding unallocated genes. The module feature gene, a first principle component module eigengene, was used to determine the link between modules and phenotype. The highest absolute correlation module was chosen for further study. (4) “Gene significance” and “module membership” analysis. Gene significance was the correlation coefficient between the expression level of a gene and phenotype which demonstrated the relationship between genes and phenotype. Module membership is the correlation coefficient between the expression value of a gene and the module eigengene of a module, which indicates the relationship between the gene and module.

Acquisition of hub genes and regulatory pathways treated with quercetin

Firstly, the candidate genes were screened by the two most positive-correlated modules from WGCNA (|GS| ≥ 0.25, |MM| ≥ 0.725), differential genes of GSE118139 (P < 0.05, |logFC| ≥ 1.5, and normalized in Genecards), and the acquired genes of compound-target-pathway network [27]. Then, the violin plot of hub genes was generated using the R packages “ggpubr” and “ggviolin”. Smaller P-values indicate more relevance between genes and phenotype, as depicted by violin plots.

GSEA, also known as Gene Set Enrichment Analysis, has fundamental differences from KEGG pathway analysis despite their similarities. More accurate and extensive gene information were obtained by using the clusterProfiler in R software (P-adjust ≤ 0.01). The gene lists were arranged by log fold change, and the samples were T2D. The final result is depicted by the “enrichplot” R package.

Additionally, to further elucidate the hub genes, the GO terms of them were annotated by the online chart platform SRplot (http://www.bioinformatics.com.cn/). Human (Homo sapiens) was used as the screening criterion.

Verification of compound interactions with through molecular docking and database

Molecular docking is an intuitive, convenient, and efficient technique for exploring potential targets and mechanisms of compounds in TCM. Its essence is to obtain relatively stable and complex conformation through the interaction with ligands and receptors, to explore the active binding mode of compounds and targets, and speculate its pharmacological action. Currently, molecular docking has gradually become the theoretical basis for predicting new drugs and related mechanisms [28]. Therefore, we used this technology to verify whether the core compounds of EZECD combined with the core targets.

Molecular docking was carried out using the following procedure. (1) Drug ligands file preparation: The molecular structure diagrams (PDB) of the compounds were retrieved from the TCMSP and PubChem databases (https://pubchem.ncbi.nlm.nih.gov/) [29]. Applying the software version 1.5.7 of Auto Dock, ligands were modified by removing water, adding hydrogen, and establishing the drug. Eventually exported as a ligand file in PDBQT format. (2) Protein receptor file preparation: the preliminary protein structure was chosen from the PDB (https://www.rcsb.org/) website, and the final receptor structure was generated by removing the water, substituting it with hydrogen, and loading the receptor’s ligand into the Pymol software [30]. Save the structure into a protein receptor file in PDBQT format using AutoDock1.5.7. (3) Selecting the docking range: After importing a ligand file and a receptor into the AutoDock 1.5.7 software, we calculated the extent of molecular docking to guarantee that the protein was contained entirely within the docking box. (4) Molecular docking and visualization: Following the completion of the aforementioned preparatory files, the PDBQT file was configured in the AutoDock 1.5.7 software using the “docking” process and “docking parameters”. The PDBQT file was subsequently converted to a PDB file. Lastly, the combined PDB file was transferred into the Pymol software for visualization. In order to provide additional evidence supporting the hypothesis.
that the actions of hub genes and compounds are related, the CTD database was performed. First of all, the relationship between genes or compounds and diseases was obtained using the CTD database [31]. Next, the hub genes and compounds were imported into “VennViewer”, the relationship between hub genes and compounds was analyzed by screening the “Chemicals-gene interaction type to “decrease” and produced a Venn diagram.

Cell culture and cell viability assay
Quercetin was obtained from Dalian meiunbio Co., Ltd. (No: MB2127, Dalian, China) and dissolved with DMSO (0.5%) in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, New York, NY, USA) with 2% fetal bovine serum at a concentration of 10 μmol/L stock solution. H9C2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), which cultured in DMEM medium containing 10% fetal bovine serum (Gibco, New York, NY, USA) at 37 °C, 5% CO₂ incubator. The culture media were changed every second day. We added 33.3 mM glucose to the medium in the high-glucose group. 5 mM glucose was added to the cells in the control group. The detailed grouping conditions are as follows: NG (normal culture medium with 5 mM glucose), high glucose (HG) (33.3 mM glucose medium), HG + Q0.01 (33.3 mM glucose medium + 0.01 μM), HG + Q0.1 (33.3 mM glucose medium + 0.01 μM), HG + Q1 (33.3 mM glucose medium + 1 μM quercetin), HG + Q10 (33.3 mM glucose medium + 10 μM).

Cell Counting Kit-8 was performed for H9C2 cells proliferation and cytotoxicity. Specifically, H9C2 cells were seeded at a concentration of 3,000 cells per well in 96-well plates. The cells were then treated with either 5 mM glucose or 33.3 mM glucose for 24, 48, and 72 hours. Next, we added 10 μL of Cell Counting Kit-8 to each well and incubated the cells for 2 hours at 37 °C. We then measured the absorbance at 450 nm using a microplate reader. To determine cellular viability, we used the optical density (OD) and calculated the inhibition rate (%) according to the following equation: inhibition rate (%) = 100% × (control cell OD-dosing cell OD)/(control cell OD – blank OD).

Quantitative polymerase chain reaction (qPCR) and western blotting analysis
The total RNA of H9C2 cells was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). The concentration of the extracted RNA was determined using an ultra-microspectrophotometer (ExCell MINIDROP), and the quality of the RNA was determined by nucleic acid electrophoresis. The RNA was reverse-transcribed to cDNA using a reverse transcription kit (Takara, Dalian, China). qPCR was performed using the SYBR Green PCR Master Mix (2×) (ExCell, Shanghai, China) and a Light Cycle 480 II (Roche, Indianapolis, IN, USA). The mRNA levels was estimated using the 2−ΔΔCt method and normalized to glyceraldehyde 3-phosphate dehydrogenase.

The western blot analysis was carried out in accordance with the previously reported techniques. In brief, the isolated proteins were run through an SDS-PAGE gel and then transferred to a PVDF membrane. (Merck Millipore, Darmstadt, Germany). Specific primary antibodies (Caspase 8, MCP-1, βAR, β-actin) were incubated with the membranes overnight at 4 °C, followed by washing three times with TBS + Tween buffer. Membranes were then incubated with the corresponding secondary antibodies for 1 h at room temperature. An enhanced chemiluminescence system was used for imaging. Band densities were analyzed using Image J software (version 1.53a; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis
Each experiment was done at least three times and data are expressed as mean ± standard error of the mean. Differences in parameters between multiple groups were evaluated with One-way Analysis of variance (Bonferroni’s Post Hoc analysis) using GraphPad Prism (Version 9.00). Differences at P < 0.05 were considered statistically significant.

Results
The construction of herb-compound-genes-disease network and analysis
EZECED was composed of 6 herbs, including Pinellia ternata, Atractyloides macrocephala rhizoma, Poria, Glycyrrhiza radix et rhizoma, Atractyloides lanceae rhizoma and Citri reticulateae pericarpium. Firstly, there were 9 compounds in Citri Exocarpium Rubrum, 12 kinds of Pinellia, 11 kinds of Poria cocos, 89 kinds of Licorice, 3 kinds of Atractylodes lancea, 4 kinds of Atractylodes macrophala after ADME screening (OB > 30% and DL > 0.18). All 126 compounds were effective because the beta-sitosterol (A) and the naringenin (B) were shared within the compound formula between Citri Exocarpium Rubrum with Pinellia or Licorice. Then a total of 274 compound genes were obtained after removing 163 unannotated and 1,646 duplicate genes (the herb names and compounds have been renamed in the Supplementary Table). Moreover, combined with T2D candidate genes and damp-heat internalization genes, 1,921 disease genes for damp-heat internalized T2D were identified. GeneCards, OMIM, DisGeNET, Pharm GKB and TTD databases were used to collect 1429, 521, 7, 42 and 18 T2D genes, as well as more than 20 genes associated with damp-heat internalization. Consequently, the 159 overlapped genes for EZECED (274 compound targets) to treat damp-heat internalized T2D (1915 targets) were identified. Finally, the herb-compound-genes-disease network was constructed in Cytoscape 3.9.1 (Figure 2A). The top three compounds were GC38, GC26, and B, each of which could interact with multiple genes according to further analysis of the degree value as well as the top 10 compounds (Table 1).

As shown in Figure 2B, we screened 5 items of BP, CC, and MF in GO enrichment analysis, respectively, which BP related to response to hormone, cellular response to nitrogen compound, response to inorganic substance, CC responded to membrane raft, membrane microdomain, side of membrane, and MF included kinase binding, protein kinase binding, protein homodimerization activity.

Construction of compound-disease targets-pathway network and screening of the hub compound
Gene Ontology terms are a gene set in and of itself, with no relationships between them defined. The key discrepancy is that not only does KEGG includes gene sets, but it also describes the intricate link between genes and metabolites. Consequently, the KEGG pathway analysis was performed to explore the mechanism of 159 intersection targets of EZECED treating damp-heat internalized T2D. KEGG showed the top 20 pathways, including pathways in cancer, lipid and atherosclerosis, AGC-RAGE signaling pathway in diabetic complications, and so on (Figure 3A). Based on the results of the study, the 98 compound-disease-targets-pathway network of EZECED in the treatment of damp-heat internalized T2D was obtained (Figure 3B). On the basis of the results by applying the “Analyze network” tool, the top three compounds were GC38 (quercetin, degree 716), C21 (wogonin, degree 310), and GC26 (kaempferol, degree 256), respectively (Table 2). Finally, the top three pathways were pathways in cancer, chemical carcinogenesis-receptor activation, and kaposi sarcoma-associated herpesvirus infection (degree 283). Therefore, the hub compound was quercetin (GC38) for EZECED in the treatment of damp-heat internalized T2D.

WGCNA for obtaining the hub genes
The weighted gene co-expression profiles based on 15113 genes and 103 samples were developed after deleting aberrant samples and screening the genes from GSE78694. Initially, with the soft threshold power set to 7, the scale independence reached 0.908, and the average connection value reached 28.925 (Figure 4A, 4B), establishing the scale-free gene network. Subsequently, 23 modules were identified based on average hierarchical clustering and dynamic tree clipping as shown in Figure 4C. After a result of correlation analysis between each module and T2D (or ND), the MEgreen showed the highest positive correlation with T2D (r = 0.39, P = 5e⁻², Figure 4D), followed by the
Figure 2 The construction of herb-compound-genes-disease network and analysis. (A) Herb-compound-genes-disease network: The degree is represented by the color and size of nodes. The hexagons represent compounds of EZECD. EZECD consists of 6 herbs and 126 compounds. 4 laurel-blue octagons represent Atractylodes macrocephala, 3 brown hexagons represent Atractylodes lancea, 11 bottle-green hexagons represent Pinellia, 7 orange hexagons represent Citri exocarpium rubrum, 11 purple hexagons represent Poria cocos, 88 bottle-blue hexagons represent Licorice. A and B in sky-blue hexagons are common compounds. T2D (the red square represent disease), and EZECD has 159 overlapped genes (the yellow triangle represent targets). (B) The bar diagram of GO enrichment analysis of interactions genes of EZECD and damp-heat internalized T2D. Left: It involves the top 5 terms of biological process (red), cellular component (blue), and molecular function (green), respectively. The change in length is the magnitude of the P-value. The dark blue bars represent the number of pathway genes. EZECD, Erzhu Erchen decoction; T2D, Type 2 diabetes; GO, Gene Ontology.

Figure 3 Quercetin (GC38) was the hub compound and the related genes were obtained. (A) The bubble diagram of KEGG pathways of EZECD-damp heat internalized T2D targets. The top 20 main pathways were shown. The red bar indicates the magnitude of the P-value. The amount of green dots along the polyline represents the number of genes in the pathway. (B) Compound-disease targets-pathway network: The 10 red squares represent the pathways, the yellow triangular represent the 98 intersection targets, and green octagons represent compounds of EZECD. The degree is represented by the color and size of the nodes. EZECD, Erzhu Erchen decoction; T2D, Type 2 diabetes; KEGG, Kyoto Encyclopedia of Genes and Genomes.
Figure 4 The outcomes of the WGCNA and the hub genes were obtained. (A) The corresponding scale-free topological model fit indices at different soft threshold powers. (B) The corresponding mean connectivity values at different soft threshold powers. (C) Dendrogram of all differentially expressed genes clustered based on the measurement of dissimilarity (1-TOM). The color band shows the results obtained from the automatic single-block analysis. (D) 23 different cell modules were obtained. Each cell contains the different modules and clinical traits correlation and p-value. The table is color-coded by correlation according to the color legend. (E) The scatter plot represents the GS and MM of the MEgreen module. (F) The scatter plot represents the GS and MM of the MEmidnightblue module. (G) The heatmap of differential genes of GSE118139. GSM3319260 and GSM3319621 were T2D samples, GSM3319260 and GSM3319621 were normal samples. (H) The overlapped genes of GSE76894 (MEgreen and MEmidnightblue modules), GSE118139 and compound-disease target-pathway genes were CASP8, CCL2, and AHR. The green circle consists of 45 genes, the purple circle consist of 101 genes, and blue circle consists of 98 genes. T2D, Type 2 diabetes; TOM, topological overlap matrix; WGCNA, weighted gene co-expression network analysis; MM, module membership; GS, gene significance.

Table 1 The top 10 compounds of EZECD

<table>
<thead>
<tr>
<th>Name</th>
<th>Compound</th>
<th>2D structure</th>
<th>Degree</th>
<th>OB</th>
<th>DL</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>GC38</td>
<td>quercetin</td>
<td></td>
<td>140</td>
<td>46.43</td>
<td>0.28</td>
<td><em>Glycyrrhiza</em> <em>radix et rhizoma</em></td>
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<td>GC26</td>
<td>kaempferol</td>
<td></td>
<td>56</td>
<td>41.88</td>
<td>0.24</td>
<td><em>Glycyrrhiza</em> <em>radix et rhizoma</em></td>
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<tr>
<td>FL9</td>
<td>Poricoic acid B</td>
<td></td>
<td>43</td>
<td>30.52</td>
<td>0.75</td>
<td><em>Poria</em></td>
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<tr>
<td>CZ1</td>
<td>wogonin</td>
<td></td>
<td>42</td>
<td>30.68</td>
<td>0.23</td>
<td><em>Atractylodis</em> <em>lanceae rhizoma</em></td>
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Table 1 The top 10 compounds of EZED (continued)

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<th>Name</th>
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<td>38</td>
<td>59.29</td>
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<td><em>Citri reticulatae pericarpium, Glycyrrhizae radix et rhizoma</em></td>
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<td>A</td>
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<td><img src="image3" alt="Structure" /></td>
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<td>36.91</td>
<td>0.75</td>
<td><em>Citri reticulatae pericarpium Pinellia ternata</em></td>
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<td><img src="image4" alt="Structure" /></td>
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<td>44.72</td>
<td>0.21</td>
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<tr>
<td>FL8</td>
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<td>0.76</td>
<td><em>Poria</em></td>
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<td>69.67</td>
<td>0.21</td>
<td><em>Glycyrrhizae radix et rhizoma</em></td>
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</table>

OB, oral bioavailability; DL, drug-likeness; EZED, Erzhu Erchen decoction.

Table 2 Top 10 active compounds degree values of EZED

<table>
<thead>
<tr>
<th>Signal</th>
<th>Effective compound</th>
<th>Degree</th>
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<td>GC38</td>
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<td>CZ1</td>
<td>wogonin</td>
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<td>kaempferol</td>
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<tr>
<td>B</td>
<td>naringenin</td>
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<td>GC20</td>
<td>licochalcone</td>
<td>164</td>
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<tr>
<td>BX11</td>
<td>beta-D-Ribofuranoside, xanthine-9</td>
<td>136</td>
</tr>
<tr>
<td>A</td>
<td>beta-sitosterol</td>
<td>136</td>
</tr>
<tr>
<td>FL9</td>
<td>Poricoic acid B</td>
<td>132</td>
</tr>
<tr>
<td>GC45</td>
<td>isorhamnetin</td>
<td>94</td>
</tr>
</tbody>
</table>

EZED, Erzhu Erchen decoction.

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MEmidnightblue module ($r = 0.35$, $P = 4e^{-4}$, Figure 4D). Further, gene significance and module membership (MM) analysis showed a significant correlation between each module eigengenes (MEgreen or MEmidnightblue) ($\text{cor} = 0.56$, $P = 7.7e^{-66}$; $\text{cor} = 0.50$, $P = 7e^{-141}$, Figure 4E, 4F), suggesting that eigengenes are highly correlated with both their respective modules, as well as T2D. Consequently, 62 and 39 hub candidate genes were filtrated in MEgreen or MEmidnightblue eigengenes using $|G6| \geq 0.25$ and $|\text{MM}| \geq 0.725$, respectively. Meanwhile, 45 differential genes in GSE118139 were obtained in GEO2R after de-duplication and normalization. As shown in Figure 4G, the heatmap of differential genes in GSE118139 was constructed. It was worth noting that CASP8, CCL2, and AHR were highly expressed in GSM3319260 and GSM3319621 (T2D). Finally, we identified the 3 overlapped genes (CASP8, CCL2, AHR) between eigengenes of the MEgreen module (62), MEmidnightblue module (39) in GSE76894, differential genes (45) in GSE118139, and genes of compound-disease target-pathway network (98) (Figure 4H).

Verification of quercetin interactions with hub genes through molecular docking

Firstly, we predicted how the quercetin (ligand) could interact with proteins Caspase-8 (PUB ID:7lvm), CCL2 (PUB ID:7so0), and AHR (PUB ID:5nj8), based on the above results through the molecular docking as shown in Table 3 and visualized in Figure 5. The results showed that quercetin could interact with Caspase-8, CCL2, and AHR. (Figure 5A–5C). In particular, Figure 6A shows quercetin forms two hydrogen bonds with LEU-27 and one hydrogen bond with ARG-33, ILE-144, and ASP-28 in Caspase-8. Similarly, in CCL2, quercetin formed two hydrogen bonds with CYS-72 and one with GLN-28 (Figure 5B), while Figure 5C shows quercetin forms two hydrogen bonds with DA-10 and three hydrogen bonds with DG-5 in AHR. It is commonly believed that the ligand-receptor binding energy values are

<table>
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<tr>
<th>Protein</th>
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<th>Protein structure</th>
<th>Compound</th>
<th>Affinity (kcal/mol)</th>
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Table 3 The binding energy of compound and hub genes (kcal mol⁻¹)

![Figure 5](https://www.tmrjournals.com/tmr)

**Figure 5 The visualization of molecular docking (3D and 2D).** The magenta molecular represents a ligand (quercetin), the yellow dotted line represents hydrogen bonds between ligand and acceptor, and the cyan, bottom-green and orange sections represent fragments of CASP8, CCL2, and AHR, respectively. (A) quercetin and CASP8. (B) quercetin and CCL2. (C) quercetin and AHR.
Figure 6 The related pathways analysis that quercetin effects on hub genes. (A) Comparative analysis of T2D and ND pancreatic islet expression of hub genes GSE76894. Higher expression of these genes was seen in T2D (red bar). Higher expression of genes was seen in ND (green bar), sorted by the P-value of AHR (0.00015), CASP8 (0.00082), and CCL2 (0.0058). (B) GSEA revealed the enriched pathways of the hub genes. AHR: Running Enrichment score of TNF signaling pathway, Lipid and atherosclerosis, NOD-like receptor signaling pathway > 0, but Glycine, serine and threonine metabolism, Butanoate metabolism, Taurine, and hypotaurine metabolism < 0. CASP8: Running Enrichment score of Tuberculosis, Type I diabetes mellitus and Viral myocarditis > 0, however Insulin secretion, Synaptic vesicle cycle, and Nicotine addiction < 0. CCL2: Running Enrichment score of TNF signaling pathway, Lipid and atherosclerosis, and NOD-like receptor signaling pathway > 0 whereas Nicotine addiction, Olfactory transduction and Circadian entrainment < 0. (C) GO enrichment analysis of AHR, CASP8, and CCL2. 10, 8, 10 terms were annotated by BP, CC, and MF independently. The circle in red represented the hub gene, and others represented GO terms which were annotated. (D) Lipid and atherosclerosis (Source: KEGG. CCL2 (MCP-1) and CASP8 were marked in red and involved in Leukocyte transendothelial migration and TNF signaling pathway, NOD-like receptor signaling pathway, the TNF signaling pathway, and apoptosis were marked in blue). T2D, type 2 diabetic; ND, non-diabetic; P, biological process; CC, cellular component; MF, molecular function; GO, Gene Ontology; GSEA, gene set enrichment analysis.
less than \(-5\) kcal\,mol\(^{-1}\), and the lower energy conformation is more stable [32]. Obviously, quercetin and Caspase 8 had the lowest binding energy \((-6.79\) kcal\,mol\(^{-1}\)), demonstrating the highest affinity between them (Table 3). As a result, it was clear from our results that quercetin had a better affinity and more stable conformation with Caspase-8 compared to other targets (CCL2 and AHR).

**Acquisition of regulatory pathways of hub genes treated with quercetin**

To examine the related pathway that hub targets of quercetin for damp-heat internalized T2D, the Violin plot, GSEA, and GO analysis were performed. Further analysis showed that the T2D groups exhibited higher expression of AHR (GS = 0.35; MM = 0.75), CASP8 (GS = 0.34; MM = 0.78), and CCL2 (GS = 0.28; MM = 0.75) (Figure 6A), sorted by GS and MM value. Therefore, CASP8, CCL2, and AHR were the hub genes of quercetin-treated damp-heat internalized T2D. Moreover, our study examined the clustering gene in T2D groups by GSEA (KEGG pathway) to understand how CASP8, CCL2, and AHR regulated the T2D-related pathway. The hub genes are involved in the diabetic-related pathways (insulin secretion, Type I diabetes) or other pathways (lipid and atherosclerosis, NOD-like receptor signaling pathway, TNF signaling pathway) (Figure 6B). In particular, the elevated expression of AHR and CCL2 in the T2D group positively regulated genes associated with lipid and atherosclerosis and NOD-like receptor signaling, as well as TNF signaling pathways, interestingly, the results illustrated how the NOD-like receptor, the TNF signaling, and apoptosis pathways are involved in lipid and atherosclerosis (Figure 6D). However, higher CCL2 expression negatively regulated the genes involved in the Insulin secretion pathway. Similarly, the overexpression of CASP8 increased the gene expressed in the Type I diabetes pathway (positively), but decreased the gene activity in the Insulin secretion pathway (negatively).

GO enrichment annotated that AHR involved transcription regular complex, B cell activation, and the active sites of CASP8 are mainly related to membrane components in CC (Figure 6C). CCL2 mostly plays a role in binding to cytokines, and CASP8 is mainly associated with the molecular functions of apoptosis and necrosis (Figure 6C). AHR was an essential transcription factor in regulating immune response, as well as in both insulin resistance and the activity of compensated β cells [33]. Researchers have discovered that CASP8 triggered and controlled apoptosis mediated by death receptors [34].

**Exploration of the relationship between quercetin and hub genes**

Subsequently, we would determine the pharmacological effect of quercetin on the hub genes (CASP8, CCL2, and AHR). Particularly, quercetin treatment for T2D was more effective than treatment for other diseases. As shown in Table 4, which showed that expression of CASP8, CCL2, and AHR may affect T2D and its complications through the CTD database. More specifically, quercetin was one of 72 compounds examined for the chemicals-gene interaction type and was found to promote the expression of AHR, CASP8, and CCL2 (Figure 7A). On the contrary, quercetin could also suppress the mRNA expression of CASP8, CCL2, and AHR interestingly, as shown in Figure 7B. Therefore, it is necessary to explore the role of quercetin for hub genes in damp-heat internalized T2D.

**Experimental verification**

To further validate the effect of quercetin on the hub genes, we conducted in vitro experiments. As depicted in Figure 8A, different doses of quercetin had no discernible effect on the proliferation of H9C2 cells \((P > 0.05)\) when 10 μM quercetin was more effective in attenuating HG induced H9C2 cells and also being non-toxic to H9C2s when treated for a period of 24 h (Figure 8B), mechanism of quercetin action at 10 μM concentration was further explored. Finally, qPCR was conducted to determine the relationship between quercetin and the hub genes (CASP8, CCL2, and AHR). As shown in Figure 8C, we found that expression of CASP8, CCL2 and AHR was increased in high glucose-induced H9C2 cells, which is consistent with our previous results (Figure 6A), and quercetin treatment decreased the mRNA levels of CASP8, CCL2, and AHR. The qPCR analysis primers are listed in Table 5. Meanwhile, the protein expression of Caspase 8, MCP-1 (CCL2) and AHR were decreased in treatment by quercetin, as shown in Figure 9. In conclusion, it is probable that quercetin of EZECD interacts with CASP8, CCL2, and AHR and suppresses their expression in damp-heat internalized T2D.

**Discussion**

Within present research, we utilized bioinformatics analysis and experimental verification to examine whether quercetin, a compound of EZECD, treats damp-heat internalized T2D to the mechanism of action through the genes CASP8, CCL2, and AHR. To begin with, quercetin, a key compound in EZECD, was obtained by using network pharmacology [35]. Moreover, the MM and GS criteria were employed to filter hub genes among eigengenes of positively correlated modules with T2D. We also analyzed overlapped genes (CASP8, CCL2, and...
Figure 7 The common compounds of CASP8, CCL2, and AHR in CTD database. (A) The blue circle represents that 749 curated compounds promotes AHR. The green circle represents that 680 curated compounds promotes CASP8. The yellow circle represents that 899 curated compounds promotes CCL2 (B) The blue circle represents that 455 curated compounds inhibits AHR. The green circle represents that 485 curated compounds inhibits CASP8. The yellow circle represents that 835 curated compounds inhibit CCL2. CTD, Comparative Toxicogenomics Database.

Figure 8 Quercetin could decrease HG induced CASP8, CCL2, and AHR expression in H9C2 cells. (A) Cell Counting Kit-8 assay of H9C2 cell viability after treatment with quercetin (μM), HG medium with DMSO (0.5%) was used as vehicle control for quercetin. (B) H9C2 cells were cultured in medium with NG (5 mM) and HG (33.3 mM) concentrations for 24–72 h in the absence or presence of indicated concentrations of quercetin. All results are expressed as mean ± SEM (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001 vs HG. (C) The mRNA levels of CASP8, CCL2, and AHR were assessed by real-time qPCR. All results are expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. SEM, standard error of the mean; HG, high glucose; qPCR, quantitative polymerase chain reaction; Q10, 10 μM quercetin.
Medicine used the mechanism in genes (HG) to decrease addition, eigengenes, in hypoglycemic antioxidant, protein receptor revealing AAGTTCAACGGCACAGTCAAGG Forward insulin Furthermore, as and quercetin, AHR Forward an CAGCAGCAAGTGTCCCAAAGAAG diabetes. resulted glucose-stimulated H9C2 secretion. negatively concentration Pharmacological signaling type 2 T2D, An expression Quercetin between both pancreatitis-mediated recent conditions, analysis GACATACTCAGCACCAGCATCAC < flavonoid, hyperglycemia hypoglycemic, secretion, quercetin-targeted in could for The quercetin yet AGAGTGGATGTGGTAGCAGAGTC induced revealed Research β anti-inflammatory type 5 in CASP8, defined which [37] years, treating shown in I and has to a CCL2, as damp-heat CASP8, and pyroptosis [39]. consistent with our findings, these data indicate that CASP8 or CCL2 can negatively affect insulin secretion. Although, another study showed that CCL2 did not affect insulin secretion, it could increase in response to inflammatory cytokines [43]. Therefore, our study showed that CASP8 or CCL2 participating in insulin secretion pathway was the mechanism of action when quercetin to treat damp-heat internalized T2D.

However, AHR and CCL2 positively modulated genes in lipid and atherosclerosis pathway, as well as/or comprising NOD-like receptor and TNF signaling pathway, as well as CASP8, which positively regulated genes in type 1 diabetes. NOD-like receptor signaling pathway, a process in which cholesterol crystals form inflammasome including NLRP3, ASC, and pro-CASP1 complex by Nrf2, resulting in pyroptosis or pro-inflammatory effects by secreting factor IL-1β and, or IL-18. There is growing acceptance of the association between T2D and the NLRP3 inflammasome [44]. The administration of quercetin has been shown in a number of trials to significantly reduce inflammation in systemic and visceral white adipose tissue, eliminate

<table>
<thead>
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<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
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<tr>
<td>CASP8</td>
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</tr>
<tr>
<td></td>
<td>Reverse GAGGATACAGCGATGAAAGGTC</td>
</tr>
<tr>
<td>CCL2</td>
<td>Forward CAGCAGCAAGTGCACCAAGAAGG</td>
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<tr>
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<tr>
<td></td>
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<td></td>
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</table>

AHR) between quercetin-targeted genes and eigengenes, revealing that they positively or negatively regulated genes in insulin secretion pathway or lipid and atherosclerosis pathway. This is the first study demonstrating that quercetin treats damp-heat internalized T2D via inhibiting CASP8, CCL2, and AHR expression.

Through network pharmacology, EZECD was composed of 6 herbs and 126 chemical compounds, among which the prime compound is quercetin. Quercetin is the primary compound of licorice, and studies have revealed that licorice has hypoglycemic effects, however the mechanism is not yet defined [36]. Pharmacological research demonstrates that quercetin, an essential flavonoid, has hypolipidemic, hypoglycemic, antioxidant, and anti-inflammatory properties [37]. In recent years, quercetin has shown dramatic anti-diabetic properties, and it can increase glucose absorption by a MAPK insulin-dependent mechanism and improve the phosphorylation of PI3K-Akt signaling pathways [38]. In addition, it has been used clinically as a major compound of QR-333 in diabetic peripheral neuropathy [39]. Consistent with the results of this study, we hypothesize that quercetin, as the hub compound of EZECD, plays an important role in treating internalized damp-heat T2D.

In addition, to quercetin treating damp-heat T2D, both CASP8 and CCL2 negatively regulated insulin secretion pathway. Our findings indicate a decrease in the expression of other genes in the insulin secretion pathway was associated with increased CASP8 and CCL2 expression in T2D patients. It is widely known that improper insulin secretion by pancreatic cells contributes to T2D. CASP8 is the chief executor of apoptosis, it regulates β cells-secreting insulin under physiological conditions, however, in type 2 diabetes, which activation resulted in β cell apoptosis [40]. An additional study demonstrated that CASP8-targeted activation fat apoptosis mice are less sensitive to glucose-stimulated insulin secretion and glucose tolerant [41]. Furthermore, through reducing insulin secretion, CCL2/CCL2 axis-mediated pancreatic cell infiltration is an important factor in chronic pancreatitis-mediated hyperglycemia [42].

Figure 9 Quercetin could decrease high glucose concentration (HG) induced CASP8, CCL2, and AHR protein expression in H9C2 cells. (A) The protein expression of Caspase8 in H9C2 treated by quercetin. (B) The protein expression of MCP-1 in H9C2 treated by quercetin. (C) The protein expression of AHR in H9C2 treated by quercetin. All results are expressed as means ± SEM (n ≥ 3). P < 0.05, **P < 0.01. HG, high glucose; Q10, 10 μM quercetin.
activation of the NLRP3 inflammasome, and improve signaling abnormalities that are indicative of insulin resistance [45]. AHR plays a significant part in the suppression of the activation of the NLRP3 inflammasome pathway in a variety of tissues, particularly the colitis, where it is most critical [46]. Higher CCL2 levels with inflammatory activation was found in hepatic macrophages, kidney, acute pancreatitis, but no research has been demonstrated how AHR or CCL2 orchestrates between inflammation and T2D or how quercetin regulates which [47–49]. Therefore, our study proposed how quercetin treats the damp-heat internalized T2D according to AHR or CCL2 positively involved in NOD-like signaling pathway. Moreover, TNF binding TNFR1 up-regulated the expression of CASP8, further apoptosis, ultimately resulting in an unstable plaque formed, which is composed of TNF signaling pathway in vascular smooth muscle cells. The combination of dietary natural flavonoids chrysanth and AHR can up-regulate pro-apoptotic cytokines TNF α and β genes to serve as chemopreventive role in human colorectal cancer cells [50]. However, it remains unclear whether quercetin regulates damp-heated internalized T2D through AHR or CCL2 genes positively regulated NOD-like receptor signaling pathway or TNF signaling pathway. Consequently, our study proposed a novel mechanism for quercetin to treat damp-heat internalized T2D.

Many biological processes are regulated by CASP8, CCL2, and AHR, which are involved in quercetin treatment for damp-heat internalized T2D. The present study showed that the binding between AHR and most the transcription factor complex could regulate a wide range of biological processes, such as the response to alcohol, the intracellular receptor signaling pathway, and the B cell activation. Evidence consistently showed that activated AHR-NQO1 signaling reversed alcoholic-induced liver damage [51]. The study also demonstrated that AHR activation influenced immunity by regulating B cell activity [52]. In addition, patients with high AHR biological activity have a greater risk of developing T2D, whereas AHR negatively regulates the initiation and progression of autoimmune responses in type 1 diabetes, which vary depending on a variety of exogenous (polyphenols) and endogenous ligands [53, 54]. These data suggest that the little research association with AHR involved biological processes and T2D, leaving a vast research space for mechanisms of action during AHR involved in T2D pathogenesis. CASP8, an apoptosis factor that primarily bound with cytokine receptor and located at cell membrane raft and mitochondrial outer membrane, and so on, also regulate all 10 types of biological processes, including the same as 3 types of AHR-regulated, as well as (cellular) response to tumor necrosis factor, (regulation of) neuron death and response to lipopolysaccharide, response to molecule of bacterial origin, response to cobalt ion. CCL2, as a ligand for CCR2, bonds only with cytokine receptor, and could moderate 6 types of biological processes, which is the same as CASP8, such as response to (cellular) tumor necrosis factor, regulation of neuron death, response to lipopolysaccharide, response to molecule of bacterial origin, and neuron death. As a result of these findings, the biological processes that CCL2 participates in are similar to those of CASP8. To examine the association of three genes involved in the pathway with the biological process, we then discuss how the CASP8 and CCL2 are involved in insulin secretion (pathway) associated with which biological process. The level of TNF-α, as a factor in TNF process, was decreased when induced the CASP8 activation and insulin secretion in mouse β Te-Tet cells [55]. The elevated level and expression of TNF-α and MCP-1 (also called CCL2) in the adipose tissue of mice fed high-fat diet, caused insulin shortage and hyperglycemia, and increased β cell apoptosis [56]. However, CCL2 in pancreatic islets increased during response to TNF and lipopolysaccharide (biological process), not through modulating insulin secretion [57]. These researchers associated with our data demonstrate that CASP8 or CCL2 is negatively involved in insulin secretion pathway more focusing on biological processes such as a response to TNF or lipopolysaccharide.

Quercetin exhibited strong bindings with CASP8, CCL2, and AHR in molecular docking. In wet verification, there was no significant decrease in cell survival occurred after administration of HG induced to h9c2 cells in CCK8, the expression level of hub genes increased significantly. Interestingly, the administration of quercetin inhibited the expression of hub genes, which may be attributed to the cellular characteristics induced by HG, which is consistent with the perspectives [58]. Additional studies have suggested that quercetin could down-regulate AHR expression, protect hepatocyte morphology, promote lipid metabolism, thereby reducing insulin resistance and improving T2D [59, 60]. The relationship between CASP8 and quercetin in pancreatic islet β cells is unclear, most studies have shown that quercetin activates CASP8, but some studies have come to the opposite conclusions [61, 62]. Clearly, the results of this paper are more supportive of the latter. For CCL2, it has been suggested that quercetin can regulate various signaling pathways such as NOD-like receptor signaling pathway to inhibit the expression of CCL2, thereby suppressing the development of diabetic nephropathy. Therefore, we speculate that quercetin could interact well with CASP8, CCL2, and AHR and then show an inhibitory effect.

**Conclusion**

In conclusion, quercetin, as the main compound in EZECD to treat damp-heat internalized T2D by improving insulin secretion response process by inhibiting the expression of CASP8 and CCL2, additionally, it also could inhibit the expression of AHR and CCL2, thereby negatively regulating atherosclerosis, as well as /or including NOD-like receptor signaling pathway, and TNF signaling pathway, consequently may reduce pancreatic islet β cells damage or apoptosis. Different from previous studies, the present paper combines the predictions of data mining with the validation of molecular docking and qPCR. The druggability of EZECD in the treatment of damp-heat internalized T2D was confirmed. However, the limitations of these methods are inherent. Some of the results of EZECD are to be verified by later experiments and clinical research validation. Therefore, this study will provide a new research approach and specific mechanism of action for exploring the experimental verification of EZECD to treat damp-heat internalized T2D.

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