Identify Molecular Mechanisms of Jiangzhi Decoction on Nonalcoholic Fatty Liver Disease by Network Pharmacology Analysis and Experimental Validation

Lei Wang, Yin Zhi, Ying Ye, Miao Zhang, Xing Ma, Hongyun Tie, Xiaokun Ma, Ni Zheng, Wei Xia, and Yanan Song

The Seventh People’s Hospital of Shanghai University of Traditional Chinese Medicine, 358 Datong Road, Pudong, Shanghai 200137, China

Correspondence should be addressed to Wei Xia; awingxia@163.com and Yanan Song; synabc.123@163.com

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Background. Jiangzhi Decoction (JZD), a traditional herb mixture, has shown significant clinical efficacy against nonalcoholic fatty liver disease (NAFLD). However, its multicomponent and multitarget characteristics bring difficulty in deciphering its pharmacological mechanisms. Our study is aimed at identifying the core molecular mechanisms of JZD against NAFLD.

Methods. The active ingredients were searched from Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and Traditional Chinese Medicine Integrated Database (TCMID). The targets of those ingredients were identified using ChemMapper database based on 3D structure similarity. NAFLD-related genes were searched from DisGeNET database and Gene Expression Omnibus (GEO) database. Then, we performed protein-protein interaction (PPI) analysis, functional enrichment analysis, and constructed pathway networks of “herbs-active ingredients-candidate targets” and identified the core molecular mechanisms and key active ingredients in the network. Also, molecular docking was carried out to predict the ligands of candidate targets using SwissDock. Finally, the human hepatic L02 cell line was used to establish the NAFLD model in vitro. The effect and key molecules were validated by Oil Red O staining, biochemical assays, and quantitative real-time PCR (qRT-PCR).

Results. We found 147 active ingredients in JZD, 1285 targets of active ingredients, 401 NAFLD-related genes, and 59 overlapped candidate targets of JZD against NAFLD. 22 core targets were obtained by PPI analysis. Finally, nuclear receptor transcription and lipid metabolism regulation were found as the core molecular mechanisms of JZD against NAFLD by functional enrichment analysis. The candidate targets PPARα and LXRα were both docked with hyperin as the most favorable interaction, and HNF4α was docked with linolenic acid ethyl ester. According to in vitro experiments, it was found that JZD had an inhibitory effect on lipid accumulation and regulatory effects on cholesterol and triglycerides. Compared with OA group, the mRNA expression levels of PPARα and HNF4α were significantly upregulated in JZD group ($P < 0.05$), and LXRα was significantly downregulated ($P < 0.001$).

Conclusion. JZD might alleviate hepatocyte steatosis by regulating some key molecules related to nuclear receptor transcription and lipid metabolism, such as PPARα, LXRα, and HNF4α. Our study will provide the scientific evidences of the clinical efficacy of JZD against NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver pathology that is characterized by the excessive accumulation of fat in the liver, including simple steatosis nonalcoholic fatty liver, nonalcoholic steatohepatitis (NASH), steatofibrosis, cirrhosis, and hepatocellular carcinoma [1]. In the face of a global obesity epidemic, NAFLD has emerged as the most common form of chronic liver disease, affecting an estimated 25% of the general population worldwide [2, 3]. Epidemiological researches have reported that NAFLD is one of the three main causes of cirrhosis [4], and NASH is rapidly becoming the leading cause of end-stage liver disease [5, 6]. However, except for lifestyle
interventions, such as exercise and dietary, there is still no approved pharmacotherapy for NAFLD. Therefore, it is important to explore and develop valid pharmacotherapies for NAFLD [7].

In recent years, traditional Chinese medicine (TCM) has been proved effective in the case of alone or integrated with western medicine and has attracted more and more people’s attention [8, 9]. Jiangzhi Decoction (JZD), a clinically used herbal formula developed in accordance with TCM pathogenesis, is composed of the following five medicinal herbs: *Trichosanthes kirilowii* Maxim. (TK), *Alisma orientale* (Sam.) Juze (AO), *Angelica sinensis* (Oliv.) Diels (AS), *Crataegus pinnatifida* Bge. (CP), and *Polygonum multiflorum* Thunb. (PM) (Table 1). Previous evidence has proved the efficacy of JZD on regulating lipid metabolism [10]. However, the molecular mechanisms of JZD are still unclear and need further exploration.

Because Chinese herbal medicines have the characteristics of multicomponent and multitargeted effects, conventional strategies can be hardly used to explore their pharmacological mechanisms. Network pharmacology, a novel approach based on systems biology, has been proved suitable for analyzing the complex relationships of various ingredients and effects in Chinese herbal medicines [11]. In this study, network pharmacology was carried out to investigate the molecular mechanisms according to screening a great deal of candidate ingredients, predicting multiple drug targets, analyzing possible signaling pathways, conducting herbs-ingredients-targets networks, and predating the possible ligands for candidate targets. Also, the NAFLD model in vitro was established to validate the inhibitory effect on lipid accumulation and the changes of expression levels of key molecules in JZD against NAFLD (Figure 1).

### 2. Methods

#### 2.1. Data Preparation

2.1.1. Searching for Active Ingredients of JZD. The active ingredients of JZD were collected from two databases. One is Traditional Chinese Medicine Systems Pharmacology (TCMSP) database [12] (http://lsp.nwu.edu.cn/tcmsp.php), which contains large number of herbal entries, drug-disease networks, and drug-target networks. A great deal of herbal information can be obtained from TCMSP database, including ingredients, molecule name, molecular weight (MW), drug-likeness (DL), human oral bioavailability (OB), half-life (HL), water partition coefficient (AlogP), number of hydrogen bond donors and receptors (Hdon/Hacc), Caco-2 permeability (Caco-2), and blood-brain barrier (BBB). The active ingredients of JZD were screened out according to the ADME parameter, and the ingredients with DL ≥ 0.18 were regarded as active ingredients [13].

If the herbal information could not be found in TCMSP database, the other database would be used. It is Traditional Chinese Medicine Integrated Database (TCMID) (http://www.megabionet.org/tcmid/), which contains 46929 prescriptions, 8159 herbs, 43413 total ingredients, 8182 drugs, 4633 diseases, 1045 prescription ingredients, 778 herbal mass spectra, and 3895 mass spectrometry of ingredients [14]. By combining the information from two databases above, the active ingredients of JZD were identified.

2.1.2. Identification of Targets of Active Ingredients. ChemMapper database (http://lilab.ecust.edu.cn/chemmapper/) is a versatile web server for exploring pharmacology and chemical structure association based on molecular 3D similarity method [15]. We searched the predicted targets of each active ingredient in JZD from ChemMapper database and screened according to the criteria of 3D structure similarity above 1.0 and prediction score above 0 [16]. The full names of targets were converted to gene symbol based on the UniProt ID in UniProt database (http://www.uniprot.org/) for further analysis.

2.1.3. Searching for NAFLD-Related Genes. DisGeNET database (http://www.disgenet.org/web/DisGeNET/menu/home) is a knowledge management platform integrating and standardizing data about disease-associated genes and variants from multiple sources, including the scientific literature [17]. Known genes of NAFLD were searched from DisGeNET database using “nonalcoholic fatty liver disease” as the keyword, and the top 30% of genes were regarded as important genes for further analysis.

In addition, Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) is an international public repository for high-throughput microarray and next-generation sequence functional genomic data sets submitted by the research community [18]. The main differentially expressed genes (DEGs) between mild NAFLD and advanced NAFLD were extracted from microarray data GSE31803 and GSE49541 in GEO database, with a cutoff value of $P < 0.05$ and fold change $|FC| ≥ 3$.

#### 2.2. Network Analysis

2.2.1. Protein-Protein Interaction (PPI) Analysis. The overlapped genes between the target genes of active ingredients and NAFLD-related genes were imported into the STRING

| Chinese name   | Pharmaceutical name | Botanical plant name | English name                  |
|----------------|----------------------|----------------------|-------------------------------|
| Gua Lou        | Trichosanthes Fructus| *Trichosanthes kirilowii* Maxim. | Snakegourd fruit          |
| Ze Xie          | Alismatis Rhizoma    | *Alisma orientale* (Sam.) Juze | Oriental Waterplantain Rhizome |
| Dang Gui        | Angelicae Sinensis Radix | *Angelica sinensis* (Oliv.) Diels | Chinese Angelica          |
| Shan Zha        | Crataegi Fructus     | *Crataegus pinnatifida* Bg. | Hawthorn fruit            |
| He Shou Wu      | Polygoni Multiflori Radix | *Polygonum multiflorum* Thunb. | Fleece flower root        |

Table 1: The herbs of JZD.
database to construct PPI network. STRING database integrates the quality-controlled protein-protein association networks of a large number of organisms. We selected the core PPI targets according to the degree score above the average value and the confidence score above 0.9.

2.2.2. Functional Enrichment Analysis. ReactomeFIViz and ClueGO, two kinds of plug-ins for Cytoscape, were used to perform functional enrichment analysis. ReactomeFIViz is a highly reliable protein functional interaction network covering around 60% of total human genes based on

**Figure 1**: The flowchart for exploring the molecular mechanisms of JZD against NAFLD.
Reactome database, the most popular and comprehensive open-source biological pathway knowledgebase [19]. ClueGO integrates Gene Ontology (GO) terms and KEGG/BioCarta pathways and creates a functionally organized GO/pathway term network [20]. \( P < 0.01 \) was regarded as the significant cutoff in this study.

2.2.3. Pathway Network Construction. Pathway networks of “herbs-active ingredients-candidate targets” were constructed using the Cytoscape 3.3.0 software. The Network Analyzer plug-in was used to identify key active ingredients and critical candidate targets based on the criterion below: nodes with degree values exceeding the average value of all nodes in the network. The degree value is the number of edges a node has in a network, which indicates how many herbs/ingredients/targets one node is related with. If the degree value of a node is larger, the node is believed to play a more important role in the network.

2.2.4. Molecular Docking. Molecular docking was carried out online using SwissDock (http://swissdock.ch/) web service. Crystal structures of candidate targets in PDB format and relative ligands in MOL2 format were uploaded. The lowest Gibbs free energy (\( \Delta G \)) was predicted in silico. The UCSF Chimera 1.14 software was used for visualizing the results and creating 3D images.

2.3. Experimental Validation

2.3.1. Cell Culture and Treatment. Human hepatic L02 cell lines were cultured in RMPI medium containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C in 5% CO2. Cells without any treatments were used as control. The cells were treated with 0.2 mM oleic acid (OA) for 24 h to establish the NAFLD model in vitro. Afterward, JZD (500 \( \mu \)g/ml) was added into the medium. After another 24 h of incubation, the cells were analyzed.

2.3.2. Oil Red O Staining. Briefly, the cells were fixed with 4% paraformaldehyde. After three times washing with PBS, the culture plates of the cells were added into 60% isopropanol and allowed to stand for 5 min. Freshly diluted oil red working solution was applied to cells for 15 min. After rinsing with 60% isopropanol, the cells were counterstaining with hematoxylin. Finally, the pictures were captured with a light microscope (OLYMPUS, Japan) at \( \times 200 \) magnification.

2.3.3. Cholesterol (TC) and Triglyceride (TG) Assays. Cells from the different groups were harvested and washed twice with PBS. The intracellular TC and TG were measured using the biochemistry assay kits in accordance with the manufacturer’s instruction (Jiancheng, Nanjing, China). The TC and TG concentrations were normalized to the total cell protein concentration.

2.3.4. Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The quality of RNA was measured by Nanodrop 2000 (Thermo Scientific, Rockford, IL, USA), and equal amounts of RNA were reverse-transcribed into cDNA using First-Strand cDNA Synthesis kits (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out using ABI 7500 System (Applied Biosystems, Foster City, CA, USA) under the following parameters: 95°C for 30 s, 95°C for 5 s (40 cycles), 60°C for 30 s, and 72°C for 15 s. The gene primer pairs used in this study were as follows: 5′-TTGGAAGACCGCTTTCTCTTGAT-3′ and 5′-GCCATGAAAACCGTCTGTG-3′ for PPARa, 5′-TGGACACCTACATCGTGCAA-3′ and 5′-CAAGGATGGCATGGAGCTCT-3′ for LXRa, 5′-CAGGCAGGAATAGCCT-3′ and 5′-GGGTGTCGTCGTCATAGCTT-3′ for HNF4a, and 5′-CCGGATCAACGTTAT-3′ and 5′-AGCCCTTCTCCAAGTGTTGGAAGAC-3′ for GAPDH. The threshold cycle (CT) of each gene was normalized to GAPDH mRNA, and the fold change was calculated by \( 2^{-\Delta\Delta CT} \) method. Each sample was run three times at least.

2.4. Statistical Analysis. The quantitative data were presented as mean ± standard deviation (SD). One-way ANOVA analysis, followed by Dunnett post hoc test, was used to determine significant differences between different groups using the SPSS software (version 21.0, Chicago, IL, USA). \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Candidate Targets of JZD against NAFLD

3.1.1. Active Ingredients of JZD. Among the five main herbs of JZD, TK, AO, and AS were searched from TCMSP database, while the other herbs, CP and PM, could not be found in TCMSP database and were searched from TCMID database. Based on the criteria of DL \( \geq 0.18 \), a total of 147 active ingredients were finally screened out in this study (Figure 2 and Supplementary Table 1). The numbers of active ingredients in TK, AO, AS, CP, and PM were 17, 15, 7, 66, and 46, respectively. There were four ingredients overlapped in two herbs, including emodin in AO and PM, epicatechin in CP and PM, and \( \beta \)-sitosterol in AS and PM. A note about those results was that ADME parameters were not provided in TCMID database. Thus, the ingredients in CP and PM did not be filtered according to the ADME parameters. Maybe it was the reason why the amounts of ingredients in CP and PM were much larger than those in TK, AO, and AS.

3.1.2. Targets of Active Ingredients of JZD. The direct targets of each chemical ingredient in JZD were identified from ChemMapper database. According to the criteria of 3D structure similarity above 1.0 and prediction score above 0, a total of 1285 targets of active ingredients of JZD were obtained (Supplementary Table 2). Our analysis showed that gallic acid, citric acid, and succinic acid were the top three active ingredients targeting 525, 481, and 440 targets, respectively.

3.1.3. Genes Related to NAFLD. A total of 333 known genes were found from DisGeNET database, and the top 30%, that were 100 genes, were chosen for further analysis. Based on
the cutoff value of $P < 0.05$ and fold change $|\text{FC}| \geq 3$, a total of 315 DEGs were extracted from microarray data GSE31803 and GSE49541. After duplicates of genes from DisGeNET database and GEO data were eliminated, a total of 401 genes were identified as NAFLD-related genes (Supplementary Table 3).

3.1.4. Targets of JZD against NAFLD. According to the search and analysis above, we obtained a total of 59 overlapped genes between the target genes of active ingredients and NAFLD-related genes, which were predicted as the candidate targets of JZD against NAFLD (Table 2).

3.2. Key Targets and Signaling Pathway of JZD against NAFLD

3.2.1. Core Targets in Protein-Protein Interaction (PPI). The 59 candidate targets were imported into the STRING database to construct the PPI network. According to the screening criteria of the degree score above the average value and the confidence score above 0.9, 22 core targets were obtained, including TNF, IGF1, IL1B, GPT, CCL2, HGF, TLR4, PPARG, GOT2, LDLR, MMP1, F2, DCN, ACE, GLUL, PPARA, THBS1, JAK2, CFTR, PLAT, OAT, and GPX8 (Figure 3). The 22 core targets may be the potential targets in the treatment of JZD against NAFLD.

3.2.2. Functional Enrichment Analysis of the Overlapped Genes. ReactomeFIViz and ClueGO pathway analyses were further performed for the overlapped genes. The top 10 significantly related pathways were shown in Figure 4(a) by ReactomeFIViz analysis, including nuclear receptor transcription pathway, glutathione conjugation, interleukin-4 and 13 signaling, regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs), and phase II conjugation. At the same time, according to ClueGo analysis, signaling pathways were divided into 13 enriched categories based on the kappa coefficient, including positive regulation of lipid metabolic process, regulation of smooth muscle cell proliferation, fatty acid transport, positive regulation of reactive oxygen species metabolic process, regulation of phosphatidylinositol 3-kinase signaling, alpha-amino acid metabolic process, regeneration, response to mechanical stimulus, muscle cell proliferation, cellular detoxification, exocrine system development, regulation of glucose transport, and cell-cell signaling involved in cardiac conduction (Figures 4(b) and 4(c)). The functional enrichment analysis indicated that nuclear receptor transcription and lipid metabolism regulation might play an important role in the treatment of JZD against NAFLD.

3.2.3. Pathway Network Construction of Herb-Ingredient-Target. To identify key molecular mechanisms of JZD against NAFLD, we further constructed herb-ingredient-target networks based on the top significantly related pathways from functional enrichment analysis (Figure 5). We found that 12 ingredients appeared in both nuclear receptor transcription
| No. | Uniprot ID | Gene ID | Gene symbol | Gene full name |
|-----|------------|---------|-------------|----------------|
| 1 | P54710     | 486     | FXYD2       | FXYD domain containing ion transport regulator 2 |
| 2 | P06396     | 2934    | GSN         | Gelsolin |
| 3 | P28472     | 2562    | GABRB3      | Gamma-aminobutyric acid type A receptor Beta3 subunit |
| 4 | P07996     | 7057    | THBS1       | Thrombospondin 1 |
| 5 | P54289     | 781     | CACNA2D1    | Calcium voltage-gated channel auxiliary subunit Alpha2delta 1 |
| 6 | P04818     | 7298    | TMY3        | Thymidylate synthetase |
| 7 | P17302     | 2697    | GJA1        | Gap junction protein alpha 1 |
| 8 | P55011     | 6558    | SLC12A2     | Solute carrier family 12 member 2 |
| 9 | P07585     | 1634    | DCN         | Decorin |
| 10 | P08729     | 3855    | KRT7        | Keratin 7 |
| 11 | P14210     | 3082    | HGF         | Hepatocyte growth factor |
| 12 | P13569     | 1080    | CFTR        | CF Transmembrane conductance regulator |
| 13 | Q96SL4     | 2882    | GPX7        | Glutathione peroxidase 7 |
| 14 | P13500     | 6347    | CCL2        | C-C motif chemokine ligand 2 |
| 15 | O94925     | 2744    | GLS         | Glutaminase |
| 16 | P21802     | 2263    | FGFR2       | Fibroblast growth factor receptor 2 |
| 17 | Q99584     | 6284    | S100A13     | S100 calcium binding protein A13 |
| 18 | Q13133     | 10062   | NRIH3       | Nuclear receptor subfamily 1 group H member 3 |
| 19 | P13716     | 210     | ALAD        | Aminovulinate dehydratase |
| 20 | P82251     | 11136   | SLC7A9      | Solute carrier family 7 member 9 |
| 21 | Q8N159     | 162417  | NAGS        | N-Acetylglutamate synthase |
| 22 | P21549     | 189     | AGXT        | Alanine-glyoxylate and serine-pyruvate aminotransferase |
| 23 | P34896     | 6470    | SHMT1       | Serine hydroxymethyltransferase 1 |
| 24 | Q08828     | 107     | ADCY1       | Adenylate cyclase 1 |
| 25 | O43708     | 2954    | GSTZ1       | Glutathione S-transferase zeta 1 |
| 26 | P51570     | 2584    | GALK1       | Galactokinase 1 |
| 27 | P15104     | 2752    | GLUL        | Glutamate-ammonia ligase |
| 28 | Q14749     | 27232   | GNMT        | Glycine N-methyltransferase |
| 29 | P36222     | 1116    | CHI3L1      | Chitinase 3 like 1 |
| 30 | Q8TED1     | 493869  | GPX8        | Glutathione peroxidase 8 (putative) |
| 31 | P11137     | 4133    | MAP2        | Microtubule associated protein 2 |
| 32 | P00750     | 5327    | PLAT        | Plasminogen activator, tissue type |
| 33 | Q9H2S1     | 3781    | KCNN2       | Potassium calcium-activated channel subfamily N member 2 |
| 34 | P04181     | 4942    | OAT         | Ornithine aminotransferase |
| 35 | Q07869     | 5465    | PPARA       | Peroxisome proliferator activated receptor alpha |
| 36 | P01130     | 3949    | LDLR        | Low density lipoprotein receptor |
| 37 | P09488     | 2944    | GSTM1       | Glutathione S-transferase mu 1 |
| 38 | P09211     | 2950    | GSTP1       | Glutathione S-transferase pi 1 |
| 39 | Q03181     | 5467    | PPARD       | Peroxisome proliferator activated receptor delta |
| 40 | P30711     | 2952    | GSTT1       | Glutathione S-transferase theta 1 |
| 41 | P08263     | 2938    | GSTA1       | Glutathione S-transferase alpha 1 |
| 42 | P03956     | 4312    | MMP1        | Matrix metalloproteinase 1 |
| 43 | Q9P2W7     | 27087   | B3GAT1      | Beta-1,3-glucuronyltransferase 1 |
| 44 | O60674     | 3717    | JAK2        | Janus kinase 2 |
| 45 | P08700     | 3562    | IL3         | Interleukin 3 |
| 46 | P12821     | 1636    | ACE         | Angiotensin I converting enzyme |
| 47 | P00734     | 2147    | F2          | Coagulation factor II, thrombin |
| 48 | Q8WTV0     | 949     | SCARB1      | Scavenger receptor class B member 1 |
pathway and lipid metabolism regulation pathway, including hyperin, emodin, emodin anthrone, questin, rhein, tricin, aloe emodin, 2-acetylemodin, epicatechin, chrysazin, chrysophanol, 4,4'-dihydroxydiphenyl methane. The results implied that those key active ingredients all participated in nuclear receptor transcription and lipid metabolism regulation, which might be the key molecular mechanisms of JZD against NAFLD.

3.2.4. Molecular Docking of Candidate Targets. According to the pathway network construction of herb-ingredient-target, we chose the candidate targets both in above two networks and closely related with liver lipid regulation that were PPARα, LXRα, and HNF4α. Based on Swissdock calculation, hyperin showed estimated ΔG of -10.13 kcal/mol and -8.91 kcal/mol for PPARα and LXRα, respectively, as the most favorable interaction, and linolenic acid ethyl ester showed ΔG of -8.49 kcal/mol for HNF4α. Figure 6 shows the visualization of the most energetically favorable binding of the ligands into the protein PPARα, LXRα, and HNF4α. As shown in Figure 6, PPARα was docked with hyperin at

![Figure 3: PPI network of 22 core targets of JZD against NAFLD. In the PPI diagram, each solid circle represents a target, and the middle of the circle shows the structure of the protein.](image-url)
Figure 4: Functional enrichment analysis from ReactomeFIViz and ClueGO. (a) The column bar graph from ReactomeFIViz. It shows the top 10 significantly related pathways, including nuclear receptor transcription pathway, glutathione conjugation, interleukin-4 and 13 signaling, regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs), and phase II conjugation. (b) The pie chart from ClueGO. It shows the enriched signaling pathway categories based on the kappa coefficient, including positive regulation of lipid metabolic process, regulation of smooth muscle cell proliferation, fatty acid transport, positive regulation of reactive oxygen species metabolic process, regulation of phosphatidylinositol 3-kinase signaling, and alpha-amino acid metabolic process. (c) The functional enrichment network from ClueGO. The node represents the signaling pathway, and the size of each node represents the enrichment significance of each signaling pathway. The larger the node is, the more significant the pathway is. The line represents the correlation between functions, and the thickness of each line represents the kappa coefficient between functions. The thicker the line is, the greater the kappa coefficient is.
Figure 5: Continued.
the binding sites of TYR 334 and MET 220 residues, LXRα was also docked with hyperin at the binding sites of GLN 222, GLU 267, and ARG 305 residues, and HNF4α was docked with linolenic acid ethyl ester at the binding sites of ARG 226 residues.

3.3. Experimental Validation of JZD against NAFLD in vitro

3.3.1. The Inhibitory Effect of JZD on Lipid Accumulation. After L02 cells were treated with 0.2 mM OA for 24 h, epithelial morphology of hepatocytes was found to be transformed into bulky lipid laden round cells, stained red due to Oil Red O stain. Compared with the OA group without JZD treatment, there was a significant reduction in the lipid content of steatotic hepatocytes in the JZD group, indicating that JZD had an inhibitory effect on lipid accumulation (Figure 7).

3.3.2. The Effects of JZD on TC and TG. Compared with the control group, the contents of TC and TG statistically increased in the OA group ($P < 0.001$). Compared with the OA group, the contents of TC and TG significantly decreased in the JZD group ($P < 0.001$) (Figure 8(a)). It showed the regulatory effects of JZD on TC and TG.

3.3.3. Changes of Expression Levels of Key Molecules in JZD against NAFLD. Compared with the control group, the mRNA expression levels of PPARα and HNF4α were significantly downregulated in the OA group ($P < 0.01$), and LXRα was significantly upregulated ($P < 0.001$). Compared with the OA group, the mRNA expression levels of PPARα and HNF4α were significantly upregulated in the JZD group ($P < 0.05$), and LXRα was significantly downregulated ($P < 0.001$) (Figure 8(b)). It implied us that JZD might...
Figure 6: Continued.
alleviate hepatocyte steatosis by regulating the mRNA expression of these key molecules from network pharmacology analysis.

4. Discussion

NAFLD is characterized by abnormal lipid metabolism and excessive lipid accumulation in hepatocytes. Most of the herbs in JZD have been reported to take part in the regulation of lipid metabolism in NAFLD or other related diseases. Some researchers showed that AO could prevent hepatic triglyceride accumulation through suppressing de novo lipogenesis and increasing lipid export and control oxidative stress markers, lipoapoptosis, liver injury panels, and inflammatory and fibrotic mediators, eventually influencing steatohepatitis and liver fibrosis [21, 22]. The components of AS have been proved to regulate lipid and glucose metabolism [23–25]. Liu et al. found that a diet formula of CP and three other herbs could alleviate hepatic steatosis and insulin resistance in vivo and in vitro [26]. Yu et al. performed a series of experiments to confirm that the active components of PM could promote the lipolysis of cholesterol and triglyceride, increase the content of HTGL, and reduce LDL and VLDL [27–29]. However, the synergetic mechanisms of all the herbs in JZD were still unclear.

In our study, nuclear receptor transcription and lipid metabolism regulation were found as the core pathways which JZD mainly participated in when alleviating NAFLD. As we know, there are 48 nuclear receptors categorized into 7 subfamilies designated as NR0-NR6 [30]. Of particular importance in NAFLD are specific members of NR1 subfamily [31]. Most potential targets of JZD in Figure 2 belong to NR1 subfamily, including PPARs (peroxisome proliferator-activated receptors, PPARα/PPARδ, PPARβ/PPARγ; NR1C1-3) and LXRα (liver X receptor α; NR1H3). PPARα activation induces the increase in fatty acid oxidation, ketogenesis, and gluconeogenesis [32]. PPARβ/δ activation exerts regulatory effects on fatty acid catabolism, reverses cholesterol transport and energy metabolism, and even reduces insulin resistance and plasma
glucose [33]. PPARγ shifts lipids from nonadipose organs such as the liver and skeletal muscles to white adipose tissue, leading to the attenuation of lipotoxicity [34]. In general, PPAR activation is thought to be beneficial in NAFLD, and clinical trials of single/dual receptor agonists are underway [31]. Another important nuclear receptor LXRα acts as the negative regulator of cholesterol metabolism through the induction of hepatocyte cholesterol catabolism, excretion, and the reverse cholesterol transport pathway [35]. Furthermore, HNF4A/HNF4α (hepatocyte nuclear factor 4α; NR2A1) also belongs to the subfamily of nuclear receptors. Previous study reported that HNF4α could prevent liver steatosis by controlling hepatic carboxylesterase 2 expression and modulating lipolysis, lipogenesis, and endoplasmic reticulum in NAFLD [36]. Therefore, they are all potential therapeutic targets for the treatment of NAFLD.

Our research found that some ingredients in JZD might be the key ones for NAFLD treatment, such as emodin, hyperin, and rhein. Many previous studies have reported that those ingredients could take part in the regulation of nuclear receptor. Emodin has been proved to increase the mRNA level of PPARγ and play a protective role in alcohol-mediated liver steatosis [37]. According to the activation of PPARγ signaling pathway, emodin could also alleviate atherosclerosis followed by promoting cholesterol efflux [38] or play other roles though regulating inflammatory response [39, 40] and nitric oxide production [41]. Furthermore, emodin has also been reported to regulate the expression of LXRα in atherosclerosis [38] and melanogenesis [42]. Hyperin is one of the chief flavonoid components of Ericaceae, Gutti-fera, Leguminosae, and Celastraceae and could remarkably induce the expression of PPARγ and attenuate inflammation of acute liver injury [43]. In addition, some studies also reported that rhein could target PPARγ signaling pathway and play anti-inflammatory activity [44, 45]. Moreover, rhein has been confirmed to ameliorate NAFLD and obesity and recover metabolic disorders through directly binding to LXRα [46, 47]. Thus, those key ingredients in JZD might improve NAFLD via regulating the nuclear receptors.

In conclusion, the multicomponent and multitarget characteristics of the therapeutic effects of JZD against NAFLD were effectively elucidated through network pharmacology approach and experimental validation. Nuclear receptor transcription and lipid metabolism regulation were found as the core molecular mechanisms by which JZD alleviated NAFLD. Therefore, our study will provide the scientific evidences of the clinical efficacy of JZD against NAFLD.

Abbreviations

AlogP: Water partition coefficient
AO: Alisma orientale (Sam.) Juzep
AS: Angelica sinensis (Oliv.) Diels
BBB: Blood-brain barrier
Caco-2: Caco-2 permeability
CP: Crataegus pinnatifida Bge
DEGs: Differentially expressed genes
DL: Drug-likeness

Figure 8: TC and TG contents (a) and relative mRNA expression levels of key genes (b) in JZD against NAFLD. N = 3 in each group. Values were expressed as mean ± standard deviation (SD). Significant differences were analyzed by one-way ANOVA with Dunnett post hoc test.
Data Availability
The data of our research can be acquired from the Supplementary Materials uploaded with this article.

Conflicts of Interest
We declare that we have no conflicts of interest.

Authors’ Contributions
Song YN and Xia W conceived the study. Wang L analyzed the data. Zhi Y, Ye Y, Zhang M, and Ma X conducted the experiments. Tie HY, Ma XK, and Zheng N made the charts. Wang L wrote the paper. Song YN and Xia W revised the paper. Lei Wang and Yin Zhi have contributed equally to this work and should be considered as co-first authors.

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Supplementary Materials

Supplementary 1. Table S1: 147 active ingredients found in Jiangzhi Decoction (JZD). The active ingredients were obtained from both Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (http://lsp.nwu.edu.cn/tcmsp.php) according to the ADME principle: drug likeness (DL) > 0.18 and Traditional Chinese Medicine Integrated Database (TCMID) (http://www.megabionet.org/tcmid/).

Supplementary 2. Table S2: 1285 targets of active ingredients of JZD. The targets were identified using ChemMapper database (http://lilab.ecust.edu.cn/chemmapper/) according to the criteria of 3D structure similarity above 1.0 and prediction score above 0.

Supplementary 3. Table S3: 401 nonalcoholic fatty liver disease- (NAFLD-) related genes. Some of NAFLD-related genes were searched from DisGeNET database (http://www.disgenet.org/web/DisGeNET/menu/home) with the top 30% of genes selected and the others from Microarray data GSE31803 and GSE49541 in Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with a cutoff value of $P < 0.05$ and fold change $|FC| ≥ 3$.

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