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Elucidating the anti-obesity phytochemicals in Chenpi and their molecular mechanisms

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ABSTRACT

Obesity has become a significant global public health issue. Previous studies have found that the Chenpi has the anti-obesity activity. However, the anti-obesity phytochemicals and their mechanisms are still unclear. This study investigated the anti-obesity phytochemicals and molecular mechanisms involved in treating obesity by Chenpi through network pharmacology and molecular docking. A total of 17 bioactive phytochemicals from Chenpi and its 475 related anti-obesity targets have been identified. The KEGG pathway analysis showed that the PI3K/Akt signaling pathway, MAPK signaling pathway, AMPK signaling pathway, and nuclear factor kappa B signaling pathway are the main signaling pathways involved in the anti-obesity effect of Chenpi. According to molecular docking analysis, the phytochemicals of Chenpi can bind to central anti-obesity targets. Based on the ADMET analysis and network pharmacology results, tangeretin exhibited the lowest predicted toxicity and potential for anti-obesity effects. In the *in vitro* lipid accumulation model, tangeretin effectively suppressed the free fatty acid-induced lipid in HepG2 cells by upregulating the PI3K/Akt/GSK3β signaling pathway based on the result of q-PCR and Western blotting. The outcomes of this research give insights for future research on the anti-obesity phytochemicals and molecular mechanisms derived from Chenpi, also providing the theoretical basis for developing anti-obesity functional foods based on Chenpi.

1. Introduction

Obesity has become a significant global public health problem, and the fundamental cause of obesity is the energy imbalance between the amount of energy intake and the amount of calories consumed[1]. Obesity is characterized by excessive accumulation of fat in the body. It will increase the prevalence of various chronic metabolic diseases, such as type 2 diabetes, dyslipidemia, cardiovascular disease, and nonalcoholic fatty liver disease[2]. The high incidence rate of these chronic metabolic diseases in obesity results from obesity-mediated inflammatory reactions and oxidative stress[3]. In clinical practice, drugs and surgery are the most commonly used treatment methods for obesity. However, most drugs have side effects, such as sibutramine[4] and orlistat[4], and surgery is unsuitable for all patients. Therefore, in recent years, many researchers have begun to screen active compounds with fewer side effects from natural products and use them for weight loss and developing nutritional and health products[5]. Various phytochemicals from fruits have great potential for controlling obesity *in vitro* and *in vivo* models[6-7].

Chenpi, also known as the aged peel of *Citrus reticulata* Blanco, is native to subtropical and tropical regions of Asia, especially China[8]. People have always consumed chenpi as a food or tea with

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health benefits, rich in secondary metabolites such as terpenoids, flavonoids, and phenols. Moreover, the polymethoxyflavones (PMSF) is also the important source of the bioactive compound exhibiting the bioactivities such as anti-cancer, anti-obesity, antioxidation, and neuroprotection[9-10]. Some anti-obesity studies have been published on the extracts of Chenpi. The *n*-butane extract of Chenpi, rich in 5-demethylated PMSF, has an improvement effect in a high-fat diet induced obesity mouse model and may be mediated through the AMPK signaling pathway[11]. Similarly, water extracts of Chenpi rich in synephrine, narirutin, hesperidin, nobiletin, and tangeretin can improve obesity by inducing brown fat generation in high-fat diet induced obesity animal experimental models[12]. A clinical trial on Chenpi extract also validated its anti-obesity effect[13]. However, the anti-obesity active phytochemicals of Chenpi and their related molecular mechanisms are still unclear.

Network pharmacology is a relatively emerging research strategy that utilizes big data and artificial intelligence[14]. It has been widely used to identify active ingredients of edible and medicinal plants and understand the overall mechanism of plants treatment for diseases, providing innovative technical and scientific support for new drug research and clinical use[15]. More and more researchers have started to use network pharmacology combined with molecular docking technology or *in vitro* and *in vivo* studies to study the molecular mechanism of phytochemicals therapy for diseases[16]. This study utilized network pharmacology to investigate the bioactive phytochemicals and their molecular mechanisms of Chenpi. The molecular docking was performed to calculate the binding affinity of the phytochemicals and targets.

An *in vitro* free fatty acid (FFA)-induced lipid accumulation cell model was widely used for studying the anti-obesity effect of the bioactive compound from the food[17-19]. High-fat-diet is a common way to improve the obesity and can increase the FFA level in in blood and liver[20]. The HepG2 cell line can be used for the *in vitro* FFA-induced lipid accumulation cell model[21]. Moreover, the network pharmacology studies focused on the *Homo sapiens* target, and *in vitro*, the human derived HepG2 cell line can be used for further study. The *in vitro* lipid accumulation study was used to further study the anti-obesity effect and its underlying molecular mechanism of tangeretin validating the results of network pharmacology. This is the first study to analyze the active phytochemicals and molecular mechanisms of Chenpi in treating obesity using network pharmacology combined with molecular docking. The finding of this research can provide the theoretical basis for developing anti-obesity functional foods based on Chenpi.

2. Material and methods

2.1 Chemicals reagents and materials

The minimum essential medium (MEM), 100 X penicillin-streptomycin solution, and HepG2 cell line were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Fetal bovine serum (FBS) was purchased from Shanghai XP Biomed Ltd. (Shanghai, China). Phosphate-buffered solution (PBS), dimethyl sulfoxide (DMSO) solution, and Oil red O staining storage solution were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The cell counting kit-8 (CCK-8) reagent kit, BeyoColor™ Pre-stained color protein marker (10−170 kDa), and the secondary anti-rabbit IgG (H+L) antibodies of Western blotting assay were purchased from Beyond Biotech Inc. (Shanghai, China). The RIPA lysis buffer, phenylmethanesulfonyl fluoride, 10X TBST solution, and pre-stained protein marker IV were purchased from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). The triglyceride test kit and non-fat powdered milk was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The FastPure cell/issue total RNA isolation kit V2 was purchased from Nanjing Vazyme Biotech Co., Ltd. (Nanjing, China). The Evo M-MLV RT Mix Kit with gDNA Clean for qPCR Ver.2 and SYBR Green Premix Pro Taq HS qPCR kit were purchased from Accurate Biotechnology Co., Ltd. (Hunan, China). The sodium oleate/palmitic acid reagent kit was purchased from Xian Kunchuang Science and Technology Develop Co., Ltd. (Xi'an, China). Tangeretin, with a purity of 99.37%, was purchased from TargetMol Chemicals Inc. (Boston, U.S.A.). The BCA protein quantification kit was purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). Isopropanol was purchased from the Damao Chemical Agent Factory (Tianjin, China). The Omni-Easy™ Protein Sample Loading Buffer (Denaturing, Reducing, 5×) was purchased from Epizyme Biotech Co., Ltd. (Shanghai, China). The primary antibodies against PI3K, p-PI3K, Akt, p-Akt, P65, p-P65, GSK-3β, p-GSK-3β, GAPDH were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). The SuperKine™ West Femto Maximum Sensitivity Substrate was purchased from Abbkine Scientific Co., Ltd. (Wuhan, China).

2.2 Collection of bioactive phytochemicals from Chenpi

TCMSP (https://old.tcmsp-e.com/index.php) was used to collect bioactive phytochemicals from Chenpi and needs to fulfill ADME screening[22]. Based on the standards of drug resistance (DL) ≥ 30% and oral bioavailability (OB) ≥ 0.18, respectively, these 2 key factors make significant contributions to the pharmacological effect of the substance and its subsequent use in drug research and development[23]. The study also supplemented the active phytochemicals of Chenpi through the research literature[24], which must include chemical information validation and anti-obesity biological activity validation.

2.3 Screening the targets of bioactive phytochemicals

The SwissTargetPrediction database (http://www.swisstargetprediction.ch/) and SEA database (https://sea.bkslab.org/) were used to collect protein targets from active plant chemicals in Chenpi[25-26]. The source of protein targets was set as Homo sapiens, and research does not focus on protein targets from other sources. All collected protein targets were converted into gene names through Uniprot database (https://www.uniprot.org/) for further analysis[27].

2.4 Screening of the obesity related genes

Obesity related genes were collected from DrugBank database (https://go.drugbank.com/), GeneCards database (https://www.genecards.org/), DisGeNET database (https://www.disgenet.org/), and OMIM database (https://www.omim.org/) using the keyword Obesity[28-31]. All collected relevant gene information was processed uniformly through the Uniprot database (https://www.uniprot.org/)[27].

2.5 Identification of overlapping gene

Venny online tool (https://bioinfogp.cnb.csic.es/tools/venny/) was used to obtain overlapping genes for the targets of active phytochemicals and disease related genes for further research[32].

2.6 Protein-protein interaction (PPI) analysis

The overlapping genes were imported into the String database (https://string-db.org/), and the model of multiple proteins was used for the protein-protein interaction analysis[33]. Moreover, the analysis results were exported as a TSV file and subsequently imported into Cytoscape to construct a network[34].

2.7 Screening of the core targets and central targets

The network of PPI analysis was analyzed using Cytoscape. The protein targets higher than the median of betweenness, closeness, and degree were collected separately, and the duplicates were removed to obtain core targets. Central targets were collected by collecting the protein targets with the top 10 degrees, closeness, maximum climate centrality (MCC), and maximum neighborhood component (MNC), respectively, and removing the duplicates[35].

2.8 Network construction of the active phytochemicals and its anti-obesity targets

The Cytoscape was used to construct a network of active phytochemicals and its anti-obesity targets from Chenpi to analyze their interactions and obtain the degree values to analyze the importance of active phytochemicals in the network[34].

2.9 Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) enrichment analysis

Metascape database (https://metascape.org/gp/index.html#/main/step1) was used to conduct GO functional enrichment analysis and KEGG pathway enrichment analysis to the core targets[36]. The items of GO enrichment analysis included cellular component (CC), biological process (BP), and molecular function (MF). The top 10 GO analysis items (BP, CC, and MF) and related KEGG pathway analysis results were displayed in the form of dot plot. Dot plot was generated by bioinformatics online platform SRplot (https://www.bioinformatics.com.cn.).

2.10 Molecular docking

The research validated the interaction between active phytochemicals with degree values greater than 50 from Chenpi and central targets through molecular docking. The purpose of molecular docking is to avoid false-positive result of network pharmacology analysis. The structures of the active phytochemicals were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and converted into Smiles using Chem draw. The structure files of Central targets were downloaded from the PDB database (https://www.rcsb.org/) and saved in PDB format. The bioactive phytochemicals and central targets were imported to AutoDockTools for pre-docking processing. Moreover, the Autodock vina was used to docking. Some of the docking results were visualized and analyzed using PyMol software and DiscoveryStudio software[37].

2.11 ADMET analysis

The SwissADME database (http://www.swissadme.ch/index.php) was used to perform machine learning to predict pharmacokinetic characteristics. The smiles of phytochemicals were input into the SwissADME database for ADME analysis to obtain relevant pharmacokinetic data[38]. The ProTox II-Prediction Of Toxicity Of Chemicals database (https://tox-new.charite.de/protox_II/index.php?site=home) was used to predict the toxicity of compounds, including predicted median lethal dose (LD_{50}), hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity.

2.12 Cell culture and treatments

HepG2 cells were cultured in MEM medium supplemented with 10% fetal bovine serum and 1% 100× penicillin-streptomycin solution at 37 °C and 5% CO_2. The study analyzed the biological activity of Chenpi in modulating the lipid synthesis pathway using a widely used lipid accumulation model[39]. This model involves incubating HepG2 cells with 0.75 mmol/L FFA mixture containing 2:1 oleate:palmitate[18,21]. More specifically, the HepG2 cells were treated with MEM essential medium for 24 h. Next the HepG2 cells were treated with MEM essential medium containing the 750 μmol/L FFA (sodium oleate:sodium palmitate, 2:1) for 24 h before harvesting the cells for further experiments[18]. And for the treatment group, the tangeretin will add with the FFA together. The ORO staining was performed on HepG2 cells with or without modeling and the result was compared with normal cells to determine whether the model has been successfully established.

2.13 Cell viability analysis

Using the CCK-8 method to detect the effect of drugs on cell viability, HepG2 cells were cultured into 96 well plates, exposed to different concentrations of tangeretin (0, 6.25, 12.5, 25, 50, and 100 μmol/L) for 24 h. After removing the previous medium and adding 100 μL MEM essential culture medium, add 10 μL CCK-8 reagent to each well. Incubate the 96-well plate at 37 °C in the dark for 30 min. Measure the absorbance at 450 nm in the FLUOstar Omega Microplate Reader (BMG Labtech, Germany). The stock solution of tangeretin was prepared by DMSO, and the working solution concentration is ensured to be below 0.1%.

2.14 Oil red O staining

After 24 h of modeling processing, the supernatant was discarded, and cells were washed twice with PBS. The Oil red O staining storage solution was diluted according to the reagent manufacturer's instructions to obtain the working solution. The cells were fixed with 4% paraformaldehyde for 30 min, then stained with Oil red O staining solution for 30 min. The result was observed, and photos were taken under the microscope after washing off the dye with 60% isopropanol.

2.15 Intracellular triglyceride (TG) assay

After 24 h of cell modeling, the supernatant was discarded, and the cells were washed twice with PBS. Then the 80 μL frozen RIPA solution with a final concentration of 2 μg/mL aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin A, 5 mmol/L of NaF, 1 mmol/L Na_3VO_4, and 1 mmol/L PMSF was added to each well and placed on ice for 30 min. The cell lysates were later used for the determination of the intracellular TG levels. The TG levels were measured using a triglyceride assay kit according to the instructions provided by the reagent vendor. According to the guidance of the reagent vendor, the BCA protein kit was used to standardize the intracellular TG content.

2.16 Quantitative real-time PCR

To evaluate the gene expression of *PI3K* (Forward, CCACGACCATCATCAGGTGAA; Reverse, CCTCACGGAGGCATTCTAAAGT), *Akt* (Forward, TCCTCCTCAAGAATGATGGCA; Reverse, GTGCGTTCGATGACAGTGGT), *GSK3β* (Forward, TGTGTGTTGGCTGAGCTGTT; Reverse, CGGGGTCGGAAGACCTTAGT), *GLUT4* (Forward, TGCGCGTCCAGCTCTTCTAA; Reverse, TAGCTCTGTTCAATCACCTTCTGAG), *FABP1* (Forward, GAGGGAGCTCTATTGCCACC; Reverse, CTCTTCCGGCAGACCGATTG), *FAS* (Forward, CTGGAAGGCGGGGCTCTA; Reverse, TCCTCGGAGTGAATCTGGGT), and *PLIN2* (Forward, GGCAGAGAACGGTGTGAAGA; Reverse, TGGCATTGGCAACAATCTGAG). Total cell RNA was extracted using FastPure cell/issue total RNA isolation kit V2 according to the manufacturer's instructions. The quantitative real-time PCR was performed using the SYBR Green Premium Pro Taq HS qPCR kit on the Exicycer 96 system (Bioneer Inc., Korea). The GenBank database was used to design primers, and the synthesis of primers was completed by BGI Tech Solutions (Beijing Liuhe) Co., Ltd. (Beijing, China). The PCR amplification conditions are 95 °C for 30 s, followed by 46 cycles of 95 °C for 10 s and 60 °C for 30 s. After the cycles, the temperature rises by 0.5 degrees per second from 60 degrees to 94 degrees. Finally, the $2^{-\Delta\Delta CT}$ analysis was performed on the data and standardize all mRNA expression levels using *GAPDH* (Forward, GGAGCGAGATCCCTCCAAAAT; Reverse, GGCTGTTGTCATACTTCTCATGG).

2.17 Western blotting analysis

HepG2 cells were cultured in a 6-well plate (8×10^5 cells/well) and treated with FFA and drug based on the description in section 2.11. The culture medium was discarded and washed by 1 mL PBS solution, and 80 μL frozen RIPA solution containing a final concentration of 2 μg/mL of aprotinin, 5 μg/mL of leupeptin, 1 μg/mL of pepstatin A, 5 mmol/L of NaF, 1 mmol/L of Na_3VO_4, and 1 mmol/L of PMSF was added to each well and placed on ice for 30 min. The supernatants were collected and use the BCA protein assay kit to determine the total protein concentration of each sample. The sample was dissolved in 5× protein sample loading buffer, vortexed, and boiled at 100 °C for 10 min. Protein samples were separated by 10% SDS-PAGE gel. All imprints were transferred onto the PVDF membrane by electrophoresis, and the membrane was sealed at room temperature for 2 h using TBST buffer containing 5% non-fat milk. The PVDF membrane was incubated with the corresponding primary antibody (PI3K, p-PI3K, Akt, p-Akt, GSK-3β, and p-GSK-3β, 1:1 000 dilution; GAPDH, 1:5 000 dilution) at 4 °C for 12 h. The PVDF membrane was washed thrice by TBST buffer for 15 min each time. Next, the PVDF membrane was incubated with the secondary anti-rabbit IgG (H+L) antibodies (1:5 000 dilution) at room temperature for 1 h. The PVDF membrane was washed by TBST buffer thrice for 15 min each time. The protein strips on the PVDF membrane were scanned by the Image Quant LAS 500 imaging system (GE Healthcare Bio-Sciences AB, Sweden) and visualized by Image J software v1.8.0 (National Institutes of Health, U.S.A.). The protein strips were standardized by GAPDH, and all results were independently repeated 3 times.

2.18 Statistical analysis

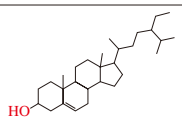
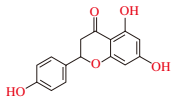
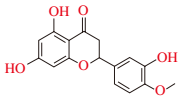
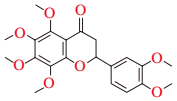
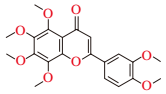
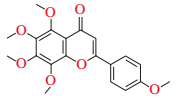
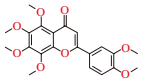
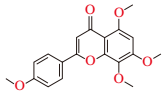
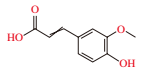
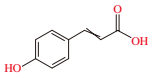
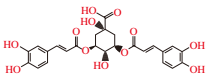
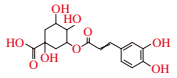
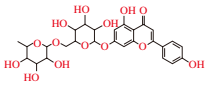
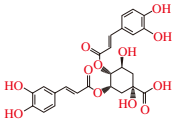
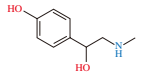
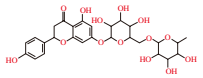
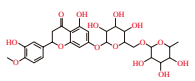
All tests for this study shall be conducted at least 3 times. One-way analysis of variance (ANOVA) is used for statistical analysis, and the data is represented as mean ± SD. Tukey's test is used for analysis on Graphpad Prism and SPSS software, with statistical significance set at $P < 0.05$.

3. Results

3.1 Phytochemicals derived from Chenpi and its targets

A total of 17 active compounds were collected from the TCMSP database[22] and research literatures[11-13] for further research, including sitosterol, naringenin, hesperetine, citromitin, nobiletin, tangeretin, 5-OH nobiletin, tetramethylisoscutellarein, ferulic acid, *p*-coumaric acid, 3,5-di-*O*-caffeoylquinic acid, chlorogenic acid, apigenin-7-rutinoside, 4,5-di-*O*-caffeoylquinic acid, synephrine, narirutin, and hesperidin. The structures and related information of these compounds are presented in Table 1, and the active phytochemicals screened from the TCMSP database must meet the screening conditions of OB ≥ 30% and DL ≥ 0.18 simultaneously. The targets of the phytochemicals were obtained based on structural similarity using the SwissTargetPrediction database[25] and SEA database[26], and a total of 584 targets were gained after de duplication.

Table 1
The chemical information and the target numbers of active phytochemicals from Chenpi.

Chemical name	Structure	Target number	Core targets	Reference/ source
Sitosterol		86	29	TCMSP
Naringenin		139	67	TCMSP/[40]
Hesperetine		118	48	TCMSP/[12]
Citromitin		35	12	TCMSP
Nobiletin		76	41	TCMSP/ [11-13,40]
Tangeretin		165	76	[11-13,40]
5-OH nobiletin		45	20	[11]
Tetramethylisoscutellarein		131	65	[13]
Ferulic acid		188	58	[13]
p-Coumaric acid		100	39	[13]
3,5-di-*O*-caffeoylquinic acid		50	19	[13]
Chlorogenic acid		57	22	[13]
Apigenin-7-rutinoside		53	16	[13]
4,5-di-*O*-caffeoylquinic acid		65	23	[13]
Synephrine		40	12	[12,40]
Narirutin		40	14	[12,40]
Hesperidin		51	15	[12,40]

3.2 Obesity related targets

Disease related targets were collected from four different disease databases[28-31], including OMIM (89), DrugBank (69), GeneCards (7 488), and DisGeNET (2 821). After removing duplicates, a total of 8 213 disease-related targets were obtained for further research.

3.3 Anti-obesity targets of Chenpi and PPI analysis

The 475 anti-obesity targets of Chenpi were obtained by intersecting the targets of active phytochemicals identified in 2.1 with the obesity-related genes collected in 2.2 (Fig. 1A)[32]. The 475 intersecting genes were imported into the String database for protein-protein interaction analysis (Fig. 1B)[33]. The PPI network consists of 474 nodes and 5 209 edges, demonstrating the complexity of the network. In this network, the proteins with the top 20 degree value are displayed in Fig. 1C. Betweenness, closeness, and degree based on the above PPI network were used to get core targets for the more meaningful and visual PPI analysis results. The screening of core targets reflects the potential targets of Chenpi involved in the anti-obesity effects[35]. The 189 core targets were obtained and needed to simultaneously meet the values of betweenness, closeness, and degree greater than its median values. The results are presented in Venn diagram (Fig. 1D). Core targets were imported into Cytoscape software to build an interaction network, and the color of each node changed from green to yellow, although the degree became smaller (Fig. 1E).

3.4 GO enrichment analysis of core targets

The Metascape database was utilized to conduct the GO enrichment analysis of 189 core targets[36]. A total of 546 BP, 100 CC, and 116 MF terms were enriched and met $P < 0.05$. The top 10 enrichment terms of BP, CC, and MF were presented in the Fig. 2A. The GO enrichment analysis results indicate that the gene target involves multiple BPs including cellular response to nitrogen compound, response to inorganic substance, cellular response to organic cyclic compound, regulation of kinase activity, response to xenobiotic stimulus, positive regulation of cell death, protein phosphorylation, etc. In the enriched CC category, the core target is involved in side of membrane, perinuclear region of cytoplasm, membrane raft, neuronal cell body, receptor complex, transcription regulator complex, vesicle lumen, etc. Moreover, based on the GO enrichment result, MF ontologies are included protein domain specific binding, kinase binding, oxidoreductase activity, transcription factor binding, protein kinase activity, protein homodimerization activity, phosphatase binding, etc.

3.5 KEGG enrichment analysis of core targets

The core targets were imported to the Metascape database for KEGG Pathway enrichment analysis[36]. A total of 167 KEGG pathway terms were enriched and met $P < 0.05$, and the top 30 signaling pathways were enriched and showed in Fig. 2B. The results of KEGG pathway enrichment analysis indicated that the molecular mechanism of Chenpi in treating obesity may involve PI3K/Akt signaling pathway, Ras signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, etc. Four signaling pathways

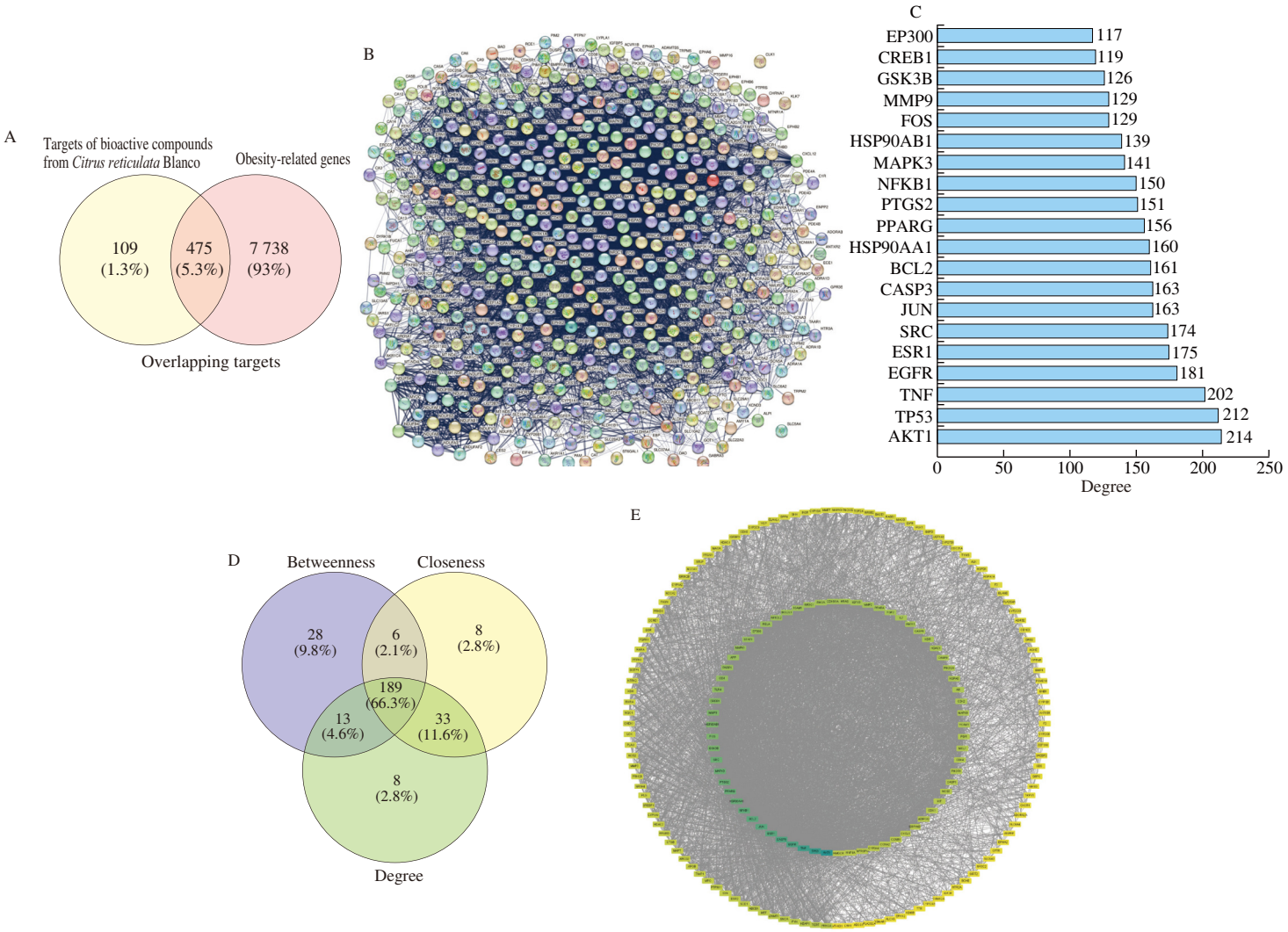


Fig. 1 (A) The Venn diagram between the targets of Chenpi and obesity related tragets. (B) PPI network of 475 overlapping genes drawn by String database. (C) The top 20 anti-obesity targets of Chenpi ranked by the degree values of PPI network. (D) The Venn diagram between the betweenness, closeness, and degree. (E) The PPI network of the 189 core targets.

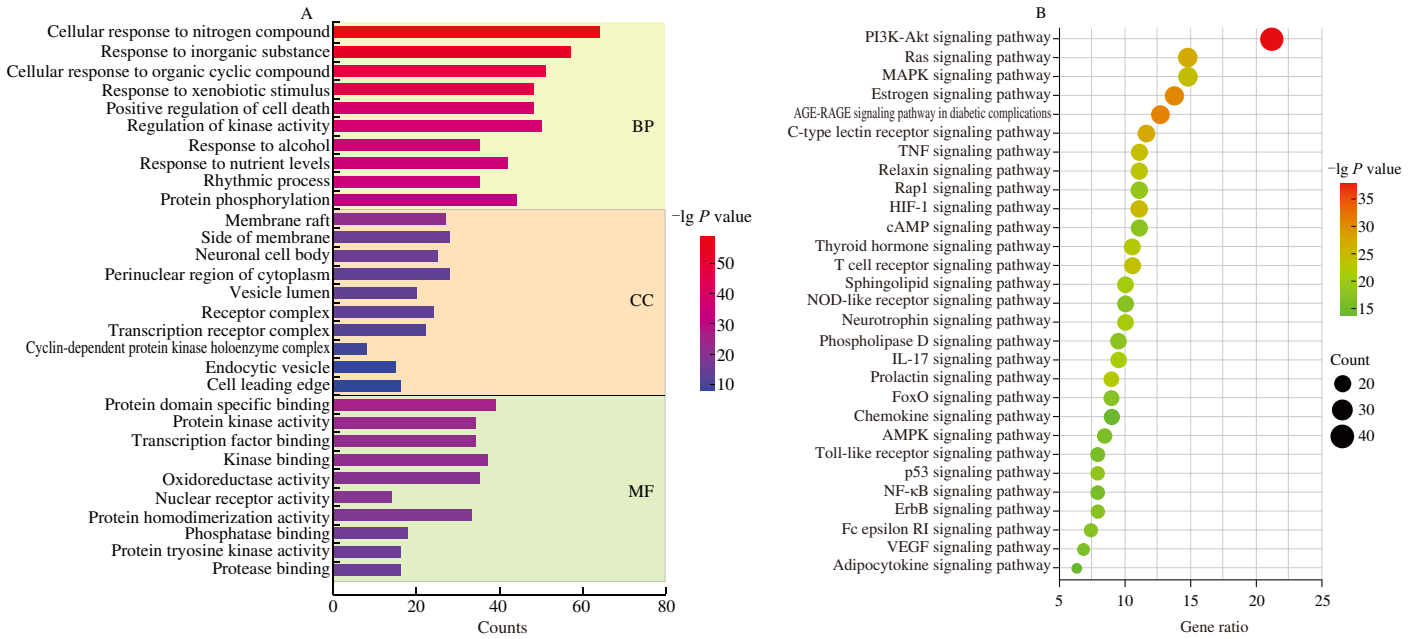
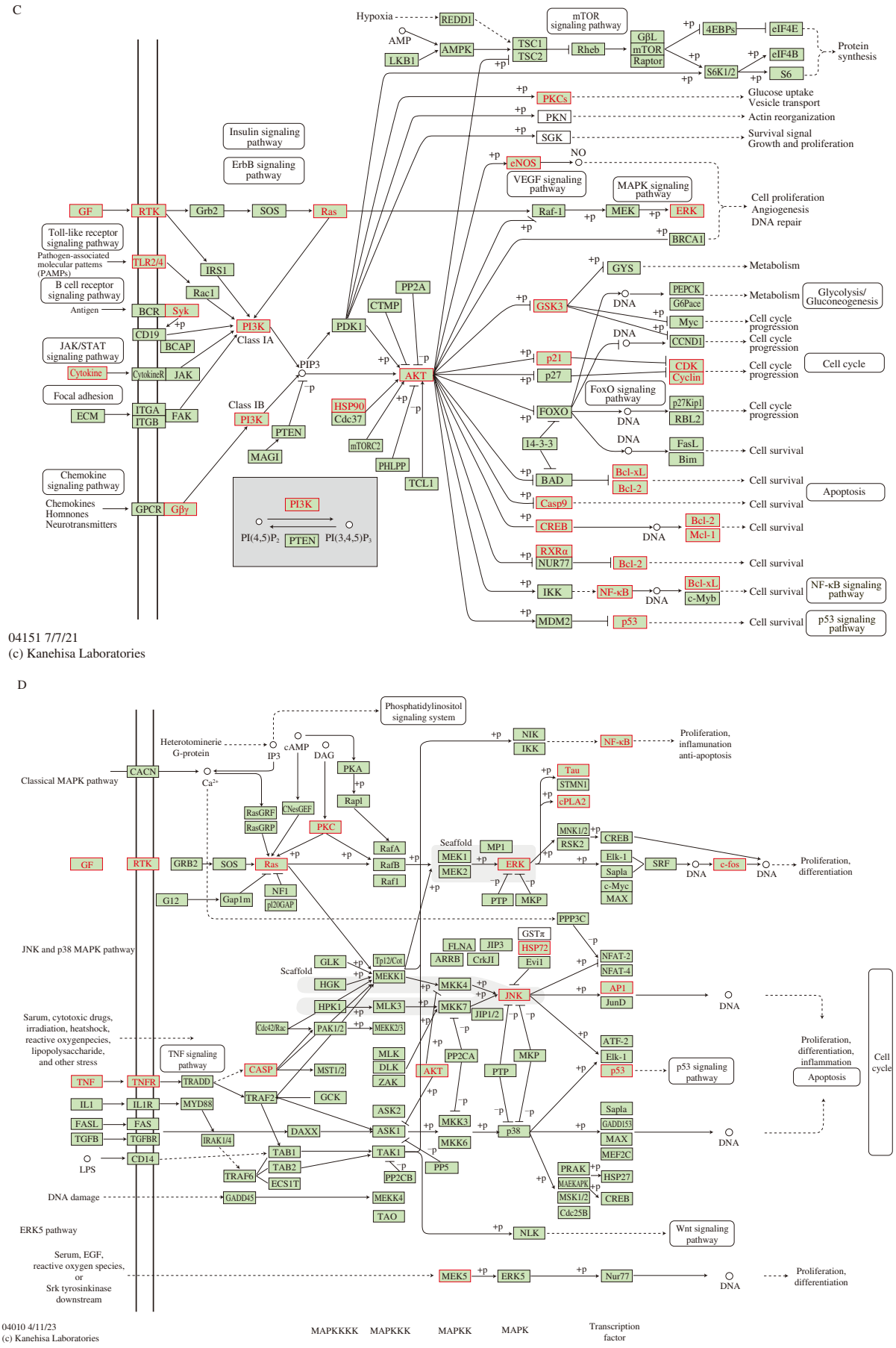


Fig. 2 (A) The GO analysis of 189 anti-obesity core targets including top 10 items of BP, CC and MF. (B) The KEGG pathway analysis of 189 anti-obesity core targets. (C) PI3K/A signaling pathway. (D) MAPK signaling pathway. (E) AMPK signaling pathway. (F) NF-κB signaling pathway.



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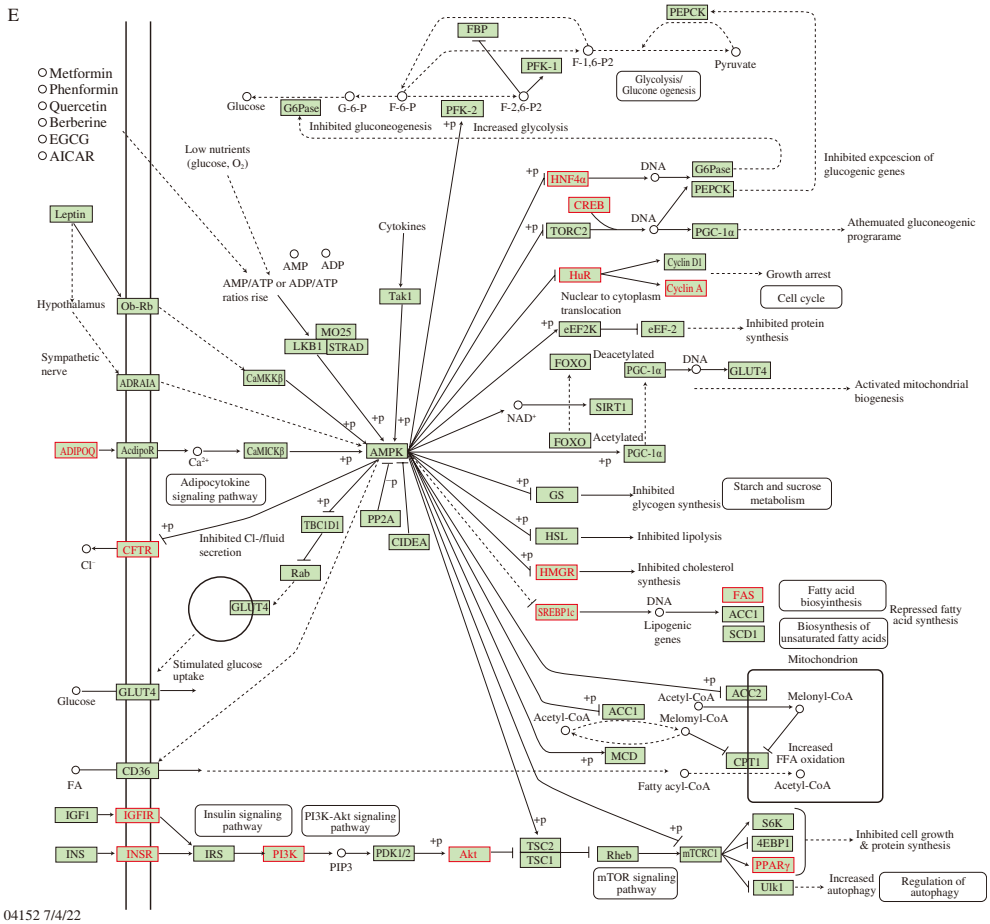

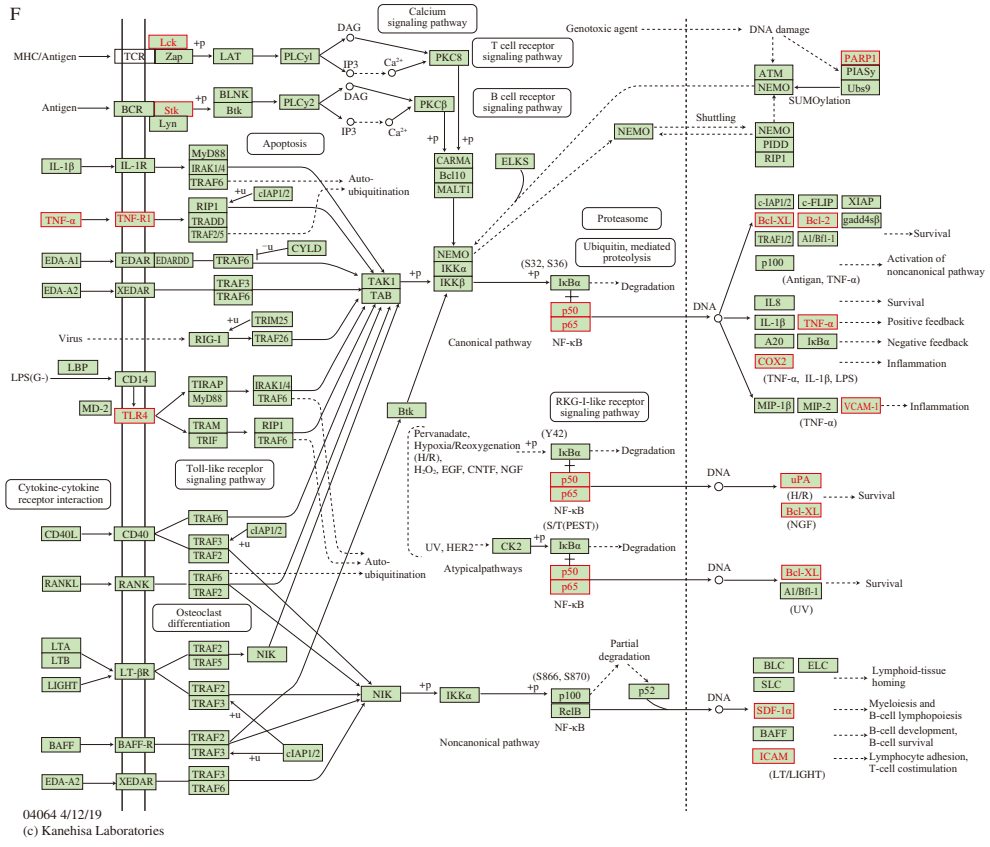

Fig. 2 (*Continued*)

may be directly involved in obesity and inflammation induced by obesity, including PI3K/A signaling pathway (Fig. 2C), MAPK signaling pathway (Fig. 2D), AMPK signaling pathway (Fig. 2E), and NF-κB signaling pathway (Fig. 2F). These signaling pathways can collectively exert the improvement effect of Chenpi on obesity.

3.6 Screening of central targets

The screening of Central targets reflects the potential targets of Chenpi in the treatment of obesity[35]. After analyzing the 475 anti-obesity targets of Chenpi through the string database, the analysis results are imported into Cytoscape for network analysis and central target prescreening are performed using the cytoHubba software. Moreover, the networks of targets with top 10 degree (Fig. 3A), closeness (Fig. 3B), MCC (Fig. 3C), and MNC (Fig. 3D) values were built respectively. The Venny online tool was used to obtain the overlapping targets of four networks as central targets, including AKT1, BCL2, CASP3, EGFR, JUN, SRC, TP53, and TNF.

3.7 Network of the compounds and anti-obesity targets

A network of phytochemicals-obesity was constructed, including 17 plant compounds from Chenpi and 189 core targets (Fig. 4A). The results showed that the network consists of 208 nodes and 782 edges.

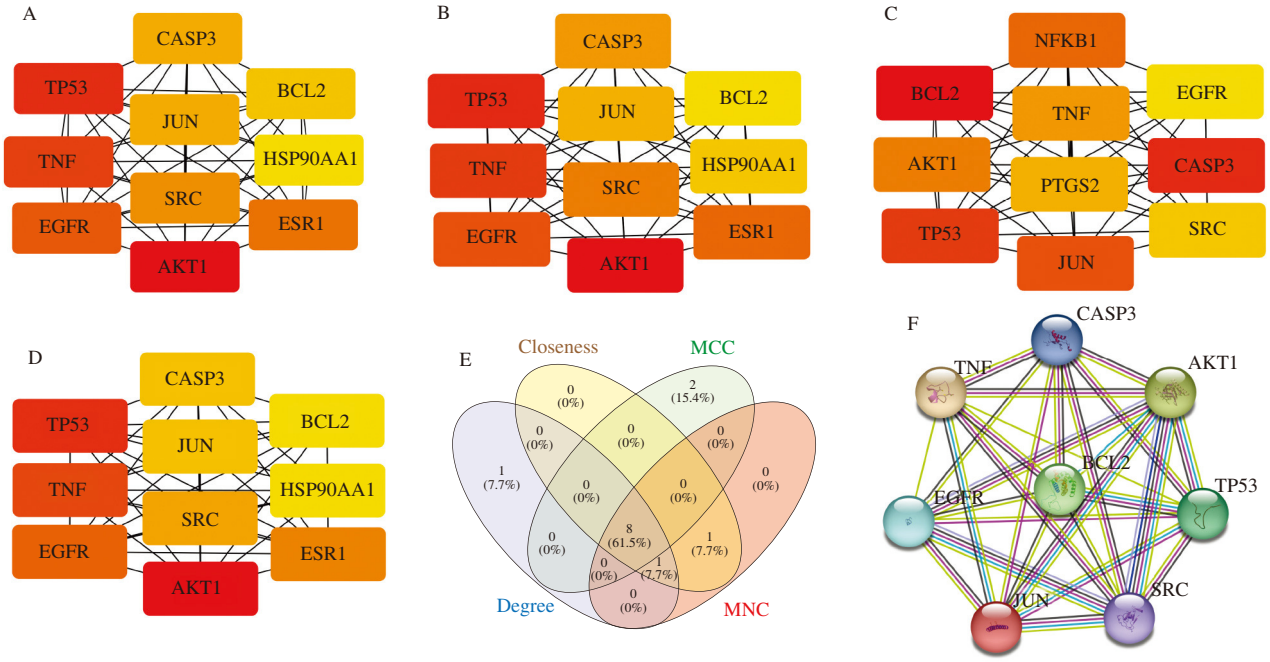

Fig. 3 (A) The network of targets with top10 degree. (B) The network of targets with top10 closeness. (C) The network of targets with top10 MCC. (D) The network of targets with top10 MNC. (E) Venn diagram between degree, closeness, MCC, and MNC. (F) The network of central targets.

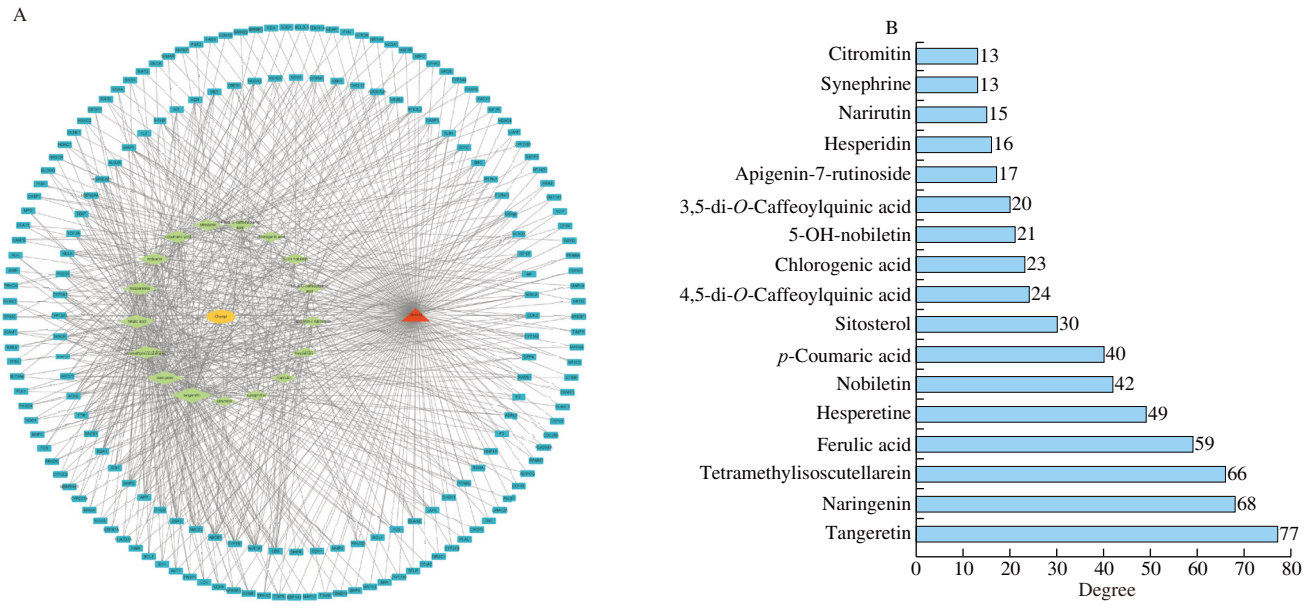

Fig. 4 (A) The network of drug-compound-target-disease. The orange node represents the Chenpi. The green nodes represent the phytochemicals derived from Chenpi. The blue nodes represent the anti-obesity targets of bioactive phytochemicals. The red node represents the disease obesity. (B) The degree value of the bioactive phytochemicals derived from Chenpi.

Each edge represents the interaction between active phytochemicals and anti-obesity core targets. The size of nodes for each phytochemical is determined by its degree value, and as the degree of nodes increases, the size of nodes also increases from small to large. The degree of nodes in a network represents the number of edges that link it to other nodes and represents the core level of that node in the network[41]. The degree values of all 17 phytochemicals from Chenpi in this network are shown in Fig. 4B, where components with a degree value greater than 50 will be used as ligands for molecular docking, including tangeretin (77), naringenin (68), tetramethylisoscutellarein (66), and ferulic acid (59). Taken together, the tangeretin has the highest degree value (77) and core targets (76), meaning that it has the highest potential of the anti-obesity effect among those bioactive compound in Chenpi.

3.8 Molecular docking

Molecular docking was performed on 4 phytochemicals with the degree value greater than 50 in Chenpi and 8 anti-obesity central targets. All selected phytochemicals have negative binding energies with central targets, indicating that they can be binding for further action. For visual analysis, the hit map was constructed for the analysis (Fig. 5A). The lower the binding affinity of phytochemicals, the greater their affinity with protein targets. Binding affinity lower than −5.0 kcal/mol indicates moderate affinity, while binding affinity lower than −7.0 kcal/mol indicates high affinity binding[35]. The molecular docking results showed that all selected phytochemicals in Chenpi can bind to AKT1 (PDBID: 4EJN), BCL2 (PDBID: 4MAN), CASP3 (PDBID: 1RE1), EGFR (PDBID: 1M17), JUN (PDBID: 5T01), SRC (PDBID: 4MXO), TP53 (PDBID: 6GGA), TNF (PDBID: 2AZ5), and have strong binding affinity.

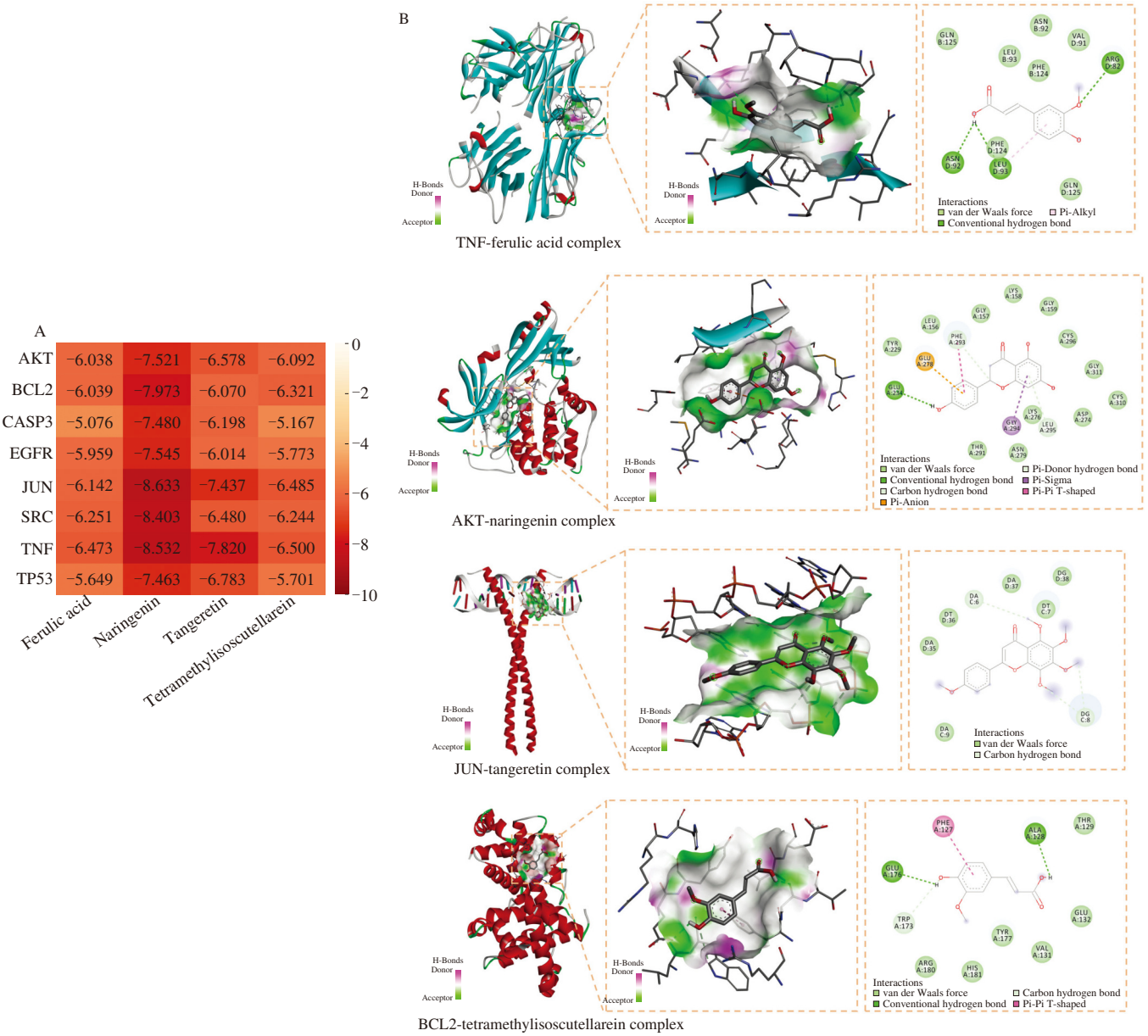

Fig. 5 (A) The hit map of molecular docking result between the central targets and the active phytochemicals from Chenpi. (B) The 2D diagram and 3D diagram of docking result between the central targets and the active phytochemicals from Chenpi.

The 3D and 2D results of some docking complexes with strong binding affinity are shown in Fig. 5B including TNF-ferulic acid complex, AKT-naringenin complex, JUN-tangeretin complex and BCL2-tetramethylisoscutellarein complex. The TNF-ferulic acid complex had 1 Pi-Alkyl interaction with LEU93, 3 conventional hydrogen bonds with ARG82, ASN 92 and LEU93, and some van der Waals forces. The AKT1-naringenin complex was stabilized by 1 Pi-Pi T-shaped interaction with PHE293, 1 Pi-Sigma interaction with GLY294, 1 Pi-Donor Hydrogen Bond with LEU 295, 1 Pi-Anion interaction with GLU 278, 1 carbon hydrogen bond with PHE 293, 1 conventional hydrogen bonds with GLU 234, and many van der Waals forces. The JUN-tangeretin complex presented 3 carbon hydrogen bond with DG8 and DA6. Moreover, the BCL2-tetranethylisoscutellarein complex had one Pi-Pi T-shaped interaction of PHE 127, 1 carbon hydrogen bond with TRP 173, and 2 conventional hydrogen bond with GLU 176 and ALA 128, and some van der Waals forces.

3.9 Results of ADMET analysis

ADMET analysis through various machine learning models is an essential tool for drug discovery and development[42]. ADME analysis and small molecule toxicology analysis were conducted using the SwissADME database and ProTox II-Prediction Of Toxicity Of Chemicals database, respectively[43]. The results showed that the 4 phytochemicals derived from Chenpi with degree values higher than 50 had excellent pharmacokinetic properties (Table 2). The pharmacokinetic characteristics of all 4 selected candidate phytochemicals are shown in Table 2 in various predictive models include gastrointestinal (GI) absorption, blood-brain barrier penetration, P-glycoprotein substrates, cytochrome P450 inhibitors, and skin permeation. Regarding compound toxicity prediction based on different kinds of toxicity model computation, tangeretin, naringenin, and tetramethylisoscutellarein all showed good biocompatibility and did not exhibit toxic behaviors such as hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity, cause only the relevant toxicity model shows active and the confidence is greater than 0.7 will be considered. Moreover, the immunotoxicity of ferulic acid should be considered in further animal studies and clinical trials. In conclusion, based on their good biocompatibility and in line with the network pharmacology results, tangeretin, naringenin, and tetramethylisoscutellarein have demonstrated promising potential.

Among these compounds, tangeretin exhibited the lowest predicted toxicity, which have the highest predictied LD_{50} and without the hepatotoxiciy, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity. Besides, the tangeretin has the most core targets among those bioactive phytochemicals in Chenpi, mentioned in the section 3.7. Taken together, tangeretin was further investigated *in vitro* cell experiment to explore its anti-obesity effect and the molecular mechanism in combating obesity.

3.10 Effect of tangeretin on cell viability

Evaluate the effect of tangeretin on cell viability using the CCK8 method (Fig. 6A). HepG2 cells did not show significant inhibitory effects when treated with tangeretin at concentrations from 0 to 25 μmol/L for 24 h compared with the control group. However, when the concentration of tangeretin reached 50 and 100, the viability of HepG2 cells significantly decreased ($P < 0.05$). Therefore, in this study's subsequent experiments, the tangerine concentration was chosen to be below 25 μmol/L.

3.11 Inhibition effect of tangeretin on lipid accumulation

To demonstrate the inhibitory effect of tangeretin on the FFA induced lipid accumulation model, HepG2 cells were treated with different concentrations of tangeretin (6.25, 12.5, and 25 μmol/L) with FFA for 24 h. The establishment of the *in vitro* lipid accumulation model and the lipid-lowering effect of tangeretin were evaluated by measuring the content of TG, as shown in Fig. 6C. Compared with the control group, the TG levels in HepG2 cells treated with FFA significantly increased, indicating successful modeling. The tangeretin treatment group can significantly reduce the TG increase caused by FFA treatment. Tangeretin inhibits the lipid accumulation induced by FFA in a concentration-dependent manner, and 25 μmol/L tangeretin has the best lipid-lowering effect. Through Oil red O staining, the degree of lipid accumulation in HepG2 cells was studied (Fig. 6B). The model group increased a large number of lipid droplets in HepG2 cells and the 25 μmol/L tangeletin treatment group can reduced the number of lipid droplets.

Table 2
ADMET profiling of phytochemicals from Chenpi.

Phytochemicals	Tangeretin	Naringenin	Tetramethylisoscutellarein	Ferric acid
GI absorption	High	High	High	High
BBB permeant	Yes	No	Yes	Yes
P-gp substrate	No	Yes	No	No
CYP1A2 inhibitor	No	Yes	Yes	No
CYP2C9 inhibitor	No	No	Yes	No
CYP2C19 inhibitor	Yes	No	Yes	No
CYP2D6 inhibitor	No	No	Yes	No
CYP3A4 inhibitor	Yes	Yes	Yes	No
Lg Kp (Skin permeation)	−6.41 cm/s	−6.17 cm/s	−6.21 cm/s	−6.41 cm/s
Predicted LD_{50}	5 000 mg/kg	2 000 mg/kg	4 000 mg/kg	1 772 mg/kg
Hepatotoxicity	Inactive	Inactive	Inactive	Inactive
Carcinogenicity	Inactive	Inactive	Inactive	Inactive
Immunotoxicity	Inactive	Inactive	Active (0.64)	Active (0.91)
Mutagenicity	Inactive	Inactive	Inactive	Inactive
Cytotoxicity	Inactive	Active (0.59)	Inactive	Inactive

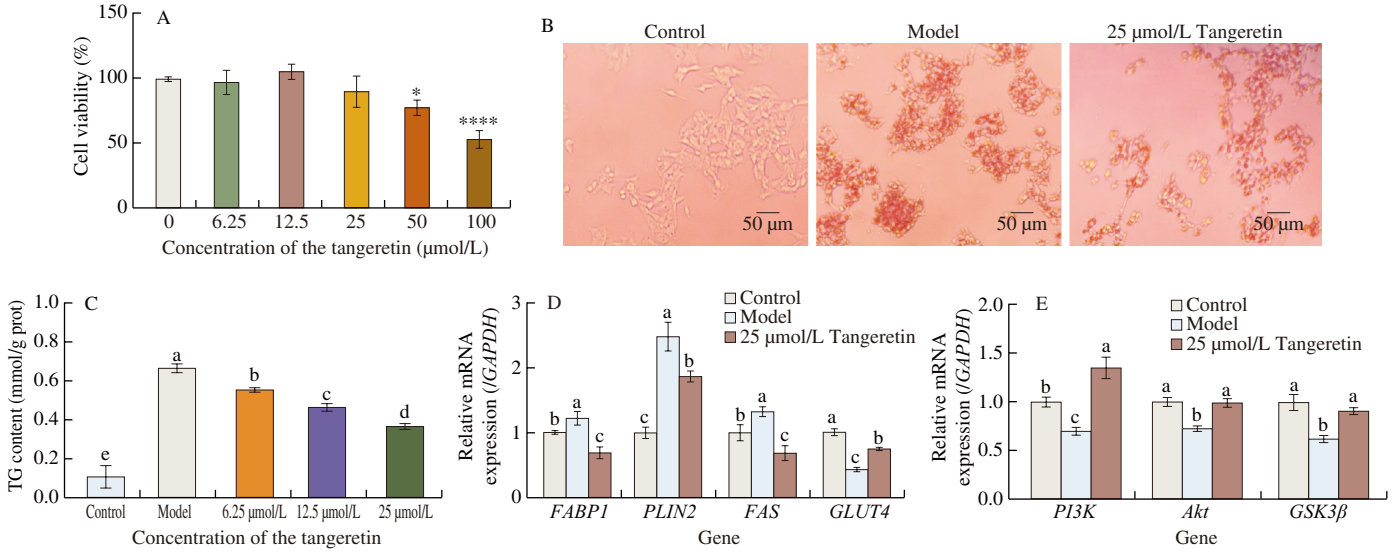

Fig. 6 (A) The effect of tangeretin on the cell viability of HepG2 cells. Statistically significant at $^{*}P < 0.05$ and $^{****}P < 0.000\ 1$ compared with the control. (B) The inhibitory effect of tangeretin on lipid accumulation in HepG2 cells. (C) Effect of tangeretin on the TG content in HepG2 cells. (D) The mRNA levels of the lipid accumulation genes in tangeretin-treated HepG2 cells including *FABP1*, *PLIN2*, *FAS*, and *GLUR4*. (E) Effect of tangeretin on the mRNA level of *PI3K/Akt/GSK3β* signaling pathway. All results are presented as mean ± SD, and the experiments were repeated as triplicates. Bars with different letters (a–e) significantly ($P < 0.05$) differ according to ANOVA and Tukey's multiple range test.

3.12 Effect of tangeretin on the gene expression of FABP1, PLIN2, FAS, and GLUT4 in HepG2 cells

The accumulation of lipid and the generation of lipid droplets are associated with various enzymes, transporters, and lipoproteins. The study investigated the expression of related genes before and after the intervention of tangeretin including *FABP1*, *PLIN2*, *FAS*, and *GLUT4* (Fig. 6D). FFA treatment resulted in significant upregulation of *FABP1*, *PLIN2*, and *FAS* genes. Tangeretin treatment can significantly reduce these effects, and the expression of *FAS* and *FABP1* genes can be reduced to lower than the control group. Moreover, tangeretin treatment can significantly inhibit the downregulation of *GLUT4* gene expression induced by FFA.

3.13 Effect of tangeretin on mRNA transcription of PI3K/Akt/GSK3β signaling pathway in HepG2 cells

To further investigate how tangeretin enters HepG2 cells and affects downstream nuclear transcription factors and lipid metabolism, we detected the gene expression levels of *PI3K*, *Akt*, and *GSK3β* in HepG2 cells treated with FFA or/and tangeretin (Fig. 6E). The results showed that FFA treatment inhibited the PI3K/Akt/GSK3β signaling pathway, but tangeretin treatment significantly upregulated the gene expression level of *PI3K* and restored the gene expression levels of *Akt* and *GSK3β* to the level of the control group.

3.14 Effects of tangeretin on protein expression of PI3K/Akt/GSK3β signaling pathway in HepG2 cells

Based on previous research, we found that tangerine can activate the PI3K/Akt/GSK3β signaling pathway in the gene level. To further explore the molecular mechanism in protein level by which tangerine alleviates FFA-induced lipid accumulation in HEPG2 cells, protein levels of PI3K, Akt, GSK3β, and their phosphorylated forms were determined by Western blotting (Figs. 7A-B). The GAPDH as the internal standard[44]. In HepG2 cells under fat accumulation conditions, the PI3K, Akt, GSK3β, and their phosphorylated proteins in this signaling pathway were significantly inhibited by FFA; however, the treatment of 25 μmol/L tangerine significantly improved the downregulation of phosphorylation of these proteins (Figs. 7C-D). Taken together, tangerine can activate the PI3K/Akt/GSK3β signaling pathway by increasing the level of protein phosphorylation of PI3K, Akt, and GSK3β, thereby reducing intracellular fat accumulation and exerting the anti-obesity effect.

4. Discussion

Chenpi is not only an easily obtainable fruit, but also a Chinese herbal medicine[40]. *In vitro*[13], *in vivo*[11-12], and clinical trials[13] have shown that Chenpi plays an important role in anti-obesity, but the relevant molecular mechanisms are still unclear. Therefore, exploring the interaction between the phytochemicals of Chenpi and the targets can screen out the phytochemicals with the best anti-obesity effect and anti-obesity molecular mechanisms of Chenpi. This study utilized network pharmacology to analyze the anti-obesity mechanism of active phytochemicals in Chenpi, and further validated the binding affinity between the phytochemicals and the anti-obesity central targets using molecular docking. Chenpi has discovered a total of 17 compounds with anti-obesity activity, and most of them have been found to have multiple therapeutic targets, including sitosterol, naringenin, hesperetine, citromitin, nobiletin, tangeretin, 5-OH nobiletin, tetramethylisoscutellarein, ferulic acid, *p*-coumaric acid, 3,5-di-*O*-caffeoylquinic acid, chlorogenic acid, apigenin-7-rutinoside, 4,5-di-*O*-caffeoylquinic acid, synephrine, narirutin, and hesperidin[11-13,22]. The components with the degree value greater than 50 include tangeretin (77), naringenin (68), tetramethylisoscutellarein (66), and ferulic acid (59). The tangeretin and naringenin are considered to be two of the main flavonoids in Chenpi[40].

Protein-protein interaction analysis indicated that AKT1, BCL2, CASP3, EGFR, JUN, SRC, TP53, and TNF are central targets for

the treatment of obesity by Chenpi. AKT1 is a critical factor in PI3K/Akt signaling pathway which is the typical signaling pathway in regulating the obesity. AKT1 can improve obesity by regulating downstream protein FOXO1, which regulates lipolysis by controlling the expression of adipose triglyceride lipase[45]. BCL2 has been shown to be associated with adipose differentiation, and its downregulation can lead to downregulation of differentiation marker genes in adipose differentiation cell models and increase the apoptosis[46]. Moreover, CASP3 can improve obesity and hyperlipidemia by regulating the cell apoptosis pathways[47]. The downregulation of EGFR expression in adipose tissue macrophages inhibits the proliferation of adipose tissue macrophages and monocyte infiltration into adipose tissue, thereby reducing the development of obesity and insulin resistance[48]. JUN is considered as one of the key therapeutic targets to regulate insulin resistance caused by type 2 diabetes. SRC is not only a potential therapeutic target for obesity but also a potential therapeutic target for diabetes[49]. TP53 polymorphisms has a significant impact on the metabolic response of high-fat diet, and this leads to increased susceptibility to metabolic diseases such as obesity and type 2 diabetes[50]. TNF is not only associated with the occurrence of obesity but also plays an important role in the immune system regulation caused by obesity[51]. Identifying anti-obesity treatment targets can promote the study of potential anti-obesity treatment mechanisms and is of great significance for preventing, treating, and managing obesity-related diseases.

KEGG pathway analysis reveals the relationship between drugs and diseases, helping to understand drugs' molecular mechanisms and regulatory networks. It plays a crucial role in network pharmacology and provides valuable information for drug development and treatment strategies[52]. The results of KEGG pathway enrichment analysis indicated that the molecular mechanism of Chenpi in treating obesity may involve pathways in cancer, lipid and atherosclerosis, PI3K/Akt signaling pathway, chemical carcinogenesis-receptor activation, pathways of neurodegeneration-multiple diseases, MAPK signaling pathway, etc. Four signaling pathways are directly associated with obesity and obesity-related diseases, including the PI3K/Akt signaling pathway, MAPK signaling pathway, AMPK signaling pathway, and NF-κB signaling pathway[53]. The downstream of the PI3K/Akt signaling pathway contains proteins such as MAPK, FOXO, NF-κB, P53, and mTOR, etc. These proteins have not only been widely proven to play a role in immune regulation and inflammatory response, but also have been shown to improve obesity through these regulatory effects[35,53]. The MAPK signaling pathway has been widely proven crucial in regulating appetite, adipogenesis, glucose homeostasis, and thermogenesis[54-55]. Moreover, the AMPK signaling pathway plays a crucial role in the development of obesity by regulating food intake, insulin sensitivity, brown adipose tissue heat production, and white adipose tissue browning[56].

Molecular docking can be used as a tool to verify the interaction between active phytochemicals and curing targets[57]. And in this research, molecular docking was used to analyze the binding stability between active compounds from Chenpi and central targets. The results showed that four active phytochemicals with the degree value greater than 50 in Chenpi could bind to eight central targets against obesity. The results of some docking complexes with strong binding affinity and their binding 2D and 3D diagram have been shown in Fig. 5B, including TNF-ferulic acid complex, AKT-naringenin complex, JUN-tangeretin complex and BCL2-tetramethylisoscutellarein complex, indicating that these complexes may play an important role in the anti-obesity activity of Chenpi. This study revealed the potential anti-obesity molecular mechanism of Chenpi through network pharmacology combined with molecular docking. However, *in vivo* and *in vitro* experiments are needed to validate the central targets and signaling pathways.

To further confirm the results of the network pharmacology study mentioned above, the researchers conducted a study to investigate the anti-obesity effects and potential molecular mechanisms of tangeretin, a key bioactive compound found in Chenpi. The study utilized an FFA induced lipid accumulation model in HepG2 cells. The results of the study demonstrated that the treatment with 25 μmol/L tangeretin

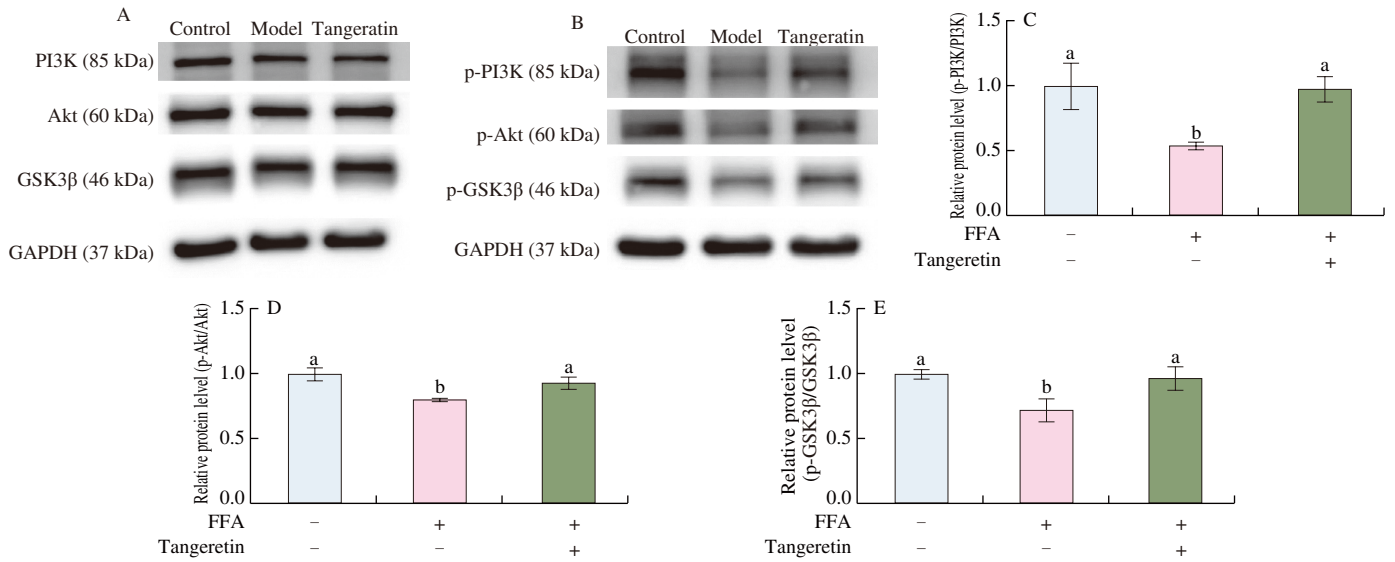

Fig. 7 (A) The PI3K/Akt/GSK3β signaling pathway related proteins in the FFA-induced HepG2 cells treating 25 μmol/L tangerine. (B) The PI3K/Akt/GSK3β signaling pathway related phosphorylated proteins. The relative protein level of (C) p-PI3K/PI3K; (D) p-Akt/Akt; (E) p-GSK3β/GSK3β. All results are presented as mean ± SD, and the experiments were repeated as triplicates. Bars with different letters significantly ($P < 0.05$) differ according to ANOVA and Tukey's multiple range test.

effectively suppressed the lipid accumulation induced by FFA in HepG2 cells. This treatment also led to a reduction in intracellular TG content and the formation of lipid droplets. Furthermore, the treatment with tangeretin was found to downregulate the expression levels of key genes associated with lipid accumulation that were upregulated by FFA, including *FABP1*, *PLIN2*, *FAS*, and *GLUT4*. Based on the findings from network pharmacology analysis, it is suggested that Chenpi and its primary active component, tangeretin, may exert their anti-obesity effects by modulating the PI3K/Akt signaling pathway. To further validate the potential mechanism of tangeretin in alleviating lipid accumulation induced by FFA in HepG2 cells, quantitative real-time PCR analysis was performed. The results demonstrated that the treatment with 25 μmol/L tangeretin effectively restored the decreased mRNA expression of *PI3K*, *Akt*, and *GSK3β* caused by FFA treatment. Moreover, the protein of PI3K, Akt, and GSK3β and its phosphorylated protein were also determined by Western Blotting. The result showed tangerine can activate the PI3K/Akt/GSK3β signaling pathway by increasing the level of protein phosphorylation of PI3K, Akt, and GSK3β. Taken together, these findings suggest that the key anti-obesity component, tangeretin, present in the tangerine peel, reduces intracellular fat accumulation and exerts the anti-obesity effect by activating the PI3K/Akt/GSK3β signaling pathway, which is consistent with the results obtained from network pharmacology analysis.

5. Conclusion

The study successfully excavated the active phytochemicals and their molecular mechanisms of Chenpi in the treatment of obesity. A total of 17 active compounds, 189 anti-obesity core targets, and 8 central targets were identified. Based on KEGG pathway analysis, the molecular mechanism of treating obesity by Chenpi may involve PI3K/Akt signaling pathway, MAPK signaling pathway, AMPK signaling pathway, and NF-κB signaling pathway. The research results also support the conclusion that the anti-obesity effect of Chenpi may be the direct or indirect synergistic effect of the multi-target and multi-signaling pathway. The molecular docking results indicate that the bioactive phytochemicals of Chenpi can bind to central targets (AKT1, BCL2, CASP3, EGFR, JUN, SRC, TP53, and TNF). Based on the ADMET analysis and the network pharmacology result, the tangeretin displayed the lowest predicted toxicity and potential for anti-obesity effects. *In vitro* lipid accumulation model, the tangeretin can downregulate the gene expression of the *FABP1*, *FAS*, *PLIN2*, and *GLUT4*. Moreover, tangerine can activate the PI3K/Akt/GSK3β signaling pathway by increasing the level of the mRNA and protein phosphorylation of PI3K, Akt, and GSK3β, thereby reducing intracellular fat accumulation and exerting the anti-obesity effect. Taken together, the tangeretin can suppressed the lipid accumulation induced by FFA in HepG2 cells through regulating the PI3K/Akt/GSK3β signaling pathway. The results of this study can provide clues for further research on the anti-obesity phytochemicals and mechanisms of Chenpi and provide a foundation for the development of modern functional foods or anti-obesity drugs based on Chenpi and its phytochemicals.

Conflict of interest

Baojun Xu is an associate editor for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare no conflict of interest.

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