



Research Article

Study on the hepatoprotective effects of *Thymus quinquecostatus* Celak. against alcoholic liver injury based on system pharmacology strategy

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Abstract: Alcoholic liver disease (ALD) is a global prevailing disorder. *Thymus quinquecostatus* Celak. (TQC) is a food additive and edible herb which possesses hepatoprotective activity against ALD. But the mechanisms involved remain largely unknown. In this study, system pharmacology strategy based on *in vivo* composition analysis, network pharmacology, molecular docking, and experimental validation was conducted to investigate the mechanisms and material basis of *Thymus quinquecostatus* Celak. extracts (TQE) against ALD. Through UPLC-QE-Orbitrap-MS/MS analysis, 41 prototypes from TQE were identified in mouse plasma. Then 19 hub ingredients and 146 related hub genes were obtained based on network pharmacology analysis. Protein-protein interaction (PPI) network, molecular docking and experimental verification study further confirmed the effect of TQE on tumor protein p53 (TP53), epidermal growth factor receptor (EGFR), AKT serine/threonine kinase 1 (AKT1), mitogen-activated protein kinase 1 (MAPK1), and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1). In conclusion, the hepatoprotective activity of TQC against ALD can be attributed to 19 main active ingredients. They might play an essential role in the management of ALD through EGFR, MAPK, and TP53 pathways.

Keywords: *Thymus quinquecostatus* Celak.; alcoholic liver injury; hepatoprotective activity; system pharmacology; molecular mechanism

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1 Introduction

Inordinate alcoholic beverage consumption could lead to alcoholic liver disease (ALD), which is characterized by the development of fatty liver, hepatitis, fibrosis, and cirrhosis^[1]. ALD is one of the most prevalent burdens throughout the world due to alcohol abuse^[2]. With the development of modern food function studies, it has been proved that edible plants play an increasingly important role in the management of chronic hepatic injury^[3,4].

Thymus quinquecostatus Celak. (TQC) is a species of thyme that belongs to the family Lamiaceae. TQC is widely distributed in Asia including China, South Korea, Japan, and India, and has been widely used as food additive and edible herb due to its nutritional and condimental values^[5,6]. Recently, TQC is attracting attention as a protective agent for hepatocytes^[7,8]. In addition, the hepatoprotective activity of TQC for chronic alcohol-induced liver injury has been indicated during the ongoing investigations in our group. The extracts could alleviate ALD partly through gut dysbiosis correction and gut-liver axis (GLA) modulation^[9].

However, the *in vivo* material basis and underlying mechanisms remain largely obscure.

With the combination of metabolic kinetics data, bioactive compounds screening, and system prediction, system pharmacology is an interdisciplinary research strategy that facilitates the study of therapeutic mechanisms elucidation^[10,11]. Recently, material basis and mechanism studies of functional foods in the management complex diseases also benefit from system pharmacology with the great development of effective analytical tools^[12,13]. Network pharmacology is an emerging strategy that aimed at investigating the complex and diverse relationships between ingredients, targets, and diseases^[14,15]. In addition, based on predicting ligand-target interactions at a molecular level, molecular docking is a computational tool to study the interactions and recognition between receptors and ingredients^[16-18]. These two potent tools can be complementarily integrated so as to provide novel insights into active compounds screening and mechanism exploration of functional foods and edible herbs. Additionally, as a complex component system,

clarifying the *in vivo* metabolic profile of plants, especially the detectable components in plasma is not only crucial for material basis elucidation but also will provide a reliable guide to network pharmacology and molecular docking studies.

Collectively, a system pharmacology strategy based on *in vivo* plant-derived metabolic profile, network pharmacology, molecular docking, and experimental validation was performed in the present study to investigate the material basis and underlying mechanism of TQC against ALD.

2 Materials and methods

2.1 TQE preparation

TQC was collected from Yulin in Shanxi province of China. The plant material was placed on cellulose filter paper in a well-aerated room and air-dried at room temperature. Then 2.0 kg plant material was repeatedly reflux extracted with 80% ethanol (*w/v* = 1: 20, 120 min) for 2 times. Finally, the solution was evaporated and 0.4 kg *Thymus quinquecostatus* Celak. extracts (TQE).

2.2 Identification of TQE-derived ingredients in blood after oral administration

2.2.1 TQE administration and plasma samples preparation

6-week-old C57BL/6N male mice purchased from Charles River Laboratory (Beijing, China) were maintained under standard experimental conditions (23 ± 2 °C, $55 \pm 5\%$ relative humidity, 12–12 h light-dark cycle). Forty-two mice were divided into two groups including normal group and ALD group ($n = 21$). For ALD group, mice were fed with alcohol-containing liquid diet (4% *v/v*) for 6 weeks. While for normal group, mice were fed with pair-fed control diet (supplemented with isocaloric maltose dextrin).

Then mice in both two groups were administered with TQE orally at a dose of 4 g/kg respectively. Blood samples were collected from the orbital vein of each mouse at 0.083, 0.25, 0.5, 1, 1.5, 2, 4 h after oral administration. Three mice (biological duplication) were set at each point-in-time. The blood samples were centrifuged at 3,500 r/min for 10 min to obtain the plasma. Equal amount of plasma fractions from each mouse in each group were combined and mixed with three volumes of methanol to remove the proteins.

2.2.2 UPLC-QE-Orbitrap-MS/MS analysis

TQE derived ingredients in mouse plasma were identified using Thermo ultra-high performance liquid chromatograph Q-Exactive-Orbitrap MS/MS (UPLC-QE-Orbitrap -MS/MS) system. UPLC separation was carried out on Waters ACQUITY UPLC HSS T3 column (2.1 mm $\times$ 100 mm, 1.8 μ m; Waters Corporation, Milford, MA, USA) at 30 °C. A binary gradient composed of water containing acetonitrile (A) and 0.1% of formic acid (B) was used in this procedure. The gradient elution program was 0–1 min, 5% A; 1–22 min, 5–98% A; 22–25 min, 98% A; 25–27 min, 98–5% A; 27–30 min, 5% A. 5 μ L was injected and flow rate was set at 0.3 mL/min. Mass spectra in the *m/z* range 100–1,500 were obtained by positive or negative ion modes. Other working MS parameters were sheath gas flow of 40 arb, auxiliary gas flow rate of 15 arb, capillary temperature of 320 °C, aux gas heater temperature of 350 °C, positive spray voltage of 3.2 kV, and

negative spray voltage of 3.0 kV. The resolution of MS is 70,000, and the resolution of MS/MS is 17,500. The compounds were identified according to the mzCloud database as well as data from previous studies.

2.3 Acquisition of ALD-related and absorbed ingredients-related targets

The ALD-related targets were collected through retrieving the databases including DrugBank (<https://www.drugbank.ca/>), Therapeutic Target Database (TTD, <http://db.idrblab.net/ttd/>), Online Mendelian Inheritance in Man (OMIM, <https://www.omim.org/>) and GeneCards (<https://www.genecards.org/>) with the keyword of “ALD”. Finally, all the available data from these four databases were merged after data deduplication and the obtained genes were considered as ALD-related therapeutic targets.

On the basis of the results from *in vivo* metabolic profile study, the detectable prototypes of TQE in both ALD and normal mouse plasma were selected as candidate ingredients. Targets associated with these candidate ingredients were identified and collected based on widely recognized databases including PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/>), SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) and Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, <http://tcmispw.com/tcmisp.php>). In addition, some reported molecular targets related to these candidate ingredients were also supplemented through literature mining. The UniProt database (<https://www.uniprot.org/>) was used to standardize gene names of the obtained ALD-related and ingredients-related targets.

2.4 Targets network construction of candidate ingredients and disease

Through Venn diagram, the intersectional targets between ALD and the candidate ingredients were identified. Based on these obtained intersectional targets, ingredient-target-ALD interaction network was constructed with Cytoscape (version 3.7.2), which is a general platform for complex network analysis and visualization.

2.5 PPI analysis

The protein-protein interaction (PPI) network of the intersectional targets was constructed by searching the STRING (<https://string-db.org/>) database, during which the minimum mutual threshold was set as high confidence (0.7). The data of PPI network was obtained and then inputted into Cytoscape (version 3.7.2) for visualization.

2.6 GO term annotation and KEGG pathway enrichment analysis

Gene Ontology (GO) functional annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were generated by using the Metascape online platform (<http://metascape.org/>). “Homo sapiens” was selected as the species. In GO annotation, gene functions were subject to three aspects, including molecular function (MF), cell composition (CC) and biological process (BP). In KEGG pathway enrichment, *P*-value < 0.05 was considered statistically significant.

2.7 Molecular docking

Molecular docking study between the obtained hub targets and their corresponding ingredients were performed using the

Discovery Studio 2016 software, which is a computational tool that can predict the interaction energy between receptors and ligands. 2D structures of the selected ingredients (small molecule ligands) were downloaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). 3D structure of the selected target proteins (macromolecule receptors) was downloaded from the Protein Data Bank (PDB) database (<https://www.rcsb.org/>). Prior to docking, the water molecules were removed from the crystal structure of receptor. The active binding pocket was set up. The pose cluster radius under top hits was set as 0.5 and other parameters remained default values. The 80% of the docking score value of the original ligand was set as threshold value to evaluate the docking results^[19].

2.8 In vivo experimental validation

2.8.1 Animals and treatment

40 male 6-week-old C57BL/6N mice purchased from Charles River Laboratory (Beijing, China) maintained under standard experimental conditions (23 ± 2 °C, $55 \pm 5\%$ relative humidity, 12–12 h light-dark cycle). The chronic ALD mouse model was established based on the Lieber-DeCarli liquid diet with some modification after adaptive feeding for one week^[20]. Briefly, 40 mice were randomly assigned into five groups ($n = 8$) as follows: Control (Con) group, ethanol (EtOH) group, ethanol + 20 mg TQE/kg/d (EtOH + TQL) group, ethanol + 40 mg TQE/kg/d (EtOH + TQM) group, ethanol + 80 mg TQE/kg/d (EtOH + TQH) group. Mice in EtOH, EtOH + TQL, EtOH + TQM and EtOH + TQH groups were fed with alcohol-containing liquid diet (4% v/v). While mice in Con group were fed with pair-fed control diet (supplemented with isocaloric maltose dextrin). For mice in EtOH + TQL, EtOH + TQM and EtOH + TQH group, daily TQE were homogenized in 0.5% CMC-Na solution and was administered orally. The animal experiment lasted for 6 weeks. Mice were euthanized at the end of the experiment, and blood and tissue samples were collected for further study. All experimental protocols were approved by the Committee on Ethics of Animal Experiments at Beijing University of Chinese Medicine (number: BUCM-2021031002-007). All experimental procedures were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.8.2 Biochemical analysis and histopathological examination

The collected blood samples were centrifuged at 3,500 r/min for 15 min at 4 °C to obtain the serum. The level of transaminase including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the content of triglyceride (TG) and total cholesterol (TC) in serum were measured with automated hematology analyzer (Beckman, USA).

Liver tissues separated from mice in each group were fixed in 4% paraformaldehyde solution overnight and then embedded in paraffin. 5 μ m sectioned samples were subsequently stained with hematoxylin and eosin (H&E). Morphology of the liver tissues were observed with an optical microscope (Nikon, Japan).

2.8.3 Quantitative PCR

Total RNA was extracted from mouse liver with TRIzol Reagent (Vazyme, China). The purity and concentration of extracted RNA were analyzed with a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). Then equal amounts of total RNA were reverse-transcribed to cDNA using HiScript 1st Strand cDNA Synthesis (Vazyme,

China). Finally, qRT-PCR analyses of the target genes were performed using SYBR® Green PCR kit (Vazyme, China) on QuantStudio™ 6 Flex system (Applied Biosystems, USA). The primers were synthesized by BGI Biotech (Beijing, China) and the sequences were summarized in Table 1. The relative mRNA expression was measured by comparative $2^{-\Delta\Delta Ct}$ method and normalized to β -actin in each sample.

Table 1 PCR primer sequences

Primer	Con
SRC	F: 5' GAACCCGAGAGGGACCTTC-3' R: 5' GAGGCAGTAGGCACCTTTTGT-3'
TP53	F: 5' AACCTACCAGGGCAACTATGGCTTC-3' R: 5' AACTGCACAGGGCAGCTCTTC-3'
PIK3R1	F: 5' CCCACTACTGTAGCCAACAAC-3' R: 5' CGTACCAAAAAGGTCCCATCA-3'
AKT1	F: 5' ATGAACGACGTAGCCATTGTG-3' R: 5' TTGTAGCCAATAAAGGTGCCAT-3'
EGFR	F: 5' CCCATGCGGAACCTACAGGAA-3' R: 5' TTGGATCACATTGGGGCAAC-3'
VEGFA	F: 5' CTGCCGTCCGATTGAGACC-3' R: 5' CCCCTCCTTGTAACACTGTG-3'
MAPK1	F: 5' GGTGTGTCCTCCAAATGCTGACT-3' R: 5' CAACTCAATCCTCTTGTGAGGG-3'
GAPDH	F: 5' -AGGTCGGTGTGAACGGATTTG-3' R: 5' -GGGTCGTTGATGGCAACA-3'

2.9 Statistical analysis

The data are displayed as mean $\pm$ standard deviation (SD). SPSS version 17.0 (SPSS Inc., Chicago, IL) was used for statistical analysis, and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was performed for multiple comparisons. $P < 0.05$ was considered as a statistically significant difference.

3 Result

3.1 Absorbed ingredients in plasma after oral administration of TQE

By comparing the pre- and post-dose mouse plasma, ingredients derived from TQE in the plasma of ALD mouse and normal mouse was analyzed. A total of 41 prototypes were identified in the plasma of both ALD mouse and normal mouse after oral administration of TQE, including 17 flavonoids, 10 phenols, 6 triterpenoids, 7 organic acids, and 1 alkaloid. The detailed information of these ingredients including retention time, formula, measured mass, mass error, and MS/MS fragments were listed in Table S1. The total ion flow diagram was showed in Figure S1.

3.2 Ingredient-target-ALD interaction network analysis

After the elimination of duplicates, 1,052 ingredient-related targets were identified from PharmMapper, TCMSP and SwissTargetPrediction, for which gene names were retrieved from the UniProt database. In parallel, 969 ALD-related target genes were identified based on DrugBank, TTD, OMIM and GeneCards databases.

A total of 241 common targets, which were associated both with eligible ingredients of TQE and ALD, were obtained by overlapping the two abovementioned targets profiles (Fig. 1). The association between the 41 TQE-derived ingredients in plasma and the 241 overlapped target genes was visualized by ingredient-target-ALD interaction network containing 281 nodes and 3,522 edges (Fig. 2).

With regard to the topological analysis of the constructed ingredient-target-ALD interaction network, 19 ingredients with a degree greater than the median were opted as the hub ingredients. Also, 146 genes with a degree greater than the median were opted as the hub targets. Based on these hub ingredients and hub targets, a core network was extracted and constructed (Fig. 3).

3.3 PPIs network analysis

PPI network was constructed based on the obtained 146 hub targets. As a result, we obtained a network including 140 nodes and 694 edges after discarding unmatched targets (Fig. 4). Nodes with a higher degree values have larger node sizes and deeper color in the network, indicating greater significance and requiring more attention. Meanwhile, the thickness of each edge indicates

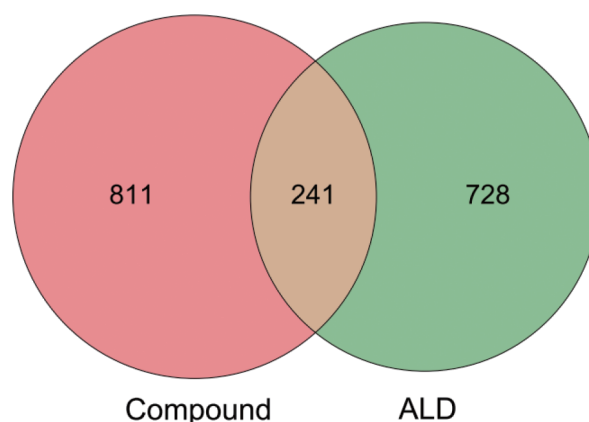


Figure 1 Venn diagram showing the intersection of identified target genes of ingredients and ALD.

the strength of that interaction. The average degree of nodes is 9.91. The target nodes with the highest degree values were SRC ($n = 44$), tumor protein p53 (TP53) ($n = 40$), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) ($n = 36$), AKT serine/

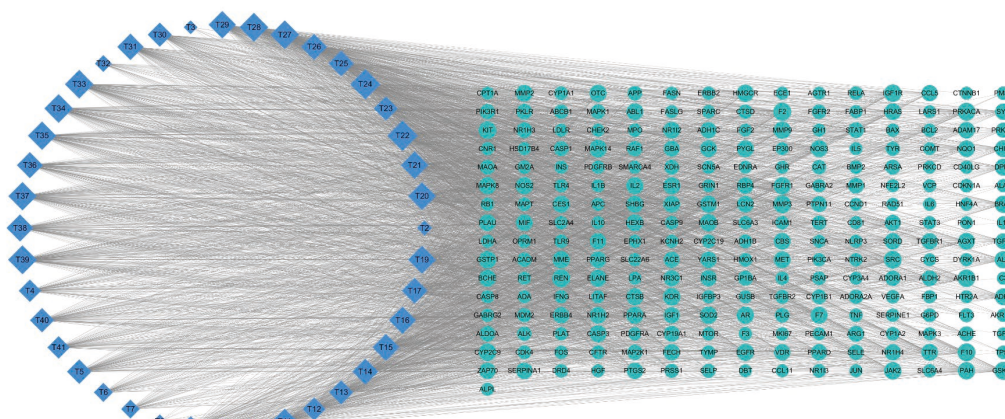


Figure 2 Ingredient-target-ALD interaction network. The blue nodes represent bioactive ingredients in TQE, and the green nodes represent the ALD-related targets of these bioactive ingredients.

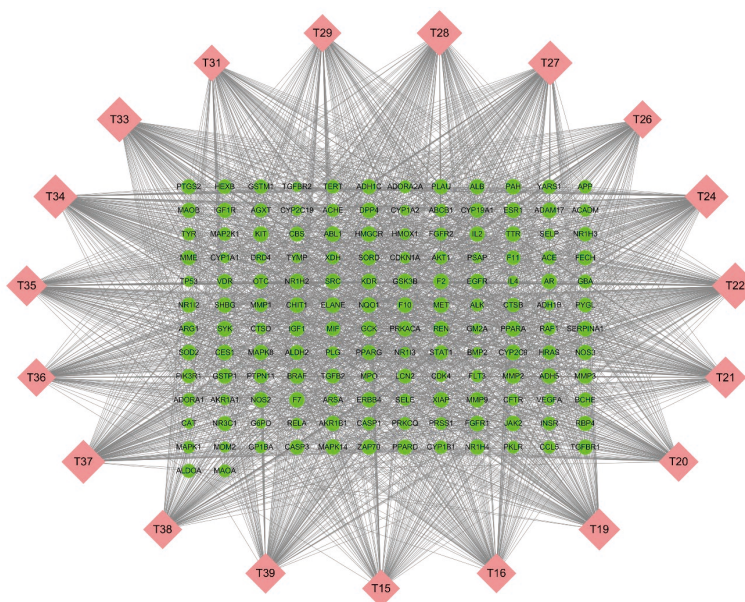


Figure 3 The extracted core ingredient-target-ALD interaction network based on hub compounds and hub targets. The pink nodes represent bioactive ingredients in TQE, and the green nodes represent the ALD-related targets of these bioactive ingredients from TQE.

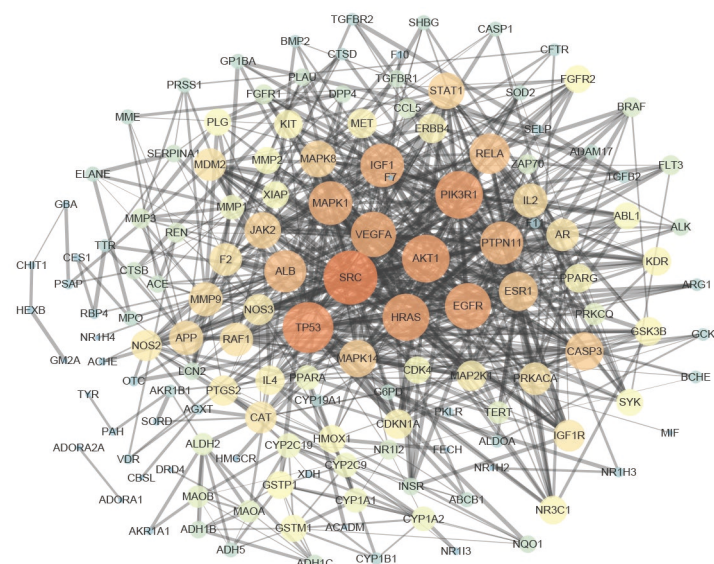


Figure 4 PPI network of hub targets. As the figure shows, the larger area and deeper color of the circle could be considered as more important in this network.

threonine kinase 1 (AKT1) ($n = 36$), HRas proto-oncogene, GTPase (HRAS) ($n = 35$), epidermal growth factor receptor (EGFR) ($n = 34$), vascular endothelial growth factor A (VEGFA) ($n = 33$), mitogen-activated protein kinase 1 (MAPK1) ($n = 31$), which may play a crucial role in the hepatoprotective activity of TQE against ALD.

3.4 GO functional annotation analysis

In order to elucidate the functions of the potential anti-ALD targets of TQE, GO functional annotation analysis were performed in this study. Top 10 GO terms subordinate to BP category, CC category, and MF category were summarized and

visualized in Fig. 5. In the BP category, most of the hub targets were involved in response to hormone (30.82%), regulation of kinase activity (28.77%) and response to extracellular stimulus (25.34%). In the CC category, the most enriched terms were correlated with protein kinase activity (21.23%), oxidoreductase activity (21.23%), protein homodimerization activity (18.49%). MF analysis revealed that most of the hub targets were associated with vesicle lumen (17.12%), receptor complex (15.75%), and membrane raft (14.38%).

3.5 KEGG pathway enrichment analysis

To clarify the crucial pathways among the hub targets in ALD

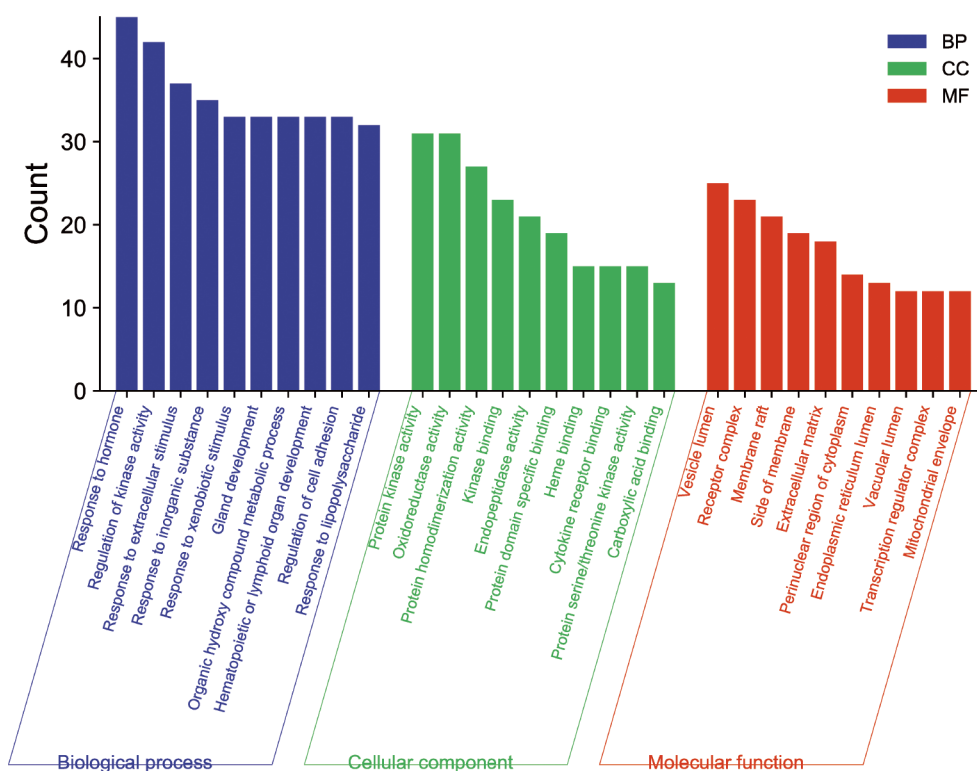


Figure 5 GO functional classification of the 146 hub targets, which was performed according to the BP, MF and CC. The top 10 generally changed GO terms in each category were displayed.

treatment, KEGG pathway enrichment analysis was also performed. The top 20 pathways were shown in Fig. 6, including pathways in cancer, HIF-1 signaling pathway, cytochrome P450 pathway, ALD pathways, calcium signaling pathway, NF-kappa B signaling pathway, etc. These signaling pathways may be involved in the hepatoprotective activity of TQE against ALD.

3.6 Molecular docking results

Top 8 targets that identified through interaction network analysis and PPIs network analysis, including MAPK1 (PDB code: 2Y9Q), SRC (PDB code: 2SRC), AKT (PDB code: 4EJN), TP53 (PDB code: 6GGA), EGFR (PDB code: 2RGP), VEGFA (PDB code: 6Z13), HRAS (PDB code: 1CTQ), and PIK3R1 (PDB code: 7CIO) were used as docking targets with their corresponding components. A docking energy greater than 80% of the original

ligand and the receptor indicates good protein-ingredients binding activity. Details of the docking information was listed in Table S2. The CDOCKER energy between the receptor and the ligand is an important index to reflect the docking capacity. The higher the binding energy is, the higher the affinity of the receptor and the ligand, and the more stable the conformation. The molecule with the highest binding energy in the docking conformation was selected to observe the binding effect by analyzing the intermolecular interaction.

As a consequence, detailed docking model of AKT1 with methyl rosmarinate, SRC with methyl rosmarinate; EGFR with rosmarinic acid, TP53 with luteolin, MAPK1 with methyl rosmarinate, VEGFA with luteolin were shown in Fig. 7. It showed that the small molecule compounds were tightly bound to the protein residues *via* various interactions. For example,

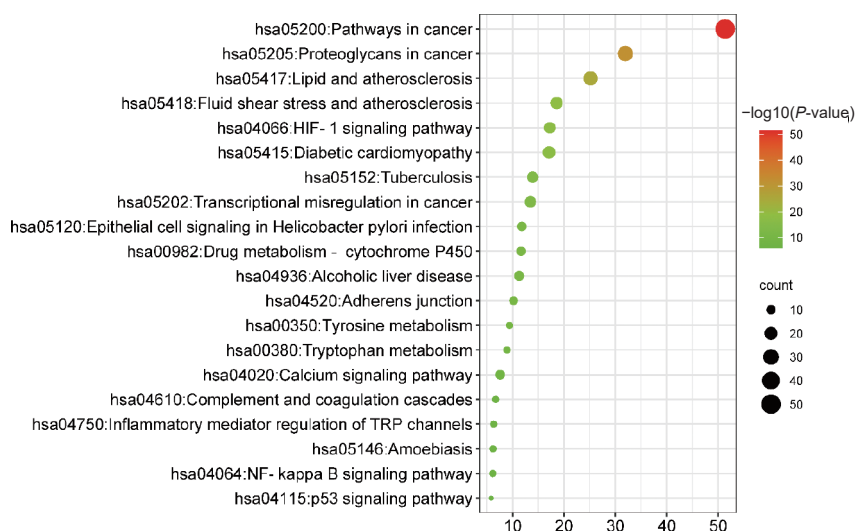


Figure 6 KEGG pathway enrichment bubble diagram of the 146 hub targets.

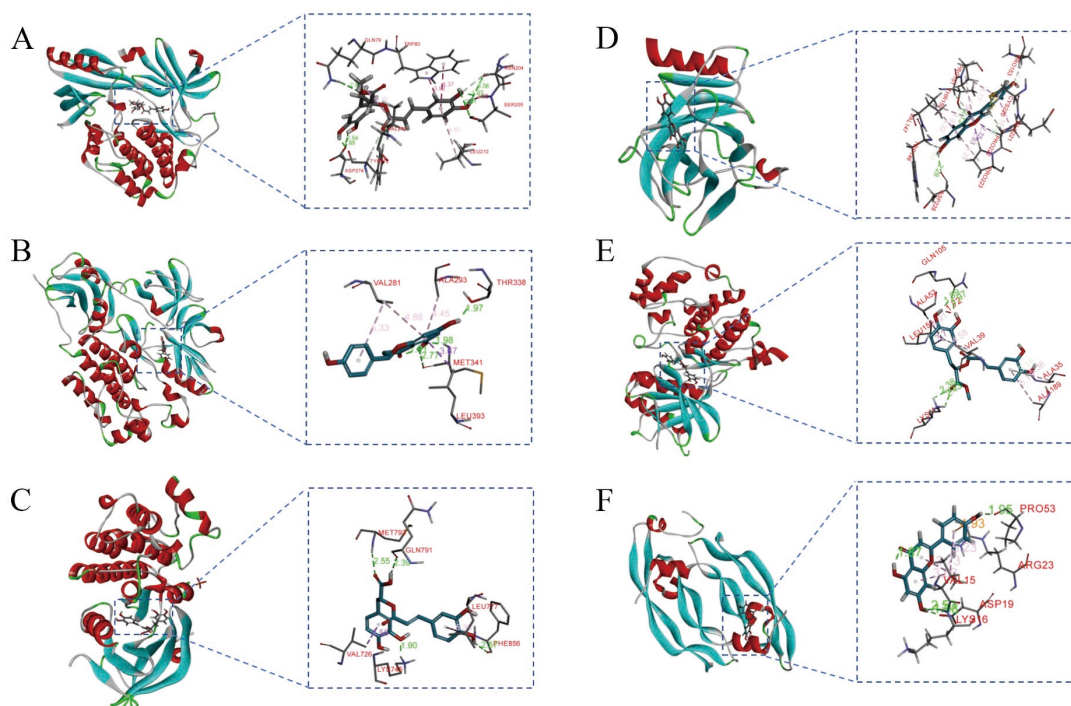


Figure 7 The docking model of the hub targets and corresponding components with the highest binding energy. (A) AKT1 with methyl rosmarinate. (B) SRC with methyl rosmarinate. (C) EGFR with rosmarinic acid. (D) TP53 with luteolin. (E) MAPK1 with methyl rosmarinate. (F) VEGFA with luteolin.

rosmarinic acid bound to EGFR through six key bonds, including four hydrogen bonds with PHE856 (2.57 Å), LYS745 (1.90 Å), GLN791 (2.39 Å) and MET793 (2.55 Å), one Pi-Alkyl bond with LEU777 (5.35 Å), one Pi-Sigma bond with VAL726 (2.49 Å).

3.7 In vivo validation results

3.7.1 Effects of TQE on alcoholic liver injury in mice

Plasma ALT and AST was elevated nearly 2 and 1.5 folds respectively due to alcohol feeding, which was significantly blunted by TQE supplementation in TQH and TQM groups (Figs. 8A and B). Alcohol intake also led to significant increase of serum TC and TG compared with those in the Con group. While significantly decreased of TC and TG were shown in the TQH and TQM groups compared with the EtOH group (Figs. 8C and D). Histopathologic examination indicated the presence of lesions including hepatocyte ballooning and lipid droplets in the liver of alcohol-fed mice. In contrast, liver lesions were reduced by TQE treatment compared with the EtOH group, especially in the TQM and TQH groups (Fig. 8E). These results indicated that TQE supplementation alleviated alcohol-induced liver injury.

3.7.2 Effects of TQE on hub targets

Bioinformatics analysis and molecular docking study have indicated that *MAPK1*, *SRC*, *AKT*, *TP53*, *EGFR*, *VEGFA*, and *PIK3R1* are the most important targets in the treatment of TQE against ALD. Thus, qRT-PCR was performed for further verification and underlying mechanism investigation. The mRNA levels of *EGFR*, *MAPK1* and *AKT* in the EtOH group were increased relative to those in the Con group due to alcohol consumption. While the mRNA levels of these targets were significantly decreased in these three TQE treatment groups with a

dose-dependent manner. The elevation of *PIK3R1* and *TP53* in EtOH group were also reversed to a certain extent in the TQE treatment groups. The mRNA levels of *VEGFA* in the EtOH group increased significantly compared with the Con group, and no significant difference was shown between EtOH and TQE treatment groups. There was no significant difference among all groups in the expression of *SRC* level (Fig. 9).

4 Discussion

In previous network pharmacology-based study, candidate active ingredients were always collected from databases. Such strategy provides a wide range of candidates and thereby leads to a difficulty to locate the key information. In addition, the majority of ingredients in plants cannot reach the specific targets *in vivo*. Therefore, screening and validation of oral bioavailability is the indispensable precondition for the identification of potential active ingredients and targets in network pharmacology-based study. In this regard, more attention has been paid to the ingredients absorbed into blood in network pharmacology studies recently^[21,22]. Compared to the strategy of databases collection combined with computational bioavailability screening, network pharmacology based on components absorbed into the blood could provide us the potential active ingredients and the key targets more accurately.

In this study, a total of 41 prototypes were identified in mouse plasma which is composed mainly of flavonoids, phenols, triterpenoids and organic acids. Within them, the ingredient-target-ALD network suggested that the therapeutic efficacy of TQE against ALD was largely attributed to 19 hub ingredients. Ursolic acid and oleanolic acid are two representative natural pentacyclic triterpenoids derived from edible plants which possess hepatoprotective activity against wide range of liver-threatening

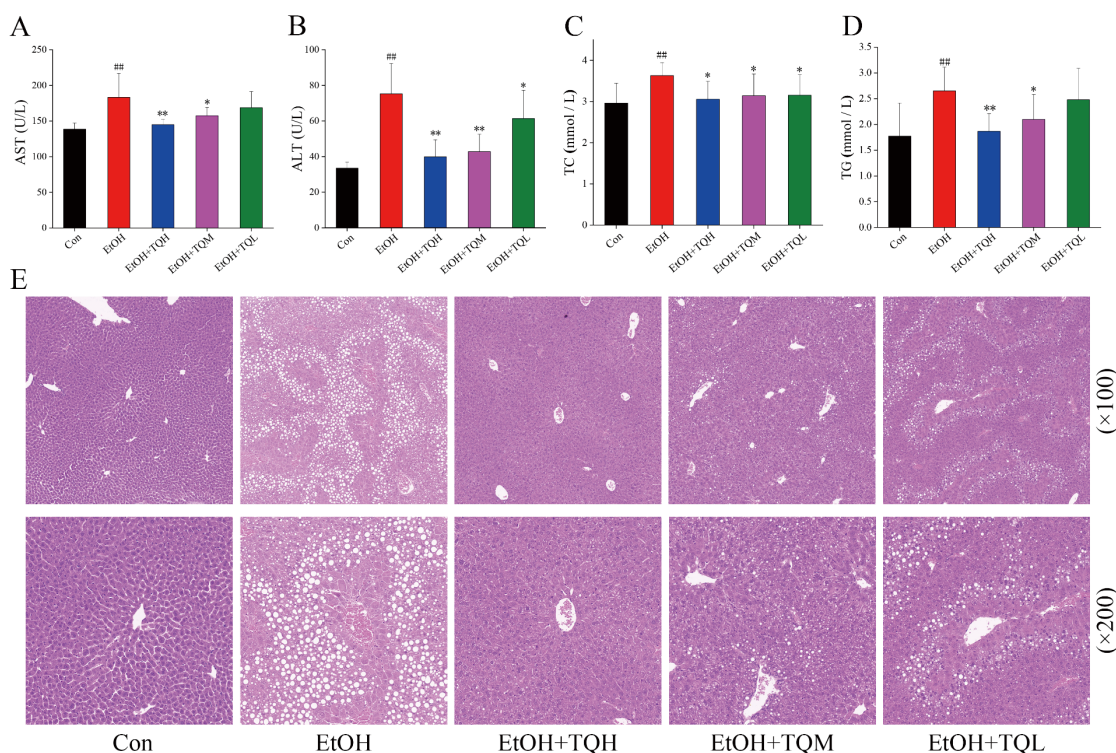


Figure 8 Effects of TQE on alcohol-induced liver injury in mice. (A) Enzymatic activities of AST in serum. (B) Enzymatic activities of ALT in serum. (C) Serum TC content. (D) Serum TG content. (E) Liver histopathology assessed by H&E staining. Data are presented as the mean $\pm$ SD. ^{*} $P < 0.05$, ^{##} $P < 0.01$, vs. Con group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, vs. EtOH group.

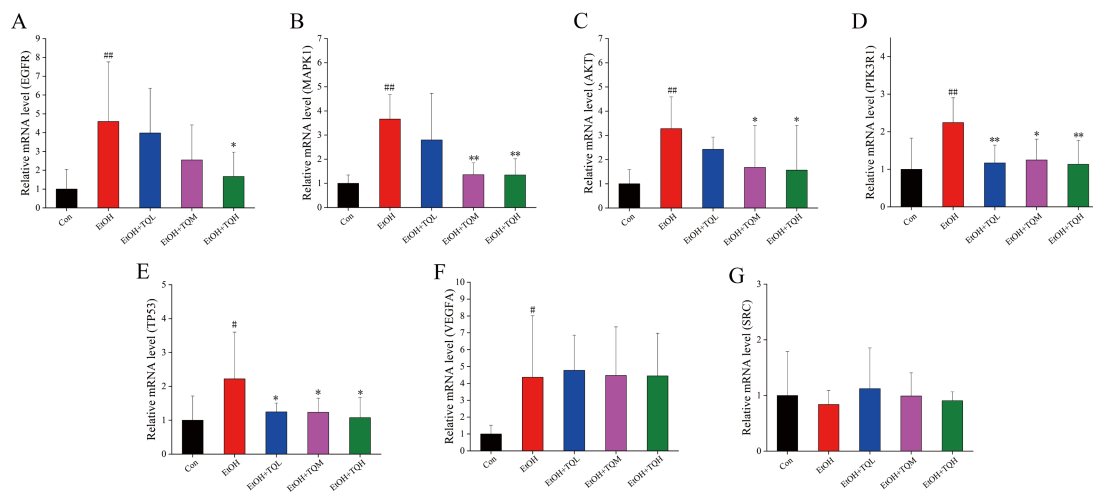


Figure 9 Effects of TQE on the mRNA levels of key targets including EGFR (A), MAPK1 (B), AKT (C), PIK3R1 (D), TP53 (E), VEGFA (F) and SRC (G). Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, vs. Con group; * P < 0.05, ** P < 0.01, vs. EtOH group.

substances^[23]. The positive effects of these two triterpenoids on ALD also have been proved^[24,25]. Flavonoids including chrysin, apigenin, daidzein, and luteolin are also reported to attenuate alcohol-induced liver injury through multiple pathways^[26-29]. Naringenin is another flavonoid compound in TQE. The evidence indicates naringenin administration increased ADH and ALDH enzyme activities, and decreased the activity of cytochrome CYP2E1 in alcohol-treated rats^[30]. Additionally, previous studies have also established the hepatoprotective capacity of several phenols against ALD including rosmarinic acid. Rosmarinic acid could protect ethanol-induced liver injury *in vitro*, and the mechanisms for protection include anti-lipid accumulation, anti-oxidative stress and anti-inflammation^[31]. Taken together, previous findings and our study suggested that these ingredients are the potential material basis for the hepatoprotective activity of TQC against ALD.

Through the integration of ingredient-target-ALD network construction, PPI analysis and molecular docking analysis, key targets such as MAPK1, SRC, AKT, TP53, EGFR, VEGFA, and PIK3R1 were screened out. EGFR is a transmembrane receptor that belongs to the family of receptor tyrosine kinases^[32]. It is one of the highly expressed proteins in hepatocytes. The EGFR machinery acts as the central hub for other growth factors, inflammatory mediators and cytokines, and thus affecting hepatic injury^[33]. Since the ligand connects to the extracellular binding domain of EGFR, the phosphorylated tyrosine site of EGFR acts as the docking site for various proteins that are involved in the activation and regulation of signal transduction cascades including PIK3R1/AKT pathways^[34]. The activation of EGFR-PI3K-AKT signaling pathway closely related to the onset and progression of liver fibrogenesis and hepatocellular carcinoma^[35,36]. It has been reported that the activation of liver EGFR-PI3K-AKT signaling pathway occurred in alcohol feed mice^[37]. ALD comprises a spectrum of pathological processes from hepatitis, steatosis, to fibrosis and finally even hepatocellular carcinoma^[38]. Suppression of EGFR-PI3K-AKT pathway could inhibit the progression of ALD.

MAPKs, also named extracellular signal-regulated kinase, are serine-threonine protein kinases that participates in various cellular activities including differentiation, apoptosis, cell survival, inflammation, and innate immunity^[39]. As the members of the MAPK family, MAPK1 is related to the proliferation,

transformation and differentiation of cell^[40]. MAPKs are important signalling pathways responsible for the formation of oxidative stress and inflammatory reactions^[41]. It is also known that p38 MAPK promote the mitochondrial apoptotic pathway, leading to ethanol-induced death of hepatocyte, suggesting an interaction between apoptosis and MAPK signalling system^[42]. Inhibiting the activation of the MAPK pathway is considered as a target for alleviating alcohol-induced liver injury^[43,44].

TP53 was another hub target obtained from the present study. It has been established that TP53 have a broad role in the pathogenesis of ALD. Activation of TP53 can be critical in ethanol-induced hepatocyte apoptosis, since genetic ablation of TP53 abrogates ethanol-induced liver damage^[45], and also important in oxidative stress, impaired survival signaling, and liver regeneration^[46]. Trough RT-qPCR analysis, we found that TQE treatment decreased the expression of TP53 protein in liver. Pathway enrichment results also showed that the TP53 signaling pathway was closely related to the hepatoprotective activity of TQE.

Collectively, these findings showed that the hepatoprotective activity of TQE against ALD act *via* multiple targets and multiple pathways consisted mainly of EGFR, MAPK and TP53 pathway. Meanwhile, several other genes and pathways may also be the targets as revealed by the GO functional enrichment and KEGG pathway analyses.

5 Conclusion

In conclusion, the material basis and mechanisms of TQC in the management of ALD were investigated with the help of system pharmacology strategy based on *in vivo* plant-derived metabolic profile, network pharmacology, molecular docking and experimental validation. The hepatoprotective activity of TQC can be attributed to 19 main active components including naringenin, rosmarinic acid, luteolin, etc. They might play an essential role in the management of ALD through the EGFR, MAPK and TP53 pathway.

Electronic Supplementary Material (ESM)

The online version contains supplementary material available at <https://doi.org/10.26599/FMH.2027.9420176>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest in this work.

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Author contribution statement

Xin Yan: Project administration, experimental design, validation, writing manuscript. Jia-Mu Ma: Project administration, data curation, experimental design. Jian-Ling Yao: Project administration, data curation, writing manuscript. Ming-Xia Li: Project administration, validation. Wei Liu: Project administration, validation. Meng-Yu Sun: Project administration, data curation. Feng Zhang: Data curation, project administration. Le-Tian Ying: Project administration, writing manuscript. Yu-Qing Yang: Data curation, writing manuscript. Yong-Qi Yang: Project administration, validation. Yun-Zi Liu: Project administration, validation. Yu Cao: Project administration, validation. Gai-Mei She: Project administration, experimental design, funding acquisition.

Informed consent

Not applicable.

Ethics statement

The animal experiments were carried out following the guidelines of the Beijing University of Chinese Medicine Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Beijing University of Chinese Medicine (Ethical code: BUCM-2021031002-007).

Use of AI statement

None.

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