

# Exploring the potential mechanisms of action of *Gentiana veitchiorum* Hemsl. extract in the treatment of cholestasis using UPLC-MS/MS, systematic network pharmacology, and molecular docking

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## Research Article

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# Abstract

## Ethnopharmacological relevance

*Gentiana veitchiorum* Hemsl. (GV) has a long history in Tibetan medicine for the treatment of the hepatobiliary disease, cholestasis. However, the mechanisms mediating its efficacy in the treatment of cholestasis have not been determined.

## Purpose

To elucidate the mechanisms of action of GV in the treatment of cholestasis, an integrated approach combining ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis with network pharmacology and molecular docking was established.

## Materials and methods

A comprehensive analysis of the chemical composition of GV was achieved by UPLC-MS/MS. Subsequently, a network pharmacology method that integrated target prediction, a protein-protein interaction (PPI) network, gene set enrichment analysis and component-target-pathway network was established. The network pharmacological results were validated through molecular docking analysis.

## Results

The UPLC-MS/MS analysis identified twenty compounds in GV. Network pharmacology identified 299 targets for the components of GV and 2999 targets for cholestatic diseases, from which 103 intersectional targets were obtained by Venn diagram. A PPI network was constructed to screen the following core proteins: AKT1, GAPDH, TNF, IL6, VEGFA, and CASP3. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses results suggested that GV may treat cholestatic disease through biological pathways related to proteolysis, cytosol, caspase complex, enzyme binding, and pathways in cancer, lipid and atherosclerosis, HIF-1, TNF, IL-17 and other key signaling pathways. The results were validated by molecular docking studies, which indicated good binding affinities between core compounds and targets, with similar binding energies to those of the positive control drugs.

## Conclusions

In this study, UPLC-MS/MS analysis and network pharmacology were used in conjunction to provide potential mechanisms of action of GV in the treatment of cholestasis.

## 1. Introduction

*Gentiana veitchiorum* Hemsl. (GV) is a plant from the genus *Gentiana* (Tourn.) L. in the family Gentianaceae, mostly found in forest margins and alpine grasslands at altitudes of 3200–4700 m, and is abundant in the southwest and northwest of China [1, 2]. As recorded in the Tibetan Plateau Medicines Illustrated and Gansu Herbal Handbook [3], GV is usually used for heat-clearing and detoxification, and mainly for treating smallpox, bronchitis, cough, headache, pharyngitis and damp-heat jaundice. Numerous studies have suggested that GV has multiple pharmacological effects, including anti-microbial, anti-oxidant, analgesic, liver-protective and anti-inflammatory properties, and it is clinically effective in the treatment of hepatobiliary diseases [4].

Cholestasis is a clinical syndrome that is characterized by impaired formation and circulation of bile acids [5]. In the absence of appropriate treatment, it may eventually lead to acute hepatotoxicity accompanied by a range of exacerbating outcomes, such as primary biliary hepatic sclerosis, hepatic failure, and biliary hepatitis [6]. The complicated etiology and complications of cholestasis have greatly hindered the development of new medicines, such that there are currently few effective treatments [7]. At present, the most effective drugs approved for the treatment of cholestasis include ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) [8]. However, their efficacy and safety are unsatisfactory, and more than 40% of patients do not respond to UDCA [9, 10]. In addition, approximately 90% of patients taking OCA exhibit a variety of side-effects [11]. It is therefore highly desirable to determine the underlying mechanisms involved in cholestasis to facilitate the search for new therapeutics.

Network pharmacology is a promising discipline for analysis of biological systems and selection of specific signaling nodes for multi-target drug molecule design. This methodology is an emerging strategy for comprehensive assessment of herbal compounds based on multidisciplinary techniques [12]. 'Network targets' play a central role in traditional Chinese medicine network pharmacology [13]. Biological networks are used to construct interactions between drug components, targets and diseases, to systematically interpret the pharmacological mechanisms of action of Chinese medicine formulations. The results provide potential new ideas for clinical treatment of diseases with Chinese medicines [14, 15].

The development of new medicines to treat cholestasis with good efficacy and few side effects is crucial and urgent, and intensified studies on the active ingredients of natural products are uniquely advantageous. Therefore, we constructed a network pharmacological method using data from ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to probe the pharmacological mechanisms by which extracts of GV affect cholestatic diseases (Fig. 1).

## 2. Materials and methods

### 2.1 Plant material

GV, a perennial plant of the genus *Gentiana* (Gentianaceae), was harvested in Yushu, Qinghai Province (97.12428E, 32.80899N). The material was identified by Associate Researcher Xiaofeng Chi and the specimens were preserved at the Northwest Institute of Plateau Biology (Xining City, Qinghai Province, China). Loganic acid was purchased from Yunnan Xili Biotechnology Co., Ltd (Kunming City, Yunnan Province, China). Swertiamarin, sweroside and gentiopicroside were purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing City, Jiangsu, China). Isoorientin and isovitexin were purchased from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China).

### 2.2 Preparation of sample solutions

The dried GV was sonicated three times in methanol (1:5 solid:solvent ratio) at ambient temperature for 30 min each time and then filtered. The combined filtrates were concentrated, dried under reduced pressure and stored in a refrigerator at 4 °C. The GV extract powder obtained by freeze-drying (5mg) was dissolved in methanol (to 10 mL in a volumetric flask) by ultrasonication. The resulting GV extract solution, at a concentration of 0.5mg/mL, was filtered through a 0.22 µm microporous filter membrane and stored in a refrigerator at 4 °C. The standards (isovitexin, sweroside, swertiamarin, isoorientin, gentiopicroside, and loganic acid; 5 mg each) were dissolved in methanol (10 mL) in a volumetric flask to obtain standard solutions (0.5mg/mL). All standards were filtered through a 0.22 µm microporous membrane and kept in a refrigerator at 4 °C.

### 2.3 Chromatography and mass spectrometry conditions

The extracts of GV were analyzed on an Acquity™ ultra performance liquid chromatography instrument (Waters, USA), and Triple TOF 5600 + time-of-flight mass spectrometer with electrospray ion source (AB SCIEX, USA). The chromatographic column consisted of a Waters, Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 150 mm). Formic acid (0.1%) in water was used as mobile phase A and formic acid (0.1%) in acetonitrile was used as mobile phase B. Linear gradient elution was as follows: 0 min 5% B; 15 min 30% B; 20 min 95% B; 22 min 95% B; the flow rate was 0.3 mL/min; the column temperature was 50 °C; the detection wavelength was 254 nm and the injection volume was 3 µL.

Full mass spectra were recorded at an  $m/z$  range of 100–1500 in negative ion mode. The MS parameters were set as follows: nebulizing gas:55 psi; curtain gas, 35 psi; ion source temperature:550°C (negative); ion source voltage, 5500 V (positive) and – 4500 V (negative); primary scan: declustering voltage, 100 V; focusing voltage:10 V; secondary scan: MS data acquisition using TOF MS ~ Product Ion ~ IDA mode with collision energy of ±40 eV.

### 2.4 Component identification analysis

The results of the GV extract analysis were summarized to obtain the chemical components and their corresponding mass spectrometry data within the retention time. The basal peak plots were compared with the control substances and their literature data to confirm the main chemical components of the GV extract.

### 2.5 Identification of relevant targets for the chemical components of GV

Several online databases were selected to predict relevant targets for the treatment of cholestasis. The PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) database (an authentic chemical database containing thorough chemical information) was first used to search for molecular information on all identified chemical components, including molecular formula, 3D molecular structure and smiles structural data. SwissTargetPrediction (<http://swisstargetprediction.ch/>), TCMSP (<https://tcm-sp-e.com/>), SEA (<http://sea.bkslab.org/>) and STITCH (<http://stitch.embl.de/>) databases were then used to obtain relevant potential targets for components of GV. Associated genes were then standardized using the Retrieve/ID plotting tool in the Uniprot database (<https://www.uniprot.org/uploadlists/>). Finally, the targets acquired from the four databases were pooled and de-duplicated using Microsoft Excel (USA) to obtain compositionally relevant targets.

### 2.6 Collection of cholestasis-related targets

Keywords such as 'cholestasis', 'cholestatic hepatic injury', 'cholestatic liver injury', 'primary biliary cirrhosis' and 'primary cholangitis' were searched in the GeneCards (<https://www.genecards.org/>) database [16]. From the search results, 'protein coding' was selected as the

criterion in the 'category' column to obtain disease-related targets, and then Microsoft Excel was used to remove duplicates.

## 2.7 Protein-protein interaction (PPI) analysis

VENNY 2.1 software (Venny 2.1.0 (csic.es)) was used to determine the overlap between GV component-related targets and cholestasis-related targets. The overlapping targets were entered into STRING 11.0 (<https://string-db.org/>) for PPI network analysis, and the interaction results were input into Cytoscape 3.8.2 for visualization and analysis. The degree data were imported into Bioinformatics (bioinformatics.com.cn) to produce pencil histograms.

## 2.8 Pathway enrichment analysis

The intersection targets were imported into David (ncicrf.gov) for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. In GO, 'Biological Process' (BP), 'Cellular Component' (CC) and 'Molecular Function' (MF) were chosen in turn. These three options, respectively, describe the molecular function that the gene product is likely to perform, the cellular context in which it is found, and the biological process in which it is involved. Enrichment of KEGG pathways was also analyzed. The precision of the two approaches was set to 'Medium', and the data obtained were summarized and processed in Microsoft Excel. Finally, visualization and analysis of the outcomes were performed in Bioinformatics.

## 2.9 Components-targets-disease-pathways network construction

In this study, the network visualization software, Cytoscape, was used to model the network in order to systematically evaluate the intricate interrelationships between compounds, targets, and diseases. The node degree is defined as the number of direct connections made between a specific node and any other node in the network. A conforming network of major components of GV acting on targets and pathways associated with cholestatic liver injury was constructed after entering the selected targets and data into Cytoscape.

## 2.10 Molecular docking simulation

To further assess the validity of the network pharmacological data, molecular docking was used to evaluate the binding energies and potential binding modes of components with targets. Components with the highest degree of freedom in the 'drug-component-disease-pathway-target' network were selected for study. Meanwhile, the positive control drugs, UDCA and OCA were included for comparative analysis.

The 3D structures of the small molecule compounds were obtained from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) database in sdf format. Hydrogen atoms and charges were added using AutoDock Vina software [17], and the structures saved in pdbqt format as ligand files. The macromolecular protein target receptor pdb format files were downloaded from the RCSB PDB (<https://www.rcsb.org/>) database. Molecular docking simulations were then performed using AutoDock Vina to analyze the binding of each protein to ligand and obtain a docking score. Finally, the generated protein-ligand complexes were imported into PyMol for visualization. The structures of complexes was checked using the Proteins (<https://proteins.plus/>) website and the interaction force between protein and ligand was analyzed using the PLIP (<https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index>) website. Proteins with lower binding energies were considered to mediate the efficacy of GV against cholestasis.

## 3. Results

### 3.1 Identification of chemical components in GV by UPLC-MS/MS

The UPLC-MS/MS total ion chromatogram of GV extract in negative ion mode is shown in Fig. 2. The molecular formulas and mass fragments were matched to known components reported in the literature and cross-referenced with controls to initially identify 20 compounds, including nine iridoids, nine flavonoids and two other compounds (Table 1).

#### 3.1.1 Iridoids

Iridoid glycosides are the main active compounds found in the Gentianaceae family, of which loganic acid, gentiopicroside, swertiamarin, sweroside and their derivative glycosides are common parent nuclei [18]. The iridoid substructure is unstable, undergoing dehydrogenative cleavage to produce the excimer ion peak  $[M - H]^-$  at low energies. Removal of functional groups from the parent ring, accompanied by loss of neutral  $H_2O$ ,  $CO_2$ ,  $CH_3OH$ ,  $CH_3COOH$  and glycosyl groups, occurs at higher energies [19]. Addition of 0.1% formic acid to the mobile phase improved peak resolution, eliminated peak dragging, and produced the adduct  $[M - H + HCOOH]^-$ , which helped to confirm the deprotonated  $[M - H]^-$  (Fig. 3).

Compounds 1 and 3 gave rise to similar deprotonated ions  $[M - H]^-$  at 3.219 and 5.196 min, with the same molecular formula  $C_{16}H_{24}O_{10}$  at  $m/z$  375.1300 and 375.1295. Compound 3 yielded secondary characteristic fragments at  $m/z$  213.0764  $[M - H - Glc]^-$  and 169.0865  $[M - H - Glc - CO_2]^-$ , and comparison with the mass spectrum of standard identified it as loganic acid, one of the major iridoid compounds found in the Gentianaceae family [20]. Compound 1 was an isomer of loganic acid, which might be formed by attachment of the sugar to the hydroxyl group through different positions, and was identified as 8-epiloganic acid, which has been reported in iridoids isolated from *Linaria cymbalaria* [21]. Compound 17, with a molecular formula of  $C_{23}H_{28}O_{13}$  and a molecular ion peak of  $m/z$  511.1475  $[M - H]^-$ , had an additional fragment ion (dihydroxybenzoyl, molecular weight 137) compared with compound 1, and was presumed to be 2'-(2",3"-dihydroxybenzoyl)loganic acid based on the spectral peak information [22].

Compound 11 appeared as an adduct  $[M - H + HCOOH]^-$  at 7.542 min. The molecular formula was  $C_{16}H_{20}O_9$  and secondary fragment ions included  $m/z$  193.0488 and 179.0550. By comparison with the retention time and MS fragmentation modes of the standards, the structure of this compound was tentatively identified as gentiopicroside. Compound 6 at 6.374 min yielded an adduct ion at  $m/z$  563.1626  $[M - H + HCOOH]^-$ , with the molecular formula  $C_{22}H_{30}O_{14}$ , and fragment ions at  $m/z$  341.1100  $[M - H - OGlc]^-$  and  $m/z$  161.0456  $[M - H - Glc - O - OGlc]^-$ , which suggested the presence of two glycosyl groups. Compound 6 was presumed to be 6'-O- $\beta$ -D-glucosyl-gentiopicroside according to the cleavage pattern [23].

Compound 7 was cleaved in a similar manner to compound 12. At 6.687 min there was an adduct of compound 7  $[M + HCOO]^-$  with  $m/z$  419.1197 and a molecular formula of  $C_{16}H_{22}O_{10}$ , which was a typical iridoid compound with secondary characteristic fragments at  $m/z$  211.0612  $[M - H - 162]^-$  and 141.0195  $[M - H - Glc - C_2H_3 - CO]^-$ . Based on comparison with the standard mass spectrum, compound 7 was identified as swertiamarin. Compound 12 was present at 7.890 min with a peak at  $m/z$  403.1239  $[M + HCOO]^-$  and a molecular formula of  $C_{16}H_{22}O_9$ , which was identified as sweroside by comparison with the standard.

The spectrum of compound 18, with the molecular formula  $C_{22}H_{32}O_{14}$ , contained an adduct of  $m/z$  565.1611  $[M - H + HCOOH]^-$  at 6.530 min. The presence of a fragment ion of  $m/z$  195.0658  $[M - H - 162 - 162]^-$  indicated there were two glucose groups, so it was tentatively identified as swertiapunimarin [24].

The spectrum of compound 20, with molecular formula  $C_{16}H_{24}O_9$ , contained a peak at 4.720 min with  $m/z$  405.1411  $[M + COOH]^-$ . The fragment ion of  $m/z$  179.0544  $[M - H - C_6H_{10}O_5 - H_2O]^-$  could be explained by loss of one molecule of glucose ligand and one molecule of  $H_2O$ , which went on to generate a fragment ion of  $m/z$  119.0338  $[M - H - C_6H_{10}O_5 - H_2O - CH_3COOH]^-$  by loss of a  $CH_3COOH$  fragment. Therefore, the structure of the compound was preliminarily identified as plantarenalloside [25].

### 3.1.2 Flavonoids

Flavonoid glycosides contain sugar groups, particularly glucose, galactose, rhamnose, and rutinose, that produce  $[M - H - 162]^-$ ,  $[M - H - 162]^-$ ,  $[M - H - 146]^-$ , and  $[M - H - 308]^-$  fragment ions, respectively;  $[M - H - 132]^-$  is the fragment ion corresponding to loss of one five-carbon sugar, such as xylose. The hexasaccharide carboside of flavone C-glycoside produces  $[M - H - 60]^-$ ,  $[M - H - 90]^-$  and  $[M - H - 120]^-$  fragment ions (Fig. 4) [26].

The spectrum of compound 14, with formula  $C_{21}H_{20}O_{11}$ , contained a deprotonated ion peak at  $m/z$  447.0953  $[M - H]^-$  and fragment ions included  $m/z$  357.0629  $[M - H - 90]^-$ , 327.0517  $[M - H - 120]^-$  and 285.0409  $[M - H - 162]^-$ , resulting from partial or total loss of the C-glucose group. The compound was presumed to be isoorientin, which has previously been found in Gentianaceae [27, 28]. Compounds 8 and 5 were very similar to the glycoside ion of compound 14. The molecular formula of compound 8 was  $C_{27}H_{30}O_{16}$  and the deprotonated ion peak at  $m/z$  609.1489  $[M - H]^-$  was observed at 6.774 min. The fragment ions at  $m/z$  447.0971  $[M - H - 162]^-$  and 429.0861  $[M - H - 162 - H_2O]^-$  were attributed to loss of a hexose unit. The strong peak at  $m/z$  327.0522  $[M - H - 162 - 120]^-$  indicated that the hexose was C-glucosyl, tentatively identifying the compound as isoorientin 4'-O-glycoside [29]. Compound 5, with molecular formula  $C_{33}H_{40}O_{21}$ , exhibited a deprotonated ionic peak at 6.613 min with  $m/z$  771.2011  $[M - H]^-$ . The fragment ion at  $m/z$  447.0967  $[M - H - 2Glc]^-$  could be due to loss of two O-glucose groups and the strong peak at  $m/z$  327.0751  $[M - H - 2Glc - 120]^-$  demonstrated the presence of a C-glucose group. Thus, compound 5 was initially identified as isoorientin-4'-diglucoside [30].

The mass spectrum of compound 15, with formula  $C_{21}H_{20}O_{10}$ , revealed a deprotonated ion peak at  $m/z$  431.0988  $[M - H]^-$  at 10.516 min. In addition, scan results showed fragment ions at  $m/z$  311.0057  $[M - H - 90]^-$  and 283.0620  $[M - H - 120]^-$ , indicating a C-glucosyl group, as well as Retro Diels-Alder reaction (RDA) cleavage ions of flavonoids at  $m/z$  161.0247, which were consistent with data for isovitexin

previously found in Gentianaceae [31]. Compound 19, with molecular formula  $C_{33}H_{40}O_{20}$ , exhibited a deprotonated ionic peak at 7.157 min. The fragment ion at  $m/z$  431.1007  $[M - H - 2Glu]^-$  could be interpreted as the loss of two O-glucose groups. Two strong peaks at  $m/z$  341.0683  $[M - H - 2Glc - 90]^-$  and 311.0569  $[M - H - 2Glc - 120]^-$  revealed the presence of another C-glucose group. In summary, these  $m/z$  fragments were similar to those of compound 15, so compound 19 was presumed to be isovitexin 7-O-diglucoside [32].

Compounds 9 and 13 at 6.930 and 8.647 min, respectively, exhibited deprotonated ionic peaks at  $m/z$  593.1522 and 593.1531  $[M - H]^-$  with the formula  $C_{27}H_{30}O_{15}$ . The fragment ions at  $m/z$  503.1245  $[M - H - 90]^-$ , 473.1126 and 473.1143  $[M - H - 120]^-$ , 341.0686  $[M - H - 90 - 162]^-$  and 311.0575  $[M - H - 120 - 162]^-$  illustrated the presence of fragmented C-glucosyl and O-glucosyl groups. The additional fragments at  $m/z$  282.0538, 281.0466 and 283.0622 were similar to those in the spectrum of compound 15, isovitexin. One was tentatively identified as isosaponarin, with a cLogP value of  $-0.697495$ , and the other as saponarin, with a cLogP value of  $-0.642387$ . A smaller value of cLogP is indicative of better hydrophilicity and an earlier peak time on the C18 column. Based on their peak times, compounds 9 and 13 were presumed to be isosaponarin and saponarin, respectively, which have previously been identified in GV [33, 34].

The spectrum of compound 10, with formula  $C_{28}H_{32}O_{16}$ , contained a deprotonated ionic peak at  $m/z$  623.1638. There was also a peak at  $m/z$  503.1224  $[M - H - 90 - 30]^-$  that could be presumed to be due to replacement of a hydrogen in isosaponarin by  $-OCH_3$ , suggesting isoscaparin-4'-glucoside [34]. Compound 16, with formula  $C_{22}H_{22}O_{11}$ , produced a deprotonated ionic peak at 11.184 min with  $m/z$  461.1092  $[M - H]^-$ , which could be attributed to generation of isoscaparin by loss of one O-glucose group from compound 10 [35].

### 3.1.3 Other categories

Compound 2 at 3.944 min produced a deprotonated ionic peak at  $m/z$  301.0929  $[M - H]^-$  with the formula  $C_{13}H_{18}O_8$ , and a fragment ion at  $m/z$  179.0555  $[Glu - H]^-$ , tentatively identifying the compound as tachioside (Fig. 5) [36].

The spectrum of compound 4, with molecular formula  $C_{16}H_{22}O_{11}$ , contained a deprotonated ion peak at 5.989 min with  $m/z$  389.1089  $[M - H]^-$ . The fragment ion at  $m/z$  345.1209  $[M - H - CO_2]^-$  could be attributed to the loss of carbon dioxide, and the fragment at  $m/z$  165.0555  $[M - H - CO_2 - Glc - H_2O]^-$  indicating subsequent loss of one molecule of glucose and one molecule of water. The structure was presumed to be secologanoside based on literature data (Fig. 5) [37].

Table 1  
Characterization of chemical constituents in GV by the UPLC-MS/MS.

| NO. | RT<br>(min) | [M-H]<br>(m/z) | MS/MS fragments                                                             | Formula                                         | Compounds                                      | References |
|-----|-------------|----------------|-----------------------------------------------------------------------------|-------------------------------------------------|------------------------------------------------|------------|
| 1   | 3.219       | 375.1300       | 375.1313,213.0761,169.0860,113.0233                                         | C <sub>16</sub> H <sub>24</sub> O <sub>10</sub> | 8-Epiloganic acid                              | [21]       |
| 2   | 3.944       | 301.0929       | 301.0927,179.0555,121.0301,119.0355,89.0265                                 | C <sub>13</sub> H <sub>18</sub> O <sub>8</sub>  | Tachioside                                     | [36]       |
| 3   | 5.196       | 375.1295       | 375.1311,213.0764,169.0865,151.0761,69.0379                                 | C <sub>16</sub> H <sub>24</sub> O <sub>10</sub> | Loganic acid                                   | [20]       |
| 4   | 5.989       | 389.1089       | 389.1106,345.1209,209.0457,<br>165.0555,121.0671,69.0382                    | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub> | Secologanoside                                 | [37]       |
| 5   | 6.163       | 771.2011       | 771.2119,447.0967,327.0517                                                  | C <sub>33</sub> H <sub>40</sub> O <sub>21</sub> | Isoorientin-4'-diglucoside                     | [30]       |
| 6   | 6.374       | 563.1626       | 563.1677,517.1612,341.1100,251.0775,<br>221.0667,179.0560,161.0456          | C <sub>22</sub> H <sub>30</sub> O <sub>14</sub> | 6'-O-β-D-glucosyl-gentiopicroside              | [23]       |
| 7   | 6.687       | 419.1197       | 419.1229,355.1060,235.0617,211.0612,<br>179.0564,141.0195,119.0355,89.0265  | C <sub>16</sub> H <sub>22</sub> O <sub>10</sub> | Swertiamarin                                   | Controls   |
| 8   | 6.774       | 609.1489       | 609.1542,489.1086,447.0971,429.0861,<br>327.0522,298.0497                   | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | Isoorientin 4'-O-glucoside                     | [29]       |
| 9   | 6.930       | 593.1522       | 593.1588,515.1253,503.1245,473.1126,<br>341.0686,311.0575,282.0538,281.0466 | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | Isosaponarin                                   | [33–34]    |
| 10  | 7.455       | 623.1638       | 623.1688,545.1358,533.1346,503.1224,<br>370.0710,341.0677,312.0643,297.0412 | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | Isoscoparin 4'-glucoside                       | [34]       |
| 11  | 7.542       | 401.1081       | 401.1109,355.1045,193.0488,179.0550,<br>119.0348,89.0260                    | C <sub>16</sub> H <sub>20</sub> O <sub>9</sub>  | Gentiopicroside                                | Controls   |
| 12  | 7.890       | 403.1239       | 403.1255,357.1199,195.0654,125.0246,81.0365                                 | C <sub>16</sub> H <sub>22</sub> O <sub>9</sub>  | Sweroside                                      | Controls   |
| 13  | 8.647       | 593.1531       | 593.1580,473.1143,431.1017,341.0686,<br>311.0575,297.0417,283.0622          | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | saponarin                                      | [33–34]    |
| 14  | 9.051       | 447.0953       | 447.0958,357.0629,327.0517,285.0409,<br>284.0334,133.0296                   | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | Isoorientin                                    | [27–28]    |
| 15  | 10.516      | 431.0988       | 431.1034,353.0699,341.0688,311.0575,<br>283.0620,281.0469,268.0391,161.0247 | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | Isovitexin                                     | [31]       |
| 16  | 11.184      | 461.1092       | 461.1107,383.0786,371.0779,341.0670,<br>298.0487,297.0421,299.0570          | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub> | Isoscoparin                                    | [35]       |
| 17  | 12.736      | 511.1475       | 511.1504,153.0189,109.0301                                                  | C <sub>23</sub> H <sub>28</sub> O <sub>13</sub> | 2'-(2",3"-<br>dihydroxybenzoyl)loganic<br>acid | [22]       |
| 18  | 6.530       | 565.1776       | 565.1811,519.1765,323.0994,195.0658,<br>125.0244,81.0362                    | C <sub>22</sub> H <sub>32</sub> O <sub>14</sub> | Swertiapunimarin                               | [24]       |
| 19  | 7.157       | 755.2066       | 755.2142,575.1452,431.1007,<br>341.0683,311.0569                            | C <sub>33</sub> H <sub>40</sub> O <sub>20</sub> | Isovitexin 7-O-diglucoside                     | [32]       |
| 20  | 4.720       | 405.1411       | 405.1415,179.0544,178.4854,<br>119.0338,89.0244                             | C <sub>16</sub> H <sub>24</sub> O <sub>9</sub>  | plantarenalosside                              | [25]       |

## 3.2 Acquisition of GV extract component-related targets

All targets of GV component compounds were obtained from SwissTargetPrediction, TCMSP, SEA, and STITCH databases, which yielded 299 GV component-related targets after de-duplication using Microsoft Excel.

## 3.3 Identification of targets related to cholestatic disease

The GeneCards database was used to obtain relevant targets using 'cholestasis', 'cholestatic hepatic injury', 'cholestatic liver injury', 'primary biliary cirrhosis' and 'primary cholangitis' as keywords. A total of 2999 relevant targets were collected after removing duplicates, using protein coding in the category column as an indicator.

## 3.4 Protein-protein interaction network

The GV component-related targets were combined with disease targets and imported into Venny 2.1 software to obtain 103 intersecting targets (Fig. 6). The PPI network was constructed by importing the intersecting genes into STRING, and targets with medium confidence (0.4) were taken for the graph. The network contained 103 nodes and 857 edges, with an average node degree value of 16.6 and PPI enrichment  $P$  value  $< 1.0e - 16$  (Fig. 7), which was later visualized using Cytoscape.

Cytoscape was utilized to analyze the network topological parameters. 'Degree' indicated the impact of each target to a certain extent, so a high 'degree' value was used to determine core targets in the PPI network. According to the results, the top 6 degrees were selected as core targets, which were AKT1, GAPDH, TNF, IL6, VEGFA and CASP3. These core targets were then used for molecular docking validation. The top ten degree values were plotted, as shown in Fig. 8.

## 3.5 Gene ontology term enrichment

To further analyze the efficacy of GV extracts in the therapy of cholestasis, 103 intersecting targets were input into the DAVID database for GO analysis. Setting  $P < 0.05$ , there were 350 items correlated with BP, 43 items correlated with CC and 92 items correlated with MF. The top ten entries were sorted by  $P$ -value from smallest to largest, and bar graphs were plotted using Bioinformatics for visualization. The results are shown in Fig. 9 and Table 2. It was found that the biological processes associated with GV treatment of cholestasis mainly included proteolysis, response to xenobiotic stimulus, and negative regulation of apoptotic processes. The cellular composition mainly included cytosol, caspase complex, and cytoplasm, and the molecular functions mainly included enzyme binding, identical protein binding and endopeptidase activity. GO enrichment results suggested that the efficacy of GV extract in the treatment of cholestatic diseases was mediated by several biological processes and molecular functions.

Table 2  
The results of GO function enrichment analysis

| No. | Category | Gene Ontology                                                                 | count | P-value     |
|-----|----------|-------------------------------------------------------------------------------|-------|-------------|
| 1   | BP       | proteolysis                                                                   | 19    | 8.80E-12    |
| 2   | BP       | response to xenobiotic stimulus                                               | 15    | 7.90E-11    |
| 3   | BP       | negative regulation of apoptotic process                                      | 19    | 3.10E-10    |
| 4   | BP       | positive regulation of protein phosphorylation                                | 13    | 1.60E-09    |
| 5   | BP       | positive regulation of cell proliferation                                     | 18    | 4.40E-09    |
| 6   | BP       | response to hypoxia                                                           | 11    | 1.50E-08    |
| 7   | BP       | execution phase of apoptosis                                                  | 6     | 1.30E-07    |
| 8   | BP       | glycolytic process                                                            | 7     | 1.40E-07    |
| 9   | BP       | positive regulation of apoptotic process                                      | 13    | 1.90E-07    |
| 10  | BP       | positive regulation of smooth muscle cell proliferation                       | 7     | 6.70E-07    |
| 11  | CC       | cytosol                                                                       | 63    | 3.5E-12     |
| 12  | CC       | caspase complex                                                               | 6     | 6.5E-11     |
| 13  | CC       | cytoplasm                                                                     | 61    | 1.3E-10     |
| 14  | CC       | extracellular space                                                           | 30    | 0.000000082 |
| 15  | CC       | cyclin-dependent protein kinase holoenzyme complex                            | 6     | 0.0000026   |
| 16  | CC       | membrane raft                                                                 | 10    | 0.000004    |
| 17  | CC       | extracellular exosome                                                         | 26    | 0.0001      |
| 18  | CC       | macromolecular complex                                                        | 13    | 0.00019     |
| 19  | CC       | nucleus                                                                       | 49    | 0.00022     |
| 20  | CC       | cell surface                                                                  | 12    | 0.00041     |
| 21  | MF       | enzyme binding                                                                | 19    | 3.1E-12     |
| 22  | MF       | identical protein binding                                                     | 31    | 6.4E-09     |
| 23  | MF       | endopeptidase activity                                                        | 9     | 0.000000011 |
| 24  | MF       | metallopeptidase activity                                                     | 8     | 0.00000015  |
| 25  | MF       | cysteine-type endopeptidase activity involved in apoptotic process            | 5     | 0.00000018  |
| 26  | MF       | cysteine-type endopeptidase activity involved in execution phase of apoptosis | 5     | 0.00000043  |
| 27  | MF       | aminopeptidase activity                                                       | 6     | 0.00000073  |
| 28  | MF       | glucose binding                                                               | 5     | 0.00000086  |
| 29  | MF       | peptidase activity                                                            | 8     | 0.0000019   |
| 30  | MF       | protein homodimerization activity                                             | 17    | 0.0000025   |

### 3.6 KEGG pathway analysis

KEGG analysis of 103 intersecting targets was performed using the DAVID database. Up to 127 pathways were obtained using  $P < 0.05$  as the screening criterion. The top 20 pathways were ranked according to  $P$ -value and visualized using Bioinformatics to draw bubble plots for analysis, as shown in Fig. 10 and Table 3. The main pathways involved in anti-cholestatic activity of GV extract were found to be Pathways in cancer, Lipid and atherosclerosis, HIF-1 signaling pathway, TNF signaling pathway, IL-17 signing pathway and Prostate cancer, etc.

Table 3  
The information of the top 20 pathways based on KEGG pathway enrichment analysis

| NO.      | Pathway                                         | P Value  | count | Genes                                                                                                                                                                                    |
|----------|-------------------------------------------------|----------|-------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| hsa05200 | Pathways in cancer                              | 1.20E-12 | 29    | HDAC1, FGF1, PTGS2, FGF2, RELA, EGFR, IKBKB, CASP7, CASP8, CCND1, TERT, CASP3, AKT1, HMOX1, MAPK1, NQO1, HSP90AA1, DAPK1, MMP1, F2, MMP9, IL2, VEGFA, CCNA2, AR, IL6, CDK4, CDK2, NFE2L2 |
| hsa05417 | Lipid and atherosclerosis                       | 1.50E-11 | 19    | HSPA8, HSP90AA1, MMP1, MMP3, TNF, MMP9, RELA, SELP, IKBKB, CASP7, IL6, CASP8, CD40LG, CASP6, CASP3, CASP1, AKT1, MAPK1, NFE2L2                                                           |
| hsa04066 | HIF-1 signaling pathway                         | 2.10E-09 | 13    | ENO1, ENO2, EGFR, RELA, HK2, HK1, VEGFA, TF, IL6, AKT1, HMOX1, MAPK1, GAPDH                                                                                                              |
| hsa04668 | TNF signaling pathway                           | 2.90E-09 | 13    | MMP3, PTGS2, TNF, MMP9, RELA, IKBKB, CASP7, IL6, CASP8, CREB1, CASP3, AKT1, MAPK1                                                                                                        |
| hsa04657 | IL-17 signaling pathway                         | 5.50E-09 | 12    | IKBKB, IL6, HSP90AA1, CASP8, MMP1, CASP3, MMP3, MAPK1, PTGS2, TNF, MMP9, RELA                                                                                                            |
| hsa05215 | Prostate cancer                                 | 7.70E-09 | 12    | IKBKB, AR, HSP90AA1, CREB1, CCND1, CDK2, MMP3, MAPK1, AKT1, MMP9, RELA, EGFR                                                                                                             |
| hsa05219 | Bladder cancer                                  | 1.20E-08 | 9     | CCND1, MMP1, CDK4, DAPK1, MAPK1, MMP9, EGFR, TYMP, VEGFA                                                                                                                                 |
| hsa04625 | C-type lectin receptor signaling pathway        | 1.90E-07 | 11    | IKBKB, IL6, CASP8, CASP1, MAPK1, AKT1, PTPN11, PTGS2, TNF, RELA, IL2                                                                                                                     |
| hsa05165 | Human papillomavirus infection                  | 5.70E-07 | 17    | HDAC1, PTGS2, TNF, EGFR, RELA, VEGFA, CCNA2, IKBKB, CASP8, CREB1, TERT, CCND1, CDK4, CASP3, CDK2, AKT1, MAPK1                                                                            |
| hsa05166 | Human T-cell leukemia virus 1 infection         | 8.70E-07 | 14    | MMP7, TNF, IL2, RELA, CCNA2, IKBKB, IL6, CREB1, TERT, CCND1, CDK4, CDK2, AKT1, MAPK1                                                                                                     |
| hsa05163 | Human cytomegalovirus infection                 | 1.00E-06 | 14    | PTGS2, TNF, EGFR, RELA, VEGFA, IKBKB, IL6, CASP8, CREB1, CCND1, CDK4, CASP3, AKT1, MAPK1                                                                                                 |
| hsa05167 | Kaposi sarcoma-associated herpesvirus infection | 1.30E-06 | 13    | TGS2, FGF2, RELA, VEGFA, IKBKB, IL6, CASP8, CREB1, CCND1, CDK4, CASP3, AKT1, MAPK1                                                                                                       |
| hsa05161 | Hepatitis B                                     | 1.50E-06 | 12    | CCNA2, IKBKB, IL6, CASP8, CREB1, CASP3, CDK2, MAPK1, AKT1, TNF, MMP9, RELA                                                                                                               |
| hsa04210 | Apoptosis                                       | 2.30E-06 | 11    | IKBKB, CASP7, CASP8, CASP6, CASP3, CASP2, MAPK1, AKT1, TNF, RELA, MCL1                                                                                                                   |
| hsa05134 | Legionellosis                                   | 2.80E-06 | 8     | HSPA8, IL6, CASP7, CASP8, CASP3, CASP1, TNF, RELA                                                                                                                                        |
| hsa05162 | Measles                                         | 2.80E-06 | 11    | IKBKB, HSPA8, IL6, CASP8, CCND1, CDK4, CASP3, CDK2, AKT1, RELA, IL2                                                                                                                      |
| hsa04151 | PI3K-Akt signaling pathway                      | 6.80E-06 | 16    | HSP90AA1, FGF1, FGF2, EGFR, IL2, RELA, VEGFA, IKBKB, IL6, CREB1, CCND1, CDK4, CDK2, AKT1, MAPK1, MCL1                                                                                    |
| hsa04218 | Cellular senescence                             | 7.90E-06 | 11    | CCNA2, IL6, CCNB1, CCND1, CDK4, CDK2, CDK1, MAPK1, AKT1, SIRT1, RELA                                                                                                                     |
| hsa05160 | Hepatitis C                                     | 8.40E-06 | 11    | IKBKB, CASP8, CCND1, CDK4, CASP3, CDK2, MAPK1, AKT1, TNF, RELA, EGFR                                                                                                                     |
| hsa05171 | Coronavirus disease - COVID-19                  | 8.60E-06 | 13    | MMP1, MMP3, F2, TNF, EGFR, IL2, RELA, SELP, IKBKB, IL6, ADAM17, CASP1, MAPK1                                                                                                             |

### 3.7 Components-targets-disease-pathways network construction

A 'drug-component-disease-pathway-target' network was established using Cytoscape, as shown in Fig. 11. In the figure, red nodes represent GV, green nodes represent cholestatic diseases, blue nodes represent core components of GV, purple nodes represent key pathways of the

disease, and yellow nodes represent common targets. Further study revealed that the top five compounds in GV in terms of freedom included compound 11 (gentiopicroside), compound 13 (saponarin), compound 15 (isovitexin), compound 18 (swertiapunimarin) and compound 20 (plantarenaloside), suggesting that these five compounds may be the core components of GV for the treatment of cholestasis.

### 3.8 Molecular docking visualization

The binding force between drugs and target proteins can be used as an important index to evaluate the mechanism of action of drugs in the treatment of diseases [38]. The five compounds with the highest target freedom in the 'drug-component-disease-pathway-target' network were used for molecular docking with six core proteins from the PPI network. The positive control drugs UDCA and OCA were added for comparative analysis.

In general, lower binding energy of the ligand to the receptor indicates that the binding conformation is more stable. The docking results revealed that the five principal components of GV bound well to all six protein receptors. Among the compounds, swertiapunimarin and isovitexin gave slightly better docking scores than the positive control drugs with AKT1, GAPDH, TNF, IL6 and CASP3, and saponarin had a similar binding effect to the six proteins as the positive control drugs. Different binding sites may affect the binding affinity of the main GV compounds to target proteins, and thus play a role in regulating cholestasis. Swertiapunimarin and isovitexin exhibited strong affinity for TNF, binding to LEU-57 and ILE-155 through hydrophobic interactions and to SER-60, SER-9 and TYR-151 through hydrogen bonding, in addition to making  $\pi$ -bonding interactions. Saponarin exhibited strong affinity for AKT1 and bound mainly through hydrophobic interactions with TRP-80, LEU-264 and TYR-272, hydrogen bonding to ASN-279, TYR-18 and ASP-274, and  $\pi$ -bonding interactions. The interacting residues are shown in the appendix, the binding energy magnitudes are shown in Table 4, the molecular docking model is shown in Fig. 12 and the 3D docking structures are shown in Fig. 13.

Table 4  
Binding energy results of molecular docking

| Ingredient            | Binding energy(kcal/mol) |       |       |      |       |       |
|-----------------------|--------------------------|-------|-------|------|-------|-------|
|                       | AKT1                     | GAPDH | TNF   | IL6  | VEGFA | CASP3 |
| Plantarenaloside      | -8.6                     | -7.2  | -9.3  | -4.3 | -7    | -7    |
| Gentiopicroside       | -8.6                     | -7.3  | -9.2  | -4.7 | -6.7  | -7.3  |
| Swertiapunimarin(L18) | -9.8                     | -8.4  | -10.2 | -5.4 | -7.2  | -8.2  |
| Isovitexin(L15)       | -10.2                    | -8.6  | -10.6 | -4.9 | -7.5  | -8.1  |
| Saponarin(L13)        | -10.3                    | -8.5  | -9.1  | -5   | -8    | -7.9  |
| OCA                   | -10.3                    | -8.3  | -8.4  | -4.7 | -7    | -7.4  |
| UDCA                  | -9.9                     | -8    | -7.9  | -4.9 | -8.3  | -7.4  |

### 4. Discussion

Cholestasis is a clinical syndrome caused by defective secretion, absorption, and flow of bile, resulting in retention of bile components in the liver and serum [39]. The condition, which is most commonly seen in patients with liver diseases such as primary cholangitis, primary sclerosing cholangitis and viral hepatitis, may be caused by a variety of factors, including toxic compounds, drug-induced liver injury, disease (viral hepatitis, bile duct obstruction, sepsis, cholelithiasis), genetic abnormalities (progressive familial intrahepatic cholestasis) and disorders of the intestinal microbiota [40]. The clinical symptoms are usually jaundice and pruritus [41]. Although development of new drugs for treatment of cholestasis is severely hampered by the complex pathogenesis and mechanism of injury, the search for drugs to treat biliary liver injury has been a focus of research in recent years.

As the concept of multi-component and multi-target treatment in traditional Chinese medicine has developed, current treatment of chronic metabolic diseases involves the use of single or compound preparations. At the same time, because of fewer adverse reactions and relatively weak drug dependence, traditional Chinese medicine has attracted increasing attention [42]. In this study, GV was the focus of research, and an extract of GV was obtained using ultrasound. The main chemical components were identified using UPLC-MS/MS. The major compounds included nine iridoid compounds and their glycosides (1, 3, 6, 7, 11, 12, 17, 18, 20), nine flavonoids and their glycosides (5, 8, 9, 10, 13, 14, 15, 16, 19) and two other compounds and their glycosides (2, 4). Among the 20 compounds resolved from the GV extract, compounds 2, 3, 5–9, 11, 12, 14–16 and 19 have previously been found in GV [43–47]. It was of interest that compounds 1, 4, 10, 13, 17, 18

and 20 had not been reported in the literature to be present in GV extracts and were identified from this plant for the first time. The main compounds identified were subsequently investigated to validate GV for the treatment of cholestasis in the field of traditional Chinese medicine and to promote the development and utilization of traditional Tibetan medicine GV.

Network pharmacology has become a promising tool for understanding complex Chinese traditional medicines [48]. A 'drug-component-disease-pathway-target' network was constructed using Cytoscape software, in which the five compounds with highest freedom were mainly iridoids (compounds 11, 18 and 20) and flavonoids (compounds 13 and 15). Many studies have shown that these two types of compounds are useful in the treatment of cholestatic diseases [49]. Han et al. [50] discovered that gentiopicoside had a therapeutic effect on cholestasis induced by  $\alpha$ -naphthol isothiocyanate (ANIT) in a comprehensive proteomic and metabolomic study. Ginsenoside Rg1 showed significant hepatoprotective activity in ANIT-induced cholestatic rats, which may be correlated with action on the Sirt5/Nrf2 pathway as well as NF- $\kappa$ B protein [51]. Shi et al. [52] found that the iridoid glycoside swertiamarin reduced ANIT-induced cholestasis by activating the farnesoid X receptor and bile acid excretion pathways. Hua et al. [53] confirmed that an iridoid glycoside, the main active component of *Veronica ciliata* Fisch., protected the liver from cholestatic liver injury by regulating oxidative stress, inflammatory response, and bile acid transport, thus playing a hepatoprotective effect. Wang et al. [54] showed that geniposide, one of the major iridoid glycosides isolated from *Gardenia jasminoides* Ellis, alleviated ANIT-induced cholestasis in rats by regulating bile acid homeostasis transporters and enzymes. Xiang et al. [55] reported that the isoflavone tectorigen reduced the activity of hepatic macrophages by activating expression of PPAR $\gamma$  to reduce the inflammatory response, and enhanced the efflux of bile acids to prevent the accumulation of bile in the liver, thus exerting therapeutic effects for the treatment of cholestasis. Zhang et al. [56] proposed that the flavone glycoside hesperidin, which is abundant in lemons and oranges, exerts protective effects against cholestasis in vivo and in vitro by activating the FXR receptor.

The core target proteins AKT1, GAPDH, TNF, IL6, VEGFA and CASP3 were predicted to be highly correlated with cholestasis. For example, AKT1 has been reported to be associated with the regulation of hepatocellular carcinoma and the progression of cholestatic liver disease [57, 58]. In addition, IL6 and TNF are inflammatory biomarkers of cholestatic liver injury, and TNF signaling may be a significant anti-cholestatic mechanism [59, 60]. Yang et al. [61] showed that expression of Pescadillo homolog 1 (Pes1) was reduced in mice with cholestatic liver disease. The low levels of Pes1 resulted in inactivation of PI3K/AKT/GSK-3 $\beta$  signaling, decreasing levels of phosphorylated PI3K and AKT proteins and subsequent signaling, ultimately downregulating expression of phosphorylated GSK-3 $\beta$ . This reduced the inhibitory effect of phosphorylated GSK-3 $\beta$  on hepatocyte apoptosis and attenuated cell damage, thus aggravating the process of cholestatic liver disease. Brandes et al. [62] demonstrated that taurine deoxycholic acid, a drug approved for cholestasis, promoted expression of related proteins in human hepatocellular carcinoma cells by inducing activation of AKT1, demonstrating for the first time that the bile acid signaling pathway could affect the expression of Rho proteins. Yao et al. [63] found that dioscin conferred good resistance to ANIT-induced acute liver injury, potentially mediated by the PI3K/Akt signaling pathway, inhibiting the expression of inflammatory factors IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , and playing a role in anti-inflammatory, antioxidant, anti-apoptosis and lipid metabolism regulation.

Liu et al. [64] showed that isovitexin inhibited the cisplatin -induced inflammatory response by inhibiting production of TNF- $\alpha$ , IL-1 and IL-6 in renal tissue, thus playing a protective role in renal injury. Wan et al. [65] found that leflunomide and/or methotrexate combined with gentiopicoside exerted hepatoprotective effects by decreasing expression of NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 to reduce the inflammatory response. Wei et al. [66] found that extracts of *herpetospermum pedunculosum* inhibited NF- $\kappa$ B signaling and restored normal levels of inflammatory factors TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 to attenuate the inflammatory response and relieve cholestatic accumulation in rats. Glycyrrhetic acid has been reported to have a protective effect against ANIT-induced cholestasis and may reduce hepatotoxicity by inhibiting NF- $\kappa$ B signaling and reducing serum TNF- $\alpha$  concentrations. The compound is considered to have potential for the treatment of cholestatic diseases [67].

In summary, based on our study of 9 iridoid compounds, 9 flavonoids and 2 other compounds identified in GV by UPLC-MS/MS, compounds 11, 13, 15, 18 and 20 are responsible for the activity of GV in the treatment of cholestasis. This study analyzed 299 GV component targets and 2999 targets potentially involved in cholestasis using network pharmacology. The results revealed that GV may act through biological pathways such as proteolysis, cytosol, caspase complex, enzyme binding, as well as Pathways in cancer, Lipid and atherosclerosis, HIF-1 signaling pathway, TNF signaling pathway, IL-17 signaling pathway and other key signaling pathways for the treatment of cholestatic diseases. The molecular docking results validated that the predicted major chemical components and key targets had good binding affinity and the binding energies were similar to those of the positive control drugs, supporting the predictions made in this study. However, the relevant target pathways need to be validated in vivo and in vitro and the specific mechanisms have yet to be elucidated.

## 5. Conclusions

In this study, the main chemical components of GV extract were analyzed by UPLC-MS/MS. Mechanisms of action were predicted by network pharmacology, and the results were verified by molecular docking. This study lays a foundation for further research on the

mechanisms of action of GV, providing suggestions for consideration. Our research group will next carry out cell and animal experiments to explore the therapeutic effects of GV extract in the cholestasis model. Reverse transcription polymerase chain reaction, western blotting and other methods will be used to further verify the key targets and pathways predicted by network pharmacology. In a nutshell, this research provides potential therapeutic approaches for the treatment of cholestatic liver disease with GV extract, and is a basis for further research into the treatment of cholestatic diseases with traditional Tibetan medicine

## Declarations

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### Conflict of interest

There is no conflict of interest between the authors of this article.

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## References

1. Wu QY, Wong ZC, Wang C, Fung AH, Wong EO, Chan GK, et al. Isoorientin derived from *Gentiana veitchiorum* Hemsl. flowers inhibits melanogenesis by down-regulating MITF-induced tyrosinase expression. *Phytomedicine*. 2019;57: 129-36. doi: 10.1016/j.phymed.2018.12.006.
2. Li S, Wan C, He L, Yan Z, Wang K, Yuan M, et al. Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS. *Revista Brasileira de Farmacognosia*. 2017;27(2):188-94. doi: 10.1016/j.bjp.2016.10.003.
3. Li S, Wan CX, Yuan MH, Liu X, Zhao Y, Zhang ZF. Study on flavonoid glycosides from *Gentiana veitchiorum*. *Chinese Traditional and Herbal Drugs*. 2016;47(15):2597-600.
4. Li S, Chemical Constituents from *Gentiana veitchiorum*. Southwest University for Nationalities, P. R. China. 2017.
5. Jansen PL, Ghallab A, Vartak N, Reif R, Schaap FG, Hampe J, et al. The ascending pathophysiology of cholestatic liver disease. *Hepatology*. 2017;65(2):722-38. doi: 10.1002/hep.28965.
6. Yang F, Wang Y, Li G, Xue J, Chen ZL, Jin F, et al. Effects of corilagin on alleviating cholestasis via farnesoid X receptor-associated pathways in vitro and in vivo. *Br J Pharmacol*. 2018;175(5):810-29. doi: 10.1111/bph.14126.
7. Chinese Society of Hepatology CSOG, Chinese Society of Infectious D. Chinese consensus on the diagnosis and management of autoimmune hepatitis (2015). *J Dig Dis*. 2017;18(5):247-64. doi: 10.1111/1751-2980.12479.
8. Wagner M, Fickert P. Drug Therapies for Chronic Cholestatic Liver Diseases. *Annual review of pharmacology and toxicology*. 2020;60: 503-27. doi: 10.1146/annurev-pharmtox-010818-021059.
9. Phaw NA, Leighton J, Dyson JK, Jones DE. Managing cognitive symptoms and fatigue in cholestatic liver disease. *Expert Rev Gastroenterol Hepatol*. 2021;15(3):235-41. doi: 10.1080/17474124.2021.1844565.
10. Floreani A, Mangini C. Primary biliary cholangitis: Old and novel therapy. *Eur J Intern Med*. 2018; 47:1-5. doi: 10.1016/j.ejim.2017.06.020.
11. Kowdley KV, Luketic V, Chapman R, Hirschfield GM, Poupon R, Schramm C, et al. A randomized trial of obeticholic acid monotherapy in patients with primary biliary cholangitis. *Hepatology*. 2018;67(5):1890-902. doi: 10.1002/hep.29569.

12. Wang X, Wang ZY, Zheng JH, Li S. TCM network pharmacology: A new trend towards combining computational, experimental and clinical approaches. *Chinese journal of natural medicines*. 2021;19(1):1-11. doi: 10.1016/S1875-5364(21)60001-8.
13. Li S, Zhang B. Traditional Chinese medicine network pharmacology: theory, methodology and application. *Chinese journal of natural medicines*. 2013;11(2):110-20. doi: 10.1016/S1875-5364(13)60037-0.
14. Liang X, Li H, Li S. A novel network pharmacology approach to analyse traditional herbal formulae: the Liu-Wei-Di-Huang pill as a case study. *Mol Biosyst*. 2014;10(5):1014-22. doi: 10.1039/c3mb70507b.
15. Lai X, Wang X, Hu Y, Su S, Li W, Li S. Editorial: Network Pharmacology and Traditional Medicine. *Front Pharmacol*. 2020; 11:1194. doi: 10.3389/fphar.2020.01194.
16. Wang M, Liu F, Yao Y, Zhang Q, Lu Z, Zhang R, et al. Network pharmacology-based mechanism prediction and pharmacological validation of Xiaoyan Lidan formula on attenuating alpha-naphthylisothiocyanate induced cholestatic hepatic injury in rats. *J Ethnopharmacol*. 2021;270: 113816. doi: 10.1016/j.jep.2021.113816.
17. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. 2010;31(2):455-61. doi: 10.1002/jcc.21334.
18. Yuan H-Y, Kwaku OR, Pan H, Han J-X, Yang C-R, Xu MJNPC. Iridoid glycosides from the Genus *Gentiana* (Gentianaceae) and their Chemotaxonomic Sense. 2017;12(10):1934578X1701201035.
19. Liang XP, Ji S, Du SB, Dong ZQ, Chen XW, Yang AF, Xie HC. Analysis of Chemical Constituents in Different Parts of *Gentiana straminea* Based on UPLC-Q-TOF-MS/MS. *Chinese Journal of Experimental Traditional Medical Formulae*. 2022;28(08)139-48.
20. Suryawanshi S, Mehrotra N, Asthana RK, Gupta RC. Liquid chromatography/tandem mass spectrometric study and analysis of xanthone and secoiridoid glycoside composition of *Swertia chirata*, a potent antidiabetic. *Rapid Commun Mass Spectrom*. 2006;20(24):3761-8. doi: 10.1002/rcm.2795.
21. Bianco A, Passacantilli P, Polidori G. 8-epiloganic acid and 7-beta-hydroxy-8-epiiridodial glucoside. *Planta Med*. 1982;46(1):38-41. doi: 10.1055/s-2007-970014.
22. Olennikov DN, Chirikova NK. Algidisides I and II, New Iridoid Glycosides from *Gentiana algida*. *Chemistry of Natural Compounds*. 2016;52(4):637-41. doi: 10.1007/s10600-016-1728-y.
23. Kikuchi M, Kakuda R, Kikuchi M, Yaoita Y. Secoiridoid Glycosides from *Gentiana scabra*. *Journal of Natural Products*. 2005;68(5):751-3. doi: 10.1021/np058017o.
24. Tan P, Liu YL, Hou CY. Structure of purplish bitter glycosides in purple red swertia. *Acta Pharmaceutica Sinica*. 1997;07:522-25.
25. Andrzejewska-Golec E, Ofterdinger-Daegel S, Calis I, Åšwiątek L. Chemotaxonomic aspects of iridoids occurring in *Plantago subg. Psyllium* (Plantaginaceae). *Plant Systematics and Evolution*. 1993;185(1):85-9. doi: 10.1007/BF00937721.
26. Wu XA, Du MQ. Rule of ESIMS-MS on C-glycosy flavonnes. *Natural Product Research and Development*. 2011;23(06):1085-7.
27. Wu QY, Wong ZC, Wang C, Fung AH, Wong EO, Chan GK, et al. Isoorientin derived from *Gentiana veitchiorum* Hemsl. flowers inhibits melanogenesis by down-regulating MITF-induced tyrosinase expression. *Phytomedicine*. 2019; 57:129-36. doi: 10.1016/j.phymed.2018.12.006.
28. Sasaki N, Nishizaki Y, Yamada E, Tatsuzawa F, Nakatsuka T, Takahashi H, et al. Identification of the glucosyltransferase that mediates direct flavone C-glucosylation in *Gentiana triflora*. *FEBS Lett*. 2015;589(1):182-7. doi: 10.1016/j.febslet.2014.11.045.
29. Schaufelberger D, Hostettmann K. High-performance liquid chromatographic analysis of secoiridoid flavone glycosides in closely related *Gentiana* species. *Journal of Chromatography A*. 1987; 389:450-5. doi: [https://doi.org/10.1016/S0021-9673\(01\)94458-9](https://doi.org/10.1016/S0021-9673(01)94458-9).
30. Li S, Wan C, He L, Yan Z, Wang K, Yuan M, et al. Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS. *Revista Brasileira de Farmacognosia*. 2017;27(2):188-94. doi: 10.1016/j.bjp.2016.10.003.
31. Huang M, Zhang Y, Xu S, Xu W, Chu K, Xu W, et al. Identification and quantification of phenolic compounds in *Vitex negundo* L. var. *cannabifolia* (Siebold et Zucc.) Hand. -Mazz. using liquid chromatography combined with quadrupole time-of-flight and triple quadrupole mass spectrometers. *J Pharm Biomed Anal*. 2015; 108:11-20. doi: 10.1016/j.jpba.2015.01.049.
32. Li S, Wan C, He L, Yan Z, Wang K, Yuan M, et al. Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS. *Revista Brasileira de Farmacognosia*. 2017;27(2):188-94. doi: 10.1016/j.bjp.2016.10.003.
33. Bergeron C, Marston A, Gauthier R, Hostettmann K. Iridoids and secoiridoids from *Gentiana linearis*. *Phytochemistry*. 1997;44(4):633-7. doi: [https://doi.org/10.1016/S0031-9422\(96\)00636-X](https://doi.org/10.1016/S0031-9422(96)00636-X).
34. Li S, Wan CX, Yuan MH, Liu X, Zhao Y, Zhang ZF. Study on flavonoid glycosides from *Gentiana veitchiorum*. *Chinese Traditional and Herbal Drugs*. 2016;47(15):2597-600.

35. Pasdaran A, Butovska D, Kerr P, Naychov Z, Aneva I, Kozuharova E. Gentians, natural remedies for future of visceral pain control; an ethnopharmacological review with an in silico approach. *Biologia Futura*. 2022;73(2):219-27. doi: 10.1007/s42977-022-00114-7.
36. Li S, Wan C, He L, Yan Z, Wang K, Yuan M, et al. Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS. *Revista Brasileira de Farmacognosia*. 2017;27(2):188-94. doi: 10.1016/j.bjp.2016.10.003.
37. Xu H, Liu T, Wang W, Su N, Yang L, Yang Z, et al. Proteomic Analysis of Hydroxysafflor Yellow A Against Cerebral Ischemia/Reperfusion Injury in Rats. *Rejuvenation Res*. 2019;22(6):503-12. doi: 10.1089/rej.2018.2145.
38. Schmidt S, Gonzalez D, Derendorf H. Significance of protein binding in pharmacokinetics and pharmacodynamics. *J Pharm Sci*. 2010;99(3):1107-22. doi: 10.1002/jps.21916.
39. van Golen RF, Olthof PB, de Haan LR, Coelen RJ, Pechlivanis A, de Keijzer MJ, et al. The pathophysiology of human obstructive cholestasis is mimicked in cholestatic Gold Syrian hamsters. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864(3):942-51. doi: 10.1016/j.bbadis.2017.11.022.
40. Mariotti V, Strazzabosco M, Fabris L, Calvisi DF. Animal models of biliary injury and altered bile acid metabolism. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864(4 Pt B):1254-61. doi: 10.1016/j.bbadis.2017.06.027.
41. Ghonem NS, Assis DN, Boyer JL. Fibrates and cholestasis. *Hepatology*. 2015;62(2):635-43. doi: 10.1002/hep.27744.
42. Zhao JF, Xu JY, Xu YE, Chen SL, Guo YX, Gao QY, et al. High-Throughput Metabolomics Method for Discovering Metabolic Biomarkers and Pathways to Reveal Effects and Molecular Mechanism of Ethanolic Extract From *Epimedium* Against Osteoporosis. *Front Pharmacol*. 2020; 11:1318. doi: 10.3389/fphar.2020.01318.
43. Li S, Wan C, He L, Yan Z, Wang K, Yuan M, et al. Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS. *Revista Brasileira de Farmacognosia*. 2017;27(2):188-94. doi: 10.1016/j.bjp.2016.10.003.
44. Li S. Chemical Constituents from *Gentiana veitchiorum*. Southwest University for Nationalities, P. R. China. 2017.
45. Li S, Zhao Y, Liu Y, Zhang ZF. Separation and preparation of three compounds from *Gentiana veitchiorum* by high-speed counter-current chromatography (HSCCC). *Journal of Southwest University for Nationalities (Natural Science Edition)*. 2016; 42(06):660-4.
46. Zou QY, Liang J, Liao X, Peng SL, Ding LS. Chemical constituents from the whole plants of *Gentiana veitchiorum*. *West China Journal of Pharmaceutical Sciences*. 2010;25(02) 512-4.
47. Cao HF, Zhao ZL, Ga W. Research progress of Tibetan medicine *Gentiana veitchiorum*. *West China Journal of Pharmaceutical Sciences*. 2014;37(06):1087-93.
48. Li S, Zhang B. Traditional Chinese medicine network pharmacology: theory, methodology and application. *Chinese journal of natural medicines*. 2013;11(2):110-20. doi: 10.1016/S1875-5364(13)60037-0.
49. Ma X, Jiang Y, Zhang W, Wang J, Wang R, Wang L, et al. Natural products for the prevention and treatment of cholestasis: A review. *Phytother Res*. 2020;34(6):1291-309. doi: 10.1002/ptr.6621.
50. Han H, Xu L, Xiong K, Zhang T, Wang Z. Exploration of Hepatoprotective Effect of Gentiopicroside on Alpha-Naphthylisothiocyanate-Induced Cholestatic Liver Injury in Rats by Comprehensive Proteomic and Metabolomic Signatures. *Cell Physiol Biochem*. 2018;49(4):1304-19. doi: 10.1159/000493409.
51. Qi MX, Liu KL, He JB. Alleviation Effect of Ginsenoside Rg1 in Rats with Cholestasis by Sirt5 Pathway. *China Pharmacist*. 2022;25(10):1718-23. DOI: 10.19962/j.cnki.Issn 1008-049X.2022.10.005.
52. Shi M, Tang J, Zhang T, Han H. Swertiamarin, an active iridoid glycoside from *Swertia pseudochinensis* H. Hara, protects against alpha-naphthylisothiocyanate-induced cholestasis by activating the farnesoid X receptor and bile acid excretion pathway. *J Ethnopharmacol*. 2022; 291:115164. doi: 10.1016/j.jep.2022.115164.
53. Hua W, Zhang S, Lu Q, Sun Y, Tan S, Chen F, et al. Protective effects of n-Butanol extract and iridoid glycosides of *Veronica ciliata* Fisch. Against ANIT-induced cholestatic liver injury in mice. *J Ethnopharmacol*. 2021; 266:113432. doi: 10.1016/j.jep.2020.113432.
54. Wang L, Wu G, Wu F, Jiang N, Lin Y. Geniposide attenuates ANIT-induced cholestasis through regulation of transporters and enzymes involved in bile acids homeostasis in rats. *J Ethnopharmacol*. 2017; 196:178-85. doi: 10.1016/j.jep.2016.12.022.
55. Xiang J, Yang G, Ma C, Wei L, Wu H, Zhang W, et al. Tectorigenin alleviates intrahepatic cholestasis by inhibiting hepatic inflammation and bile accumulation via activation of PPARgamma. *Br J Pharmacol*. 2021;178(12):2443-60. doi: 10.1111/bph.15429.
56. Zhang G, Sun X, Wen Y, Shi A, Zhang J, Wei Y, et al. Hesperidin alleviates cholestasis via activation of the farnesoid X receptor in vitro and in vivo. *Eur J Pharmacol*. 2020;885:173498. doi: 10.1016/j.ejphar.2020.173498.
57. Yang X, Feng Y, Liu Y, Ye X, Ji X, Sun L, et al. Fuzheng Jiedu Xiaoji formulation inhibits hepatocellular carcinoma progression in patients by targeting the AKT/CyclinD1/p21/p27 pathway. *Phytomedicine*. 2021; 87:153575. doi: 10.1016/j.phymed.2021.153575.

58. Kassouf T, Sumara G. Impact of Conventional and Atypical MAPKs on the Development of Metabolic Diseases. *Biomolecules*. 2020;10(9). doi: 10.3390/biom10091256.
59. Nabih ES, El-Kharashi OA. Targeting HMGB1/TLR4 axis and miR-21 by rosuvastatin: role in alleviating cholestatic liver injury in a rat model of bile duct ligation. *Naunyn Schmiedebergs Arch Pharmacol*. 2019;392(1):37-43. doi: 10.1007/s00210-018-1560-y.
60. Liu B, Zhang J, Shao L, Yao J. Network pharmacology analysis and molecular docking to unveil the potential mechanisms of San-Huang-Chai-Zhu formula treating cholestasis. *PLoS One*. 2022;17(2):e0264398. doi: 10.1371/journal.pone.0264398.
61. Yang RJ. Effect of Pes1 on mice with cholestatic liver disease via PI3K/AKT/GSK-3 $\beta$  signaling pathway. *ANHUI MEDICAL UNIVERSITY*. 2019;54(10):1511-5.
62. Liu B, Zhang J, Shao L, Yao J. Network pharmacology analysis and molecular docking to unveil the potential mechanisms of San-Huang-Chai-Zhu formula treating cholestasis. *PLoS One*. 2022;17(2):e0264398. doi: 10.1371/journal.pone.0264398.
63. Yao H. Protective effects and mechanisms of dioscin on liver injury. Dalian Medical University. 2017. DOI: 10.26994/d.cnki.gdlyu.2017.000294.
64. Liu S, Zhang X, Wang J. Isoviteixin protects against cisplatin-induced kidney injury in mice through inhibiting inflammatory and oxidative responses. *Int Immunopharmacol*. 2020; 83:106437. doi: 10.1016/j.intimp.2020.106437.
65. Wan Z, Li H, Wu X, Zhao H, Wang R, Li M, et al. Hepatoprotective effect of gentiopicroside in combination with leflunomide and/or methotrexate in arthritic rats. *Life Sci*. 2021; 265:118689. doi: 10.1016/j.lfs.2020.118689.
66. Wei X, Fan X, Feng Z, Ma Y, Lan X, Chen M. Ethyl acetate extract of *herpetospermum pedunculatum* alleviates alpha-naphthylisothiocyanate-induced cholestasis by activating the farnesoid x receptor and suppressing oxidative stress and inflammation in rats. *Phytomedicine*. 2020; 76:153257. doi: 10.1016/j.phymed.2020.153257.
67. Yan M, Guo L, Yang Y, Zhang B, Hou Z, Gao Y, et al. Glycyrrhetic Acid Protects alpha-Naphthylisothiocyanate- Induced Cholestasis Through Regulating Transporters, Inflammation and Apoptosis. *Front Pharmacol*. 2021; 12:701240. doi: 10.3389/fphar.2021.701240.

## Figures

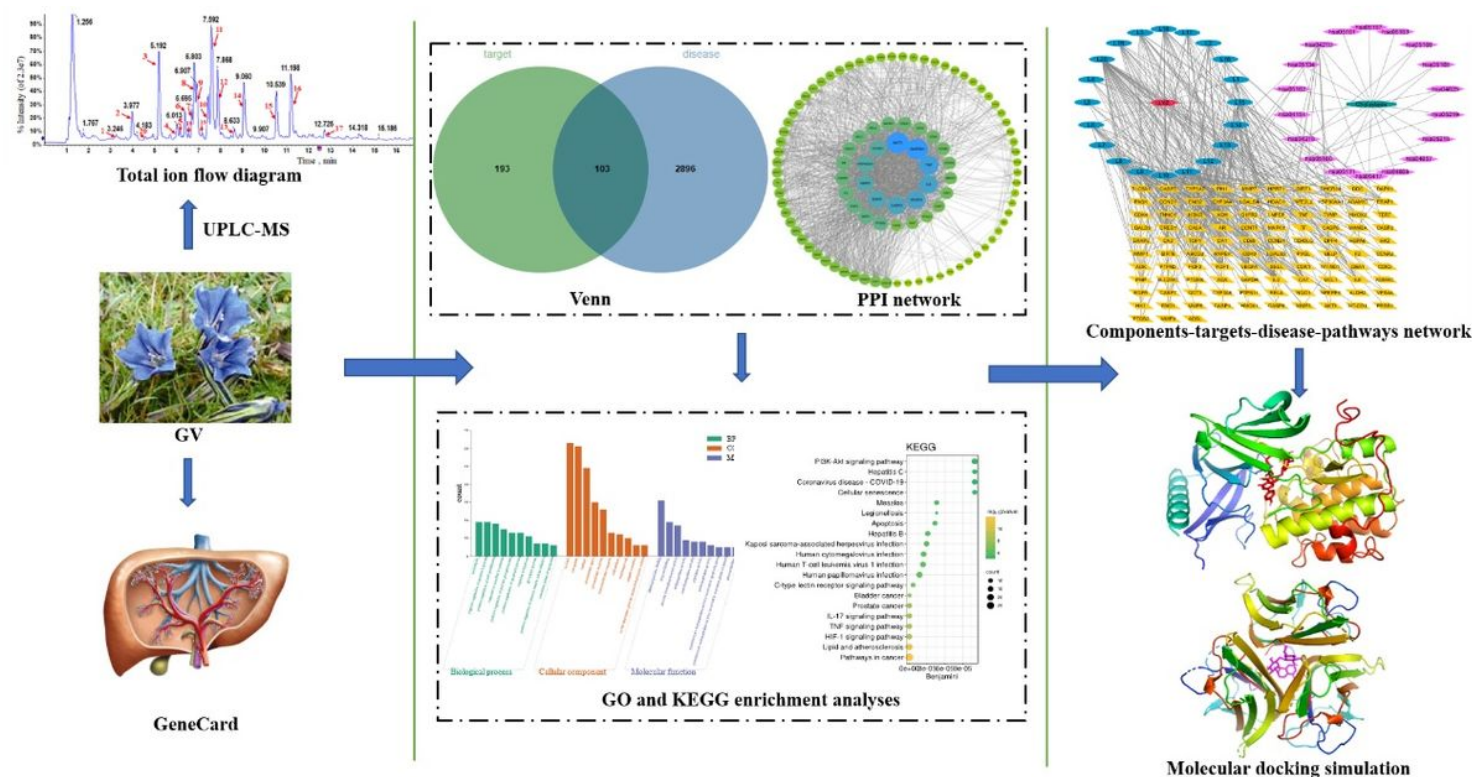


Figure 1

The mechanism of GV in treatment of cholestasis based on the UPLC-MS/MS and systematic pharmacology strategies.

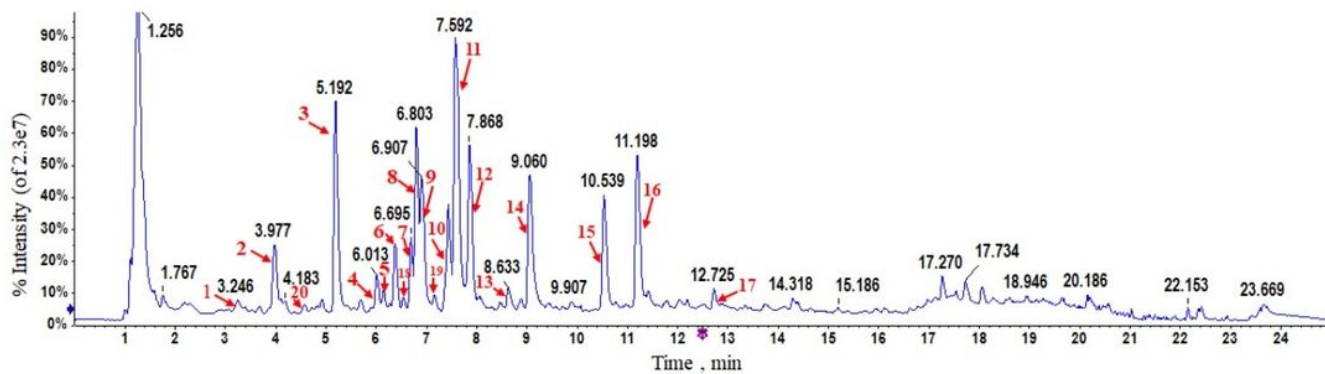


**Figure 2**

UPLC/MS total ion flow diagram of GV extracts

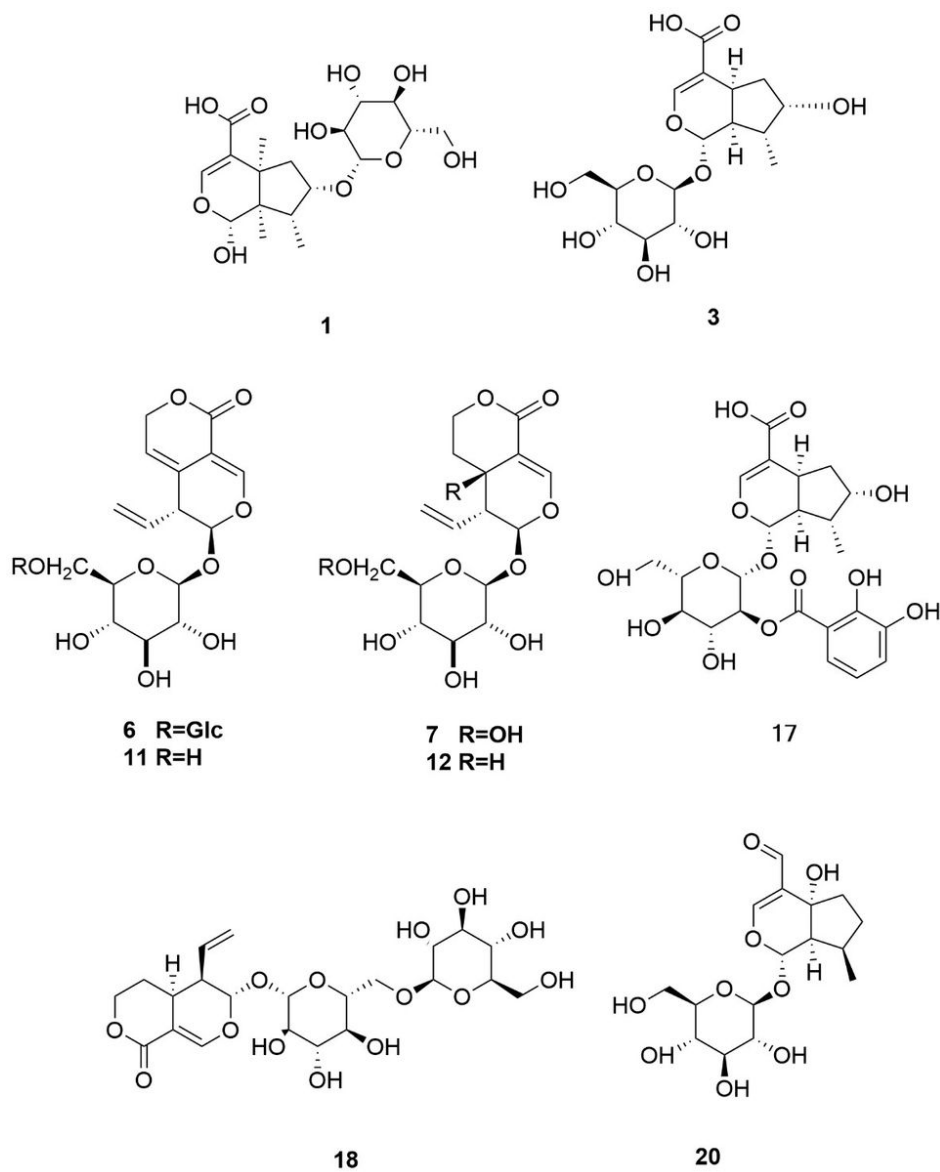
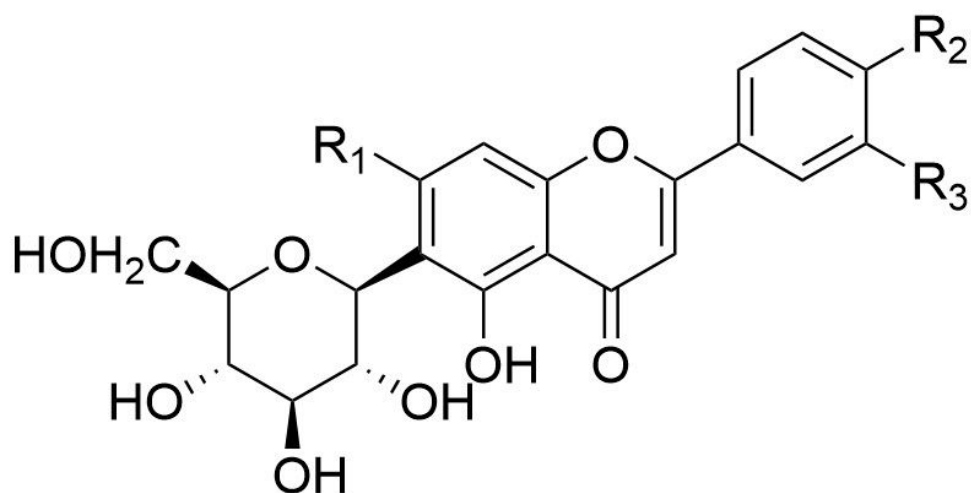


Figure 3

Structural analysis of Iridoid compounds



**5** R<sub>1</sub>=OH;R<sub>2</sub>=OGlc6-1Glc;R<sub>3</sub>=OH

**8** R<sub>1</sub>=OH; R<sub>2</sub>=OGlc;R<sub>3</sub>=OH

**9** R<sub>1</sub>=OH;R<sub>2</sub>=OGlc;R<sub>3</sub>=H

**10** R<sub>1</sub>=OH; R<sub>2</sub>=OGlc; R<sub>3</sub>=OCH<sub>3</sub>

**13** R<sub>1</sub>=OGlc;R<sub>2</sub>=OH;R<sub>3</sub>=H

**14** R<sub>1</sub>=R<sub>2</sub>=R<sub>3</sub>=OH

**15** R<sub>1</sub>=R<sub>2</sub>=OH;R<sub>3</sub>=H

**16** R<sub>1</sub>=OH;R<sub>2</sub>=OH;R<sub>3</sub>=OCH

**19** R<sub>1</sub>=OGlc6-1Glc; R<sub>2</sub>=OH;R<sub>3</sub>=H

Figure 4

Structural analysis of flavonoids

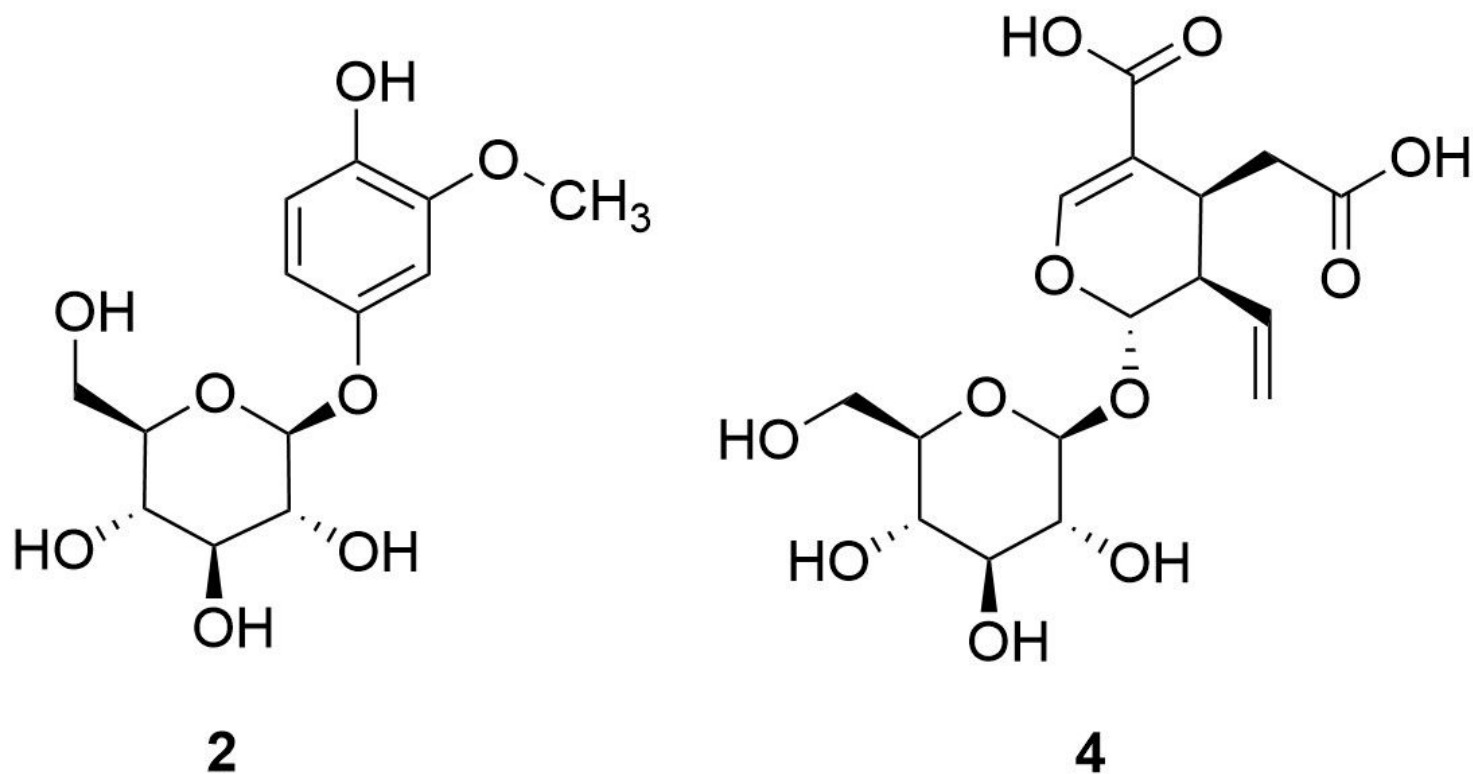


Figure 5

Structural analysis of other types of compounds

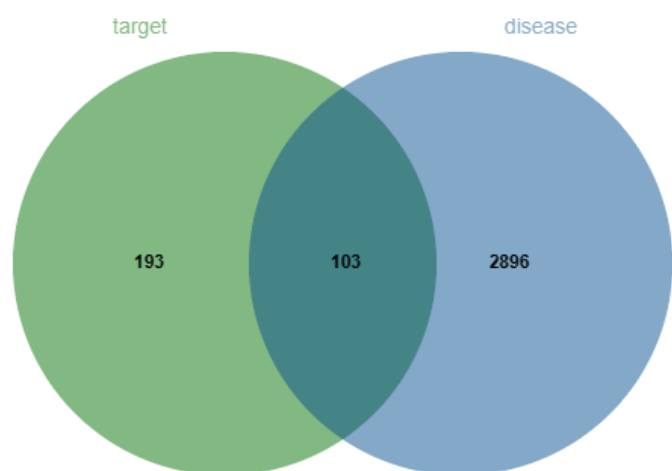


Figure 6

Venn diagram of targets' intersection of GV's extracts and cholestasis.

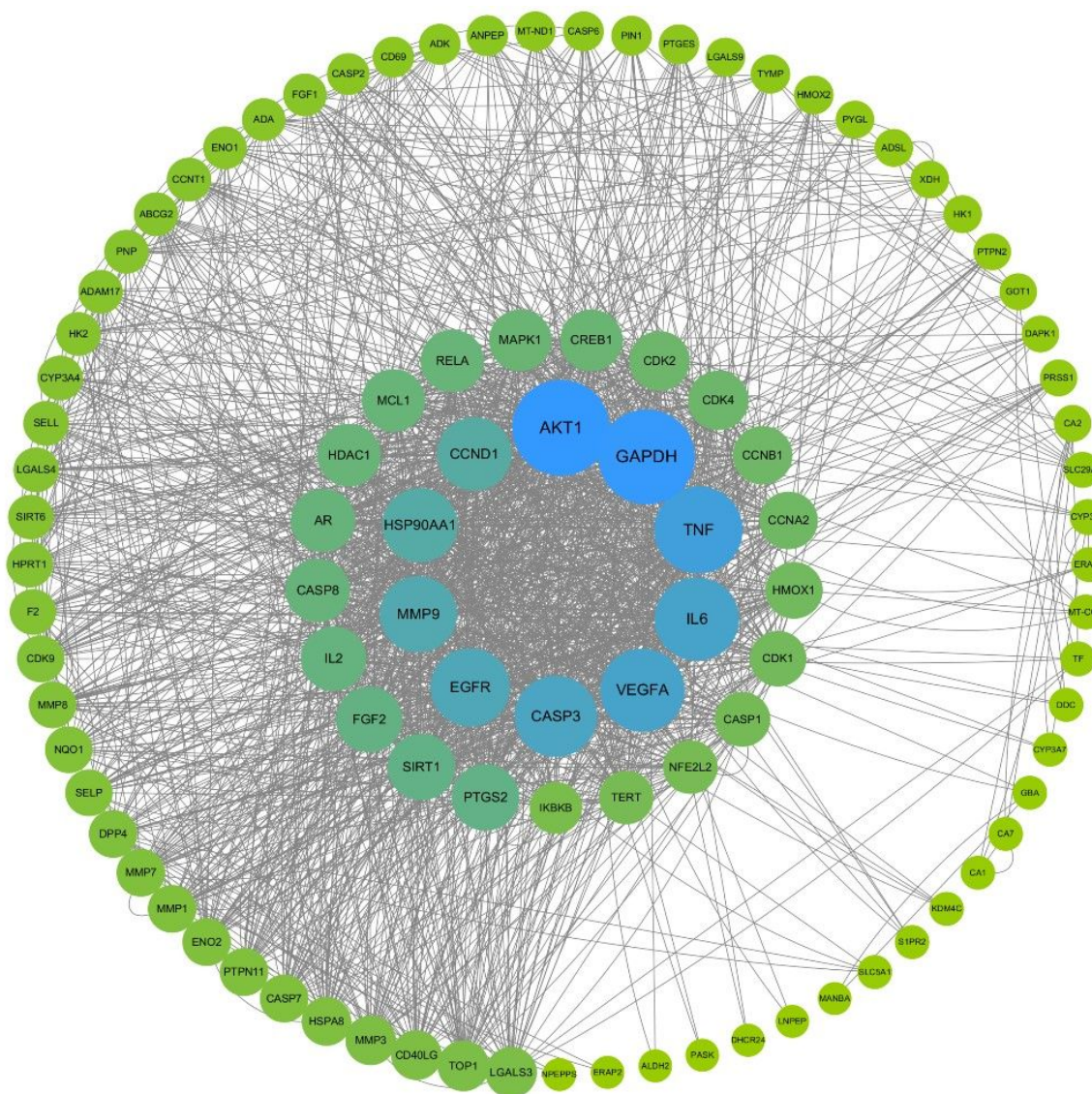


**Figure 7**

Protein-protein interactions network diagram. The PPI for the 103 major pivotal or core key targets of GV for cholestasis are sorted by color and size according to degree, from large and blue to small and green.

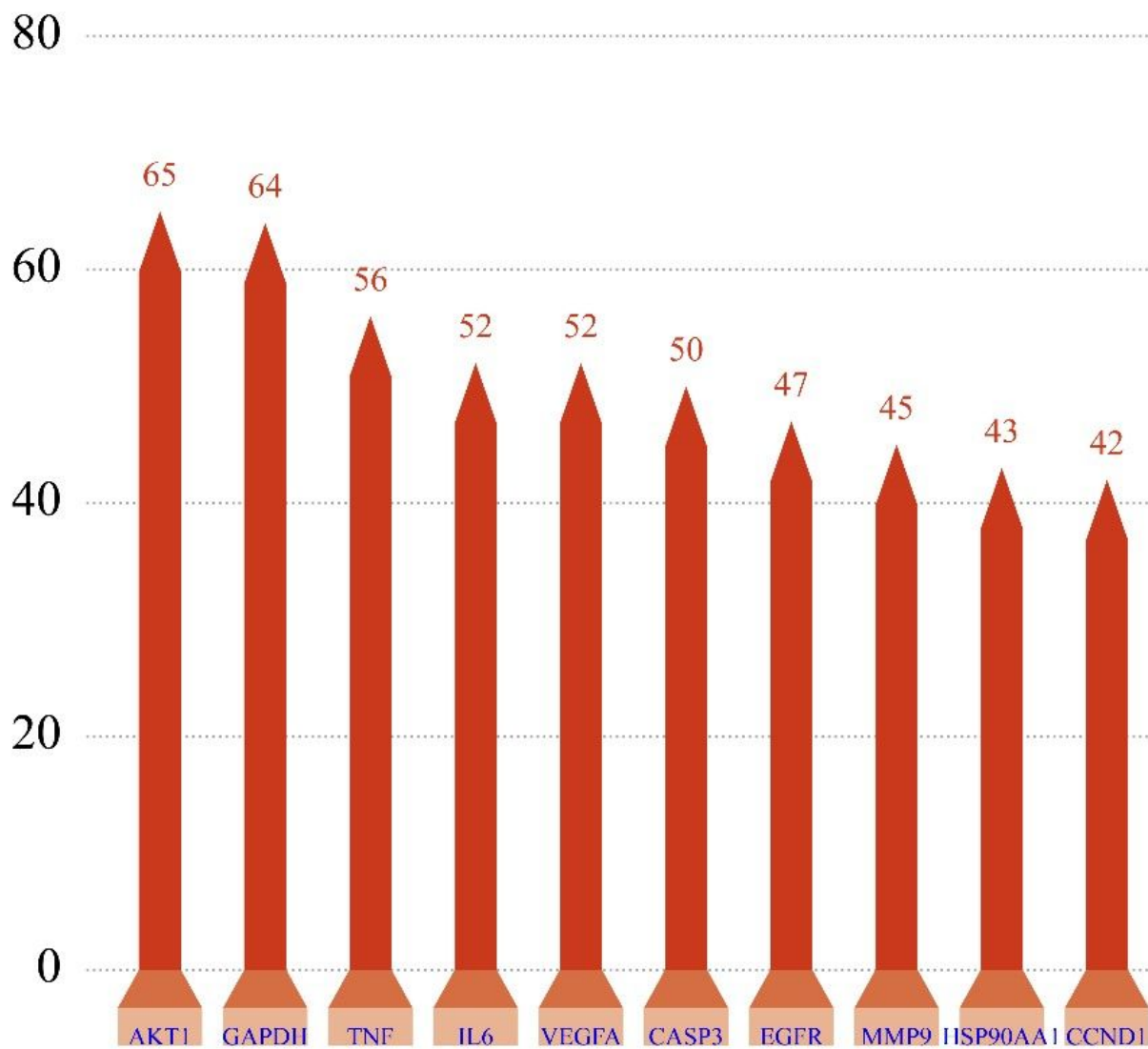


**Figure 8**

Histogram of key target proteins of GV in the treatment of cholestasis.

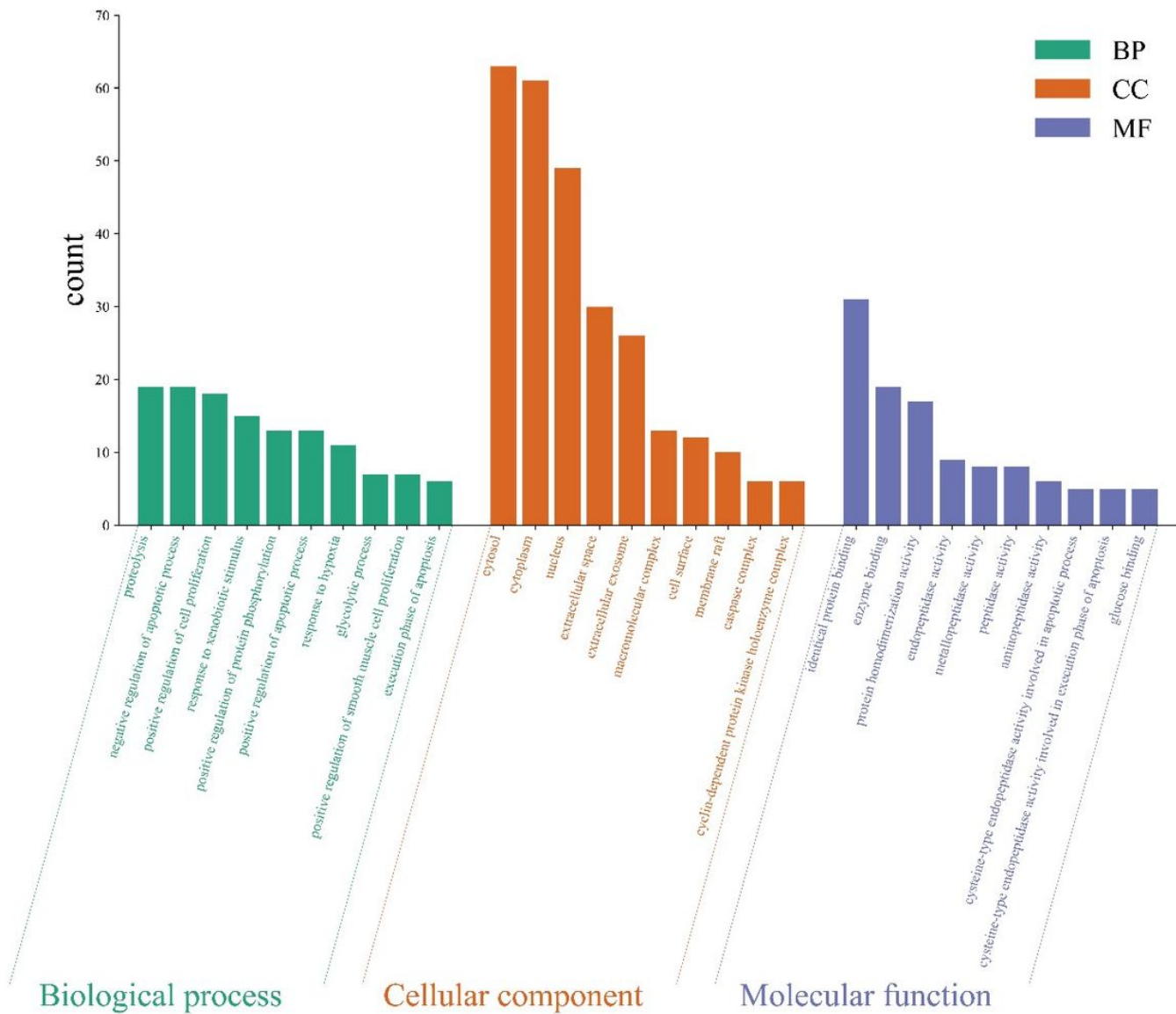


**Figure 9**

The bar graph of Gene ontology (GO) function enrichment analysis. Biological process (BP) refers to the process that is completed through a variety of molecular activities; Cell composition (CC) refers to the position of the cell structure where the gene product performs its function; Molecular function (MF) refers to the activity of gene products of target genes at the molecular level.

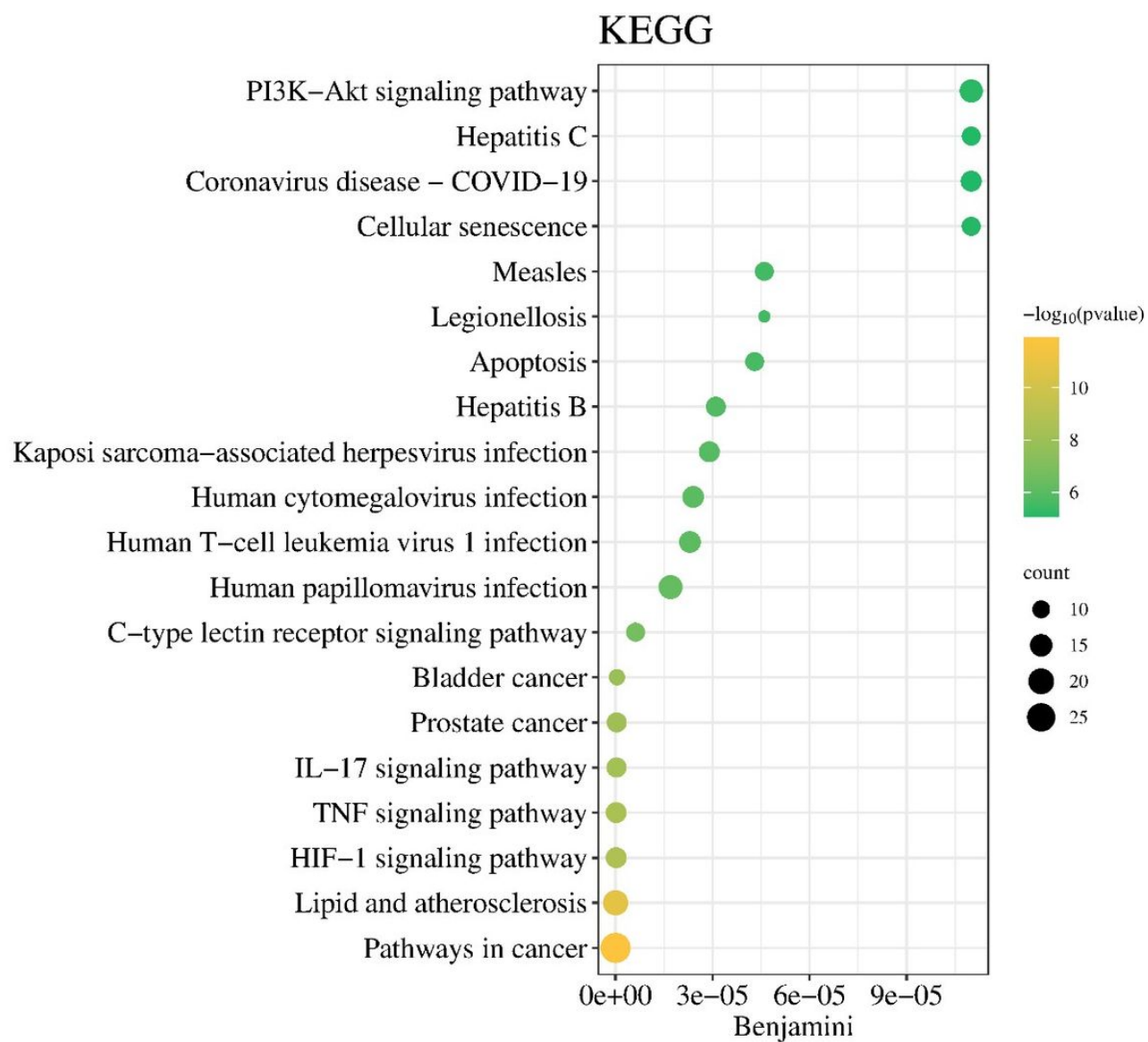


**Figure 10**

The bubble chart of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

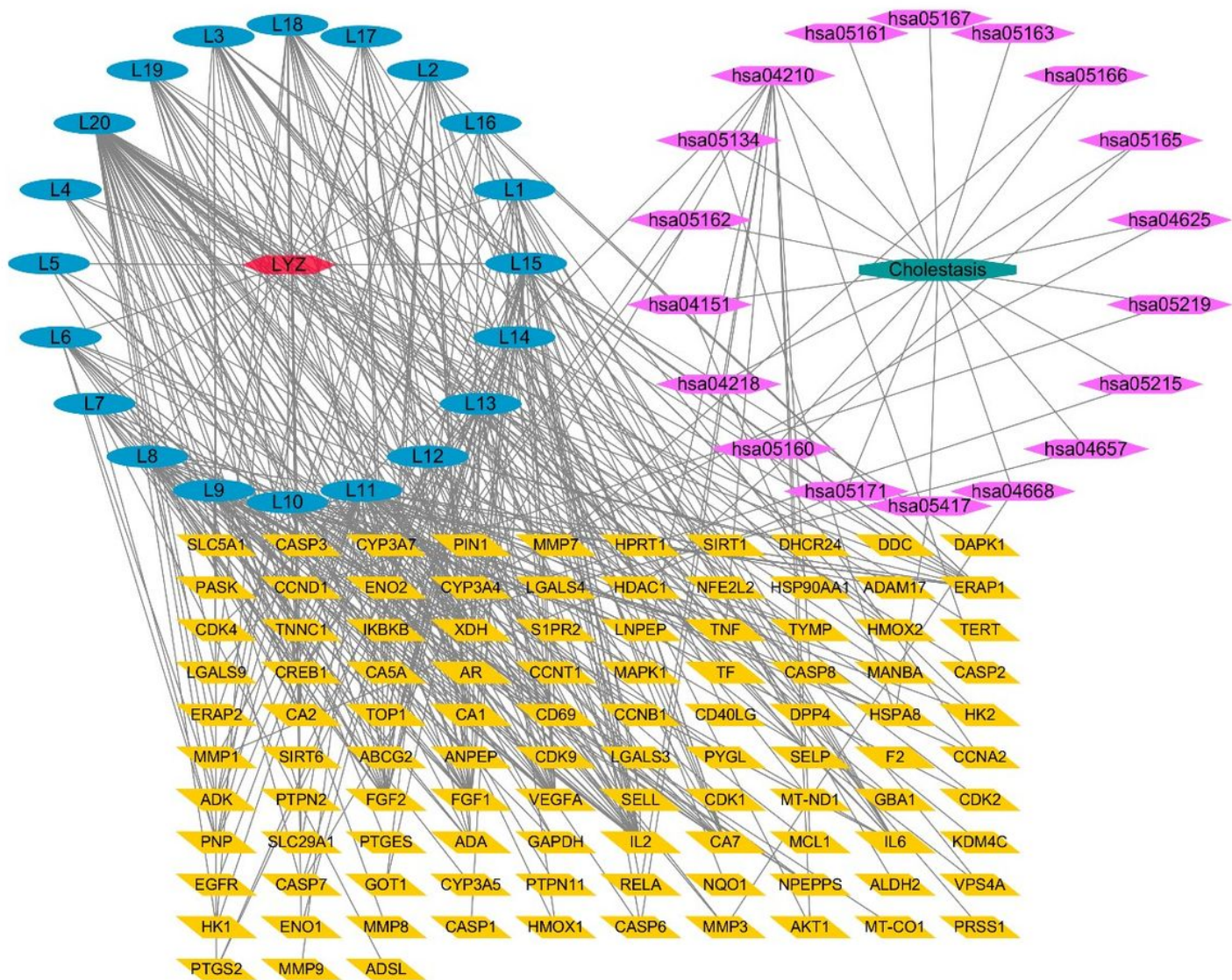


**Figure 11**

The network diagram of 'drug-component-disease-pathway-target'. In the figure, red nodes represent GV, green nodes represent cholestatic diseases, blue nodes represent core components of GV, purple nodes represent key pathways of the disease, and yellow nodes represent common targets.

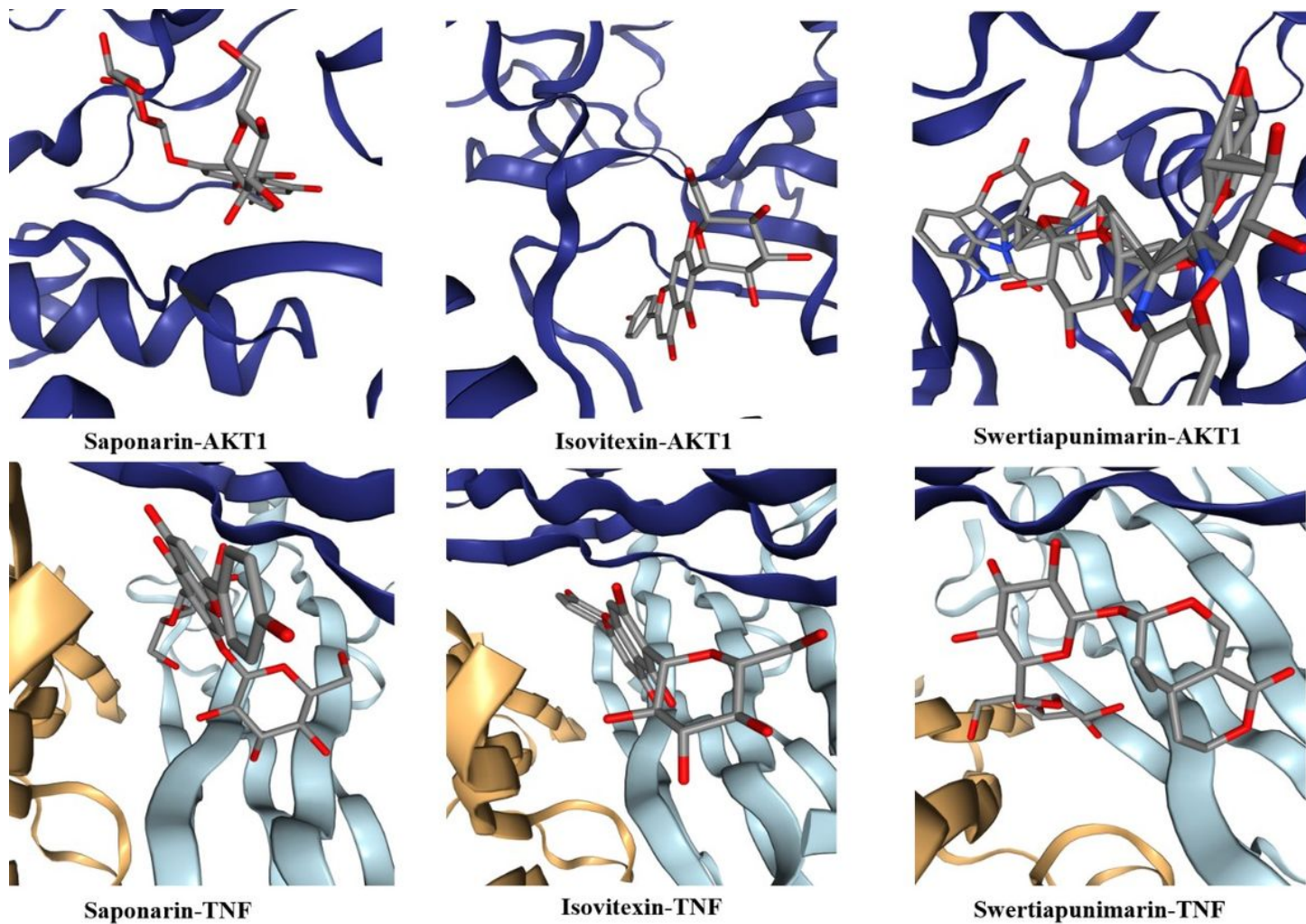


**Figure 12**

Molecular docking analysis of interactions between compounds and protein receptors.

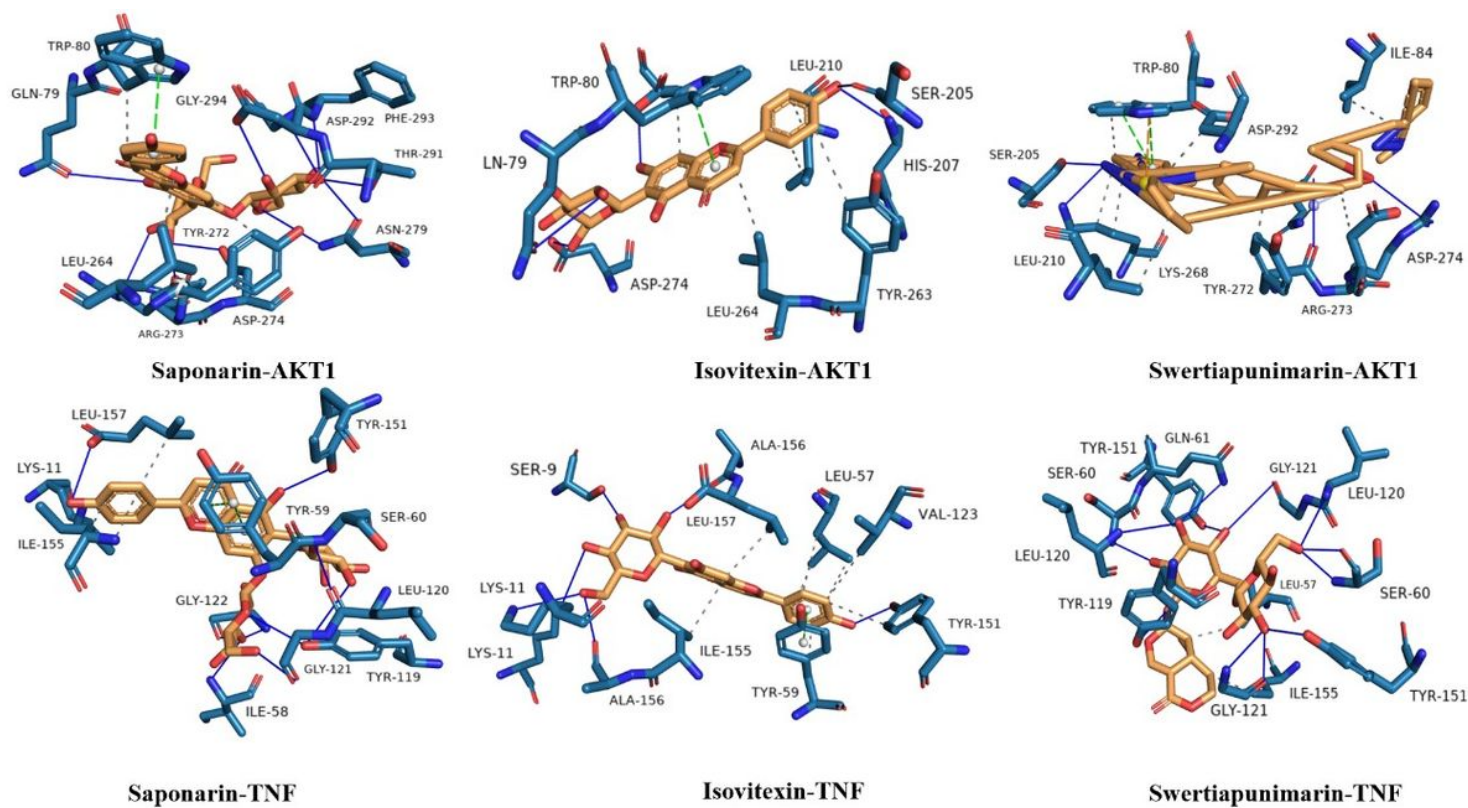


**Figure 13**

The 3D docking structures between the compounds and protein receptors.

Black dashed line denotes hydrophobic interactions, blue solid line denotes hydrogen bonding, and green dashed line denotes  $\pi$ -stacking.

## Supplementary Files

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