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# Network Pharmacology Study of the Hepatoprotective Effects of Quercetin-Containing Traditional Chinese Medicine, *Anoectochilus roxburghii*, and Validation of Quercetin as an Anti-Liver Injury Agent in a Mouse Model of Liver Injury

Authors' Contribution:
Study Design A
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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**Background:** *Anoectochilus roxburghii* (Orchidaceae) (AR) has been widely used to treat liver injury in China, but its underlying mechanisms remain elusive. Network pharmacology was utilized to assess the hepatoprotective effects of quercetin (Que)-containing AR, and to validate the anti-liver injury effects of Que in a mouse model of liver injury.

**Material/Methods:** Network pharmacology analysis was performed to determine bio-active compounds in AR. The core therapeutic targets of AR against liver injury were identified using a protein–protein interaction network. Biological function and pathway enrichment were analyzed based on the identified core therapeutic targets. The hepatoprotective effects of Que in a mouse model of liver injury induced by $CCl_4$ were assessed to verify the reliability of network pharmacology analysis.

**Results:** Seven bio-active compounds of AR met drug screening criteria and 17 core therapeutic targets of AR against liver injury were identified. Biological function analysis demonstrated that the therapeutic effects of AR against liver injury were chiefly associated with the suppression of inflammation and immunity; and pathway enrichment analysis showed that nuclear factor-kappa B (NF-κB) and tumor necrosis factor (TNF) signaling pathways were associated with the inflammatory responses. Experimental validation in a mouse model showed that AR exerted anti-inflammatory effects by regulating the NF-κB signaling pathway, a finding that also confirmed the reliability of network pharmacology analysis.

**Conclusions:** The bio-active compounds identified in AR and the elucidation of their mechanisms of action against liver injury provide a theoretical basis for designing agents that can prevent or suppress liver injury.

**MeSH Keywords:** Drug-Induced Liver Injury • Medicine, Chinese Traditional • Systems Biology • Validation Studies

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## Background

The liver, as the main metabolic organ in humans, plays vital roles in immune defenses, metabolism, resistance to pathogens, and detoxification [1]. Liver injury is one of the major threats to human health, affecting more than 10% of the world's population. For example, viral hepatitis, obesity and metabolic syndrome, and acute liver injury caused by toxins or alcohol are likely to result in the development of liver fibrosis, cirrhosis, and eventually cancer [2]. At present, synthetic drugs are not ideal for the treatment of liver injury because most of them have adverse side effects [3]. In recent years, many compounds in traditional Chinese medicine (TCM) have been studied for the complementary and alternative treatment of liver injury [4].

*Anoectochilus roxburghii* (AR), a perennial herb of the genus *Anoectochilus* in the Orchidaceae, is a precious plant in TCMs and is known as the "king of medicine" and "golden grass" [5]. According to the theory of TCM, AR shows various health effects, such as removing heat to cool the blood, damp-inhibiting and wind-dispersing properties, calming the liver, as well as lowering blood pressure [6]. AR has been used to treat liver injury, diabetes, and tumors, showing anti-inflammatory and antiviral effects [7,8]. Currently, various forms of AR, including oral liquid [9], capsules [10], and combination agents [11], are used in clinics to treat a variety of acute and/or chronic types of liver injury, and have shown good curative effects with negligible side effects [7–11]. Previous studies have revealed that polysaccharides, flavonoids, glycosides, organic acids, steroids, alkaloids, and nucleosides in AR are the main bio-active compounds, playing a protective role against liver injury [12]. However, potential liver-protecting compounds in AR and their mechanisms of action remain to be systematically elucidated.

Network pharmacology is an emerging discipline based on systems pharmacology, which takes "disease-gene-target-drug" as research network to reveal the effects of drugs on different diseases and the mechanisms of drug-body interactions from a holistic perspective [13–16]. For example, the mechanism of action of paeony in the treatment of ulcerative colitis has been studied based on network pharmacology, with results showing that the interactions of active ingredients of paeony and 70 targets related to ulcerative colitis were primarily responsible for reducing the stimulation of inflammatory responses in the intestinal tract through the tumor necrosis factor (TNF) signaling pathway [14]. In addition, active components of Gegen Qinlian decoction and their mechanisms of action in the treatment of type 2 diabetes have been analyzed by the network pharmacological method [15]. The present study therefore utilized network pharmacology to study the hepatoprotective effects of quercetin (Que)-containing AR, and to validate the protective function of Que in a mouse model of $CCl_4$-induced liver injury. This study may provide a scientific basis for the mechanism of action of AR in preventing and suppressing liver injury and a reliable theoretical basis for the development of new liver-protecting drugs.

## Material and Methods

### Screening of major active ingredients and targets of AR

The major bio-active ingredients of AR were determined based on previously identified chemical components [5,17–21]. The names of these bio-active ingredients were imported into the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP) (*http://lsp.nwsuaf.edu.cn/*) to identify targets of the active ingredients of AR, and the names of target genes were matched using the UniProtKB database (*https://www.uniprot.org/*). Screening criteria included oral bio-availability (OB) ≥30%, drug-like property (DL) ≥0.18, Caco-2 cell permeability ≥0, and drug-like principles [22–27].

### Prediction of liver injury related targets

As previously described [3], liver injury related targets were predicted by entering keywords, such as "liver disease" "hepatic damage", "alcoholic liver", "non-alcoholic liver", "fatty liver", "liver fibrosis", "oxidative damage", "steatosis", and "lipid metabolism" into the Therapeutic Target Database (TTD) (*http://bidd.nus.edu.sg/group/ttd/*), PharmGKB (*http://www.pharmgkb.org*), and Comparative Toxicogenomics Database (CTD) (*http://ctdbase.org/*). The liver injury related targets identified by each database were combined and the repetitions removed.

### Construction of protein–protein interaction (PPI) and component-target (C-T) networks

The potential targets of AR against liver injury were identified by the intersection of the targets related to the bio-active ingredients of AR and the targets related to liver injury. These potential targets were imported into the String database (*https://string-db.org/*) to construct a protein-protein interaction (PPI) network. The network was screened based on a confidence score >0.7 to determine a PPI network with high reliability, which was imported into Cytoscape V 3.7.2 software (*https://cytoscape.org/*). Using the NetworkAnalyzer tool, topology network was analyzed based on three parameters: "Degree", "Betweenness Centrality", and "Closeness Centrality". Finally, the liver injury related core targets were obtained. The threshold value of the core node in the PPI network was the median of each parameter.





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Using Cytoscape 3.7.2 software, the core targets were associated with the pharmaceutically active ingredients to construct a compound-target (C-T) network, and to subsequently analyze the key pharmaceutically active ingredients of AR.

### GO and KEGG enrichment analyses

Biological function enrichment analysis of gene ontology (GO) and signaling pathway analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG, *http://www.kegg.jp/*) for identifying hub targets were performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID, v 6.8) (*https://david.ncifcrf.gov/*). The signaling pathways identified by KEGG enrichment analysis were screened based on a false discovery rate (FDR) ≤0.05. As the FDR increases, the relationship between various targets in the signaling pathways and the pharmacological action of related drug components becomes closer.

### Animal model and treatment

Twenty healthy male BALB/c mice, weighing 20±2 g, were randomly divided into four groups. Mice in two of the groups were treated with high-dose (120 mg/kg; Que-H group) or low-dose (60 mg/kg; Que-L group) Que every day for 10 days by gavage, whereas mice in the other two groups, the normal control (Con) and the $CCl_4$ groups, were treated daily by gavage with the same volume (0.2 mL) of normal saline. About 6 h after the last gavage, mice in the $CCl_4$ and the Que groups were intraperitoneally injected with $CCl_4$ solution (10 mL/kg, 0.2% in soybean oil) to induce acute liver injury, whereas mice in the Con group were injected with the same volume of soybean oil. After a 12-h fast, serum samples were collected. The animals were anesthetized with ether and sacrificed by direct heart puncture, and their livers were removed.

All procedures involving experimental animals were evaluated and approved by the Animal Ethics Committee of Fujian Agriculture and Forestry University and followed the guidelines for experimental animal welfare (Permit No. PZCASFAFU20190328).

### Biochemical analyses and histopathological examination

Serum samples were centrifuged at 3,000 rpm for 10 min and stored at –20°C until further analyzed. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentration were measured by ELISA (Jiancheng, Nanjing, China). Liver samples were soaked in formalin, embedded in paraffin, and sectioned, and the sections were stained with hematoxylin and eosin (H&E) and examined under an optical microscope.

### Western blotting analysis

Proteins were extracted from liver tissue using the Radio Immunoprecipitation Assay (RIPA) lysate (Solarbio, Beijing, China) in an ice bath according to the manufacturer's instructions. Total protein content was determined using BCA test kits (Solarbio). Protein samples (40 μg) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels, electrophoresed, and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) [28]. The membranes were blocked with 5% skim milk at room temperature for 2 hours, washed, and incubated at 4°C for 12 hours with diluted antibodies to nuclear factor-kappa B (NF-κB) p65 (1: 1000, #82425, Cell Signaling Technology, Inc), phosphorylated IκBα (p-IκBα) (1: 1000, #28595, Cell Signaling Technology, Inc), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1: 1000, #51745, Cell Signaling Technology, Inc). After washing with TBST (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween-20), the membranes were incubated with HRP-linked secondary antibody (1: 1000, 70657, Cell Signaling Technology, Inc) at room temperature for 50 min and their chemiluminescence determined (Advansta, Menlo Park, CA, USA). The gray values of the protein were analyzed using QuantityOne 4.6 software (Bio-Rad, Hercules, CA, USA) to determine the expression level of the target proteins relative to GAPDH.

### Statistical analysis

Results are reported as mean±standard deviation (SD) and differences between groups analyzed by one-way analysis of variance (ANOVA) and Tukey's test. All data were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) software, with a P-value <0.05 regarded as statistically significant.

## Results

### Major active ingredients of AR

Analysis of AR yielded a total of 43 chemical components. Screening criteria identified seven of these compounds as active ingredients of AR, including four flavonoids, Que, quercetin-7-O-β-D-glucoside, isorhamnetin, and rhamnazin; and 3 sterols, campesterol, lanosterol, and stigmasterol (Table 1).

### Target screening

Based on the TCMSP database, a total of 188 nonredundant bio-active compound-related targets were identified. The official names of these targets were matched through the UniProtKB database and used to construct a network of the seven bio-active compounds and the 188 targets related to these bio-active compounds (Figure 1). Of the seven bio-active compounds,





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**Table 1.** The major bio-active compounds in *Anoectochilus roxburghii* (AR).

| Mol ID | Chemical compound | MW | Hdon | Hacc | OB (%) | Caco-2 | DL |
|---|---|---|---|---|---|---|---|
| MOL000098 | Quercetin | 302.25 | 5 | 7 | 46.43 | 0.05 | 0.28 |
| MOL005921 | Quercetin-7-O-β-D-glucoside | 300.28 | 5 | 6 | 49.57 | 0.13 | 0.27 |
| MOL000354 | Isorhamnetin | 316.28 | 4 | 7 | 49.6 | 0.31 | 0.31 |
| MOL000351 | Rhamnazin | 330.31 | 3 | 7 | 47.14 | 0.53 | 0.34 |
| MOL000493 | Campesterol | 400.76 | 1 | 1 | 37.58 | 1.31 | 0.71 |
| MOL001979 | Lanosterol | 426.8 | 1 | 1 | 42.12 | 1.52 | 0.75 |
| MOL000449 | Stigmasterol | 412.77 | 1 | 1 | 43.83 | 1.44 | 0.76 |

MW – molecular weight; Hdon – hydrogen-bond donors; Hacc – hydrogen-bond acceptors; OB – oral bioavailability; Caco-2 – Caco-2 cell permeability, DL – drug-likeness.

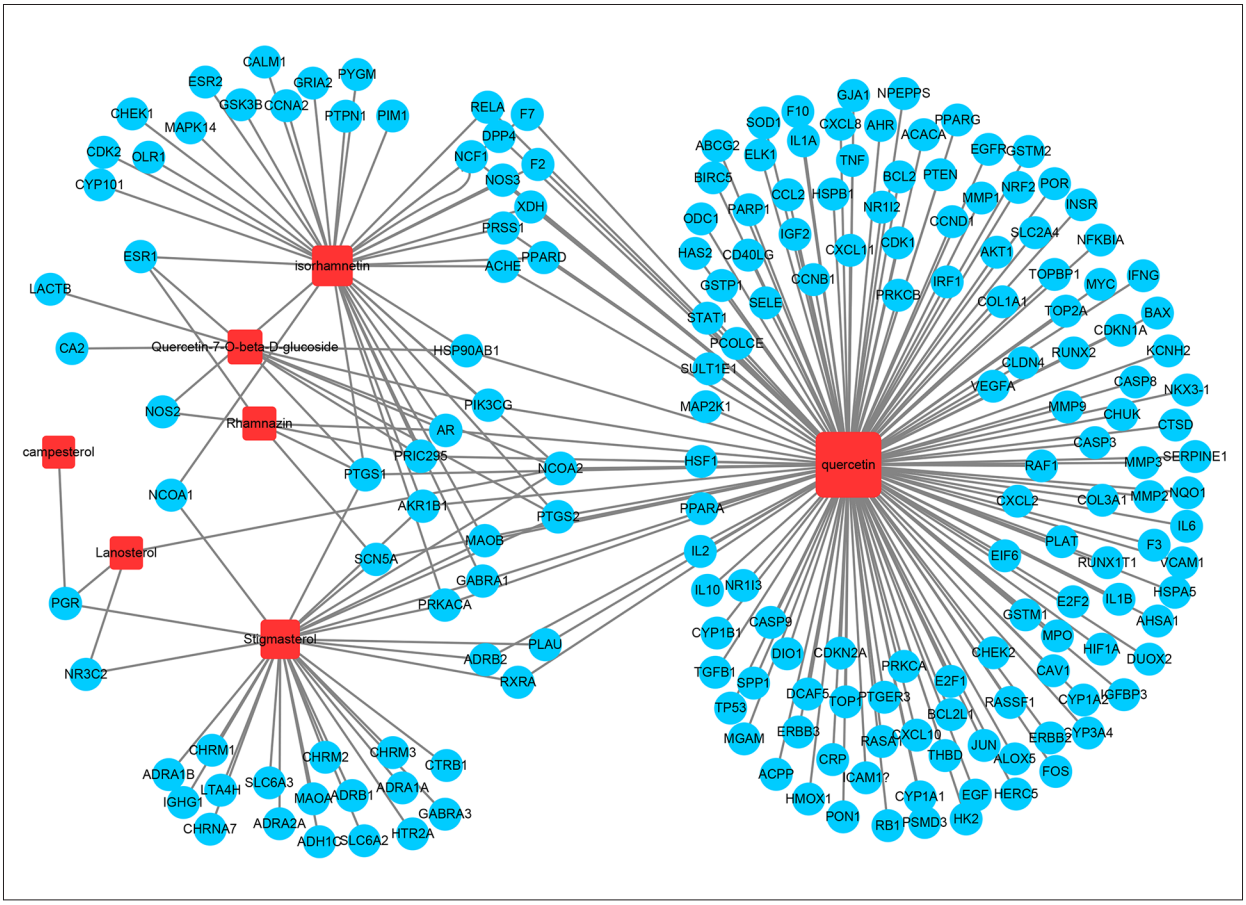


**Figure 1.** Compounds-targets network. The red nodes represent the active compounds in *Anoectochilus roxburghii* (AR), and the blue nodes represent the targets of AR compounds. The edges represent the relationships between the active compounds of AR and the corresponding targets.





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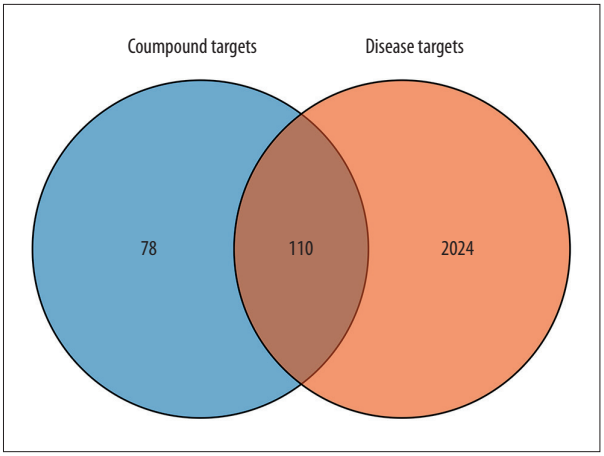


**Figure 2.** Venn diagram of *Anoectochilus roxburghii* (AR) bio-active compounds-related targets and liver injury-related targets.

Que had the greatest number of targets, followed by isorhamnetin and stigmasterol. A total of 2,134 targets related to liver injury were screened using the CTD, TTD, and PharmGKB databases. Based on the intersection between the bio-active ingredients and disease targets, 110 targets of AR related to liver injury were identified (Figure 2).

### Analysis of hub targets

To better understand the mechanism by which AR protects the liver, a PPI network with 110 targets was constructed using the String database, which was composed of 101 nodes and 736 edges. Subsequently, all the nodes in the PPI network were analyzed according to three topological parameters (degrees, degrees of centrality, and closeness centrality). Targets with values greater than the median were selected as hub targets to construct the core hub node of AR for the treatment of liver injury. This PPI network composed of 101 nodes and 736 edges was imported into Cytoscape software to draw the interaction network diagram (Figure 3A). The first screening yielded an initial interaction network diagram composed of 52 nodes and 495 edges (Figure 3B), whereas the second screening yielded a core interaction network composed of 17 nodes and 114 edges (Figure 3C). The 17 core targets were IL6, JUN, TNFA, TP53, VEGFA, EGFR, IL1B, MYC, MMP9, EGF, CCND1, CXCL8, PTGS2, MAPK14, FOS, IL10, and RELA (Table 2).

### Compound-target network

To better clarify the relationship between the bio-active ingredients and the core targets, an interaction network was constructed between the active ingredients and the core targets (Figure 4). Four active ingredients of AR were found to be critical to the core target. Que had the largest number of liver injury related targets (16), followed by isorhamnetin (3), stigmasterol (1), and quercetin-7-O-β-D-glucoside (1). These high-degree nodes in the network had more component-target interactions, which are likely to play a more important role in liver protection.

### GO and KEGG enrichment analyses

GO functional enrichment for the 17 core targets resulted in the identification of 26 biological processes, most of which

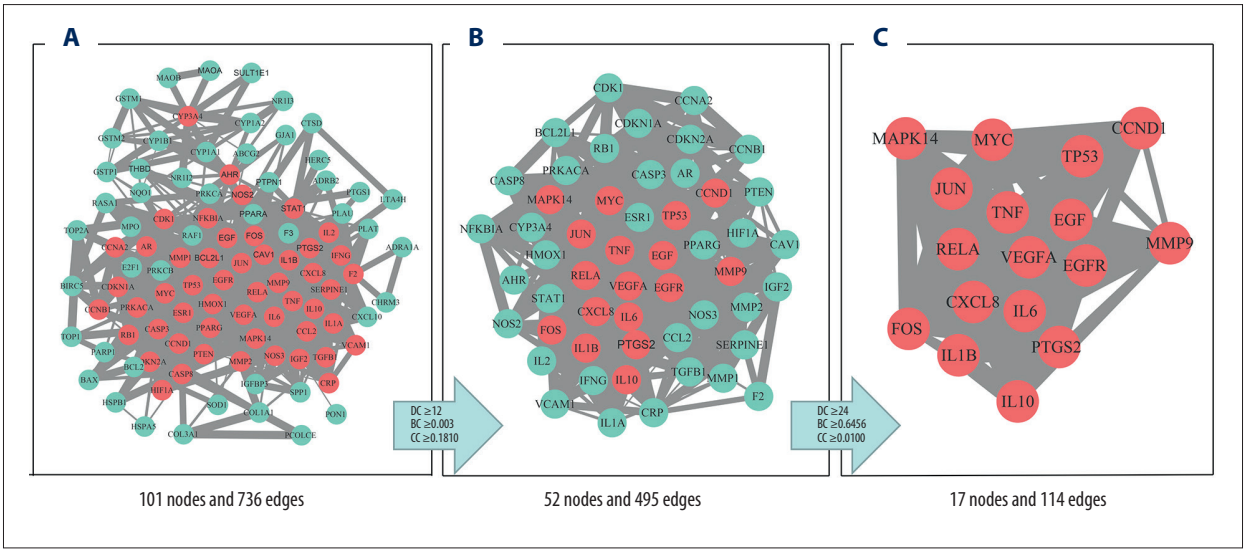


**Figure 3.** Processing of the topological analysis of the protein–protein interaction (PPI) network. The blue nodes represent the original input targets, and the red nodes represent the selected targets after topological analysis. The width of edges represents combined score. (**A**) original input targets in the PPI network; (**B**) sub-filtered targets in the PPI network based on the degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC) values; (**C**) hub targets in the PPI network.





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**Table 2.** Seventeen hub targets of *Anoectochilus roxburghii* (AR) against liver injury identified by topological analysis.

| Uniprot ID | Gene symbol | Protein name | Degree |
|---|---|---|---|
| P05231 | IL6 | Interleukin-6 | 38 |
| P05412 | JUN | Transcription factor AP-1 | 37 |
| P01375 | TNFA | Tumor necrosis factor | 37 |
| P04637 | TP53 | Cellular tumor antigen p53 | 37 |
| P15692 | VEGFA | Vascular endothelial growth factor A | 35 |
| P00533 | EGFR | Epidermal growth factor receptor | 29 |
| P01584 | IL1B | Interleukin-1 beta | 29 |
| P01106 | MYC | Myc proto-oncogene protein | 28 |
| MMP9 | MMP9 | Matrix metalloproteinase-9 | 26 |
| P01133 | EGF | Pro-epidermal growth factor | 26 |
| P24385 | CCND1 | G1/S-specific cyclin-D1 | 26 |
| P10145 | CXCL8 | Interleukin-8 | 26 |
| P35354 | PTGS2 | Prostaglandin G/H synthase 2 | 25 |
| Q16539 | MAPK14 | Mitogen-activated protein kinase 14 | 24 |
| P01100 | FOS | Proto-oncogene c-Fos | 24 |
| P22301 | IL10 | Interleukin-10 | 24 |
| Q04206 | RELA | Transcription factor p65 (NF-κB p65) | 24 |

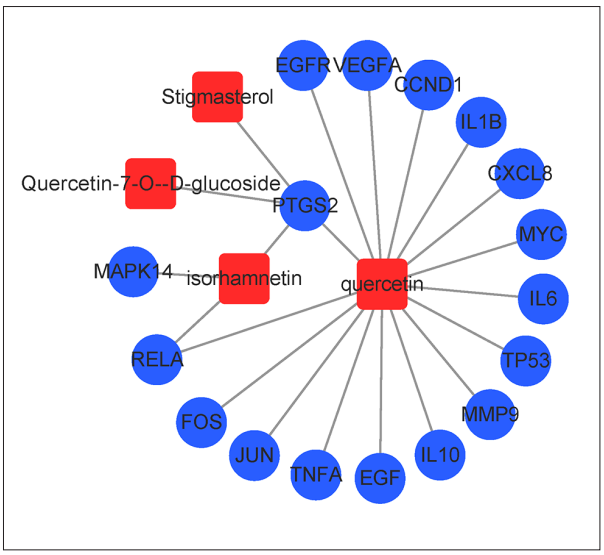


**Figure 4.** Network of bio-active compounds and core targets of *Anoectochilus roxburghii* (AR) against liver injury. Red square nodes denote bio-active compounds of AR, and blue round nodes denote the hub targets of AR against liver injury.

are involved in biological processes, are cell components, or have molecular functions as classified (Figure 5A). The biological processes mainly included positive regulation of transcription from RNA polymerase II promoter, positive regulation of transcription, DNA templates, responses to drugs, negative regulation of apoptotic process, inflammatory responses, positive regulation of gene expression, negative regulation of cell proliferation, positive regulation of smooth muscle cell proliferation, positive regulation of protein phosphorylation, cellular response to lipopolysaccharide, angiogenesis, positive regulation of nitric oxide biosynthesis, positive regulation of the ERK1 and ERK2 cascades, aging, response to muscle stretching, response to glucocorticoids, positive regulation of MAP kinase activity, positive regulation of epithelial cell proliferation, liver regeneration, and positive regulation of fever generation. Cell components included those in the extracellular space, whereas molecular functions included protein binding, transcription factor binding, enzyme binding, transcription regulatory region of DNA binding, and cytokine activity. These findings suggest that AR treatment of liver injury may be the result of a complex of multiple pathways with synergistic effects.

KEGG enrichment analysis identified 36 signaling pathways (Figure 5B), including the signaling pathways involving TNF, Toll-like receptors, mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)-Akt, forkhead box O (FoxO), and nucleotide-binding and oligomerization domain (NOD)-like receptor. Other signaling pathways included those involved in the development of hepatitis B, hepatitis C, non-alcoholic fatty liver disease (NAFLD), pancreatic cancer, colorectal cancer, prostate cancer, microRNAs in cancer, endometrial cancer, and bladder cancer.





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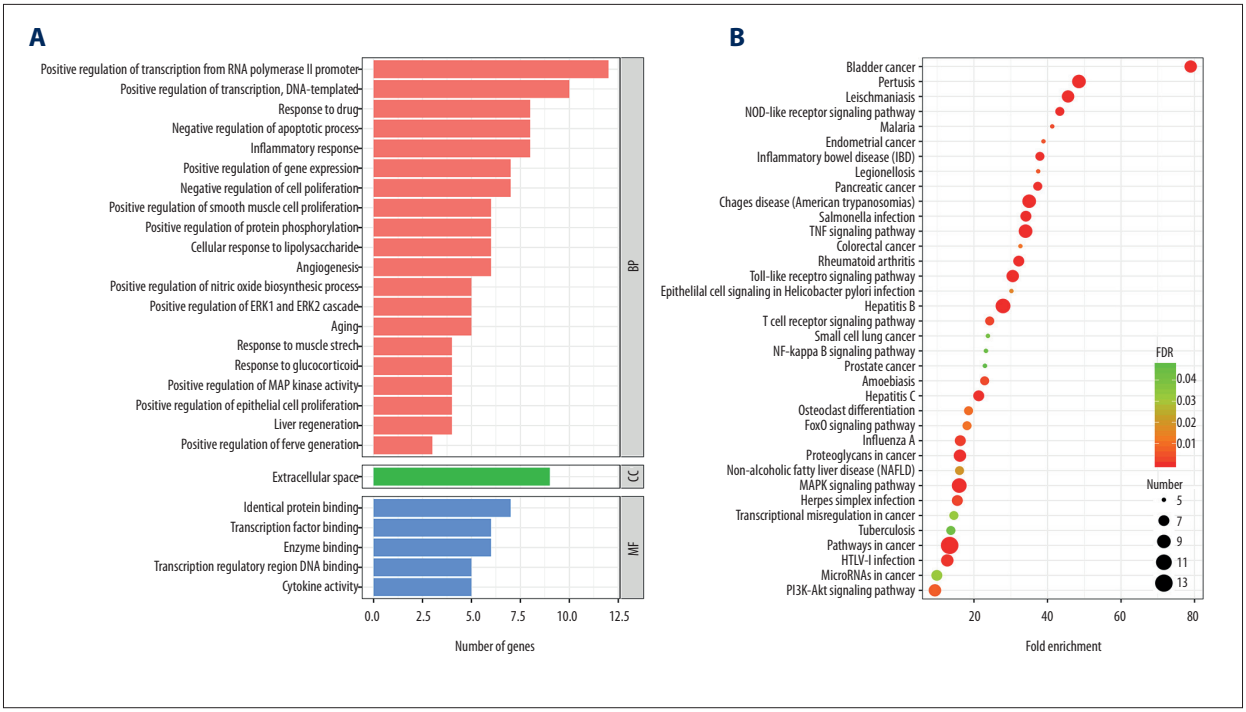


**Figure 5.** Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of hub targets related to liver injury. (**A**) GO enrichment analysis of hub targets for biological processes (BP), cellular components (CC) and molecular functions (MF). (**B**) KEGG enrichment analysis of hub targets.

### Protective effect of Que against liver damage

To confirm the results of network pharmacological analysis, Que, the key active ingredient in AR, was selected to determine whether it could prevent or alleviate liver injury induced by $CCl_4$ in mice. H&E staining (Figure 6) of normal control livers showed a normal hepatic lobular structure, with the hepatic cell bundles radiating from the central vein. Although most cells had a single central nucleus, some were binuclear (Figure 6A). Treatment with $CCl_4$ resulted in marked histopathological changes, such as swelling of hepatocytes in the central lobe of the liver, high cytoplasmic vacuolization, large areas of cell necrosis, and massive mononuclear cell infiltration (Figure 6B). Morphologic analysis of the mice treated with Que showed significant improvements. Compared with $CCl_4$ treated mice, low dose Que slightly reduced the area of injury, necrotic cells, and inflammatory infiltration (Figure 6C), whereas high dose Que showed significant improvement in cellular structure, significant reduction inflammatory cell infiltration, and disappearance of necrotic hepatocyte plaques (Figure 6D). The results indicated that Que could improve the histopathological changes in the liver induced by $CCl_4$.

### Effect of Que on serum ALT and AST levels

Compared with control mice, the serum levels of ALT and AST were significantly higher in mice treated with $CCl_4$ (Figure 7). Treatment with Que resulted in significant reductions in ALT and AST levels (Figure 7). Moreover, these liver-protecting effects of Que were found to be dose-dependent.

### Effect of Que on NF-κB signaling pathway in liver tissue

To further confirm the results of network pharmacological analysis, the mechanism by which AR suppresses inflammation in $CCl_4$-induced liver injury was investigated. Because the generation of pro-inflammatory cytokines, like TNF-α and interleukin 6 (IL-6), could induce activation of the NF-κB signaling pathway, the effects of Que on the levels of expression of NF-κB p65 and p-IκBα were assessed in these mice. The levels of expression of both NF-κB p65 and p-IκBα were significantly higher in liver tissue from $CCl_4$-treated than control mice. Treatment with Que, especially at the higher dose, reduced the expression of NF-κB p65 (Figure 8A, 8B) and p-IκBα (Figure 8A, 8C) compared with $CCl_4$-treated mice. These results indicated that the liver-protective effects of Que were closely related to its anti-inflammatory activity, consistent with the results of network pharmacological analysis.

## Discussion

Screening of AR for compounds that suppressed liver injury identified seven key active components, including four





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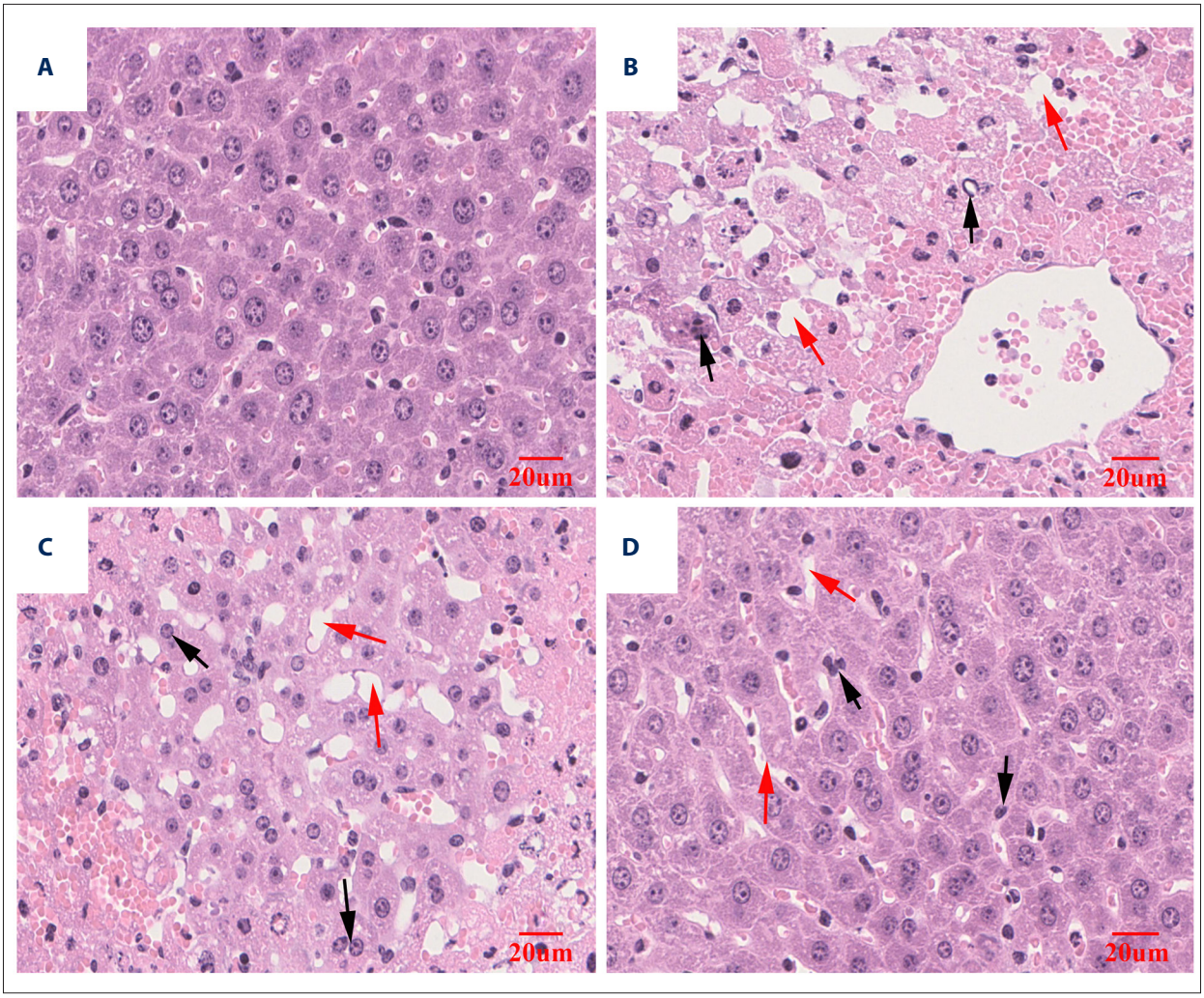


**Figure 6.** Histological analysis of liver injury induced by carbon tetrachloride ($CCl_4$) and the effects of quercetin (Que) in mice. (**A**) Control mice; (**B**) Mice treated with $CCl_4$; (**C, D**) Mice treated with (**C**) low dose (60 mg/kg) or (**D**) high dose (120 mg/kg) Que prior to treatment with $CCl_4$. The red arrows indicate vacuoles and the black arrows indicate inflammatory cell aggregates. (hematoxyline and eosin (H&E) staining; magnification, 100×).

flavonoids and three sterols. Flavonoids, such as Que, quercetin-7-O-β-D-glucoside, isorhamnetin, and rhamnazin, have been shown to reduce blood cholesterol concentration, liver toxicity, liver damage, and liver fibers [27–30], whereas sterols, such as campesterol, lanosterol, and stigmasterol, can lower cholesterol level, protect the liver, and have anti-inflammatory and anti-tumor effects [31,32]. The synergistic effects of these active ingredients are likely responsible for the liver-protecting effects of AR.

Topology analysis identified 17 hub targets related to liver injury. After the GO functional enrichment of the core targets, it was speculated that AR may suppress liver injury by the negative regulation of apoptosis, inflammatory responses, cell responses to lipopolysaccharides, liver regeneration, transcription factor binding, and cytokine activity. KEGG pathway enrichment analysis indicated that the core targets related to liver injury could participate in 36 signaling pathways. These included signaling pathways related to inflammation, such as the NF-κB, TNF, and MAPK signaling pathways; signaling pathways related to liver disease, such as hepatitis B, hepatitis C, and NAFLD; and signaling pathways related to immunity, such as Toll-like receptor and NOD-like receptor signaling pathways. PI3K-Akt and FoxO signaling pathways are involved in the cell proliferation, differentiation, and apoptosis, and other signaling pathways are related to the cancer. Therefore, various compounds in AR might act on multiple targets and pathways, and these compounds might play a pivotal role in the prevention and treatment of liver injury, mainly through signaling pathways involving inflammation, immunity, cell proliferation and differentiation, and liver disease related modules.





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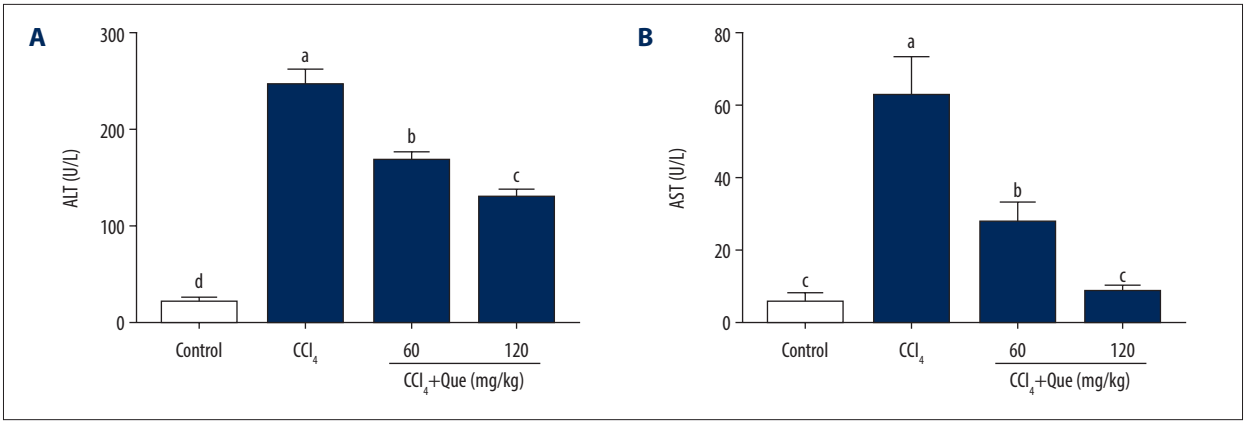


**Figure 7.** Effects of carbon tetrachloride ($CCl_4$) and quercetin (Que) on the activities of serum (**A**) aspartate aminotransferase (AST) and (**B**) alanine aminotransferase (ALT). Con, control mice; $CCl_4$: mice treated with 0.2% $CCl_4$ (10 mL/kg). All data were expressed as mean±standard deviation (SD) (n=5). Columns labeled with different letters indicate significant difference among groups ($p<0.05$).

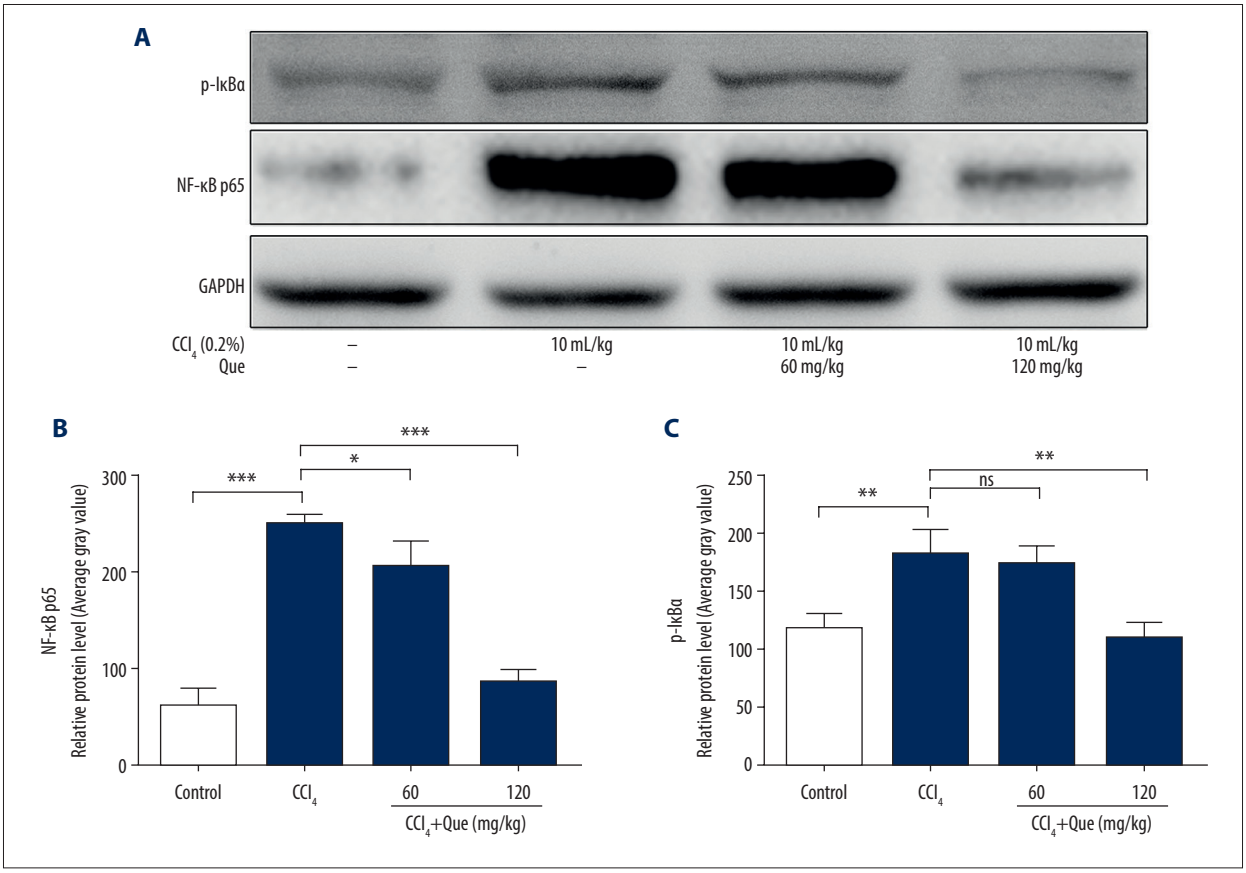


**Figure 8.** Effects of carbon tetrachloride ($CCl_4$) and quercetin (Que) on protein expression of nuclear factor-kappa B (NF-κB) p65 and phosphorylated IκBα (p-IκBα) in hepatic tissue of mice. (**A**) Western blotting analysis of NF-κB p65 and p-IκBα. (**B**) Quantification of the relative protein expression levels of NF-κB p65 and p-IκBα. All data were expressed as mean ±standard deviation (SD) (n=5). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with the mice treated with carbon tetrachloride ($CCl_4$).





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To confirm the reliability of the results of network pharmacological analysis, Que, the most important active ingredient in AR, was selected for experimental validation. Que significantly reduced ALT and AST levels, and alleviated pathological changes of liver injury induced by $CCl_4$. These findings indicated that Que could protect against liver injury induced by $CCl_4$. Que also reduced the levels of expression of NF-κB p65 and p-IκBα elevated in response to $CCl_4$ treatment, indicating that the Que in AR could exert liver-protecting effects by regulating the NF-κB pathway signaling [33–35]. Moreover, experimental validation showed that the liver-protecting effect of the active ingredients of AR was closely related to the suppression of inflammation, further confirming the reliability of the results of systematic network pharmacological screening, as well as the prediction of the molecular mechanism in this study.

## Conclusions

The liver-protecting effects of AR were likely due to the synergistic effects of various compounds, such as flavonoids and sterols. The ability of AR to suppress liver injury was associated with various biological activities, including anti-inflammatory and anti-immune activities. An important limitation of the present study was that $CCl_4$-induced liver injury in mice does not mimic liver injury induced by steatosis and general liver disease. Moreover, many of the signaling pathways screened in this study require further investigation.

### Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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### Conflicts of interest

None.

### Abbreviations

**ALT** – alanine aminotransferase; **ANOVA** – one-way analysis of variance; **AR** – *Anoectochilus roxburghii*; **AST** – aspartate aminotransferase; **CTD** – Comparative Toxicogenomics Database; **DL** – drug-like property; **FoxO** – forkhead box O; **GAPDH** – glyceraldehyde-3-phosphate dehydrogenase; **GO** – gene ontology; **H&E** – hematoxylin and eosin; **KEGG** – Kyoto Encyclopedia of Genes and Genomes; **MAPK** – mitogen-activated protein kinase; **NOD** – nucleotide-binding and oligomerization domain; **NF-κB** – nuclear factor-kappa B; **OB** – oral bio-availability; **PI3K** – phosphoinositide-3-kinase; **p-IκBα** – phosphorylated IκBα; **PPI** – protein–protein interaction; **Que** – quercetin; **TCM** – Traditional Chinese Medicine; **TCMSP** – Traditional Chinese Medicine Systems Pharmacology Database; **TNF** – tumor necrosis factor; **TTD** – Therapeutic Target Database.

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