Mechanisms exploration of Angelicae Sinensis Radix and Ligusticum Chuanxiong Rhizoma herb-pair for liver fibrosis prevention based on network pharmacology and experimental pharmacology

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Available online 20 Apr., 2021

[ABSTRACT] Angelicae Sinensis Radix (Danggui) and Ligusticum Chuanxiong Rhizoma (Chuan Xiong) herb-pair (DC) have been frequently used in Traditional Chinese medicine (TCM) prescriptions for hundreds of years to prevent vascular diseases and alleviate pain. However, the mechanism of DC herb-pair in the prevention of liver fibrosis development was still unclear. In the present study, the effects and mechanisms of DC herb-pair on liver fibrosis were examined using network pharmacology and mouse fibrotic model. Based on the network pharmacological analysis of 13 bioactive ingredients found in DC, a total of 46 targets and 71 pathways related to anti-fibrosis effects were obtained, which was associated with mitogen-activated protein kinase (MAPK) signal pathway, hepatic inflammation and fibrotic response. Furthermore, this hypothesis was verified using carbon tetrachloride (CCl₄)-induced fibrosis model. Measurement of liver functional enzyme activities and histopathological examination showed that DC dramatically reduced bile acid levels, inflammatory cell infiltration and collagen deposition caused by CCl₄. The increased expression of liver fibrosis markers, such as collagen 1, fibronectin, α-smooth muscle actin (α-SMA) and transforming growth factor-β (TGF-β), and inflammatory factors, such as chemokine (C-C motif) ligand 2 (MCP-1), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 in fibrotic mice were significantly downregulated by DC herb-pair through regulation of extracellular signal-regulated kinase 1/2 (ERK1/2)-protein kinase B (AKT) signaling pathways. Collectively, these results suggest that DC prevents the development of liver fibrosis by inhibiting collagen deposition, decreasing inflammatory reactions and bile acid accumulation, which provides insights into the mechanisms of herb-pair in improving liver fibrosis.

[KEY WORDS] Angelicae Sinensis Radix; Ligusticum Chuanxiong Rhizoma; Liver fibrosis; Herb-pair; Network pharmacology

Introduction

Liver fibrosis is the result of dysregulated wound-healing responding to a variety of pathological stimuli, characterized by the accumulation of extracellular matrix (ECM) [1]. If left untreated, liver fibrosis will lead to cirrhosis and even liver malignancy, which is a significant cause of morbidity and mortality worldwide. Modern pharmacological studies highlight critical biological mechanisms underlying fibrotic processes, including but not limited to activated hepatic stellate cells (HSCs), increased hepatic inflammation, unbalanced immune response and the interaction between hepatic non-parenchymal cells [2,3]. Despite considerable amounts of efforts have been made to treat liver fibrosis, the clinical management of this disease is still challenging because of the poor understanding of its pathogenesis and thus lack of effective therapeutic options. Hence, it is of the utmost importance to identify vital mechanisms and discover innovative therapeutic drugs for the treatment of liver fibrosis.

Over the past thousands of years, Traditional Chinese medicine (TCM) has been used in clinical practice and makes a great contribution to the management of human diseases. A number of clinical trials and experimental studies have proved that TCM or herbal drugs exert unique advantages...
due to their synergistic drug combination and fewer adverse effects. Compared with traditional formulas, herb-pair (the compatibility of two certain herbs), which is characterized with more simplified form and clearer pharmacological effects/mechanism, is attracting growing attentions. Among abundant TCM, Angelicae Sinensis Radix (Danggui), the dried radix of Angelica sinensis (Oliv.) Diels (Umbelliferae) and Ligusticum Chuanxiong Rhizoma (Chuanxiong), the dried root of L. chuanxiong Hort. (Umbelliferae), are most popular herbal medicines used for improving various vascular diseases including chronic liver disorders [4,5]. In addition, Angelicae Sinensis Radix and Ligusticum Chuanxiong Rhizoma have been widely applied in medicine, food therapy and health preserving and protection. The combination of Angelicae Sinensis Radix and Ligusticum Chuanxiong Rhizoma is not only a classic formula, but also is a famous herb-pair (DC herb-pair) commonly used in Chinese medicine to improve blood circulation, prevent inflammation and alleviate pain [6,7]. According to the difference of compatibility ratio, DC herb-pair can be divided into Fo-Shou-San, Guixiong Powder and Yiqi powder. The current understanding of its effects on blood stasis, thrombosis, local ischemia, and its bioactivities on the regulation of hepatic inflammation and bile acid metabolism has shed novel lights for its potential application in the treatment of liver fibrosis [8,9]. Several experimental studies have reported that DC herb-pair and some prescriptions containing DC herb-pair, such as Siwu decoction and Taohong Siwu Decoction, exerted blood-tonifying, migraines relief, improved blood flow and iron metabolism in vivo [10,11]. DC and these prescriptions have also been traditionally used to improve blood stasis and treat cardiovascular and liver diseases, including but not limited to hepatic necroinflammation and fibrosis [12,13]. However, molecular mechanisms of DC herb-pair in the prevention of liver fibrosis development are still indecisive.

Network pharmacology has become an efficient way to systematically investigate the pharmacological effects and potential mechanisms of TCM by constructing the "drug-component-targets" network and screening out the nodes involved in pathogenetic process. In recent years, researchers are paying more attention on multi-ingredients and multi-targets system in TCM and have successfully applied network pharmacology approach to identify the molecular mechanisms of TCM formula in various diseases. Researches showed that the prediction and verification of targets of some herb-formulas, such as Qingluo Tongbi, Erzhi Pill, Yinchenhao decoction have been established with the application of network pharmacology, which offer the guidance for developing novel drug targets and studying the underlying mechanism for the treatment of liver diseases [14-16].

In this study, both network pharmacology analysis and experimental pharmacology verification were applied. At first, we carried out the network pharmacology approaches to predict the targets and mechanisms of DC in the treatment of liver fibrosis. Then, we used experimental pharmacology method to validate and evaluate the effects of different dosage of DC on carbon tetrachloride (CCL4)-induced hepatic fibrosis in mouse model, and explored the underlying mechanisms.

Materials and Methods

Materials and chemicals

Formaldehyde and phosphate buffer saline (PBS) solution were provided by Beijing Solarbio Technology Co., Ltd. (Beijing, China). Ferulic acid and ligustrazine were purchased from Innochem Technology Co., Ltd. (Beijing, China). iQ™ SYBR Green Supermix were purchased from Bio-Rad (Hercules, CA). CCl4 and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against phosphorylated ERK1/2 (p-ERK1/2) (sc-7383), ERK1 (sc-271269), ERK2 (sc-1647) were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against phosphorylated protein kinase B (p-AKT) (4060), total-AKT (p-AKT) (4685), β-Actin (4970) were purchased from Cell Signaling Technology (MA, USA).

Preparation of DC extract

The raw herbs, including the root of Angelicae Sinensis Radix (Danggui) and Ligusticum Chuanxiong Rhizoma (Chuanxiong) were purchased from Beijing, China, and were authenticated by Dr. WANG Dai-Jie and then kept in our laboratory. By reviewing a TCM database containing DC, we found that the most frequent and commonly used ratio for DC herb-pair was 1 : 1 (m : m). Danggui and Chuanxiong (60 g, mass ratio 1 : 1) were sliced and then extracted with 480 mL distilled water (sample : solvent, 1 : 8, V/W) under reflux for 40 min. Extracted liquid was transferred to a container and then the remaining was extracted again with 360 mL distilled water (sample: solvent, 1 : 6, V/W) under reflux for 40 min. The extractions were combined and concentrated with a rotary evaporator at 45 °C until the final volume was 60 mL. Finally, DC extract was suspended in distilled water, aliquoted into sterile tubes and stored at 4 °C.

A qualitative analysis of the major ingredients in the DC was performed using a Shimadzu High Performance Liquid Chromatography (HPLC) system with reference standards. The chromatographic separations of DC were performed on a Kromasil C18 column (4.6 mm × 250 mm, 5 μm) at 25 °C. The mobile phases consisted of eluent A (0.1% formic acid in water, V/W) and eluent B (acetonitrile) with a flow rate set at 1.0 mL·min⁻¹. A linear gradient program was performed as follows: 13%−21% B at 0−20 min, 21% B at 20−23 min, 21%−13% B at 23−28 min, 13% B at 28−38 min. A 10 μL aliquot of each sample solution was injected into the HPLC system for analysis at 280 nm.

Network pharmacology-based prediction of the potential effects of DC on liver fibrosis

Targets for compound of DC and liver fibrosis

Firstly, all of compounds of Angelicae Sinensis Radix and Ligusticum Chuanxiong Rhizoma were obtained by searching the TCM systems pharmacology database and liter-
total RNA isolation. Isolated total RNA was pared for sections or frozen in liquid nitrogen for total RNA isolation. During liver functional enzyme activities and livers were pre-weighted and sacrificed. Blood was collected for measurement of serum liver functional enzyme activities and total bile acid levels.

Measurement of serum liver functional enzyme activities and total bile acid levels

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bile acid (TBA) and direct bilirubin (DBIL) were determined following the manufacturer’s instructions of corresponding kits as previously described [19].

Histopathology and Masson’s Trichrome staining

Mice were sacrificed and the livers were immobilized with 4% formaldehyde and embedded in paraffin. Tissues were cut into 4.5-μm sections and stained with hematoxylin and eosin (H&E) and Masson’s Trichrome as previously described [5]. Images were obtained using Olympus 1X81 microscope with a 20X objective (Carl Zeiss, Germany). The degree of hepatic injury was evaluated and histological changes of inflammatory infiltration were scored. Quantification of collagen deposition in Masson’s Trichrome staining (the percentage of blue collagen area relative to the total tissue area) was conducted using Image J software.

Quantitative real-time PCR

Total RNA from mouse livers was isolated by Trizol reagent following manufactures instruction and quantified using 2000 Nanodrop Spectrophotometer from Thermo (DE, USA). After extraction, 1 μg of RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Kit from Vazyme Technology Co., Ltd. (Nanjing, China). Quantitative real-time PCR (qPCR) was performed as described previously [18]. The primer sequences for qPCR had been listed in Table 1. The results were normalized using HPRT1 as an internal control.

Western blotting

Total protein of mouse liver samples was extracted with RIPA buffer (Beyotime, China) and were quantified by BCA Protein Assay Kit and prepared with 4X loading buffer (Beyotime, China). After resolved in 10% SDS-PAGE gel, proteins were transferred to the PVDF membrane, blocked 5% milk at room temperature for 1 hour, add incubated in specific primary antibodies at 4 °C overnight. After rinsing with TBST for 3 times, proteins were incubated with corresponding secondary antibody, detected on a Bio-Rad Gel Doc XR’ Imaging System (CA, USA) and analyzed by Quantity One software.

Statistical analysis

Data from at least three independent experiments in all studies are expressed as the mean ± SEM and repeated. Statistical analysis was performed using two-tailed Student’s t test between two groups or One-way analysis of variance with Tukey’s post-hoc test among multiple groups using GraphPad Prism 5 software (GraphPad, San Diego, CA). P value ≤ 0.05 was defined statistically significant.
Results

Network pharmacology prediction

In order to better investigate its potential mechanism of DC herb-pair against liver fibrosis, the network pharmacology approach was employed to predict its potential targets.

Collection of active compounds and potential targets of DC herb-pair

A total of 125 and 189 compounds were obtained from Angelicae Sinensis Radix and Ligusticum Chuanxiong Rhizoma from the TCMSP database, respectively. A total of 11 major ingredients were then selected as the candidate bioactive compounds in DC herb-pair when taking OB and DL into consideration. It is worth noting that although 5-hydroxymethylfurfural and chlorogenic acid were not detected in the DC after filtering by OB and DL values, they have been reported to exhibit remarkable pharmacological activities as bioactive compounds [19, 20]. Collectively, 13 ingredients were used to construct the network for analysis (Table 2). A total of 202 targets related to Angelicae Sinensis Radix and Ligusticum Chuanxiong were obtained by uploading the Smiles format of the candidate compound to Swiss Target Prediction. Combining the targets related to active compounds of DC herb-pair, the drug-compound-targets network was established. As shown in Fig. 1A, 217 nodes (2 composition of DC herb-pair, 13 active compounds and 202 potential targets) and 682 edges (the interactions between different nodes) were included in the drug-compound-targets network.

Table 1  List of primers used for qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>GACCTCAAGAGCTCTAACATCC</td>
<td>GTCATCACAGACAGATG</td>
</tr>
<tr>
<td>α-SMA</td>
<td>GTCATCCACAGACAGATG</td>
<td>GTCATCACAGACAGATG</td>
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<tr>
<td>Collagen 1</td>
<td>TGAACGTGGGTGTAAGGTC</td>
<td>CCATTTTACAGGGAACAT</td>
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<tr>
<td>Fibronectin</td>
<td>CTATAGGATTGGAGACACGTGG</td>
<td>CTGAGGATTGGAGACACGTGG</td>
</tr>
<tr>
<td>PCNA</td>
<td>CCGAGACCTTAGCCACATGG</td>
<td>CTCCTCTTATTCACATTAC</td>
</tr>
<tr>
<td>e-Myc</td>
<td>GGAATACTAGCCTCGACTAC</td>
<td>CTGCTGTTGCTGGTGATA</td>
</tr>
<tr>
<td>H19</td>
<td>CATCCAGCCTTGTGAAC</td>
<td>GGATAGCACCATTCTTT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TTCACAACACCTAAGACACCTCC</td>
<td>GCCATACAGTCGAGCAGCAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>CTCCAACAGACCTGCTTATAC</td>
<td>GACCTCAAGAGCTCTAAACATCC</td>
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<td>TNF-α</td>
<td>GACCTCAAGAGCTCTAACATCC</td>
<td>GTCATCACAGACAGATG</td>
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<tr>
<td>IL-1β</td>
<td>AATCTCACAGCAGACATC</td>
<td>AGCCAGGTTACATCATCATCC</td>
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<tr>
<td>CYP7A1</td>
<td>CAGAGCATATAGACACAGTAGT</td>
<td>GTGACAAGGCACATCATCC</td>
</tr>
<tr>
<td>HPRT1</td>
<td>CAGACTTTGTTGGATTGAAA</td>
<td>GCTCATTAGGCTTTGTAT</td>
</tr>
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Table 2  List of 13 ingredients selected as the candidate compounds in DC herb-pair

<table>
<thead>
<tr>
<th>ID</th>
<th>Compound name</th>
<th>OB/%</th>
<th>DL</th>
<th>Medicinal herb</th>
</tr>
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<tbody>
<tr>
<td>MOL000360</td>
<td>Ferulic acid</td>
<td>39.56</td>
<td>0.06</td>
<td>Danggui/Chuanxiong</td>
</tr>
<tr>
<td>MOL000358</td>
<td>β-sitosterol</td>
<td>36.91</td>
<td>0.75</td>
<td>Danggui</td>
</tr>
<tr>
<td>MOL000449</td>
<td>Stigmasterol</td>
<td>43.83</td>
<td>0.76</td>
<td>Danggui</td>
</tr>
<tr>
<td>MOL000748</td>
<td>5-Hydroxymethylfurfural</td>
<td>45.07</td>
<td>0.02</td>
<td>Danggui</td>
</tr>
<tr>
<td>MOL001955</td>
<td>Chlorogenic acid</td>
<td>11.93</td>
<td>0.33</td>
<td>Danggui</td>
</tr>
<tr>
<td>MOL001494</td>
<td>Mandenol</td>
<td>42.00</td>
<td>0.19</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL002135</td>
<td>Myricanone</td>
<td>40.60</td>
<td>0.51</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL002140</td>
<td>Perifolylone</td>
<td>65.95</td>
<td>0.27</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL002151</td>
<td>Senkyunone</td>
<td>47.66</td>
<td>0.24</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL002157</td>
<td>Wallchilide</td>
<td>42.31</td>
<td>0.71</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL000359</td>
<td>Sitosterol</td>
<td>36.91</td>
<td>0.75</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL000433</td>
<td>Folic acid</td>
<td>68.96</td>
<td>0.71</td>
<td>Chuanxiong</td>
</tr>
<tr>
<td>MOL002202</td>
<td>Tetramethylpyrazine</td>
<td>20.01</td>
<td>0.03</td>
<td>Chuanxiong</td>
</tr>
</tbody>
</table>
network. Pink arrows represented the ingredients of DC, yellow circles represented the active compound of DC, and green circles represented the targets of these compounds.

**PPI, GO-BP and KEGG pathway enrichment analysis**

To elucidate underlying effects of DC herb-pair on liver fibrosis, we sought for the targets of liver fibrosis and overlapped with the targets of DC herb-pair. 1028 targets were obtained related to liver fibrosis from OMIM and Genecard database with the organism limited to “Homo sapiens”. Comparing the potential targets of DC herb-pair with the 1028 candidate targets relating to liver fibrosis, there was an overlap containing 46 targets, such as AKT, epidermal growth factor receptor (EGFR) and signal transducer and activator of transcription 3 (STAT3), suggesting the hepatoprotective effects of DC herb-pair against liver fibrosis. In the String database, the PPI network was established with 46 elements and the results were imported into Cytoscape software to modified the interaction network. The PPI selected targets were obtained from using median values to determine key targets and constructed the big hub nodes as the main targets that may cause the effect of DC on liver fibrosis, based on “degree” and “closeness certainty” \[21\]. The threshold values were degree ≥ 12 and closeness ≥ 0.016 and the details were shown in Fig. 2A, which consisted of 46 nodes and 424 edges. The complex PPI network showed that there was affected and restrict relationship among these targets involved in liver fibrosis. The top 5 targets were AKT1, heat shock protein 90 kDa alpha A1 (HSP90AA1), EGFR, phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) and sarcoma gene (SRC).

To further identify the functions of these targets, a network of GO-BP was constructed and analyzed. ClueGO was used for enrichment analysis of the 46 targets. The threshold was selected as \( P < 0.05 \) and 43 GO terms were retrieved. As shown in Fig. 2B, 43 biological processes were mainly involved in MAPK signal pathway, fibrotic response and inflammation. According to manual characterization, all terms were further divided into three categories, in the group of
MAPK signal pathway: ERBB signaling pathway (GO:0038127), EGFR pathway (GO:0007173) and protein tyrosine kinase (PTK) activity (GO:0004713); in the group of fibrotic response: positive regulation of blood vessel endothelial cell migration (GO:0043536), epithelial cell migration (GO:0010631), and regulation of focal adhesion assembly (GO:0051893); in the aspect of autography: positive regulation of autophagy of mitochondrion (GO:1903599). Based on above, we speculated that the major mechanisms underlying anti-fibrotic effects of DC herb-pair may be the intricate multi-path synergy between these functions.

Subsequently, KEGG pathways was performed to evaluate relevant pathways. Totally 46 targets were found to be involved in 71 pathways and top 20 pathways of enrichment were selected for bubble visualization analysis. As shown in Fig. 2C, nodes with higher enrichment indicated a more important role played in this network. It was not difficult to find that ERBB is relatively significant signaling pathway (gene ratio: 21.2% with a P-value of 4.67E-23) among the Top 20 pathways, followed by VEGF, neurotrophin, T cell receptor, NF-kappa B (NF-κB), Fc epsilon RI, estrogen and MAPK signaling pathways, which play crucial roles in anti-fibrotic effects of DC herb-pair. Targets involved in more pathways might play more important roles in the crosstalk and interaction between those different pathways. Therefore, these results indicated that the anti-liver fibrosis effect of DC was closely related to the activities of these signaling pathways.
Animal experiment verification

To further validate the results obtained from network pharmacology, we used mouse models for pharmacological studies in vivo. Considering that CCl₄ was widely used to induce chronic fibrotic liver injury, we prepared the DC herb-pair samples and selected CCl₄-treated mice as experimental models.

DC herb-pair attenuated CCl₄-induced liver fibrosis in mice

Previous studies presented that bioactive aromatic acids (ferulic acid, chlorogenic acid and caffeic acid) and phthalides (ligustrazine, senkyunolide I, senkyunolide H, senkyunolide A, butylphthalide, and butylidenephthalide) were found and simultaneously quantified by using HPLC method [22]. Compared with other active ingredients, ferulic acid in Angelicae Sinensis Radix and ligustrazine in Ligusticum Chuanxiong Rhizoma were believed to be two representative compounds and regarded as markers of quality control [23]. We conducted a qualitative analysis of the main components in DC by using HPLC system with reference standards and reconciled the previous study. As shown in Fig. 3, in addition to 5-hydroxymethylfurfural, caffeic acid, chlorogenic acid, and senkyunolide H, ligustrazine (peak 1) and ferulic acid (peak 2) were also identified from DC herb-pair samples. Combined with the prediction of network pharmacology, we proposed that these two ingredients may be the more important active ingredients in this DC herb-pair.

Later, we evaluated the in vivo effects of DC herb-pair in CCl₄-induced liver fibrosis. After sacrificed, livers and spleens of mice were weighted for calculating the ratio of tissue and body weight. The ratios of liver or spleen weight over body weight of CCl₄ mice were increased, and were slightly decreased after DC herb-pair treatment (Fig. 4A and 4B). To further investigate whether DC herb-pair improved liver function, serum levels of biochemical indicators of liver function and TBA were determined by corresponding kits. As shown in Fig. 4C and 4D, serum levels of AST and ALT were markedly decreased in DC herb-pair treatment groups compared with elevated levels in CCl₄ model group. Although serum ALP was not changed after DC administration (data not shown), serum TBA and DBIL showed a significant reduction compared to CCl₄ model group (Fig. 4E and 4F). Histological analysis further revealed that inflammatory cell infiltration and severe damage in livers of CCl₄ mice were markedly attenuated by DC herb-pair treatment (Fig. 5A). Fragmented hepatic nuclei and the formation of collagenous fibrous are usually observed in fibrotic area. As shown in Fig. 5B, Masson’s trichrome staining showed that DC treatment significantly decreased the CCl₄-caused abundant deposition of collagen in a dose-dependent manner. These results were consistent with the predictions of network pharmacology and indicated that DC herb-pair alleviated CCl₄-mediated hepatotoxicity, bile acid accumulation and fibrotic liver injury.

DC herb-pair abrogated ERK1/2 and AKT signaling pathways in mice

Based on the observations of GO and KEGG analysis, MAPK and EGFR/ERBB were shown to be the most significantly changed signaling pathways. Generic MAPK signaling pathway consists of four distinct cascades, including the ERK1/2, Jun amino-terminal kinases (JNK1/2/3), p38-MAPK and ERK5 and aberrant activation of MAPK signaling has been associated with various liver diseases. The EGFR/Pi3K/AKT signaling pathway was also reported to be required for fibrosis pathogenesis, and the inhibition of AKT signaling could inhibit the activation and migration of HSCs [24]. Furthermore, our previous studies also showed that ERK1/2 and AKT signaling pathways was responsible for conjugated bile acids-mediated bile duct proliferation, inflammation and cholestatic liver injury in both hepatocytes and cholangiocytes [25-27]. To further elucidate the effects of DC herb-pair on MAPK/EGFR signaling pathways, the protein levels of p-ERK1/2, total ERK1/2 (t-ERK1/2), p-AKT and t-AKT were determined by western blot analysis. As shown in Fig. 6A, we found that CCl₄-induced the activation and phosphorylation of ERK1/2 and AKT were significantly and dose-dependently abolished in the presence of DC herb-pair.

DC herb-pair inhibited target fibrotic genes expression in mice

Based on the observations that DC herb-pair inhibited ERK1/2-AKT activation, we next investigated the effects of DC herb-pair on the expression of fibrotic genes and downstream targets in vivo using qPCR assay. Activation of transforming growth factor-β (TGF-β) promotes liver fibrosis by activating HSCs and subsequently activated HSCs triggered

![Fig. 3 Representative HPLC chromatograms of DC aqueous extract. 1, Ligustrazine; 2, Ferulic acid](image-url)
the expression of fibrotic markers, such as alpha-smooth muscle actin (α-SMA), collagen1 and fibronectin [2]. To identify the role of DC herb-pair in the progress of liver fibrosis, we examined the expression of fibrotic genes in livers. Expressions of TGF-β and its downstream targets, α-SMA, fibronectin and collagen1, were sharply increased in CCl4 groups but dramatically decreased in DC herb-pair treatment groups (Fig. 7A−7D). Interestingly, although only high dose of DC significantly downregulated TGF-β levels, there was a striking decrease in hepatic α-SMA expression in DC herb-pair treated groups (Fig. 7B). Additionally, increasing of cell proliferation, especially for HSCs and vessel endothelial cells, participates in liver fibrosis progression. Proliferating cell nuclear antigen (PCNA) is known as a molecular marker for cell proliferation, and c-Myc, a transcription factor of oncogene MYC, links growth factor stimulation and cellular proliferation [28]. To further determine whether DC herb-pair prevented cell proliferation caused by CCl4, we compared hepatic PCNA and c-Myc levels in different groups. As shown in Fig. 7E−7F, hepatic PCNA and c-Myc levels in CCl4 group were significantly increased compared to control group, but were markedly decreased after DC herb-pair treatment.

Liver fibrosis is often accompanied with chronic inflammation and high levels of pro-inflammatory chemokines and cytokines, such as chemokine (C-C motif) ligand 2 (MCP-1), tumor necrosis factor-α (TNF-α), IL-1β and IL-6 [29]. It has been reported that c-Myc induced the expression of long non-coding RNA (lncRNA) H19 by allele-specific binding and H19 was increased during periods of cell proliferation and liver fibrosis [30]. Our most recent study further showed that hepatic H19 level was closely correlated with macrophage ac-

![Fig. 4](image-url) Anti-fibrotic effects of DC herb-pair on mice with CCl4-induced liver injury. (A) The ratio of liver and body weight. (B) The ratio of spleen and body weight. (C−F) Serum levels of AST, ALT, TBA and DBIL. Values are presented as the mean ± SEM. Statistical significance: **P < 0.01, ***P < 0.001 vs control group; *P < 0.05, ##P < 0.01, ###P < 0.001 vs CCl4 group (n = 6).
tivation and hepatic fibrosis by increasing the expressions of IL-6 and MCP-1 and promoting the macrophages recruitment [2]. Combining the results of network pharmacology and previous findings, we hypothesized that the regulation of inflammatory responses might contribute to the effects of DC herb-pair on liver fibrosis and then evaluated the expression of these pro-inflammatory chemokines and cytokines in different groups. Supporting our hypothesis, we found that CCl\textsubscript{4}-induced H19 expression was significantly and dose-dependently suppressed by DC herb-pair treatment (Fig. 8A). CCl\textsubscript{4} treatment significantly increased mRNA levels of MCP-1 and IL-6, which was markedly inhibited by DC herb-pair administration (Fig. 8B and 8C). Although no significant decrease was observed in the expression of TNF-\textalpha and IL-1\beta in DC low group, medium and high doses of DC were significantly downregulated these pro-inflammatory cytokines caused by CCl\textsubscript{4} (Fig. 8D and 8E).

It is well recognized that bile acids play a vital role in the development of cholestasis and liver fibrosis by activating bile acid receptors and modulating downstream pathways [18]. Given the observation that DC herb-pair decreased the serum TBA levels induced by CCl\textsubscript{4}, we measured the hepatic expression of cholesterol 7 alpha-hydroxylase A1 (CYP7A1), the rate-limiting enzyme of bile acid synthesis. Hepatic CYP7A1 levels increased by CCl\textsubscript{4} was significantly suppressed by DC herb-pair treatment at medium and high doses.

Fig. 5 Liver tissue histopathological changes. (A) Representative images of HE staining (20 ×). Inflammatory cell infiltration in livers (Black arrows). The injury score in each group are shown. In brief, 0, no inflammation or fibrosis; 1, confluent or intralobular inflammation; 2, focal necrosis or portal fibrosis; 3, serious confluent inflammation or bridging fibrosis; 4, cirrhosis. (B) Representative images of Masson’s trichrome staining (20X). The relative collagen fiber content in each group are shown. Values are presented as the mean ± SEM. Statistical significance: ***P < 0.001 vs control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs CCl\textsubscript{4} group (n = 6).
Fig. 6  Effects of DC herb-pair on ERK1/2 and AKT signaling pathways. (A) Protein levels of p-ERK1/2, t-ERK1/2, p-AKT and t-AKT were determined by western blot analysis and normalized with t-ERK1/2 and t-AKT. β-Actin served as a loading control. Representative images are shown. Values are presented as the mean ± SEM. Statistical significance: *P < 0.01 vs control group; **P < 0.05, ***P < 0.01, ****P < 0.001 vs CCl4 group (n = 3)

Fig. 7  Effects of DC herb-pair on the mRNA expression of fibrotic genes. (A–F) The mRNA expression of TGF-β, α-SMA, collagen1, fibronectin, PCNA and c-Myc in mice were determined by qPCR and normalized using HPRT1 as an internal control. Values are presented as the mean ± SEM. Statistical significance: *P < 0.05, ***P < 0.001 vs control group; *P < 0.05, **P < 0.01, ***P < 0.001 vs CCl4 group (n = 6)
Discussion

Previous studies indicated that DC herb-pair contributes to the pharmacological effects including improving blood circulation, preventing atherosclerosis, regulating menstruation and relieving pain. In the current study, we used network and experimental pharmacology to evaluate the underlying mechanism of DC herb-pair in the prevention of liver fibrosis (Fig. 9). As the results of network pharmacology, 13 compounds with standard-compliant OB, DL or activities were screened out and predicted as active ingredients of DC herb-pair. The major bioactive compounds in DC were also identified by HPLC analysis. Previous studies have demonstrated that ferulic acid and ligustrazine improve liver injury through the reduction of inflammatory signaling pathways and oxidative damage in different animal models [31, 32]. Recently, our unpublished studies indicated that ferulic acid and ligustrazine might inhibit HSC activation and decrease collagen deposition (data not shown). Whether and how ferulic acid and ligustrazine are involved in the progression of bile acid synthesis and fibrosis remain to be further examined. Subsequently, 46 important targets, 20 pathways and the PPI network were obtained through Cytoscape software. The PPI network showed a complex interlaced network and indicated that several typical targets and pathways, including AKT, EGFR, MAPK and TNF signaling pathway have high probabilities of combination with active ingredients, which may

Fig. 8 Effects of DC herb-pair on the mRNA expression of inflammatory genes. (A−F) The mRNA expression of H19, MCP-1, IL-6, TNF-α, IL-1β and CYP7A1 in mice were determined by qPCR and normalized using HPRT1 as an internal control. Values are presented as the mean ± SEM. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001 vs control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs CCl4 group (n = 6); 0.05, ##P < 0.01, ###P < 0.001 vs CCl4 group (n = 6)
contribute to DC-induced anti-fibrotic effects. Finally, these mechanisms were verified by in vivo experiments. By constructing the drug-ingredient-targets network, we demonstrated that multiple ingredients in DC affected on multi-targets and predicated signaling pathways in the prevention of liver fibrosis, but further experimental evidence is required.

Many factors have been demonstrated to affect the efficacy of herb-pair, such as solvent, the ratio of drugs, boiling time and temperature. It has been reported that Ligusticum Chuanxiong Rhizoma significantly prolonged the half-life of distribution and increased the absorption of Angelicae Sinensis Radix, which exerted a synergic action when used together [33]. A combination of DC herb-pair (3 : 2) is known as classic formula, Fo-Shou-San, first recorded in Pu Ji Ben Shi Fang, the monograph concerning TCM and has been used in the treatment of blood deficiency, blood stasis and ischemic cerebral vascular disease for about thousand years [34]. A combination of DC herb-pair in different ratio also have been recorded such as Yiqi powder (10 : 7), Shengma powder (3 : 50) and Muhuang decoction (1 : 1) [35]. In addition, researchers reviewed a database of DC herb-pair and found that most frequent ratio was 1 : 1 and this combination was commonly used in blood deficiency syndrome, cardiovascular disorders, gynecology and chronic liver diseases [36, 37]. Concurrently, different ratio of DC herb-pair have also been reported to exert multiple pharmacological effects, including anti-neurovascular headache, anti-tumor and anti-carcinogenesis [38, 39]. Further studies are still required to elucidate the best ratio of DC herb-pair against liver fibrosis and the exact way of this herb-pair-decreased ERK1/2 and AKT signaling in fibrotic mouse model.

The essence of liver fibrosis in TCM theory is primarily liver-blood stasis [9]. It has been reported that DC herb-pair with different proportions or in different prescriptions have effects on promoting blood circulation and dissolving blood stasis, which further regulate physiological responses, such as inflammation responses and vascular endothelial cell proliferation [7, 40]. As expected, our HE staining results demonstrated that different dosage of DC herb-pair attenuated CCl₄-induced inflammatory cell infiltration (Fig. 5A), which further suggests the anti-inflammatory effects of this herb-pair against liver fibrosis. Our qPCR results further showed that the mRNA expressions of PCNA and c-Myc were reduced in DC treated group (Figs. 7E−8F), which was consistent with these findings and further verified our hypothesis. Recently, we demonstrated that H19 level was closely correlated with macrophage activation, hepatic inflammation and fibrosis progression [2]. As shown in Fig. 7F and 8A, the expression of hepatic H19 and its direct target, c-Myc in CCl₄ group was very high but significant decreased after DC herb-pair treatment. Consistently, we also found that DC herb-pair dramatically suppressed the mRNA expression of different pro-inflammatory cytokines or chemokines in livers (Fig. 8B−8E). These observations favor the hypothesis that DC herb-pair might also directly improve blood circulation, decrease the hepatic accumulation of inflammatory factors and therefore have effective therapeutic effects on liver fibrosis.

Sphingosine 1-phosphate (S1P) can activate specific S1P receptors (S1PRs) and play a critical role in various cellular processes [41]. Previously, we found that both conjugated bile acids and S1P promoted cholangiocyte proliferation and aggravated bile acid ligation (BDL)-induced liver fibrosis by activating ERK1/2 and AKT signaling pathways [42]. It has been shown that ERK1/2 activation further resulted in the
production of S1P in nucleus [43]. Endogenous metabolites involved in thiamine metabolism and sphingolipid metabolism could be regulated closer to normal level after DC herb-pair intervention [4]. Interestingly, our network pharmacology results indicated the involvement of MAPK, AKT and S1P pathways in the effects of DC herb-pair against liver fibrosis. Consistently, in vivo study demonstrated that DC herb-pair significantly downregulated the mRNA expression of CYP7A1 and sphingosine kinase 2 (data not shown) and significantly inhibited ERK and AKT signaling pathways, leading to decreased serum levels of TBA (Fig. 8F). These studies suggest that cross-talk between DC herb-pair and S1P/bile acids-mediated downstream signaling pathways play a pivotal role in regulating liver fibrosis, which remain the subject of further study.

Conclusion

In summary, we applied network and experimental pharmacology to investigate the effects and mechanism of DC herb-pair in the prevention of liver fibrosis. More than 40 targets and 20 pathways were obtained and three main mechanisms, including the MAPK signaling pathway, anti-fibrotic response and anti-inflammation were suggested to contribute to the hepatoprotective effect of DC herb-pair. Finally, changes in signaling pathways and downstream targets involved in these three mechanisms were validated by in vivo experiments. Based on our findings from previous and current studies, we propose that DC herb-pair significantly inhibited cell proliferation, decreased bile acid accumulation, alleviated hepatic inflammation and subsequently protected liver fibrosis through the inhibition of ERK1/2-AKT signaling pathway. Overall, our results provide novel insights into the pharmacological mechanisms of DC herb-pair or TCM prescriptions containing DC herb-pair in the prevention of liver fibrosis development.

References


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