Network Pharmacology and Molecular Docking Reveal the Mechanism of Isodon Ternifolius (D. Don) Kudo Against Liver Fibrosis

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Abstract

Background

Isodon ternifolius (SanYe Xiang ChaCai, *SYXCC*) is a traditional Chinese medicine commonly used in the treatment of chronic hepatitis B in China. Many studies have demonstrated the hepatoprotective or anti-fibrotic effects of *SYXCC*, but its pharmacological basis and mechanism remain unclear. In this study, we used in vitro models to validate the predicted results and reveal the potential mechanism of action and active ingredients with the help of network pharmacology methods and molecular docking.

Methods

We obtained the chemical structures of *SYXCC* by literatures. Potential targets of *SYXCC* were predicted by Swiss Target Prediction. The disease targets were collected through the databases of Gene Card. PPI protein interaction network was obtained using STRING database. Signaling pathway enrichment analysis was performed on drug-disease targets with of DAVID database. In vitro, human umbilical vein endothelial cell was used to validate the results predicted by network pharmacology.

Results

90 pathways related to liver fibrosis were obtained by KEGG enrichment. We were interested in the top 25 core target proteins involved in the TLR4, MAPK and PI3K-Akt signaling pathways associated with liver fibrosis. RT-PCR result showed that *SYXCC* could reduce EGFR, ERK1, Akt1, VEGFR-2, ERK2 mRNA expression.

Conclusion

In the study, our results suggest that a multitude of active ingredients of *Isodon ternifolius* play an important role in the liver fibrosis disease network. Inhibition of angiogenesis was a new potential pharmacological mechanism for the anti-hepatic fibrosis activity of *Isodon ternifolius*.

1. Introduction

Liver fibrosis (LF) is a reversible damage repair response, which is characterized by the accumulation of extracellular matrix (ECM). If the injury was acute or self-limiting, these changes were transient and the liver structure could return to its normal composition. However, if the insult is sustained, the liver parenchyma is replaced by fibrotic scar tissue, a process in which ECM and MMPs have recognized key functions^[1]. Chronic liver diseases (CLDs) are characterized by long-term chronic hepatic parenchymal cell injury, a persistent inflammation response and a sustained activation of LF and damage repair response, so LF is present in most chronic liver diseases^[2]. The prevalence and mortality of CLDs could be compared with major public health issue related to common chronic diseases, such as diabetes, tuberculosis, and cardiovascular diseases. Previous studies have shown that 844 million people worldwide suffer from CLDs, with 2 million deaths per year^[3]. Currently, curing LF remains a huge

challenge globally, as drug treatment strategies can only slow down the process of LF, but not cure it. Therefore, it is necessary to explore drugs for the treatment of LF.

Traditional Chinese medicine (TCM) has a long history of preventing and treating diseases. TCM has curative effects in the treatment of liver diseases, especially in the field of anti-fibrosis, which has significant advantages and great development value. *SYXCC*, also known as the San Jie Mei in the Chinese folk^[4]. The genus of *SYXCC* is Lamiaceae, which is sweet, slightly bitter, and cool. It has the effects of clearing heat and detoxification, promoting dampness and soothing the liver^[4]. It is used for treat enteritis, acute and chronic hepatitis and early cirrhosis of the liver^[5]. Chinese patent medicine containing *SYXCC*, “Fufang sanyexiangchacai Pian”, were used to treat acute and chronic hepatitis B^[6, 7]. Previous studies have found that *SYXCC* has anti-LF effect, the mechanism may be through inhibiting the activation of TLR4/NF- κ B signaling pathway^[8, 9] but its anti-fibrosis mechanism still needs further study.

1. Network pharmacology and molecular docking

1.1 The source of chemical constituents and the prediction of drug targets in *SYXCC*

We searched the herbal databases from TCMSP (Traditional Chinese Medicine Database and Analysis Platform) (<https://tcmsp-e.com/>), ETCM (The Encyclopedia of Traditional Chinese Medicine) (<http://www.tcmip.cn/ETCM/>), and TCMID (Traditional Chinese Medicines Integrated Database) (<http://47.100.169.139:8000/tcmid/>) for hand-collected data on the ingredients of *SYXCC*. If no, we have collected the ingredients of *SYXCC* through literature search. The compound structures were drawn using ChemBioDraw Ultra 14.0, saved in the mol2 format and converted into 3D structures by ChemBio3D Ultra 14.0, and saved mol2 format file after minimizing MM2 energy. Subsequently, with the help of Swiss Target Prediction (STP) (<http://swisstargetprediction.ch/>) database to predict the drug target, and get the drug target genes.

1.2 Target genes of LF

GenCard integrates gene-centric data from 150 network sources, including genomics, transcriptomics, proteomics, genetics, clinical and functional information. Liver fibrosis target genes were obtained from GenCard database(<https://www.genecards.org/>).

1.3 Network construction

1.3.1 Compound-disease target network

The online tool Venny2.1(<https://bioinfogp.cnb.csic.es/tools/venny/>) was used to draw Venn diagrams to obtain target genes of compound-disease intersection. The visualization software Cytoscape3.7.1 was used to construct the compound-disease target network.

1.3.2 Construction of Protein-protein Interaction (PPI) Network and Core Target Screening

The common target of *SYXCC* of compound-LF was imported into String database (<https://string-db.org/>), and Homo sapiens was selected. Finally, the PPI network is constructed, and the network is constructed, and the results were exported and saved as files.

1.3.3 GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis and construction of core target pathways

The compound of *SYXCC*-LF common target genes were enriched by GO function and analyzed by KEGG pathway through the DAVID6.8(<https://david.ncifcrf.gov/>) biological information annotation database. GO enrichment analysis includes Molecular Function (MF), Cellular Component (CC), and Biological Process (BC). *P*-Value and FDR have been used to assess the correlation, set the parameters *P*-Value ≤ 0.05 FDR ≤ 0.05 . The core target was imported into KEGG enrichment and the relevant enrichment pathway was obtained through the website (https://www.genome.jp/kegg/tool/map_pathway1.html).

1.4 Molecular docking

Autodock 4.2 is an automated program for predicting the interaction between ligands and biological macromolecular targets, which is used in drug research and development^[10]. We obtained the main enrichment pathways through enrichment analysis of the KEGG pathway, and used Autodock 4.2 to verify the molecular docking of the core targets related to the middle value of the protein interaction network with the chemical components of *Isodon ternifolius*.

1.4.1 Acquisition and processing of target protein

The 3D structure of candidate target is downloaded in the PDB protein database (<https://www.rcsb.org/>), and the result was saved in the pdb format, and then the visualization software PyMOL (TM) Molecular Graphics System, Version 2.4 was used to remove the water in the target protein Molecule and separation of small molecules.

1.4.2 Docking steps and result evaluation

(1) Ligand and receptor file preparation before docking: Small molecule ligand compounds and core proteins are imported into AutoDockTools (v1.5.6) for hydrogenation, charge calculation, charge distribution and save as “pdbq”; (2) Grid file preparation: After opening the Grid option to import the receptor and ligand, select the Grid Box, adjust the center of the box to wrap the macromolecular protein, and save the output as “gpf”; (3) Parameter file preparation: Open Docking to import receptor and ligand files, set the parameters to default values, and operate as follows: Docking/Search Parameters/Genetic Algorithm Parameters, Docking/Docking Parameters, Output/Genetic Algorithm output is saved as “dpf”; (4) Put the prepared protein files, compound files and corresponding folders into the corresponding folders, and use AutoDock (v1.5.6) for molecular docking; (5) Evaluation of results: The docking possibility and stability are evaluated by the spatial position and binding energy after docking. We choose the binding energy ≤ -5.0 as the evaluation of receptor-ligand binding^[11].

2. Network Pharmacology And Molecular Docking Results

2.1 Compound of *SYXCC*

Ethyl acetate extraction and n-butanol extraction from the roots of *SYXCC* were isolated and identified 25 compounds, following 3-carboxy formate-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene (1); 3-carboxy-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene (2); 1-acetate-2,6-dipiperonyl-3,7-dioxabicyclo[3,3,0]-octane (3); 2-(3,4,5-trimethoxyphenyl)-6-(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3,3,0]-octane (4); syringylglycerol- β -syringaresinol ether 4",4- m-di-O- β -D-glucopyranoside (5); clemastanin A (6); 3-(3,4-dihydroxyphenyl)acrylic acid 1-(3,4-dihydroxyphenyl)-2-methoxycarbonyl-ethyl ester (7); oxyneolignans A (8); 19-hydroxytatarol (9); (-)-lambertic acid (10); isodoternifolin B (11); longikaurin A (12); maoyecrystal F (13); 2 α -hydroxy oleanolic acid (14); tormentic acid (15); myricetin 3-neohesperidoside (16); 3,4-dimethoxy-1-hydroxylphenyl-O- β -D-glucopyranoside (17); 2,6-dimethoxy-p-hydroquinone-1-O- β -D-glucopyranoside (18); 3,4, α -trihydroxyl-methyl phenylpropionate (19); erythro-l-c-syringyl-glycerol (20); *E*-P-hydroxyl ethyl cinnamate (21); caffeic acid (22); 3, 4-dihydroxyphenacyl alcohol (23); protocatechuic acid (24); Syringic acid (25) (The constituents of *Isodon ternifolius* (D. Don) Kudo were derived from Wang. K's master's thesis in Guangxi Normal University, 2018.)

2.2 Network construction of prediction targets and disease targets of *SYXCC*

The Swiss Target Prediction platform database was used to predict the target of *SYXCC*. Compounds 13, 19, 20, and 23 were eliminated due to no prediction results, and the remaining 21 compounds were predicted to have 395 targets (Probability ≥ 0 , delete duplicate targets). Using "liver fibrosis" as the search term, GeneCards retrieved 3794 LF target genes. The common target of compounds-hepatic fibrosis was imported into Cytoscape 3.7.2 for network visualization (Figure 2). This network has a total of 319 nodes and 626 edges.

2.3 Target protein interaction network construction and core target screening

The 298 predicted targets of *SYXCC* for the treatment of LF were imported into the STRING database (<https://string-db.org/>), and then the protein interaction network was constructed. The target point is hidden without network interaction, and other parameters are the default values. (Fig 3).

2.4 Screening GO function enrichment and KEGG signal pathway analysis

In order to explain the possible role of the candidate targets, the targets were used for GO function enrichment and KEGG pathway analysis through the DAVID6.8 database. GO enrichment analysis yielded a total of 219 data results. 23 results are used to describe the location of gene products in cells, such as cellular components (CC) such as endoplasmic reticulum, mitochondria, or nucleus. 33 results were used to describe the functions of individual gene products, such as molecular functions (MF) such as binding activity and catalytic activity. 164 results are used to describe an orderly biological process (BP) with multiple steps, such as cell growth, proliferation, apoptosis, and signal transduction.

2.5 Molecular docking

Due to the drug targets of *SYXCC* are mainly enriched in the MAPK signaling pathway and the PI3K-Akt signaling pathway (Figure 7), the core targets of this study are TNF (PDB ID:1EXT), Akt1(PDB ID:4EKL), MAPK1(PDB ID:6GLD), EGFR (PDB ID:5d41), TLR4(PDB ID:2z65).

3. Experiment

3.1 Materials & Methods

3.1.1 Medicinal materials preparation

The dried roots of *SYXCC* were purchased from Guangxi xianju Chinese Medicine Technology Company (20190501, Guangxi Province, China) in October 2019. The *SYXCC* of rhizome was soaked in 10 times waters for 30 minutes, then boiled for 60 minutes. Gauze was used to filter the herb residue and the filtrate was collected. The above process was repeated three times. 95% ethanol was used for extraction. After standing for 24 hours, the sediment was filtered off and the filtrate was collected. The filtrate was concentrated into an extract and refrigerated at -80°C. *SYXCC* extract was prepared with DMEM solution and used after filtration.

3.1.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were Cell Bank of Typ Culture Collection of Chinese Academy of Sciences (Shanghai, China). HUVECs were cultured in DMEM complete medium (GIBCO, cat # 11965092) containing 10 % fetal bovine serum FBS (GIBCO, cat # 10099-141) in 5 % CO₂, 37 °C, 100 % saturated humidity CO₂ incubator.

3.1.3 MTT assay

HUVECs were seeded in 96 well plates with 1×10^4 cells/well and cultured in DMEM medium containing different concentrations (2ug/ml, 5ug/ml, 10ug/ml, 40ug/ml, 75ug/ml) of *SYXCC* extract for 24h. Each group was repeated three times. MTT cell proliferation and cytotoxicity kit (China, Solarbio, cat# M1020) was used to detect cell viability and proliferation inhibition rate. After 24 h of culture, 20 µL of MTT (5 mg/mL) was added to each well. After 4 h culture, the supernatant was centrifuged, and 150 µL of DMSO was added to each well to dissolve the crystalline particles. The absorbance was measured at 570nm.

3.1.4 Scratch healing assay

Scratch healing assay used to assess changes in migration of HUVECs. Different groups of cells were seeded into 6-well plates. After the cells have grown to full, the central portion of the cells was removed by scoring the central area of cell growth with tip, and then the cells continue to be cultured with a culture medium containing different concentrations of *SYXCC*. After 24 hours, the cells were observed under

microscope whether migrated to the central scratch area, and photographed. Image J software was used to analyze migration and calculate migration rate.

3.1.5 Tube formation assay

To observe microvasculature, HUVECs treated by different concentration were seeded in 96-well plates with matrix adhesive with substrate gel (Solarbio, cat# 356234) and cultured at 37°C in a 5% CO₂ incubator. After 6 hours of culture, the vascular network was imaged by inverted microscope at 10x and vascular network was quantified by Image J's package with angiogenesis analyzer.

3.1.6 Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted by TriQuick Reagent Total RNA Extraction Kit (Solarbio, R1100) and stored at -80 °C. RNA was reverse transcribed to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, RR036A) according to the instructions and stored at -80°C. The predicted target genes EGFR, VEGFR-1, ERK1, ERK2, Akt mRNA were amplified by Veriti™ 96-Well Thermal Cycler Applied Biosystems® in Real-Time PCR System (Agilen, AriaMx). PCR conditions: protein denaturation 95 °C for 30s, 40 cycles, each cycle 95 °C for 5s, 60 °C for 10s, 72 °C for 30s. β-ACTIN was used as the internal reference to correct the target Ct value, and $2^{-\Delta\Delta}$ was used to calculate the relative expression of mRNA. All primers were synthesized by Sangon Biotech in Shanghai (Table1).

Table 1 mRNA primer

Gen	Gen bank	primer	Sequence (5'->3')	Length
EGFR	NM_001346897.2	F	GTCTCCAGTGTCCAAGCCAG	20
		R	TACGCCCTTCACTGTGTCTG	20
VEGFR-2	NM_002253.4	F	AGACCGGCTGAAGCTAGGTA	20
		R	CGATGCTCACTGTGTGTTGC	20
Akt1	NM_001014431.2	F	TCCCCCAGTTCTCCTACTCG	20
		R	TCCCTCCAAGCTATCGTCCA	20
ERK1	NM_001040056.3	F	GACTCCAAAGCCCTTGACCT	20
		R	GGACTGGCCACCTCATC	18
ERK2	NM_002745.5	F	TCGGTGTTCTTCTTCCCAGT	20
		R	CGGCTCAAAGGAGTCAAAGTG	21

3.1.7 Statistical analysis

All data are presented as mean ± standard deviation (SD) and were evaluated using one-way analysis variance. $p \leq 0.05$ or $p \leq 0.01$ was considered statistically significant. All statistical analyses were performed

using SPSS 26.0.

3.2 Experimental results

3.2.1 Effects of *SYXCC* on proliferation of HUVECs in vitro.

In aim of evaluating the effect of *SYXCC* extract on the proliferation viability of HUVECs, we experimented with a variety of concentrations of *SYXCC* extract (2µg/ml, 5µg/ml, 15µg/ml, 45µg/ml, 75µg/ml) to treat HUVECs. MTT results showed that IC₅₀ was 11.91µg/ml.

3.2.2 *SYXCC* extract inhibits migration and angiogenesis of HUVECs in vitro

Many evidences show that angiogenesis is involved in the development of liver fibrosis. Hypoxia and inflammation are the main triggers of angiogenesis. Fibroblasts were located at the intersection between angiogenesis and fibrosis, and play a key role in angiogenesis^[13, 14]. In the pharmacology network, we found that the mechanism of the anti-fibrotic of *SYXCC* may be related to angiogenesis. In vitro, we investigated whether *SYXCC* has inhibitory effect on angiogenesis. Scratch healing and tube formation assays demonstrate that *SYXCC* extracts inhibited the migration of HUVECs in a dose-dependent manner and significantly inhibited the formation of blood vessels.

3.2.3 *SYXCC* extract inhibits activation of PI3K/Akt and ERK1/2 Signaling Pathways in Vitro

We have predicted that multiple *SYXCC* ingredients were involved in the regulation of PI3K/Akt and ERK1/ERK2 signaling pathways via network pharmacology. It has been demonstrated that ERK1/2 and PI3K/Akt were important kinase cascade signaling pathways in vivo, regulating HSCs of activation, proliferation, differentiation or intrahepatic vessels, resulting in the progression of liver fibrosis^[15-20]. Akt, also known as protein kinase B (PKB), was a serine/threonine protein kinase that plays an important role in the development of alcoholic hepatitis, including promoting cell proliferation, migration, and transcription, and at the same time damage apoptosis. It was found that Akt1 and Akt2 in the Akt family are important in the pathological development of alcoholic hepatitis, and the mechanism is to regulate the occurrence of inflammatory response and fibrosis in the progression of alcoholic hepatitis^[21]. It was found that PI3K inhibitors could inhibit the activation of ERK and the expression of c-fos^[22]. PIK3R1 or Akt3 mice or HSC cells were knocked out, the expression of α-SMA and collagen I are reduced, and the degree of LF is reduced^[20]. As the main signal transduction pathway involved in the phenotypic response of liver myofibroblasts, the ERK signaling pathway plays an important role in the progression of LF^[15]. When Raf kinase inhibitory protein (RKIP) was blocked in rat liver tissue and primary HSCs, the ERK signaling pathway was activated to promote the production of α-SMA, MMP-1, MMP-2 and collagen, aggravate LF^[23]. It has been reported that when fed with choline deficiency and supplemented with methionine diet, the degree of LF in ERK knockout mice was relieved, the expression of fibrosis marker α-SMA protein was reduced, and the percentage of proliferation and activated T cells was reduced^[17].

Epidermal growth factor receptor (EGFR) was a transmembrane receptor tyrosine kinase that was activated by variety of signaling pathways to mediate cell proliferation and differentiation^[24]. EGFR was important in the progression of LF^[25, 26]. EGFR was up-regulated in LF. EGFR was blocked, it could alleviate the progress of LF by reducing lipid deposition, anti-oxidative stress, inhibiting HSCs activation and the release of inflammatory factors^[25-27]. VEGF was the most important pro-angiogenic cytokine and plays a central role in angiogenesis because it was related to all the steps of angiogenesis: increasing vascular permeability, inducing endothelial cell proliferation, and regulating the diameter of new blood vessel lumen^[28]. VEGF signaling pathway was blocked, it could effectively inhibit angiogenesis^[29, 30]. VEGF plays an important role in the progression of LF, promoting the activation, proliferation and collagen production of HSC^[31]. In the liver, VEGF and its receptor tyrosine kinases VEGFR1(Flt-1) and VEGFR2(Flt-2) were not only expressed in the endothelial cells and hepatocytes of the liver, but also expressed in mesenchymal cells, especially in activation of HSC^[32, 33].

PCR suggested that *SYXCC* significantly inhibited the activation of PI3K, Akt, ERK1/2 and reduced ERFG, VEGFR-2 mRNA expression in vitro. The results showed that inhibition of angiogenesis by regulating PI3K/Akt and ERK1/2 signaling pathways may be a new mechanism of anti-hepatic fibrosis.

4. Discussion

Network pharmacology found that the *SYXCC* through the MAPK pathway^[34], TNF pathway^[35], PI3K-Akt pathway^[36], TLR4/NF- κ B pathway^[37], VEGF pathway^[31], HIF-1^[38], which are involved in the regulation of viral infection, inflammatory response, angiogenesis, and initiate the progress of fibrosis. Our previous studies have shown that *SYXCC* has the effects of protecting the liver and anti-LF, could down-regulate the activation of the TLR4 signaling pathway, and reduce interleukin-1 β (IL-1 β), interleukin-6(IL-6), tumor necrosis factor- α (TNF- α)^[8, 9] but its mechanism and pharmacological basis are still unclear. In this study, we used network pharmacology and molecular docking technology to revealed the mechanism and potential active ingredients of *SYXCC* against LF, and verified it through in vitro experiments. The extract of *SYXCC* could inhibit the formation of blood vessels, possibly by inhibiting ERK1/ERK2 signaling pathway and the PI3K/Akt signaling pathway, inhibiting angiogenesis with inhibiting the progression of LF.

More and more evidences show that angiogenesis plays a crucial role in the progression of LF. Hepatic angiogenesis occurs in CLDs by inflammation and progressive fibrosis^[13]. HSC is the core of the pathogenesis of LF, and plays an important role among liver inflammation, fibrosis and angiogenesis. Quiescent HSCs could be stimulated by a variety of growth factors, pro-inflammatory mediators produced by damaged resident cell types, and hypoxia to promote the formation of new blood vessels, which in turn could become a bridge between acute and chronic inflammation^[39]. Evidence shows that under clinical and experimental conditions, liver angiogenesis and fibrosis were closely related^[36, 38]. Hypoxia, inflammation and HSC were considered to be the main causes of angiogenesis. Pro-fibrosis cells were located at the intersection between inflammation, angiogenesis, and fibrosis, and also play a key role in

angiogenesis^[13, 14]. The TLR4 family is a type α transmembrane protein, which mediates the recognition of pathogen-related molecular patterns (PAMP) and damage-related molecular patterns (DAMP) from dead or damaged cells, initiates immune responses, and induces inflammation. It was found that LF is related to TLR4. TLR4 activates NF- κ B, IRF-3, AP-1, etc., promotes the release of inflammatory factors and pro-fibrotic factors, and activates hepatic HSCs in the progress of LF.^[40] TNF binds to its ligands TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) activates downstream NF- κ B, MAPKs and Akt signaling pathways, and promotes the release of inflammatory factors^[35]. Wree etc, found that TNF and IL-17 activate NLRP3 inflammasomes to amplify the inflammatory response in the progression of LF.^[41] We have previously confirmed that *SYXCC* could inhibit the activation of TLR4/NF- κ B pathway and reduce the production of IL-6 and TNF- α in inhibiting the progression of LF^[8, 9].

ERK1/2 and PI3K/Akt were regulated by a variety of cell growth factors, including VEGF, PDGF, EGF, etc.^[42], and produce fibrosis through a variety of mechanisms, including promoting inflammation, activated endothelial cells releasing fibrosis enhancing molecules and activated HSCs^[43, 44], up-regulate α -SMA, collagen, integrin, fibronectin, IFN- α , IL-6, IL-10^[31]. Once the liver was damaged, HSCs autocrine or paracrine cell growth factors such as VEGF, PDGF, EGF, which growth factors bind to the tyrosine kinase receptor on the cell membrane to transmit the activation signal to GTPase Ras, and then Ras is recruited to the plasma membrane and binds to Raf^[48]. Ras was the common upstream molecule of ERK1/2 and PI3K/Akt signaling pathways^[45]. The activation signal was phosphorylated through the Ras/Raf/MEK/ERK cascade, and then also phosphorylates the phosphatidylinositol 3-kinase-Akt-p70 S6 kinase (PI3K-Akt-p70 S6k) cascade, which phosphorylates its substrate and expresses various transcription factors involved in cell proliferation and differentiation, secrete pro-fibrosis factors and angiogenesis in the progress of LF^[15, 46]. The PI3K/Akt signaling pathway regulated the synthesis of MMP and the degradation of extracellular matrix through the activation of HSCs in the progression of LF. Type I collagen was the main component of ECM and the main collagen secreted by activated HSC. PI3K regulated the transcriptional expression of collagen and plays an important regulatory role in the synthesis of collagen^[47].

In the drug-disease network, we found 3-carboxy formate-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene (2), 19-hydroxytatarol (9), (-)-Lambertic acid (10), 2 α -hydroxy oleanolic acid (14), tormentic acid (15), E-P-hydroxyl ethyl cinnamate (21), play an important regulatory role. Some compounds have been reported to have therapeutic effects on liver disease, such as ursolic acid and oleanolic acid. Oleanolic acid and ursolic acid were classified as pentacyclic triterpene acid compounds, which have been reported to treat acute and chronic liver diseases. Its mechanism includes anti-oxidative stress^[48-51], inhibition of HSC activation and reduction of the production of pro-fibrotic factors^[52, 53], reduction of blood lipids, inhibition of liver lipid synthesis and promotion of lipid metabolism^[54], , and improvement of intestinal flora^[55, 56]. Tormentic acid could prevent LF by blocking PI3K/Akt/mTOR and NF- κ B signaling pathways to inhibit HSC activation^[57]. 19-hydroxytatarol could antioxidant by inhibiting mitochondrial lipid peroxidation^[58]. 3-carboxy formate-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene (1) was first

discovered as a component of *SYXCC*, which could relieve inflammation and reduce LPS-induced RAW264.7 cells secrete PGE2 and TNF- α ^[4]. The molecular docking results also showed that 2 α -hydroxy oleanolic acid (14), tormentic acid (15), 1-acetylacetylonyl-2e, 6e-dipiperonyl-3, 7-dioxabicyclo [3, 3, 0]-octane (3), 19-hydroxytatarol (9), longikaurin A (12) could bind to the core target.

Network pharmacology is based on the theory of systems biology. It integrates the biological system network and the drug action network, and then analyzes the regulatory role of the drug in the disease network, emphasizing the overall regulatory role. The research method of network pharmacology is consistent with the multi-target and multi-path mechanism of TCM. Among them, 1-acetylacetylonyl-2e-6e-dipiperonyl-3, 7-dioxabicyclo[3, 3, 0]-octane (3) has anti-inflammatory activity^[12]. longikaurin A (12) activate the ROS/JNK/c-Jun signaling pathway by inhibiting the expression of Skp2, resulting in the induction of cycle arrest and apoptosis of liver cancer cells^[59]. Particularly, (-)-lambertic acid was docked by multiple core target proteins, indicating that (-)-lambertic acid may be a potential active ingredient in *SYXCC*. It has been reported that (-)-lambertic acid exists in *Lambertae* plants such as *Isodon macrophylla*^[60], *Isodon amethystoides*^[61], which has anti-tumor^[62] and antioxidant effects^[61]. However, there is still no report on the effect of (-)-lambertic acid on liver disease. In the future, we will further explore the effect and mechanism of (-)-lambertic acid on anti-liver and anti-fibrosis. We concluded that *SYXCC* inhibits LF through multiple compound and multiple drug targets.

5. Conclusion

Network pharmacology and molecular docking technology explored the mechanism of anti-hepatic fibrosis and potential active ingredients of *SYXCC*. The experiment verified that the anti-LF mechanism of the *SYXCC* might be the inhibition of intrahepatic blood vessels. We concluded that: (1) *SYXCC* multi-target regulation TLR4, PI3K/Akt, ERK1/ERK2, inhibits the synthesis and release of inflammatory cytokines, fibrosis-related factors, and inhibits the formation of blood vessels. (2) The most potential activities of *SYXCC* include 19-hydroxytatarol, (-)-lambertic acid, longikaurin A, 2 α -hydroxy oleanolic acid, tormentic acid, which are easily combined with TLR4, TNF, MAPK, EGFR.

Abbreviations

LF: Liver Fibrosis; TCM: Traditional Chinese Medicine; TNF: Tumor Necrosis Factor; MAPK1: Mitogen-activated Protein Kinase 1; EGFR: Epidermal Growth Factor Receptor; TLR4: Toll-like Receptors; PPI: Protein Protein Interaction; ECM: Extracellular Matrix; CLDs: Chronic Liver Diseases; TCMSP: Traditional Chinese Medicine Database and Analysis Platform; ETCM: The Encyclopedia of Traditional Chinese Medicine; TCMID: Traditional Chinese Medicines Integrated Database; STP: Swiss TargetPrediction; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular Function; CC: Cellular Component; BP Biological Process; SYXCC: *SYXCC*; HUVECs: Human Umbilical Vein Endothelial Cells; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; HSC: Hepatic Stellate Cells; PAMP: Pathogen-related

Molecular Patterns; DAMP: Damage-related Molecular Patterns; TNFR1: TNF Receptor 1; TNFR2: TNF Receptor 2; PKB: Protein Kinase B; RKIP : Raf Kinase Inhibitory Protein.

Declarations

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Not applicable

Author's contributions

Le Qin: methodology, software, Formal analysis, Writing - Original Draft; **Zhipin Zhou**: Project administration, Supervision, Conceptualization; **Huang Hui**: Writing - Review & Editing, Formal analysis; **Yong Chen**: Investigation, Data Curation; **Jingjing Wang**: Visualization, Investigation; **Runang Nong**: Software, Writing - Review & Editing.

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Authors statement

Each of the coauthors has seen and agrees with each of the changes made to this manuscript in the revision and to the way his or her name is listed.

Competing interests

The authors declare that there are no conflicts of interest.

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Figures

Figure 2

Protein-protein interaction network (PPI) of common target proteins of SYXCC and liver fibrosis. (A) The original PPI network graph derived from STRING data has 298 nodes, 626 edges, and average node degree value is 29.6. (B) The initial PPI network map was visualized by cytoscape software, the darker colour, the greater number of ineractions with liver fibrosis network target proteins. (C) The top 25 target protein interaction network graphs were obtained by topological network structure analysis. The deeper the color was, the more target proteins interacted with liver fibrosis network.

Figure 3

GO and KEGG enrichment analysis. GO enrichment analysis includes (A) The top 10 terms of biological process (BP). (B) The top 10 terms of Molecular function (MF). (C) The top 10 terms of cellular component (CC). KEGG pathway enrichment analysis (D) The top 25 terms of signaling pathways, including MAPK, TNF, PI3K-Akt, Toll-like receptor, ErbB, HIF-1, VEGF, HBV, HCV, etc. Different colors represent different adjusted p-values, and dot size represents numbers of genes enriched in each signaling pathway.

Figure 1 consists of six panels. Panels (a) through (e) show 3D molecular docking models of Lambircic acid (C10) or 2a-hydroxyoleanoic acid (C14) into the binding pockets of Akt1, EGFR, MAPK1, TLR4, and TNF, respectively. The ligands are shown in green stick representation, and the protein binding pockets are shown as semi-transparent surfaces. Panel (f) is a schematic diagram showing the docking of five receptors (MAPK1, TLR4, Akt1, TNF, and EGFR) into the binding pocket of Lambircic acid (C10). The receptors are represented by red circles, and the binding pocket is represented by a dashed box. The diagram shows the relative positions of the receptors and the binding pocket, with labels for each receptor and the binding pocket.

PI3K-AKT SIGNALING PATHWAY

The diagram illustrates the PI3K-AKT signaling pathway, showing the flow of information from various receptors to downstream effectors. Key components include:

- Upstream Activators:** RTK, TLR2/4, BCR, CD19, JAK, FAK, GPCR, and others.
- Core Pathway:** PI3K (Phosphoinositide 3-kinase) is activated by these receptors, leading to the production of PIP3 and the activation of AKT (Protein Kinase B).
- Downstream Effectors:** AKT regulates a wide range of cellular processes, including:
 - Metabolism:** Via PKC, PKD, S6K, and others.
 - Cell Cycle:** Via FOXO, GSK3, p21, p27, and others.
 - Cell Survival:** Via BAD, CREB, RXRα, IKK, MDM2, and others.
 - Cell Proliferation:** Via ERK, MEK, and others.
- Regulation:** The pathway is tightly regulated by negative feedback loops, such as PTEN inhibiting PI3K, and various other proteins like PDK1, PDK2, and others.

Legend:

- $\rightarrow$: Activation
- $\dashv$: Inhibition
- $\rightarrow$ (with DNA): DNA binding

Molecular docking. (A) (a, b, c, d) Docking model with the lowest binding energy between SYXCC ingredient and core targets of TNF, Akt, MAPK1, EGFR and TLR4. (f) Binding energy -5 was used as evaluation criterion for drug docking with core targets. Compounds 10, 12, 14 and 15 could bind to multiple core targets, and TLR4, MAPK1 and Akt1 could bind to ingredients of SYXCC most. Red dots represent core targets, and the larger the dots, the more ingredients are combined. Green triangle

represents components that could dock with core targets, and size of dots represents numbers of docking with core targets. Yellow triangle represents components that fail to dock with core targets. (B) Map of PI3K-AKT signaling pathway derived from KEGG URL (<https://www.genome.jp/kegg/>). Core targets TNF, Akt1, MAPK1, EGFR and TLR4 were involved in PI3K-AKT signaling pathway, regulating cell proliferation, DNA damage and angiogenesis.

Figure 5

Effect of different concentration of SYXCC on HUVECs activity. (Note: SYXCC represents *Isodon ternifolius*)

Figure 6

Effect of SYXCC extract on cell migration ability and tube formation. In vitro, human umbilical vein endothelial cells were treated with blank control, VEGF (10ng/ml), low-dose SYXCC (10μg/ml), medium-dose SYXCC (20μg/ml), high-dose SYXCC (40μg/ml), or medium-dose SYXCC (10ng/ml) + VEGF (10ng/ml). (A) Scratch healing assays. (B) Tube formation assays (n=3). (C) Quantitative analysis of scratch healing assays (n=3). (D) Quantitative analysis of tube forming assays (n=3). All data are expressed as mean ± SD. #P < 0.05 (VS VEGF group), ## P < 0.01 (VS VEGF group). (SYXCC: SYXCC, L: low dose, M: medium dose, H: high dose)

Figure 7

Effect of SYXCC on expression of core target genes. In vitro, human umbilical vein endothelial cells were treated with blank control, VEGF (10ng/ml), low dose SYXCC (10 μ g/ml), medium dose SYXCC (20 μ g/ml), high dose SYXCC (40 μ g/ml) or medium dose + VEGF (10ng/ml). (A, B, C, D, E) EGFR, ERK1, Akt1, VEGFR-2, ERK2 mRNA expression was analyzed by PCR (n=3). (SYXCC: SYXCC, L: low dose, M: medium dose, H: high dose)

Supplementary Files

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