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Original Research

Effects of *Salvia miltiorrhiza* and *Radix astragali* on the TGF-β/Smad/Wnt pathway and the pathological process of liver fibrosis in rats

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Abstract: Traditional Chinese medicine has made some progress in the study of liver fibrosis, and provides valuable experience for clinical treatment of liver fibrosis. The aim of this study was to investigate the rationality of compatibility use of *Salvia miltiorrhiza* and *Radix astragali* on liver fibrosis in rats. For this purpose, the rat model of liver fibrosis was treated with single or different compatibilities of herbals extracts for 4 weeks. Saline and colchicine were set as a negative and positive control, respectively. Liver histopathology, liver function, and expressions of key proteins in the TGF-β/Smad/Wnt pathway were assessed. Results showed that compared with colchicine, herbal extracts showed better ability to reduce deposition of α-SMA and type I collagen, and improve liver function. The effect of *R. astragali* extracts and 1:1 compound on improving liver fibrosis and liver function was relatively better than other treatment options. The compound groups showed a particularly significant effect on reducing Cyclin D1 expression. It was concluded that the 1:1 compatibility use of *S. miltiorrhiza* extracts and *R. astragali* extracts can preferably attenuate liver fibrosis by regulating the expression of TGF-β1 and Cyclin D1.

Key words: Compatibility of Chinese Medicine; Cyclin D1; Liver Fibrosis; *Radix astragali*; *Salvia miltiorrhiza*.

Introduction

Liver fibrosis is a pathological process with hyperplasia of connective tissue in the liver. Liver fibrosis could be caused by various injuries such as hepatitis virus infection, alcohol abuse, aflatoxin, and the use of particular medicine. Active prevention and rational treatment of liver fibrosis is a key link to avoid cirrhosis (1). With the rapid development of molecular biology and biotechnology, the understanding of the mechanism implicated in the occurrence and development of liver fibrosis has been further explored. There are literatures that have confirmed that inflammatory response, extracellular matrix formation, and hepatic stellate cell activation are involved in the pathogenesis of liver fibrosis (2-4). At present, western medicine is the lack of effective treatment. Although colchicine has certain efficacy, it cannot be widely used in clinical practice for a long time because of its low efficacy and large side effects (5). It is necessary to develop other effective and low side effect treatment methods for liver fibrosis.

In recent years, traditional Chinese medicine (TCM) has made some progress in the study of liver fibrosis and provides valuable experience for clinical treatment of liver fibrosis and the prevention of chronic liver disease. Using of *S. miltiorrhiza* (Dan Shen) extracts and *R. astragali* (Huang Qi) extracts have achieved therapeutic effects on liver fibrosis (6-10). However, the impact of different compatibilities of these two herbs on liver fibrosis is currently unknown. In order to improve the therapeutic effect of *S. miltiorrhiza* and *R. astragali*, this study explored the rationality of compatibility use of these herbs for liver fibrosis.

Materials and Methods

Experimental animals and herbal extract preparation

In total, 64 SPF male Sprague-Dawley rats (body weight: 42.5 ± 6.3 g) were provided by the Experimental Animal Center of Nanjing University of Chinese Medicine (Certificate no. SYXK 2018-0027). The animal work was approved by the Ethics Committee for Experimental Animal of Nanjing University of Chinese Medicine (approval number JN2018033). The rats were individually housed with high-fat corn diet (79.5% corn flour + 20.0% lard + 0.5% cholesterol) and clean water ad libitum. Dried powdered *S. miltiorrhiza* or *R. astra-*

gali were immersed in 10-fold (w/v) distilled water for 1 h and boiled at 120±5ºC for 150 min. The resultant extracts were collected by centrifugation and filtration. The filtrates were concentrated *in vacuo* into fine spray-dried powder. This process yielded approximately 250 g dry powder from 1000 g dried crude herbs. The spray-dried powder of *S. miltiorrhiza* or *R. astragali* was dissolved with water and mixed into 1:1, 1:2, or 2:1 composite preparations for use (final extract concentration equal to 1 g crude herbs/mL).

Preparation of the animal model

After adaptive feeding for 1 week, 64 rats were randomly divided into 8 groups (8 rats/group) and received different interventions: blank control group (no modeling or intervention), negative control group (modeling + intervention of normal saline), positive control group (modeling + intervention of colchicine), *S. miltiorrhiza* group (modeling + intervention of *S. miltiorrhiza* extracts), *R. astragali* group (modeling + intervention of *R. astragali* extracts), and 3 compound preparation group (modeling + intervention of *S. miltiorrhiza* extracts and *R. astragali* extracts at the ratio of 1:1, 1:2, or 2:1). For induction of liver fibrosis, rats were intraperitoneally treated with a 40% solution of CCl4 (Yangtze River Pharmaceutical Group, Shanghai, China) in olive oil (0.4 g/kg body weight, three times a week for total 4 weeks). After a one-week washout to eliminating acute effects of CCl4, rats received 4 weeks of interventions by daily gavage. Gavage of 1 mL 0.9% saline was set as a negative control. Colchicine (0.1 mg/kg body weight, purchased from Sigma-Aldrich, St. Louis, MO, USA) was set as a positive control. Rats of TCM treatment groups received herbal extracts by gavage at a single daily dose of 1 g extract solution/100 g body weight. At the end of treatment, rats were anesthetized with sodium pentobarbital (40 mg/kg body weight via intraperitoneal injection) and sacrificed. Blood and liver were collected from each rat. Blood samples were centrifuged at 3000 g for 15 min to separate serum without hemolysis. Liver samples were rinsed with cold normal saline and cut in half. One half was fixed in 4% paraformaldehyde for histopathological examination, and the other half was frozen at -80ºC for RT-PCR and Western blot.

Liver histopathology

After fixation for 24 h, liver tissues were washed with PBS, dehydrated in ethanol, and embedded in paraffin. Tissues were then cut into 5 μm thick sections and were used for HE staining and Masson's trichrome staining. Ten visual fields at a magnification of 200 times were randomly selected for each section.

Detection of α-SMA and type I collagen expression by immunohistochemistry

Liver tissue slices were dewaxed, dehydrated, and processed with antigen retrieval routinely. Tissue slices were then immersed in 3% H_2O_2 at room temperature for 10 min and blocked with 0.1% bovine serum albumin at room temperature for 30 min. After the serum was decanted, rabbit anti-α-SMA (1:400) (Boster Biological Technology, Wuhan, China) and rabbit anti-type I collagen (1:400, Boster) were added directly and incubated overnight at 4ºC. After incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:200, Servicebio Technology Co., Ltd., Wuhan, China) at 37ºC for 30 min. The slices were added with DAB, counterstained with hematoxylin, dehydrated with ethanol, transparent with xylene, and sealed with neutral gum. Positive areas within brownish yellow fine granular precipitation in the nucleus were observed under light microscopy (× 200).

Determination of serological indexes of liver tissue

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γGGT), total bilirubin (TBILL), and direct bilirubin (DBIL) were measured by Au480 Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA). Serum hyaluronic acid (HA), type III procollagen (PIIIP), and type IV collagen (IVC) were measured by the SN-682 radioimmunoassay system (Shanghai Nucleus Research Institute Rihuan Instrument Factory, Shanghai, China). Radioimmunoassay reagents were purchased from the Beifang Institute of Biotechnology (Beijing, China).

Detection of mRNA by fluorescence quantitative PCR

The total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Gene Company Limited, Shanghai, China). Using HiscriptII reverse transcription Kit (Vazyme Biotechnology Co., Ltd., Nanjing, China), cDNA was synthesized with the total RNA in 20 μL volume of the reaction system. The reaction conditions were as follows: 42ºC for 3min, 60ºC for 15min, and 85ºC for 5min. Using cDNA as a template, SYBR qPCR Master Mix (Vazyme) was applied for PCR amplification in 20 μL volume of the reaction system. The reaction conditions were as follows: 95ºC for 2 min followed by 40 cycles of 60ºC for 5 s and 95ºC for 10 s. GAPDH was used as the internal reference gene of the experiment, and the relative expression was expressed in the form of $2^{-\Delta\Delta CT}$, where ΔCT=CT (objective gene) - CT (GAPDH). There were two parallel holes for each group and experiments were repeated three times. The sequences of each primer were shown in Table 1.

Western Blot

A small amount of tissue blocks was placed in the spherical part of the homogenizer. The tissue blocks

Table 1. Primer sequences for PCR.

Primer	Sequence (5' to3 ')
GAPDH forward	GCCAGCCGAGCCACAT
GAPDH reverse	TACGACCAAATCCGTTGACTCC
TGF-β1 forward	AACGCAATCTATGACAAA
TGF-β1 reverse	ACAGTTGACTTGAATCTC
Smad2 forward	CCAGAAGGCATATAGGAA
Smad2 reverse	ATCGCACTATCACTTAGG
β-catenin forward	GAAGTTCTTGGCTATTAC
β-catenin reverse	TGTAGGTTCTCATTATGT
Cyclin D1 forward	TAAGATGAAGGAGACCATTC
Cyclin D1 Reverse	CAGAAGCAGTTCCATTTG

were cut up with clean scissors as much as possible, and then a proper amount of cell lysate was added. The cell supernatant was separated by high-speed centrifugation, and the protein concentration was quantified by the Bradford method. Protein was separated from 10% polyacrylamide gel by electrophoretic separation, transferred to the nitrocellulose membrane and incubated overnight at 4 degrees with primary antibodies. The primary antibodies were used at 1:1000 as follows: rabbit anti-TGFB1, rabbit anti-Cyclin D1, anti-Smad2, rabbit anti-β catenin (all from Bioss Inc., Beijing, China). After PBS washing, membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG (1:200) for 2 h and expressions of the target proteins were detected by the ECL system. GAPDH was used as the internal reference for evaluating the expression of the target protein. The electrophoresis bands on the X-ray films were processed and semi-quantified by Image J software.

Statistical method

All the experimental data were analyzed with GraphPad prism 8. ANOVA test with Tukey's post-hoc analysis was used to compare the mean differences among groups. In the experimental data, the measurement data were expressed as mean ± SEM. A P-value <0.05 was considered as statistically significant.

Results

Effect of *S. miltiorrhiza* and *R. astragali* on liver histopathology

Compared with the normal group, the model group rats were depressed in spirit, disordered and lusterless in hair, decreased in activity and slow in the velocity of weight gain. At the sampling time, the liver color of rats in each experimental group became light in varying degrees with small particles on the surface. The luster of the hair recovered significantly with increased activity and body weight.

HE staining and Masson staining (Figure 1A and Figure 1B) showed that the structure of liver lobule in the blank control group (i.e. normal group) was intact and the hepatic cord was arranged orderly with occasional blue staining in the wall of the tube, suggesting that only a small amount of collagen tissue was deposited in the wall of central vein in normal liver tissue. In the negative control group (i.e. model group), hepatocyte edema, fatty degeneration, loss of normal structure of liver lobule, enlargement of the portal area, a large number of collagen tissue deposition in the central vein wall, and formation of false lobule were observed. Treatment with colchicine or herbal extracts improved liver pathology. Basically, the normal structure of liver lobule, gradual ordered arrangement of liver cord, unobvious fatty degeneration of cells, only a small amount of collagen deposition in the portal area without pseudo lobule structure were observed in the compound preparation group. From the perspective of histopathology, *S. miltiorrhiza* extracts, *R. astragali* extracts, and their combinations had similar improvement effects. Comparatively speaking, the liver structure was more complete and the collagen deposition was less in the *R. astragali* group and the 1:1 compound group.

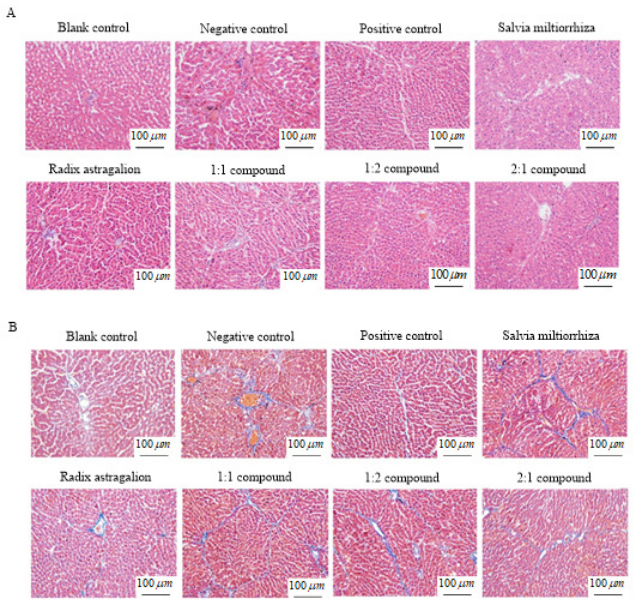

Figure 1. Effect of herbal extracts on histopathology of liver tissues by (A) HE staining (× 200) and (B) Masson's trichrome staining (× 200). Blue areas represent collagen fibers in the liver.

Effect of *S. miltiorrhiza* and *R. astragali* on liver fibrosis

Next, we studied the fibrosis status of liver tissues in each group of rats. The expression of α-SMA (Figure 2A) and type I collagen (Figure 2B) were detected by immunohistochemistry. Compared with the blank control group, the cells in the negative control group were plump and most of the nuclei were stained brown.

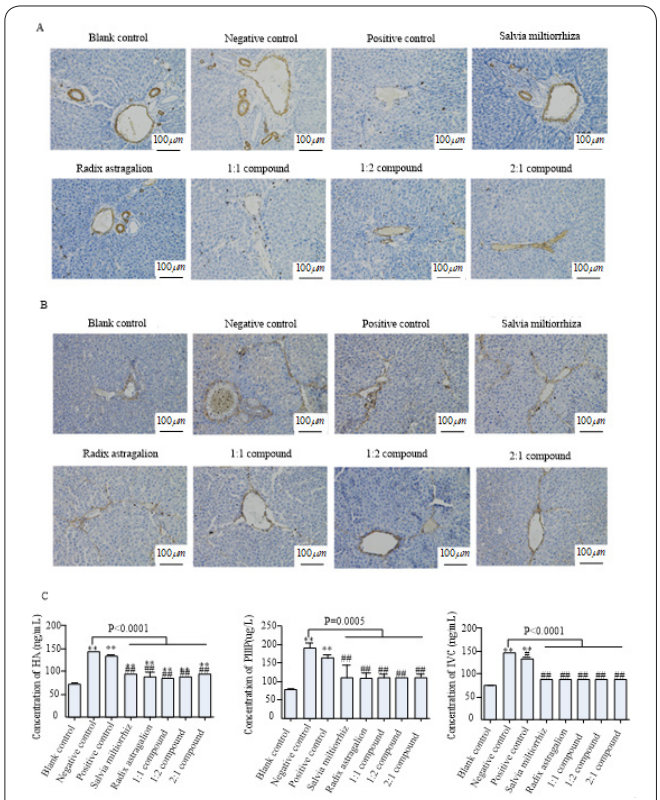

Figure 2. Effect of herbal extracts on liver fibrosis. (A) Immunohistochemical detection for α-SMA (× 200). (B) Immunohistochemical detection for collagen I (× 200). (C) Serum concentrations of liver fibrosis markers including HA, IVC, and PIIIP. $^{**}P$<0.01 compared with the blank control; $^{\#\#}P$<0.01 compared with the negative control.

Compared with the negative control group, the expression of α-SMA and type I collagen in each experimental group decreased in varying degrees, and the nucleus was light stained. Among them, the *R. astragali* group and the 1:1 compound group appeared to have the least expression of α-SMA and type I collagen. The serum markers of liver fibrosis, HA, PIIIP, and IVC, were also tested (Figure 2C). Compared with the blank control group, colchicine treatment could only reduce the serum concentration of IVC (P<0.05), but could not reduce the serum concentration of HA and PIIIP (both P>0.05), while all herbal extracts groups significantly improved the increase of these fibrosis markers (all P<0.01). Compared with the colchicines group (i.e. positive group), serum levels of HA and IVC were significantly lower in all herbal extracts groups (all P<0.01), while PIIP level was only significantly lower in the *R. astragali* group and the 1:1 compound group (both P<0.05). These results suggested that herbal extracts, especially *R. astragali* extracts and 1:1 compound, could effectively attenuate liver fibrosis. However, the difference between the TCM treatment groups was not significant.

Effect of *S. miltiorrhiza* and *R. astragali* on liver function

To investigate the effect of herbal extracts on liver function, the serum levels of representative markers were detected. As shown in Figure 3, AST, γGGT, TBILL, and DBIL were not sensitive enough to reflect liver function. Although these indexes increased after CCl4 modeling, there was no significant difference compared with the blank control group. Serum levels of ALT and ALP were significantly different in all groups, which were alleviated in the negative control group and reduced in the TCM treatment groups (all P<0.05). Colchicine could only reduce the serum level of ALP, but not ALT. The effect of *R. astragali* extracts, 1:1 compound, and 1:2 compound on improving liver function was relatively better than other treatment options, although the difference was not significant using Tukey's post-hoc multiple comparisons.

Effect of *S. miltiorrhiza* and *R. astragali* on TGF-β/Smad/Wnt pathway

Growing evidence suggests that TGF-β/Smad signaling is a central regulatory mechanism of fibrogenesis (11). TGF-β/Smad signaling is closely connected with the Wnt pathway in regulating fibroblast proliferation, myofibroblast differentiation, and extracellular matrix deposition (12-14). Next, we detected the effect of herbal extracts on the expression of several key mediators in the TGF-β/Smad/Wnt pathway (Figure 4). The results showed that the expression levels of TGF-β1, Smad2, β-catenin, CyclinD1 in the negative control group were significantly up-regulated compared with that in the blank control group (all P<0.05). Although treatment with herbal extracts could reduce the levels of Smad2 and β-catenin, the reduction was generally not significant compared with the negative control group. The expression of TGF-β1 was significantly reduced in some herbal extracts groups, but the effect was not consistent at the level of mRNA and protein. However, herbal extracts could significantly reduce Cyclin D1 expression, and the effect of compatibility groups was better than that of single prescription groups, but the difference did not reach a significant level.

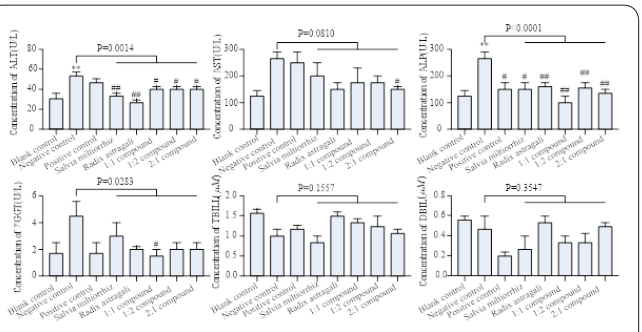

Figure 3. Effect of herbal extracts on liver function. Serum concentrations of liver function markers including ALT, AST, ALP, γGGT, TBILL, and DBIL were detected. $^{**}P$<0.01 compared with the blank control; $^{\#}P$<0.05 compared with the negative control; $^{\#\#}P$<0.01 compared with the negative control.

Discussion

Liver fibrosis is the common pathological basis of all chronic liver diseases and runs through the process of the occurrence and development of chronic liver diseases. It is the necessary stage for the transformation of chronic liver diseases to cirrhosis, and also the final reversible process (15, 16). TCM exhibits potential for the treatment of liver fibrosis and has been used as

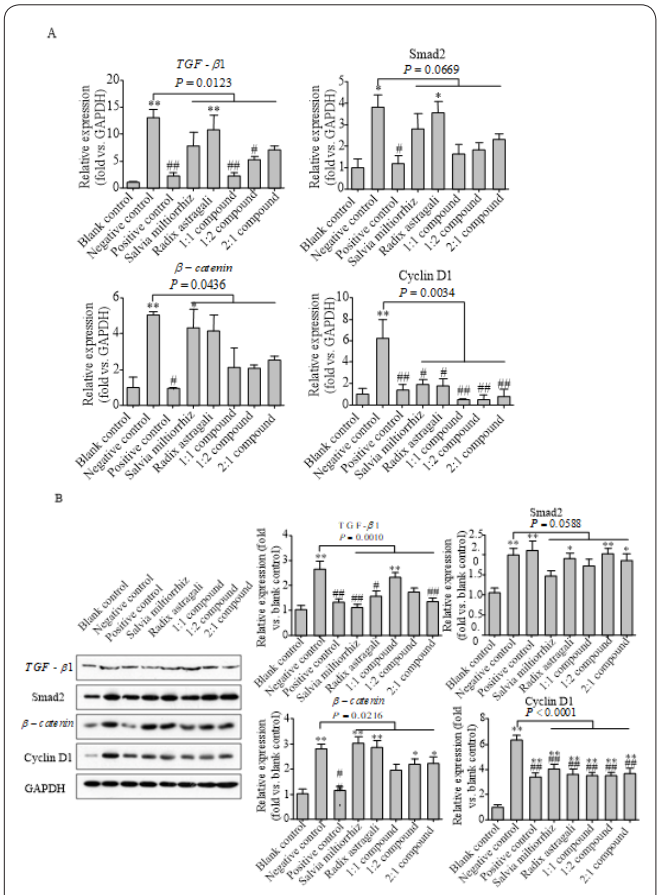

Figure 4. Effect of S. miltiorrhiza and R. astragali on the TGF-β/Smad/Wnt pathway. expression of TGF-β1, Smad2, β-catenin, and Cyclin D1 was detected by RT-PCR and Western blot. *P<0.05 compared with the blank control; **P<0.01 compared with the blank control; #P<0.05 compared with the negative control; ##P<0.01 compared with the negative control.

conventional or complementary medicines worldwide (17). A formula containing two or more herbs is a characteristic of TCM, which is supposed to obtain better curative efficacy. Herb pairs are the most fundamental and centralized representative form of TCM compatibility (18). However, compatibility without validation would be harmful, so the dosage ratio must be determined carefully. There have been many studies reporting favorable therapeutic effects of *S. miltiorrhiza* to *R. astragali* . This study investigated the rationality of compatibility use of *S. miltiorrhiza* and *R. astragali* on hepatic fibrosis in rats. The results showed that 1:1 compound could preferably attenuate liver fibrosis and improve liver function.

S. miltiorrhiza and *R. astragali* are commonly used tonic herbs in TCM for the treatment of liver diseases. Salvianolic acids are the primary active components of *S. miltiorrhiza* and the primary active component of *R. astragali* is astragalosides. These actives components were found to significantly inhibit CCl4-induced liver fibrosis in vivo and TGF-β-stimulated activation of hepatic stellate cells (19-22). The combined use of *S. miltiorrhiza*, *R. astragali*, and other Chinese or Western medicines can also effectively treat liver fibrosis, liver cirrhosis and hepatocellular carcinoma caused by various reasons (8, 23, 24). Those results of improving liver fibrosis and liver function were consistent with what we reported here. However, there are some inconsistencies in the regulation of Smad signal. Some literatures reported that that herbal treatment could significantly change the expression and phosphorylation of Smad2/Smad3 (21, 23, 24), but our study only found that the trend of Smad2 expression decreased, though not significantly. This may be related to the different compatibility formula adopted in different studies, further indicating that the compatibility of drugs often results in interaction and requires more caution.

Our study also found that *S. miltiorrhiza* and *R. astragali* could regulate the expression of Cyclin D1, a key factor of Wnt/β-catenin signaling. This effect has never been reported for these two herbs. Cyclin D1 expression is closely related to the activation of hepatic stellate cells and liver regeneration (25, 26). Interestingly, the regulatory effect of herbal extracts on β-catenin was far less obvious than that on Cyclin D1 expression. This suggests that other signaling pathways should also be involved to regulate Cyclin D1 expressions, such as interleukin-8, Ras/mitogen-activated protein kinase, and phosphoinositide 3-kinases (PI3K) (27-30).

Understanding the paths is of particular importance in molecular biology (31). If these paths are related to pathogenesis, they are more important (32-36). Medicinal plants have been reported to be very effective in this regard (37, 38). There is also a lot of research on liver complications (39-41). In this research, we used two medicinal plants for inhibition of liver fibrosis

In summary, we explored the therapeutic effect of different compatibility of *S. miltiorrhiza* and *R. astragali* CCl4-induced liver fibrosis in rats and found that 1:1 compound could effectively attenuate liver fibrosis and improve liver function. The protective effect appears to be related to inhibiting expression of TGF-β1 and Cyclin D1. Of note, the protective effect on other types of fibrogenesis and the exact mechanism remains to be elucidated.

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Author Contributions

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. T Cao, B Xue, and Y Lu conceived and designed the study. T Cao, J He, M Zhu, J Cheng, B Ye, N Fang, and Y Cui collected and analyzed the data. T Cao, J He, B Xue, and Y Lu wrote the manuscript. All authors read and approved the manuscript for publication.

Conflict of interest

No conflict of interest associated with this work.

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