A novel Chinese Medicine JY5 Formula Alleviates Hepatic Fibrosis Through Inhibiting Notch Signaling Pathway

Yadong Fu
  Shanghai University of Traditional Chinese Medicine

Zhun Xiao
  Shanghai University of Traditional Chinese Medicine

Xiaoting Tian
  Shanghai Institute of Materia Medica CAS: Shanghai Institute of Materia Medica Chinese Academy of Sciences

Wei Liu
  Shuguang Hospital

Yonghong Hu
  Shuguang Hospital

Jing Fang
  Shuguang Hospital

Siqi Gao
  Shanghai University of Traditional Chinese Medicine

Dingqi Zhang
  Shuguang Hospital

Yongping Mu
  Shuguang Hospital

Hua Zhang
  Shuguang Hospital

Yiyang Hu
  Shuguang Hospital

Chenggang Huang
  Shanghai Institute of Materia Medica CAS: Shanghai Institute of Materia Medica Chinese Academy of Sciences

Jiamei Chen (✉ cjm0102@126.com)
  Shuguang hospital

Ping Liu
  Shanghai University of Traditional Chinese Medicine

Research

Abstract

Background: Advanced liver fibrosis can lead to cirrhosis, resulting in an accelerated risk of liver failure and hepatocellular carcinoma. It is necessary to develop an effective antifibrotic strategy. It has been reported that Fuzheng Huayu formula (FZHY) had a remarkable anti-hepatic fibrosis effect. Here, We obtain a new anti-fibrotic composition, which consists of the main active ingredients of FZHY formula and investigate its mechanism of pharmacological action.

Methods: The main active ingredients of FZHY through the quantitative analysis in FZHY extracts and FZHY-treated plasma and liver in rats were investigated. The best anti-fibrotic composition of the main active ingredients was studied through the uniform design and validation experiments *in vivo* and its mechanism was evaluated in CCl_4- and BDL-induced liver fibrosis models in rats and mice and TGF-β1-indued LX-2 cells activation model in vitro.

Results A novel composition, namely JY5 formula, which consisted of Salvianolic acid B, schisantherin A and amygdalin, the main active components of FZHY, could significantly alleviated hepatic hydroxyproline content and collagen deposition in CCl_4- and BDL-induced fibrotic liver in rats and mice. Further studies showed that JY5 could inhibit the activation of hepatic stellate cells (HSCs) through inactivating Notch signaling *in vivo* and *in vitro*.

Conclusions: We found a novel composition JY5 formula, which had an anti-hepatic fibrotic effect through inhibiting Notch signaling pathway, consequently suppressing HSCs activation. These results may provide some adequate scientific basis for the clinical research and application of JY5 formula, as a potential new therapeutic candidate for liver fibrosis.

Background

Liver fibrosis is a common pathological feature of chronic liver diseases, including chronic viral hepatitis, metabolic associated fatty liver disease, cholestatic liver disease, etc. It is a consequence of an abnormal wound healing response, characterized by excessive deposition of extracellular matrix (ECM), especially of collagen. If the injury persists, liver fibrosis can progress to cirrhosis, hepatocellular carcinoma, ultimately leading to liver failure [1]. The effective treatment for liver fibrosis is critical to block the progression of chronic liver diseases. Some clinical studies [2] have shown that liver fibrosis, even early cirrhosis, is reversible, which provided reliable evidence for undertaking clinical researches on anti-hepatic fibrosis drugs. With several kinds of animal models of liver fibrosis becoming more and more mature, the pathogenesis of liver fibrosis has been deeper understanding in the last a few decades [3]. A number of anti-hepatic fibrosis drug studies have been conducted in recent years, some of which have been researched in clinical trials [4]. However, there are not any clinically approved or effective medical therapies aimed specifically at hepatic fibrosis so far.

Traditional Chinese medicine (TCM) has remarkable clinical effects on the treatment of liver fibrosis. Among these, Fuzheng Huayu formula (FZHY) is the first Chinese medicine in the field of hepatology, finishing the phase II clinical trial of anti-hepatic fibrosis post hepatitis C in USA [5]. However, there are some challenges in TCM, such as multi-component, multi-target, ill-defined active ingredients and mechanisms, to some extent, which limit their clinical application and in-depth research. In recent years, based on these questions, our group

has conducted a large amount of researches to reveal the mechanisms of FZHY in the prevention and treatment of chronic liver diseases, including inhibiting inflammatory response, protecting hepatocytes (to relieve hepatocyte damage and to inhibit hepatocyte apoptosis), inhibiting hepatic stellate cells (HSCs) activation, reducing collagen deposition, inhibiting Kupffer cells (KCs) activation, inhibiting liver sinusoidal endothelial cells (LSECs) capillarization and angiogenesis, promoting liver regeneration, etc [6, 7]. We further studied the effect of anti-fibrosis of different compounds of FZHY. For instance, phenolic acids in Danshen play a prominent role in inhibiting the inflammatory response, protecting hepatocytes and inhibiting HSCs activation [8–12]. Amygdalin, as one of the major active compounds of peach kernel, has a main role in inhibiting the inflammatory response, reducing collagen deposition and inhibiting HSCs activation [13, 14]. The lignan compounds from Schisandrae take a prominent position in protecting hepatocytes and inhibiting HSCs activation [15, 16]. These findings suggest that the related components in FZHY may be potential anti-fibrotic bioactive ingredients.

Notch signaling is a highly conservative pathway during evolution. Notch receptors interact with the ligands on the surface of adjacent cells, then cleave inside of the cell membrane and translocate into the nucleus, followed by regulating transcription of multiple target genes. Previous studies [17] have shown that as an important inter- or intra-cellular signaling pathway, Notch plays an important role in liver development and pathophysiology. Notch has a great impact on the occurrence and development of hepatic fibrosis, which can interact with TGF-β, Hedgehog, Hippo signaling pathways to mediate cell–cell interactions. Activation of HSCs is a critical cellular event in liver fibrosis. Studies [18] have shown that HSCs transdifferentiate into myofibroblasts accompanied by activation of Notch signaling pathway. After Notch activity levels are suppressed, this process could be reversed. In addition, with the progression of rat liver fibrosis induced by carbon tetrachloride (CCl_4) and bile duct of ligation (BDL), Notch signaling pathway is activated significantly. Efficient inhibition of Notch pathway can significantly mitigate rat liver fibrosis, and reduce hepatocyte apoptosis to some extent [19]. Thus, targeting Notch signaling pathway can regulate activation of HSCs, and thereby suppress the occurrence and development of hepatic fibrosis. Therefore, Notch promises to be one of potential therapeutic targets for the treatment of hepatic fibrosis.

In this study, we found that salvianolic acid B, schisantherin A and amygdalin were the main active ingredients of FZHY formula through quantitative analysis in FZHY extracts and FZHY-treated plasma and liver in rats. Then, a novel composition, namely JY5, was obtained through the uniform design and validation experiments. The anti-hepatic fibrosis efficacy of JY5 is comparable to that of FZHY in CCl_4-induced hepatic fibrosis in rats. Further studies demonstrated that JY5 alleviated liver fibrosis by inhibiting the activation of HSCs via the inhibition of Notch signaling pathway.

Materials And Methods

Animals

Adult *Wistar* or Sprague-Dawley (SD) rats (male, weighed 160-180g, SPF) were purchased from Shanghai Xipuer-Bikai Laboratory Animal Co., Ltd, and fed in the Laboratory Animal Center at School of Pharmacy, Fudan University. Adult C57/BL6 mice (male, aged 6-8weeks, weighed 18-20g, SPF) were purchased from Shanghai Southern Model Biotechnology Co., Ltd, and maintained in Shanghai Research Center of the

Southern Model Organisms. Rats and mice were housed under constant conditions (ambient temperature 25 ± 2 °C, relative humidity 40–60% and 12/12h light-dark cycle) with free access to standard diet and water. All rat experiments were approved by the Institutional Animal Care and Use Committee (IACUC), School of Pharmacy, Fudan University (Ethical approval code No. 2018-07-SZYD-LP-01). All mice experiments were approved by IACUC at Shanghai Research Center of the Southern Model Organisms (Ethical approval code No. 2019-0031).

Drugs

Reference standard: Salvianolic acid B, salvianic acid, salvianic acid A, rosmarinic acid, gypenoside XLIX, ginsenoside Rb3, amygdalin, schisantherin A, schisandrol A, schisandrol B, deoxyschizandrin, schisandrin B, tanshinone, cryptotanshinone, adenosine and cordycepin were purchased from Shanghai Standard Technology Co. Ltd. (Shanghai, China).

Fuzheng Huayu (FZHY) decoction: A mixture of salvia miltiorrhiza at 533g, peach kernel at 133 g and gynostemma pentaphylla at 400g was heated to boiling with water for 2h for the first time and 1.5h for the second time, respectively. The combined decoction was filtered, and concentrated to the relative density at 1.20 g/ml (50~55 °C). After cooling down, the decoction was precipitate by adding 70% alcohol, and then the filtrate 1 were generated after filtration and concentration. A combination of cordyceps mycelium powder at 267g, and schisandra chinensis at 133 g was heated with 70% alcohol for 2h for the first time and 1.5h for the second time, respectively, and the combined decoction was filtered and concentrated as filtrate 2. Pollen pini at 133g were infiltrated with 50% alcohol for 4h for the first time and 2h for the second time, respectively, and the combined decoction was filtered, and concentrated as filtrate 3. The filtrates 1-3 were combined and concentrated to 800ml at the 2g raw drug/mL.

A pharmacokinetic study of FZHY decoction in rats

This study was consistent to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). The experimental protocol was shown in Supplementary material: material and methods 1.1.

Instrumentation

There were 16 compounds determined in various biosamples by an UltiMate 3000 ultrahigh-performance liquid chromatograph linked to Qactive qdrupole electrostatic field orbital trap high resolution mass spectrometer, connecting to electrospray ionization source (Thermo Scientific, US). The operating parameters were set as shown in Supplementary material: material and methods 1.2.

Experimental liver fibrosis models

CCl_4-induced liver fibrosis rat model, BDL-induced liver fibrosis rat model and CCl_4-induced liver fibrosis mice model were used in this study. And the experimental protocols were shown in Supplementary material: material and methods 1.3.

Cell culture

The immortalized human hepatic stellate cell line (LX-2) was provided by Institute of Liver diseases, Shanghai University of Traditional Chinese Medicine. 1.25×10^5 LX-2 cells were seeded on 6 cm-dishes or 6-well plates and maintained in DMEM medium containing 1% P/S and 10% FBS under the special condition (37°C and 5% CO2 in the incubator). After postinoculation for 24h, all LX-2 cells except the control group, were treated with TGF-β1 (5ng/mL), and simultaneously added with different concentrations of JY5 respectively: Low-dose group (salvianolic acid B 8 μm, amygdalin 0.25 μM, schisantherin A 1 μM), Medium-dose group (salvianolic acid B 16 μM, amygdalin 0.5μM, schisantherin A 2 μM) and High-dose group (salvianolic acid B 32Mm, amygdalin 1μM, schisantherin A 4 μM). SB431542 (10 μM), one TGF-β receptor inhibitor, was used as a positive control. After incubation for 24h, LX-2 cells were lysed and collected for Western blot and RT-qPCR analysis.

Serum biochemistry analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBil), direct bilirubin (DBil) and total bile acids (TBA) levels were measured using the TBA-40FR automatic biochemistry analyzer (Toshiba Medical, Tokyo, Japan) at Science and Technology Experiment Center, Shanghai University of TCM.

Histopathological and immunohistochemical (IHC) analysis

Liver injury and fibrosis were assessed with hematoxylin and eosin (H&E) and Sirius Red staining using 4 μm thick paraffin-embedded liver sections. IHC stainings of Col-I, Col-IV, α-SMA and Desmin were performed. The detailed protocols were shown in Supplementary material: material and methods 1.4.

Hepatic hydroxyproline (Hyp) content assay

According to the kit instructions, hydroxyproline content in liver tissue was detected by using Hydroxyproline Testing Kit-Alkaline Hydrolysis Method (Cat No. A030-2, Nanjing Jiancheng Bioengineering Institute (NJBI), Nanjing, China).

Western blot analysis

Total proteins in Liver tissues or cells were extracted using RIPA lysis buffer containing proteinase and phosphatase inhibitor (Cat No. P0013B, Biyuntian Biotechnology Co., Ltd., Shanghai, China). Total proteins concentration was determined using BCA protein assays kit (lot.TD265229, Thermo Fisher Scientific). Denatured at 100 °C for 5 min, 30-50μg proteins were mixed into sample loading buffer and loaded into each lane. Proteins samples were separated by 10% SDS-PAGE, then transferred into polyvinylidene fluoride (PVDF) membranes by wet transfer. Blocking was performed with 5% BSA in 1% PBST at room temperature for 60 min, before incubation with primary antibody (see Table S1) overnight at 4°C on a rocker. The following day, after incubation with fluorescence-labeled second antibody (see Table S1) avoiding light for 1 h at room temperature. The PVDF membranes were scanned using the Odyssey infrared scanner (LI-COR Biosciences). The greyscale values relative to GAPDH of the target proteins were analyzed using Image-J.1.51j8 software.

qRT-PCR analysis

Total RNA in liver tissues or cells was extracted and reverse-transcribed respectively using Nucleic Acid Purification Kit (Code: NPK-201F, lot. 742100, TOYOBO CO., LTD. Osaka, Japan) and ReverTra Ace qPCR RT Kit (Code: FSQ-301, Lot.616800, Osaka, Japan). The Specific operation was performed referring to the instructions. The qRT-PCR primers sequences information was listed in Table S2. The following a two-step approach PCR cycling program was: at 95°C for 60s, 40 cycles of 95°C for 15 s and 60°C for 60s, followed by melt curve analysis. The GAPDH gene was used as the internal reference for normalization of the target genes. The relative mRNA expression of each group was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data were analyzed by using the SPSS 21.0 software package. All the measurement data were expressed as means ± standard deviation (SD). Comparisons between multiple groups were analyzed by the one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test. $p<0.05$ was considered statistically significant. In addition, the pharmacokinetic parameters were calculated by noncompartmental analysis in WinNonlin software with a sparse sampling algorithm (Pharsight 6.2, NC, USA).

Results

Quantitative analysis in FZHY decoction and FZHY-biological samples

The chemical structure and concentration of 16 compounds in FZHY decoction (2 g/mL) were displayed in Fig.S1 and Table S3, respectively. The concentration−time curves of compounds in the plasma and liver after oral administration of FZHY decoction (20 g/kg) in rats was shown in Fig. 1a and 1b, and the corresponding PK parameters were shown in Table 1. The summary of the contents and AUCs of compounds in the FZHY decoction or FZHY- biological samples were shown in Fig. 1c.

The PK parameters of 11 compounds in the portal vein plasma, systemic plasma and liver, following oral administration of FZHY decoction in rats.

Compounds	Portal vein plasma			Systemic plasma		
	T_{max}(h)	C_{max}(ng/ml)	AUC(h*ng/ml)	T_{max}(h)	C_{max}(ng/ml)	AUC(h*ng/ml)
Salvianolic acid A	0.167	26.7 ± 11.2	108.7 ± 9.1	0.167	33.1 ± 14.9	122.3 ± 5.7
Salvianolic acid B	0.167	74.8 ± 36.4	144.4 ± 7.3	0.167	61.5 ± 42.1	55.9 ± 18.6
Danshensu	0.167	279.8 ± 58.8	1515.9 ± 64.6	0.5	206.6 ± 41.6	1362 ± 95
Gypenoside XLIX	0.5	21.5 ± 7.3	21.3 ± 3.5	0.5	22.4 ± 9.8	26.4 ± 3.8
Amygdain	0.167	347.7 ± 82.5	254.7 ± 23.2	0.167	286.2 ± 44.2	262.8 ± 23.9
Rosmarinic acid	0.167	27.1 ± 8.1	51.9 ± 4	0.167	15.4 ± 4.9	45.5 ± 4.7
Schisandrol A	0.167	68.2 ± 7.2	62 ± 8.6			
Schisandrol B	0.5	43.4 ± 3	215.1 ± 49.3	0.5	35.9 ± 0.6	84.3 ± 19.8
Deoxyschizandrin	0.167	4.4 ± 1.8	15.6 ± 1.1			
Schisandrin B	0.5	29.9 ± 11	89.4 ± 15.7			
Schisantherin A	0.5	1657.7 ± 584.3	8542.1 ± 566.2	0.5	2094.4 ± 681	8672.6 ± 1093.6
	Liver					
Compouds	T_{max}(h)	C_{max}(ng/g)	AUC(h*ng/g)			
Amygdain	7	185 ± 24.1	4423.2 ± 599.8			
Schisandrol A	0.5	42.5 ± 8.4	96.4 ± 13.7			
Schisandrol B	14	29.9 ± 1.8	578.7 ± 50.3			
Schisandrin B	0.5	18.7 ± 1.7	24.1 ± 4.6			
Schisantherin A	0.5	1011.1 ± 322.6	1070.3 ± 103.4			

There were 16 compounds determined in the FZHY decoction, including 6 compounds derived from Salvia miltiorrhiza, 5 compounds derived from Schisandra chinensis, 2 compounds derived from Gynostemma pentaphylla, 2 compounds from Cordyceps mycelium and 1 compound from Gynostemma pentaphylla. Among the compounds, salvianolic acid B was of the highest content in FZHY decoction, followed by danshensu, amygdalin, schisantherin A and salvianolic acid A, as the members of the high content group (concentration ≥ 1000 μg/ml). Adenosine, gypenoside XLIX, rosmarinic acid, and schisandrol A were subjected to the middle content group (100 μg/mL⪅concentration⪅1000 μg/mL), and the concentrations of the rest compounds, including schisandrol B, deoxyschizandrin, ginsenoside Rb3, schisandrin B, tanshinone I, cryptotanshinone and cordycepin, were very low(concentration ≤ 100μ g/ml)

Portal vein blood is the first site after gut absorption but before hepatic disposition, which is responsible for transferring the substances to the liver post-dose. Following oral administration of FZHY in rats, there were 11 compounds accurately detected in the portal vein plasma, where tanshinone I, cryptotanshinone, cordycepin, ginsenoside Rb3 and adenosine were undetected, probably due to the low content in the formula or the poor physicochemical property. The T_{max}s of all the compounds were within 0.5 h in portal vein plasma, indicating of the fast absorption. Schisantherin A exhibited the maximum exposure in the portal vein plasma, followed by danshensu (AUC ≥ 1000h*ng/mL). There were 4 compounds,including amygdalin、schisandrol B, salvianolic acid B, and salvianolic acid A belonged to the middle exposure group (100 h*ng/mL、concentration、1000 h*ng/ml).The rest 5 compounds were subjected to the low exposure group (AUC ≤ 100 h*ng/mL).

After hepatic disposition, the compounds were transported to the systemic plasma, which is responsible for delivering the substances to the other organs, except liver. Compared to those in the portal vein plasma, there were 8 compounds determined in the systemic plasma, where schisandrol A, schisandrin B, and deoxyschizandrin were undetected. Similar to those in the portal vein plasma, the absorption of those compounds was quick, and schisantherin A and danshensu were attributed to the high exposure group (AUC ≥ 1000 h*ng/mL), and schisantherin A were of the highest exposure in the systemic plasma. The middle exposure group (100 h*ng/mL、AUC、1000 h*ng/ml) included of amygdalin and salvianolic acid A, and the rest compounds belonged to the low exposure group (AUC ≤ 100 h*ng/ml).

Following oral administration of FZHY decoction, there were only 5 compounds detected in the liver. The T_{max}s of schisandrol A, schisandrin B and schisantherin A were at 0.5 h, similar to those in the plasma. In contrast, those of schisandrol B and amygdalin were at 14 h and 7 h, respectively, consistent to their multi-peak phenomenon in the concentration-time curves. Contrary to those in the plasma, amygdalin exhibited the highest hepatic exposure, followed by schisantherin A, whose exposure were more than 1000 h*ng/g. Schisantherin A belong to the middle exposure group (100 h*ng/g、AUC、1000 h*ng/g), and the hepatic exposure of schisandrol A and schisandrin B was very low (AUC ≤ 100 h*ng/g). Finally, salvianolic acid B, schisantherin A and amygdalin, who were of the maximum quantity in the FZHY decoction, plasma and liver, respectively, were selected to combined to evaluate the anti-hepatic fibrosis effect in vivo.

JY5 significantly alleviate hepatic injury and collagen deposition in CCl$_4$-induced rat and mouse liver fibrosis

Compared with the control group, the levels of serum ALT and AST were significantly increased in the CCl$_4$ group. After treatment with JY5 or FZHY, the levels of ALT and AST were significantly decreased (Fig. 2a, 2b and Fig. S2d, S2e). The serum AST level was decreased in the SORA group compared with the CCl$_4$ group (Fig. 2b).

H&E staining showed that the hepatic lobular structure was severely collapsed with more complete pseudo-lobules formed in the CCl$_4$ group. And fibrous tissue became denser, and hepatocytes were disordered and ballooning degeneration. There were a large number of inflammatory cells infiltration surrounding the hepatic sinusoid, central vein and portal tract. The above lesions were obviously attenuated with less pseudo-lobules and inflammatory cells infiltration, after treatment with JY5 or FZHY or SORA (Fig. S2a and Fig. 2c, upper panel).

SR staining showed that compared to the control group, the collagen deposition was obviously increased in the CCl_4 group. The fibrotic septum became significantly widened and distributed from the portal tract to the periphery in a reticular manner, forming pseudo-lobules with varying sizes. In contrast, the collagen deposition was obviously decreased, the fibrotic septum became narrower, and pseudo-lobules structures were observed less in the JY5 or FZHY or SORA treated groups (Fig. S2a and Fig. 2c, middle panel). Both the hepatic Hyp content and collagen deposition were significantly increased in the CCl_4 group, compared to the control group. The above indicators were significantly reduced after the intervention with JY5 or FZHY or SORA (Fig. S2b, S2c and Fig. 2d, 2e). These results demonstrated that JY5 formula had a significantly anti-liver fibrosis comparably to that of FZHY.

IHC staining showed that compared with the control group, numerous Col-Ⅰ expression were visible in the fibrotic septum in the CCl_4 group. By contrast, JY5 and SORA could significantly reduce Col-Ⅰ expression in the liver tissue (Fig. 2c, lower panel). In addition, qRT-PCR results showed that Col-Ⅰ mRNA expression was significantly more elevated in the CCl_4 group than that in the control group. Whereas compared to the CCl_4 group, Col-Ⅰ mRNA expression was significantly reduced in JY5 treated group (Fig. 2f).

Consistent with the CCl_4-induced rat liver fibrosis model, JY5 could significantly alleviate hepatic injury and collagen deposition in CCl_4-induced liver fibrosis in mice (Fig. 3).

JY5 significantly alleviate hepatic injury and collagen deposition in BDL-induced rat liver fibrosis

Compared with the sham group, the levels of ALT, AST, TBil, DBil, TBA and ALP were significantly increased in the BDL group. After JY5 or DAPT treatment, the levels of ALT, AST, TBil, DBil, TBA and ALP were significantly decreased (Fig. 4a-4f).

Consistent with the CCl_4-induced liver fibrosis, JY5 could reduce the hepatic Hyp content and collagen deposition, meanwhile down-regulate the expressions of Col-Ⅰ and Col-IV in BDL-induced rat liver fibrosis (Fig. 4g-4i). These results suggested that JY5 could significantly alleviate hepatic injury and collagen deposition in BDL-induced liver fibrosis in rats.

JY5 significantly represses the activation of HSCs in vivo

Both in the CCl_4-induced rat and mouse liver fibrosis experiments, IHC staining showed that numerous α-SMA and Desmin expression were visible in the fibrotic septum in the CCl_4 group. By contrast, both α-SMA^{+} cells and Desmin^{+} cells were decreased in the JY5 and SORA treated group (Fig. 5a, 5d). Western blot and qRT-PCR showed that the α-SMA expression was significantly elevated compared to the control group in the CCl_4 group. Whereas compared to the CCl_4 group, both α-SMA protein and mRNA expressions were significantly reduced in JY5 and SORA treated groups (Fig. 5b, 5c, 5e and 5f). Similarly, in the BDL-induced liver fibrosis experiment, the treatment effect of JY5 was consistent with these results in the CCl_4-induced liver fibrosis experiments (Fig. 5g-5i). These results demonstrated that JY5 significantly represses the activation of HSCs in CCl_4 -and BDL-induced liver fibrosis.

JY5 significantly inhibit the activation of Notch signaling pathway in vivo

In the CCl$_4$-induced liver fibrosis rat experiment, qRT-PCR showed that the mRNA expressions of Notch2, Notch3, Notch4, Jagged1, Jagged2 and recombination signal binding protein-κB (RPB-κB) were significantly more up-regulated in the CCl$_4$ group than these genes in the control group. Whereas compared to the CCl$_4$ group, Notch2, Notch3, Notch4, Jagged1, Jagged2 and RPB-κB mRNA expressions were significantly reduced in JY5 and SORA treated groups (Fig. 6a). Western blot showed that the protein expression of RPB-κB was significantly increased compared to the control group in the CCl$_4$ group. RPB-κB protein expression was significantly more reduced in JY5 and SORA treated groups than that in the CCl$_4$ group (Fig. 6b). While in the CCl$_4$-induced liver fibrosis mice experiment, JY5 could not only decrease the expressions of Notch2, Notch3, Notch4, Jagged1 and RPB-κB, but also down-regulate the expression of Dll1 (Fig. 6c and 6d). Consistent with the CCl$_4$-induced rat liver fibrosis model, JY5 could decrease the expressions of Notch2, Notch3, Notch4, Jagged1 and RPB-κB in the BDL-induced liver fibrosis model. In addition, the expressions of Dll1, Dll4 and Jagged 2 were significantly decreased after treatment with JY5 (Fig. 6e and 6f). These results suggested that JY5 could significantly inhibit the activation of Notch signaling pathway in CCl$_4$ -and BDL-induced liver fibrosis.

JY5 might inhibit activation of LX-2 cells induced by TGF-β1 via regulating Notch signaling pathway

LX-2 cells were activated by TGF-β1 to observe the effect of JY5 at various concentrations *in vitro*. qRT-PCR showed that the mRNA levels of α-SMA, Col-Ⅰ, Notch3 and Jagged1 were significantly elevated in the TGF-β1 treated cells compared to the control cells. Whereas a-SMA、Col-Ⅰ、Notch3 and Jagged1 mRNA expressions were significantly reduced after treatment with various concentrations of JY5. And Col-Ⅰ、Notch3 and Jagged1 mRNA expressions were significantly decreased in the high-dose of JY5 treated group, compared to the low-dose of JY5 treated group (Fig. 7b-7e). Western blot results showed that the protein expressions of a-SMA and RBP-κB were significantly increased after treated with TGF-β1. Compared to the TGF-β1 group, both a-SMA and RBP-κB protein expressions were significantly reduced in various concentrations of JY5 treated groups. Among of these, the protein expression of RBP-κB was significantly reduced in the high-dose of JY5 treated group compared to the middle-dose and low-dose of JY5 treated groups (Fig. 7a and 7f). The above results suggested that JY5 might inhibit the activation of LX-2 cells induced by TGF-β1 via regulating Notch signaling pathway.

Discussion

Liver fibrosis is an abnormal repair response to tissue damage, characterized by excessive deposition of ECM, leading to the persistence and development of pathological scar. Hepatic fibrosis is common in most chronic liver diseases process, which is a clinically important problem to be urgently solved. In order to develop effective anti-hepatic fibrotic drugs, researchers have conducted a large number of basic and clinical studies. Despite achieving certain results in recent years, it has been shown that the most were stay at preclinical or clinical trials stages, even some finally ended up in failure with severe toxic side effects [20]. TCM has remarkable clinical effects in the treatment of liver fibrosis, which is closely correlated with its characteristics of multi-ingredients compatibility and multi-targets. But the ingredients of TCM are complex, and their mechanisms are not very clear, which somewhat increases the complexity of studies of TCM. As the intensive

development of multidisciplinary crossover study, active ingredients screening, extraction and purification from Chinese herbs provide a new approach for TCM formula research.

Salvianolic acid B is the most main water-soluble phenolic acid compound. Numerous studies [8, 9, 12, 21] have indicated that salvianolic acid B exerted significant anti-hepatic fibrosis effect through the following mechanisms: inhibiting the activation of HSCs via downregulating TGF-β1/Smads signaling pathway, protecting hepatocytes from apoptosis via inhibiting death receptor pathway, stabilizing the mitochondrial membrane and regulating NF-κB/IκBα signaling pathway. Amygdalin is considered to be the most major ingredient of peach kernel. It has been shown that [13, 14] amygdalin can inhibit the activation of HSCs via downregulating TGF-β/CTGF signaling pathway, and induce activated HSCs apoptosis via upregulating Bax gene expression, subsequently exerting anti-hepatic fibrotic effect. Lignans are reported to be the main bioactive components of Schisandrae. Studies [15] have shown that these lignans could suppress inflammation, protect hepatocytes and inhibit the activation of HSCs via downregulating TGFβ/Smads and MAPK signaling pathway.

In this study, we respectively measured the content of various compounds in FZHY extract, plasma and liver in rats after intragastric administration with FZHY. We obtained three main bioactive ingredients of FZHY, salvianolic acid B, which was the highest content in FZHY extract, schisantherin A, which was the maximum exposure in plasma and amygdalin, which was the highest hepatic exposure in the liver. Then we conducted uniform design and validation experiments to explore their composition with best ratio treating in rat hepatic fibrosis model. Therefore, we got a new composition, namely JY5, which had an anti-fibrotic effect as effective as FZHY formula. Further studies have showed that JY5 could significantly decrease serum ALT and AST levels and inhibit inflammation reaction, at the same time, reduce collagen deposition in CCl_4-induced or BDL-induced liver fibrosis models.

The activation of HSCs is a pivotal event in liver fibrosis. Under persistent stimulations from CCl_4 and BDL, HSCs are largely activated and transformed into myofibroblasts, which causes excessive ECM accumulated in the liver, eventually leading to hepatic fibrosis formation. Activated HSCs, as one of the main sources of hepatic ECM, can secrete Col-▢ and Col-▢ proteins. It is well known that α-SMA is a specific marker of activated HSCs. This study suggested that JY5 significantly reduce mRNA and protein expressions of α-SMA, and decreased Col-▢mRNA expression in CCl_4- and BDL-induced liver fibrosis in rats and mice. The results were further confirmed in TGF-β1-indued LX-2 cells activation. JY5 could significantly down-regulate the mRNA and protein expressions of α-SMA in activated LX-2 cells induced by TGF-β1. Other than that, JY5 can downregulate Col-▢ mRNA expression in a dose-dependent manner *in vitro*. These results suggested that JY5 significantly inhibit the activation of HSCs.

Notch signaling is a highly conserved pathway evolutionarily, which influences intercellular signal transduction and cell fate decisions, and regulate growth and development homeostasis of multiple tissues and organs in body, in particular, the progress and development of diseases [22]. Notch signaling pathway mainly consists of 4 Notch receptors (Notch1, Notch2, Notch3, Notch4), 5 Notch ligands (Jagged1, Jagged2, Dll1, Dll3, Dll4) and the transcriptional regulatory elements of downstream signals [23]. Previous studies [24]have shown that Notch play an important role in the progress and development of hepatic fibrosis, which can interact with other signaling pathways, such as TGF-β, Hedgehog, Hippo, etc. TGF-β1 can promote proliferation and activation of

HSCs-T25 cells through regulating Notch signaling pathway [25]. Jagged1 gene was successfully interfered by using rAAV1-Jagged1-shRNA in CCl_4-indued liver fibrosis, resulting in alleviating liver fibrosis [26]. In addition, the knockout of RBP-κB gene, which is considered to be the key transcription factor of Notch pathway, can inhibit the proliferation and activation of HSCs to alleviate CCl_4-indued liver fibrosis in mice [27]. It can be seen that the blockade of Notch pathway can effectively inhibit activation of HSCs, which in turn attenuate liver fibrosis. In our study, the expressions of Jagged1, Jagged2, Notch2, Notch3, Notch4, RBP-κB were significantly increased in CCl_4- and BDL-induced liver fibrosis in rats and mice. While after intervention with JY5, the expressions of these Notch-related genes and proteins were significantly decreased. This is further confirmed by LX-2 cells activation induced by TGF-β1 experiments *in vitro*. These results suggested that JY5 might exert anti-fibrotic effect through regulating Notch signaling pathway to inhibit the activation of HSCs.

In this study, we respectively used SORA and DAPT as the positive controls in the experiments. SORA, as a multi-receptor tyrosine kinase inhibitor, can inhibit the proliferation of multiple tumor cells and promote cells apoptosis, which is commonly used for treatment of hepatocellular carcinoma (HCC) clinically [28]. DAPT, as a γ-secretase inhibitor, can block the release of Notch intracellular domain (NICD) into intracellular to inhibit the activation of Notch signaling pathway [24]. In accord with reported studies and previous researches [29, 30], our results show that both SORA and DAPT have significant anti-hepatic fibrosis effects. Interestingly, SORA also can significantly down-regulate mRNA expressions of Jagged1, Jagged2, Notch2, Notch3, Notch4, RBP-κB and the protein expression of RBP-κB. We speculate that SORA exerts anti-hepatic fibrosis effect, which might be related to regulation of Notch pathway. But the precise mechanism remains to be further investigated. In addition, the reported studies [19] have shown that DAPT inhibit liver fibrosis through blocking Notch pathway. This conclusion is further confirmed in our study. However, it is important to note that γ-secretase inhibitors, as a nonspecific Notch blocker, have severe side effects in the clinical trial [24]. So the clinical applications of DAPT could be limited to a certain. Compared with SORA and DAPT, JY5 may exist synergistic anti-fibrosis effect targeted on multiple pathways, not specifically blocking a particular target or pathway, which confers its relatively higher safety and more effectiveness .

However, the main targets and specific modalities, by which JY5 regulate Notch pathway, should be to further investigated. In addition, given that JY5, as a component of TCM compounds, has shown the good efficacy in two different liver fibrosis models, we speculate that JY5 might alleviate hepatic fibrosis through other mechanisms. In response to these issues, a series of related studies to elaborate the compatibility mechanisms of anti-liver fibrosis effect of JY5 will be further carried out. Thus, these studies will provide more adequate scientific basis for the clinical research and application of JY5 formula.

Conclusions

In summary, we obtained a novel anti-hepatic fibrosis component of TCM compounds, namely JY5, through the uniform design and validation experiments *in vivo*, and explored its part of mechanisms for the first time. Our study has shown that JY5 maybe exert anti-hepatic fibrotic effect through regulating Jagged/Notch/RBP-κB signaling pathway to inhibit the activation of HSCs. JY5 formula may be a potential new therapeutic candidate for liver fibrosis.

Abbreviations

FZHY, Fuzheng Huayu formula; SORA, sorafenib; HSCs, hepatic stellate cells; ECM, extracellular matrix; TCM, traditional Chinese medicine; KCs, Kupffer cells; LSECs, liver sinusoidal endothelial cells; CCl_4, carbon tetrachloride; BDL, bile duct of ligation; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBil, total bilirubin; DBil, direct bilirubin; TBA, total bile acids; Hyp, hydroxyproline; IHC, immunohistochemical; H&E, hematoxylin & eosin; SR, sirius red; α-SMA, α-smooth muscle actin; RBP-κB, recombination signal binding protein-κB; HCC, hepatocellular carcinoma; NICD, Notch intracellular domain.

Declarations

Ethics approval and consent to participate

All rat experiments were approved by the Institutional Animal Care and Use Committee (IACUC), School of Pharmacy, Fudan University (Ethical approval code No. 2018-07-SZYD-LP-01). All mice experiments were approved by IACUC at Shanghai Research Center of the Southern Model Organisms (Ethical approval code No. 2019-0031).

Consent for publication

Not applicable

Availability of data and materials

All data and materials in the current study are included in this published article and its additional information files.

Declaration of competing interests

The authors declare that they have no competing interests.

Authors' contributions

Ya-dong Fu, Zhun Xiao, Xiao-ting Tian, Cheng-gang Huang, Jiamei Chen and Ping Liu wrote the manuscript and were involved with project concept and submission; Ya-dong Fu, Zhun Xiao, Xiao-ting Tian completed the main part of the experiment; Wei Liu, Yong-hong Hu and Jing Fang participated in the pharmacokinetic experiment; Si-qi Gao and Yong-Ping Mu participated in the in vivo experiment; Ding-Qi Zhang, Hua Zhang and Yi-yang Hu participated in the in vitro experiment; Cheng-gang Huang, Jiamei Chen and Ping Liu revised the manuscript and were responsible for final approval. All authors read and approved the final manuscript.

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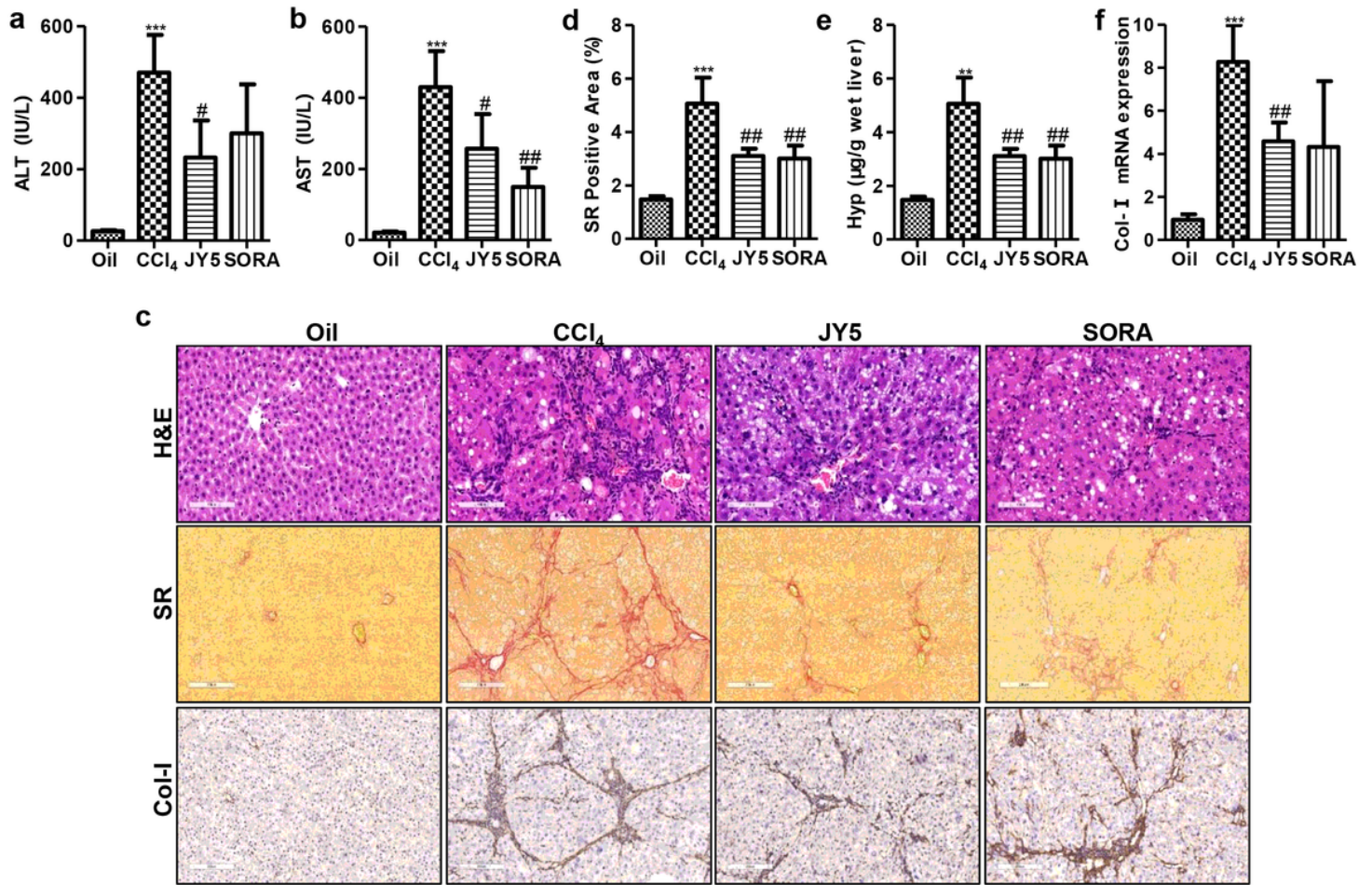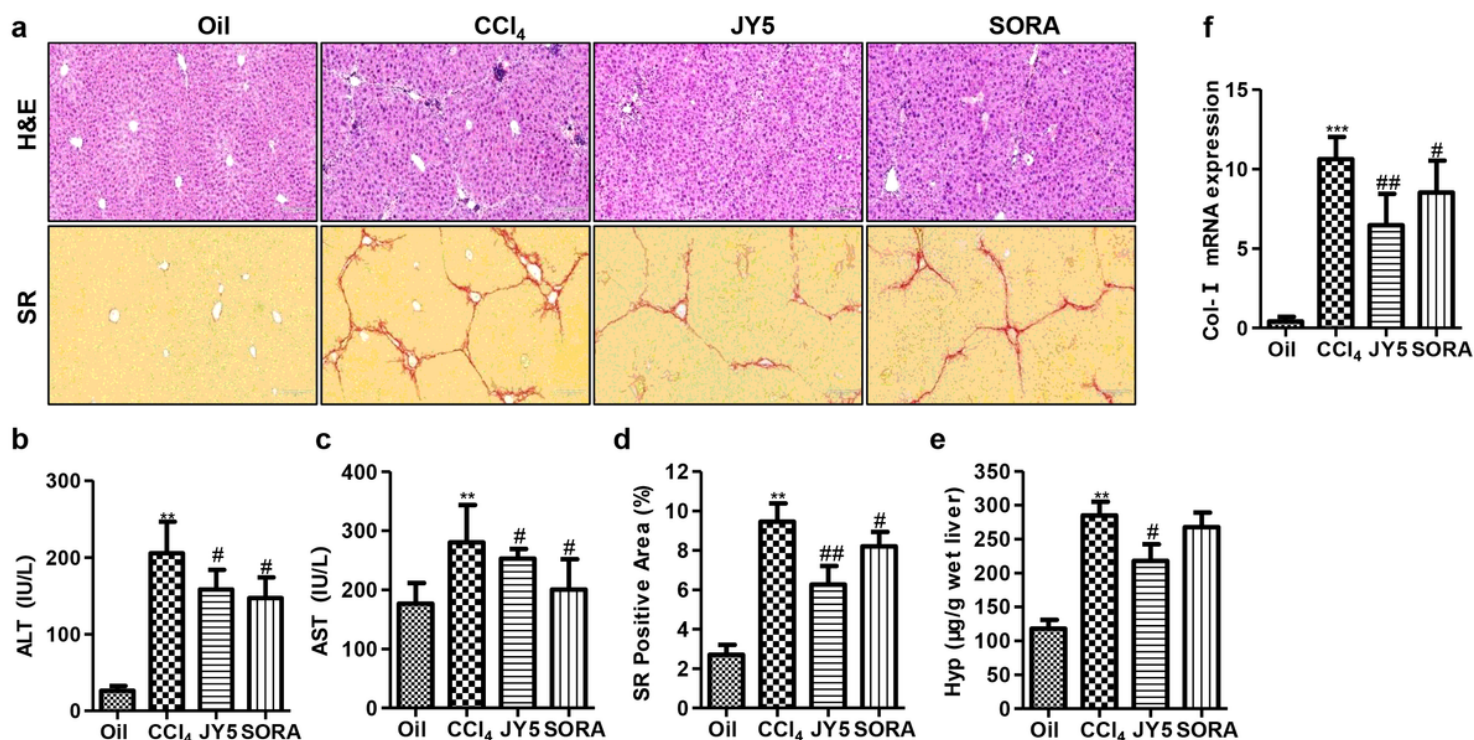
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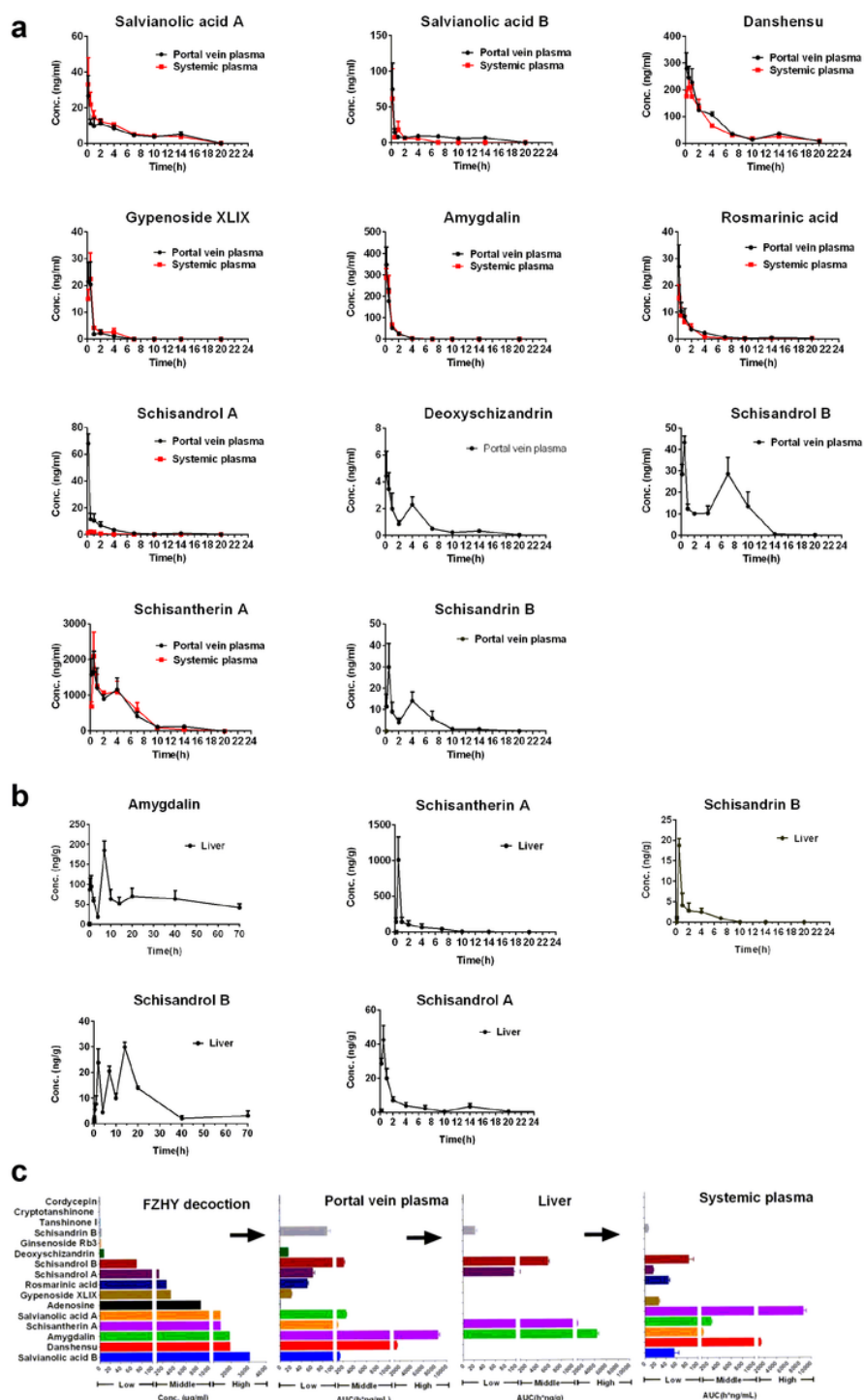
Figure 1

Quantitative analysis in FZHY decoction and FZHY-biological samples. The concentration−time curves of compounds in the plasma (a) and liver (b) after oral administration of FZHY decoction (20 g/kg) in rats (n=5). (c). The summary of the contents and AUCs of compounds in the FZHY decoction or FZHY- biological samples.
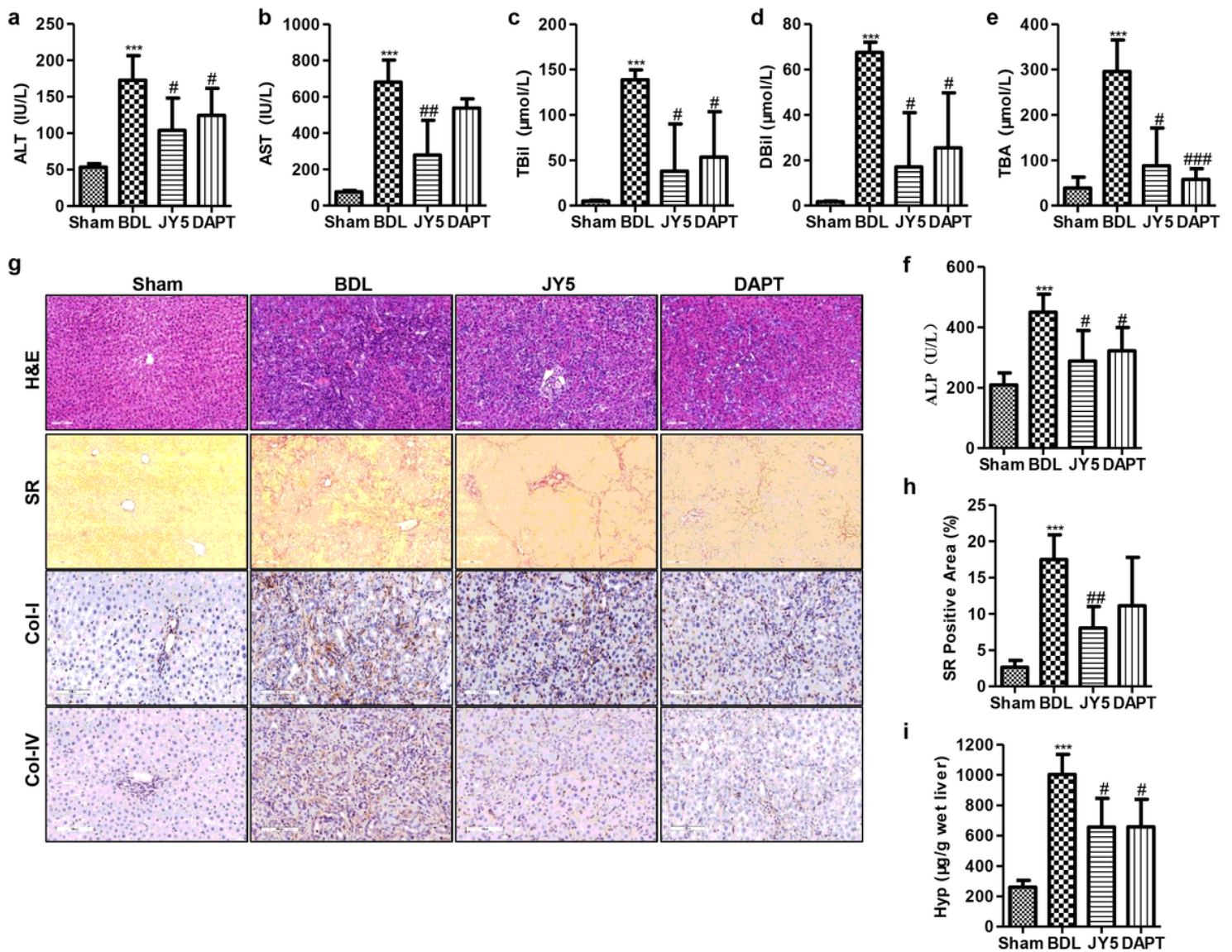
Figure 2

JY5 significantly alleviates hepatic inflammatory injury and collagen deposition in CCl4-induced rat liver fibrosis. The activity of serum ALT (a) and AST (b) were measured in each group. (c). H&E staining (200×), SR staining (100×) and IHC staining of COL-Ⅰ(100×), and semi-quantitative analysis (d) of collagen disposition (%) in SR-stained liver sections. (e). Hydroxyproline content in wet liver tissue was detected via alkaline hydrolysis. (f). The mRNA levels of COL-Ⅰin liver tissue was analyzed by qRT-PCR. **p<0.01, ***p<0.001 vs. the control group, #p<0.05, ##p<0.01 vs. the CCl4 group. Oil: the control group; CCl4: the CCl4 group; JY5, the JY5 treated group; SORA: the SORA treated group.
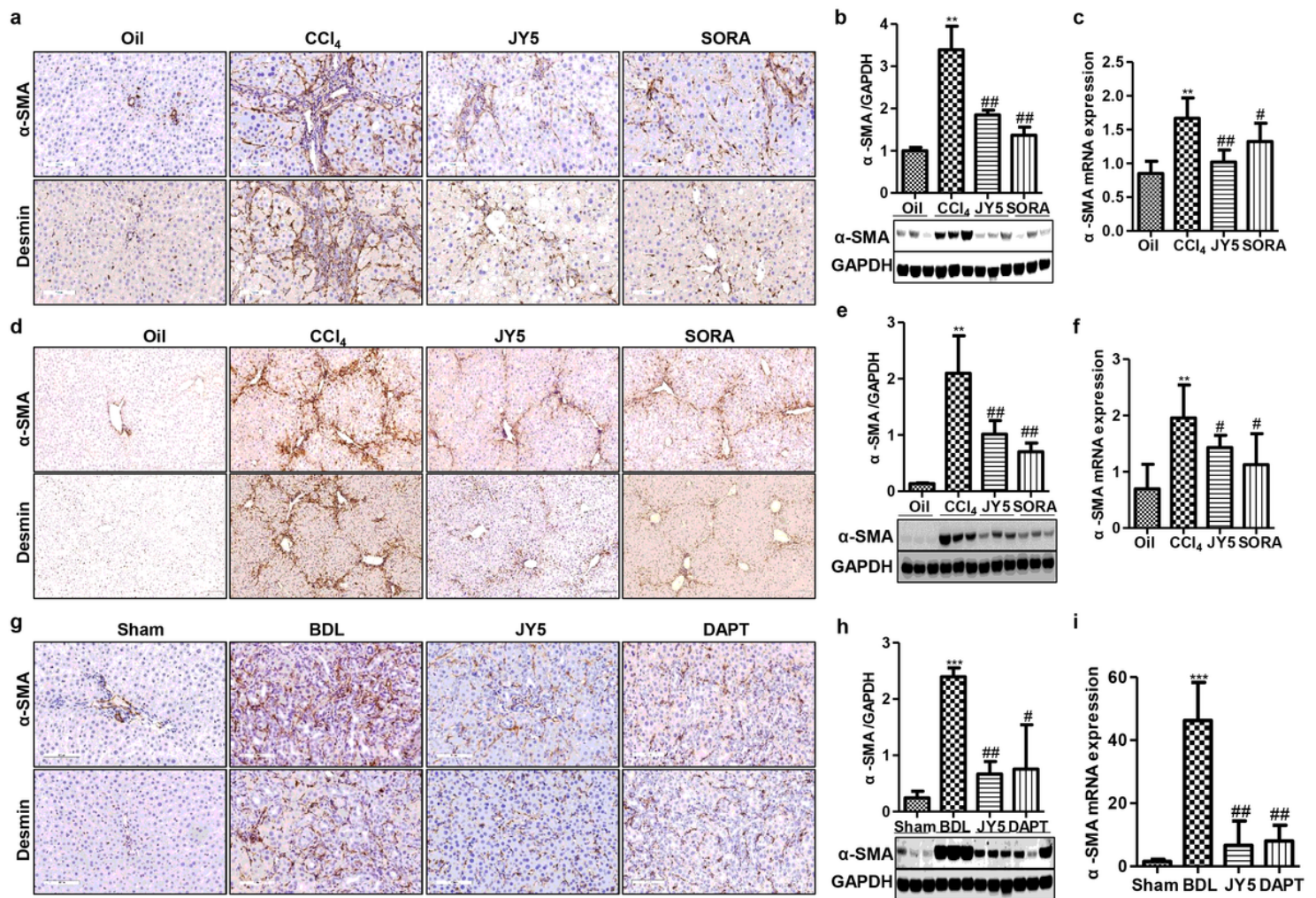
Figure 3

JY5 significantly alleviates hepatic inflammatory injury and collagen deposition in CCl4-induced mice liver fibrosis. (a). H&E (200×) and SR (100×) staining in liver tissue. The activity of serum ALT (b) and AST (c) were measured in each group. (d). semi-quantitative analysis of collagen disposition (%) in SR-stained liver sections. (e). Hydroxyproline content in wet liver tissue was detected via alkaline hydrolysis. (f). The mRNA expression of COL-Ⅰin mice liver tissue were analyzed by qRT-PCR. **p<0.01, ***p<0.001 vs. the control group, #p<0.05, ##p<0.01 vs. the CCl4 group. Oil: the control group; CCl4: the CCl4 group; JY5, the JY5 treated group; SORA: the SORA treated group.
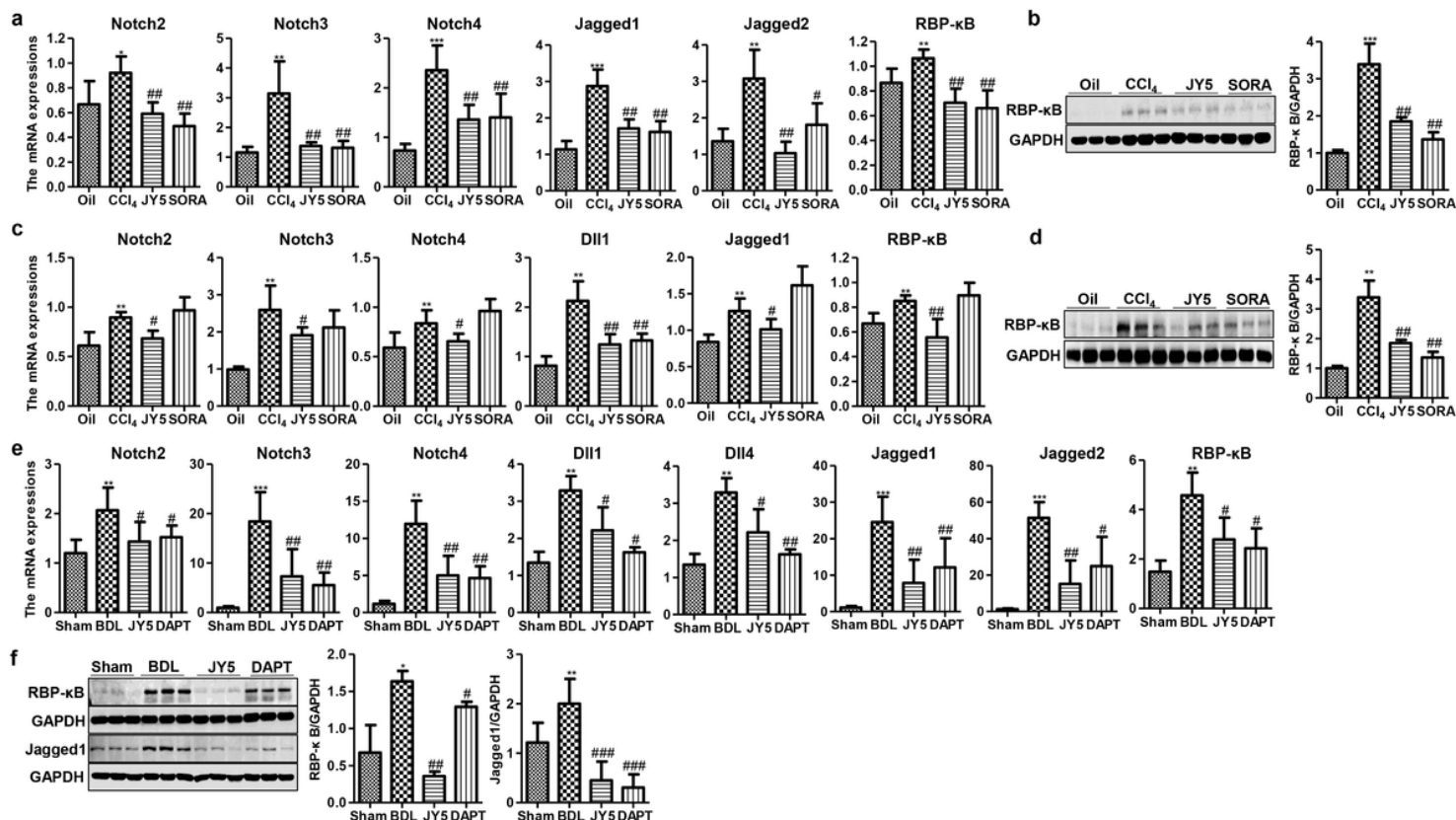
Figure 4

JY5 significantly alleviates hepatic inflammatory injury and collagen deposition in BDL-induced rat liver fibrosis. The levels of serum ALT (a), AST (b), TBil (c) DBil (d), TBA (e) and ALP (f) were measured in each group. (g). H&E staining (200×), SR staining (100×) and IHC for COL- and COL- (200×) staining, and semi-quantitative analysis (h) of collagen disposition (%) in SR-stained liver sections. (i). Hydroxyproline content in wet liver tissue was detected via alkaline hydrolysis. ***p<0.001 vs. the Sham group, #p<0.05, ##p<0.01 vs. the BDL group. Sham: the Sham group; BDL: the BDL group; JY5, the JY5 treated group; DAPT, the DAPT treated group.
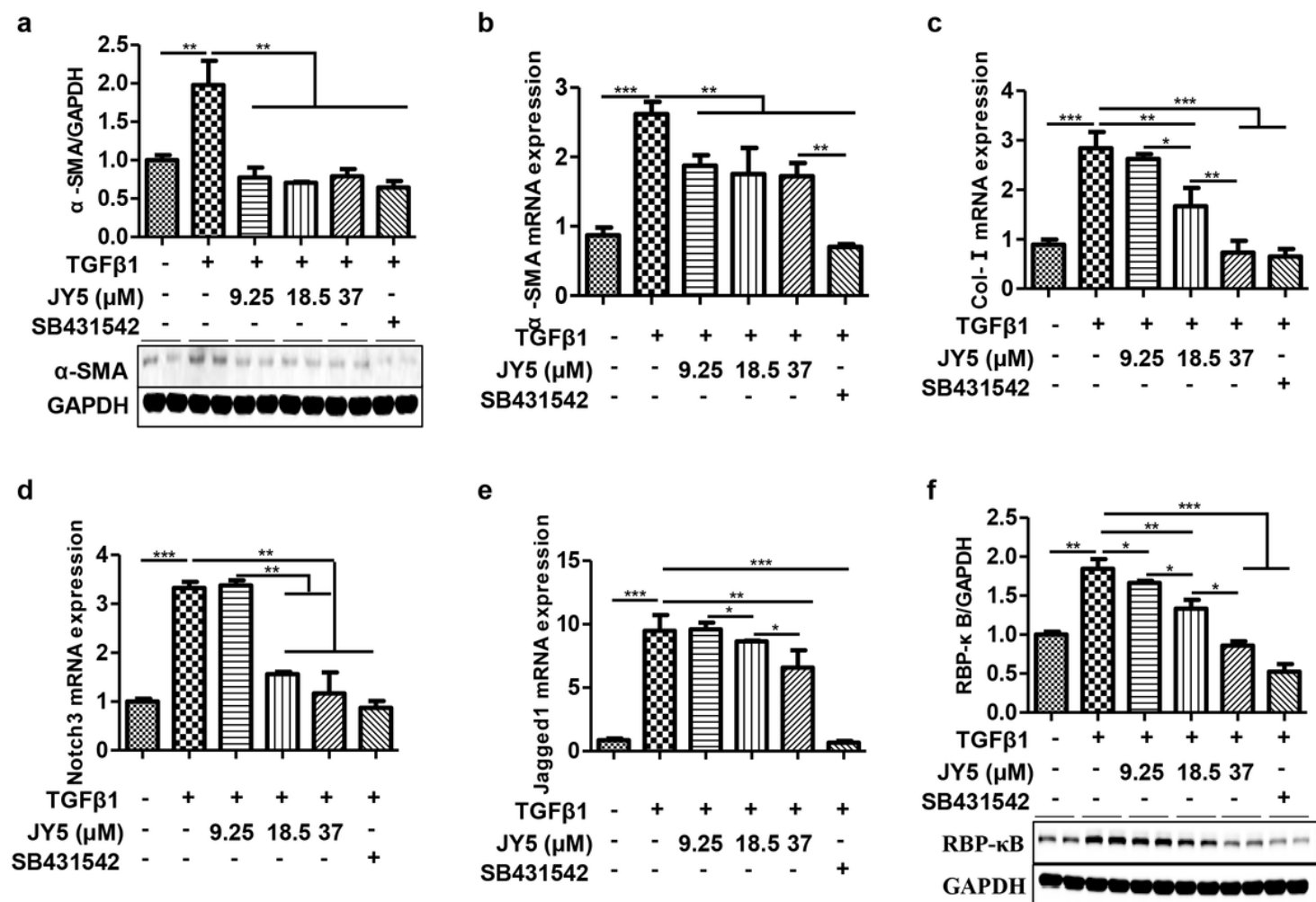
Figure 5

JY5 inhibits the activation of HSCs in vivo. (a). In the CCl4-induced rat liver fibrosis experiment, representative images of IHC (100×) staining for α-SMA and Desmin. The protein and mRNA levels of α-SMA were respectively analyzed by Western blot (b) and qRT-PCR (c). (d). In the CCl4-induced liver fibrosis mice experiment, representative images of IHC (100×) staining for α-SMA and Desmin in liver tissue from mice treated with the various treatments. The protein and mRNA levels of α-SMA in mice liver tissue were respectively analyzed by Western blot (e) and qRT-PCR (f). (g). In the BDL-induced rat liver fibrosis experiment, representative images of IHC (200×) staining for α-SMA and Desmin in liver tissue. The protein and mRNA expressions of α-SMA were respectively analyzed by Western blot (h) and qRT-PCR (i). **p<0.01, ***p<0.001 vs. the control group or Sham group, #p<0.05, ##p<0.01 vs. the CCl4 group or BDL group. Oil: the control group; CCl4: the CCl4 group; SORA: the SORA group; Sham: the Sham group; BDL: the BDL group; DAPT: the DAPT group; JY5, the JY5 group.
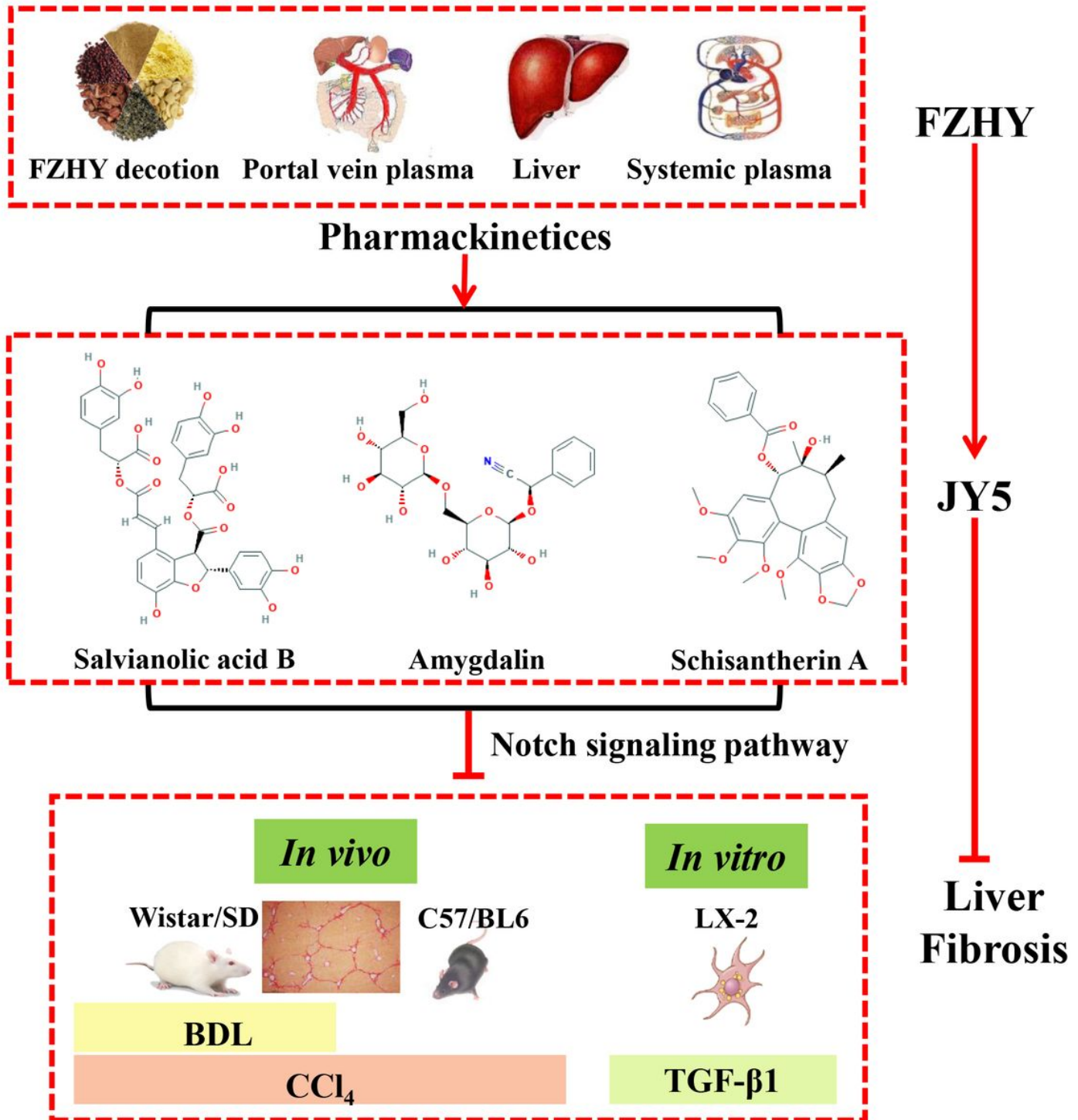
Figure 6

JY5 may ameliorate liver fibrosis through inhibition of Notch signaling pathway in vivo. (a). In the CCl4-induced rat liver fibrosis experiment, the mRNA levels of Notch2, Notch3, Notch4, Jagged1, Jagged2 and RBP-κB were measured by qRT-PCR. (b). Western blot and quantitative analyses of RBP-κB protein. (c). In the CCl4-induced liver fibrosis mice experiment, the mRNA levels of Notch2, Notch3, Notch4, Dll1, Jagged1 and RBP-κB were measured by qRT-PCR. (d). Western blot and quantitative analyses of RBP-κB protein. (e). In the BDL-induced rat liver fibrosis experiment, the mRNA levels of Notch2, Notch3, Notch4, Dll1, Dll4, Jagged1, Jagged2 and RBP-κB were measured by qRT-PCR. (f). Western blot and quantitative analysis of Jagged1 and RBP-κB protein. *p<0.05, **p<0.01, ***p<0.001 vs. the control group or Sham group, #p<0.05, ##p<0.01, ###p<0.001 vs. the CCl4 group or BDL group. Oil: the control group; CCl4: the CCl4 group; SORA: the SORA group; Sham: the Sham group; BDL: the BDL group; DAPT: the DAPT group; JY5, the JY5 group.

Figure 7

JY5 inhibits the activation of LX-2 cells induced by TGF-β1 via repressing Notch signaling pathway. (a). Western blot and quantitative analysis of RBP-κB protein in cell lysate. The mRNA expressions of α-SMA (b)、Col-Ⅰ(c)、Notch3 (d) and Jagged1 (e) were measured by qRT-PCR. (f) Western blot and quantitative analyses of RBP-κB protein. *p<0.05, **p<0.01, ***p<0.001.

Figure 8

A brief diagram of this study was shown. JY5 formula, which consists of the main active ingredients of FZHY, could exert anti-hepatic fibrotic effect through regulating Notch signaling pathway and inhibiting the activation of HSCs.

Supplementary Files

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- Supplementarymaterial.docx