Liver fibrosis is a pathological process characterized by excess deposition of extracellular matrix (ECM) that are mainly derived from activated hepatic stellate cells. Previous studies suggested that ligustroflavone (LF) was an ingredient of *Ligustrum lucidum* Ait. with activities of anti-inflammation and anti-oxidation. In this study, we investigated whether LF had any effect on liver fibrosis. In our study, we established a mouse model of carbon tetrachloride (CCl₄)-induced liver fibrosis and used TGF-β₁-stimulated human hepatic stellate cell line (LX-2) to explore the effect of LF and associated underlying mechanism. LF was used *in vivo* with low dose (L-LF, 5 mg·kg⁻¹, i.p., 3 times each week) and high dose (H-LF, 20 mg·kg⁻¹, i.p., 3 times each week) and *in vitro* (25 μmol·L⁻¹). Histopathological and biochemical assays investigations showed that LF delayed the formation of liver fibrosis; decreased AST, ALT activities and increased Alb activity in serum; decreased MDA level, Hyp content and increased GSH-Px concentration, SOD activity in liver tissues. Moreover, immunohistochemical, immunofluorescent and Western blot results showed that LF reduced the expressions of hepatic stellate cells specific marker proteins, including collagen I and α-SMA in vivo and *in vitro*. In addition, LF markedly suppressed TGF-β₁-upregulated protein expressions of Tj/R I, Tj/R II, P-Smad2, P-Smad3 and Smad4 in LX-2 cells. Taken together, these findings demonstrated LF could decrease histopathological lesions, ameliorate oxidative injury, attenuate CCl₄-induced liver fibrosis, which may be associated with down-regulating the TGF-β/Smad signaling pathway.

### ABSTRACT
Liver fibrosis is a pathological process characterized by excess deposition of extracellular matrix (ECM) that are mainly derived from activated hepatic stellate cells. Previous studies suggested that ligustroflavone (LF) was an ingredient of *Ligustrum lucidum* Ait. with activities of anti-inflammation and anti-oxidation. In this study, we investigated whether LF had any effect on liver fibrosis. In our study, we established a mouse model of carbon tetrachloride (CCl₄)-induced liver fibrosis and used TGF-β₁-stimulated human hepatic stellate cell line (LX-2) to explore the effect of LF and associated underlying mechanism. LF was used *in vivo* with low dose (L-LF, 5 mg·kg⁻¹, i.p., 3 times each week) and high dose (H-LF, 20 mg·kg⁻¹, i.p., 3 times each week) and *in vitro* (25 μmol·L⁻¹). Histopathological and biochemical assays investigations showed that LF delayed the formation of liver fibrosis; decreased AST, ALT activities and increased Alb activity in serum; decreased MDA level, Hyp content and increased GSH-Px concentration, SOD activity in liver tissues. Moreover, immunohistochemical, immunofluorescent and Western blot results showed that LF reduced the expressions of hepatic stellate cells specific marker proteins, including collagen I and α-SMA in vivo and *in vitro*. In addition, LF markedly suppressed TGF-β₁-upregulated protein expressions of Tj/R I, Tj/R II, P-Smad2, P-Smad3 and Smad4 in LX-2 cells. Taken together, these findings demonstrated LF could decrease histopathological lesions, ameliorate oxidative injury, attenuate CCl₄-induced liver fibrosis, which may be associated with down-regulating the TGF-β/Smad signaling pathway.

### KEY WORDS
Ligustroflavone; Liver fibrosis; HSCs; TGF-β/Smad

### CLC Number
R965

### Document code
A

### Article ID
2095-6975(2021)03-0170-11

### [Received on]
23-Apr.-2020

### [Research funding]
This work was supported by the National Natural Science Foundation of China (Nos. 81770432 and 81970245) and the Science and Technology Program of Shaanxi Province, China (Nos. 2019PT-23 and 2019ZDLSF04-03-01).

### [Corresponding author]
Tel/Fax: 86-29-84772221, E-mail: xxqqli@fmmu.edu.cn

These authors have no conflict of interest to declare.
most effective cytokine for the transformation and proliferation of HSCs \[12\]. TGF-β/Smad signaling pathway plays a key role in the progression of liver fibrosis by promoting transdifferentiation and migration of HSCs \[13\]. Thus, blocking TGF-β/Smad signaling pathway may serve as an effective strategy for inhibiting activation of HSCs \[14\]. The activated HSCs are major targets for antifibrotic therapy. If the activation of HSCs can be inhibited, or even the activation state of HSCs can be reversed to a static state, it can provide an effective clinical treatment of liver fibrosis.

Ligustri Lucidi Fructus (LLF), the fruit of *Ligustrum lucidum* Ait. is a major component among kidney-tonifying herbal prescriptions \[15\]. Modern pharmacological studies have found that LLF has antioxidant \[16\], anti-osteoporosis \[17\], anti-tumor effects \[18\] and so on. Ligstroflavone (LF) is a natural flavonoid glycoside from the LLF. This compound provides significant pharmacological properties, including anti-inflammatory \[19\], and anti-complementary effects \[20\]. In addition, LF is a calcium-sensing receptors (CaSR) antagonist and has protective effects against diabetic osteoporosis in mice \[21\]. LF can also reduce necroptosis in rats’ brains after ischemic stroke through targeting RIPK1/RIPK3/MLKL pathway \[22\]. However, the effects of LF on liver fibrosis, thus far, have not been reported.

Therefore, in this study we investigated the antifibrotic effects LF on the human hepatic stellate cell line LX-2 and

---

**Fig. 1** LF protected against in CCl\(_4\)-induced liver injury. (A) The molecular structure of LF. (B) Schematic representation of the liver fibrosis model in C57BL/6J mice induced by intraperitoneal CCl\(_4\) (diluted at 1 : 4 in olive oil) that was administered twice a week for 6 weeks. Meanwhile, LF was intraperitoneally injected with for 6 weeks. (C) The time course of the body weight gained during the experiment. (D) body weight, liver weight, liver/body weight. Results were shown as the means ± SEM (\(n = 6–7\)). *P < 0.05, **P < 0.01 vs the CCl\(_4\) group; *P < 0.05, **P < 0.01 vs control group
carbon tetrachloride (CCl₄)-induced hepatic fibrosis in mice and elucidated the potential mechanisms underlying its function in vitro and in vivo.

**Materials and Methods**

**Induction of hepatic fibrosis by CCl₄**

Male C57BL/6J mice (twenty-eight male, weighing between 18 g and 22 g) were supplied by the Experimental Animal Research Center of the Fourth Military Medical University (FMMU). The animals were kept under room temperature (25 ± 1 °C), atmospheric moisture (50% ± 10%), a regular 12-hour dark-light cycle, and were fed with standard laboratory food and water. To create animal models of liver fibrosis, C57 mice were intraperitoneally (i.p.) injected with CCl₄ (diluted at 1 : 4 in olive oil) or olive oil alone (2 mL/kg body weight) twice a week for 6 weeks [21]. Mice were randomly divided into five groups (n = 7/group); (i) Control group; (ii) LF group; (iii) CCl₄ group and (iv) CCl₄ + L-LF group; (v) CCl₄ + H-LF group; We used two different doses of LF to assess its efficacy. Mice were administered LF with low dose (L-LF, 5 mg·kg⁻¹) and high dose (H-LF, 20 mg·kg⁻¹) [21] by intraperitoneal injection for 6 weeks, either alone or after CCl₄ administration. Mice were then sacrificed 24 h after the last injection of LF. Blood samples were collected from the orbit of the mice for further biochemical analysis. Some portions of liver tissues were fixed in 10% formalin for paraffin preparation while other portions were snap-frozen in liquid nitrogen and then stored at −80 °C until use. All animal study procedures were verified by the Institutional Animal Care and Use Committee of FMMU and were performed according to the approved guidelines.

**Biochemical analysis assay**

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin (Alb) were detected to assess liver function. The glutathione peroxidase (GSH-Px) concentration, superoxide dismutase (SOD) activity and malondialdehyde (MDA) level in liver tissues were measured to estimate the lipid peroxidation products and antioxidants. As a major component of collagen proteins, Hydroxyproline (Hyp) content was used to assess the degree of liver fibrosis. They were all measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using a half-automatic biochemical analyser, according to the manufacturer’s instructions.

**Tissue sectioning and histopathology**

The fixed paraffin tissues were dehydrated in a graded ethanol series and were embedded in paraffin wax. Tissues were placed on glass slides, and paraffin was removed with xylene. Then, the tissue sections were subjected to haematoxylin and eosin, Masson’s Trichrome and Sirius Red staining to evaluate the severity of fibrosis. Examinations were performed and photographs were captured with a light microscope (DM2000 LED, Leica, Solms, Germany). Image J software (National Institutes of Health, Maryland, USA) was used to calculate the ratio of the area with positive expression to the total field of Masson’s Trichrome and Sirius Red staining.

**Immunohistochemistry staining**

Liver specimens fixed in 10% buffered formalin were embedded in paraffin blocks. Liver sections were then processed using a standard immunostaining protocol. After routine deparaffinization, blocked endogenous peroxidase was treated with 0.3% H₂O₂ for 10 min and blocked in 5% BSA for 60 min. Then, the sections were incubated with anti-α-SMA antibody (1 : 100, Abcam, MA, USA) or anti-COL 1A1 antibody (1 : 200, Santa Cruz Biotechnology, Texas, USA) overnight at 4 °C, and were incubated with biotin-conjugated goat anti-mouse IgG for 1 h at room temperature. The sections were incubated with the SABC (Streptavidin Biotin complex, Boster, USA) for 30 min, developed with DAB complex, Boster, USA) for 30 min, developed with DAB (3,3′-diaminobenzidine, Boster, USA) and counterstained with haematoxylin. The sections were observed and photographed with a microscope. Image J software (NIH) was used to calculate the ratio of the area with positive expression to the total field of α-SMA and COL 1A1 Immunohistochemistry staining.

**Cell culture**

The human immortalized HSC LX-2 cells was obtained from Prof. YUE Zhen-Sheng, Department of Hepatobiliary Surgery, Xijing Hospital of FMMU. The cells were cultured in High Glucose DMEM (HyClone, Utah, USA) supplemented with 10% FBS (Gibco, California, USA), 100 U·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin (Solarbio, Beijing, China) at 37 °C and 5% CO₂ in a fully humidified atmosphere.

**Immunofluorescence staining**

5 ng·mL⁻¹ TGF-β₁ was added in a medium of LX-2 cells with LF (25 μmol·L⁻¹) for 24 h. After incubation, the cells were fixed in 4% paraformaldehyde in PBS for 10 min and were permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature. Nonspecific binding sites were blocked by incubating the cells with 2% bovine serum albumin for 1 h. The fixed cells were incubated with a primary antibody against α-SMA (1 : 100, Abcam), COL 1A1 (1 : 200, Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation with fluorescence (FITC)-conjugated goat anti-rabbit IgG (1 : 50, Cowin Biosciences, Beijing, China) for 70 min at room temperature. The cellular nuclei were stained with DAPI for 10 min. All samples were imaged with a light microscope (DM2000 LED, Leica). Image J software (NIH) was used to calculate the ratio of the area with positive expression to the total field of α-SMA and COL 1A1.

**Western blotting**

Protein from each sample was subjected to 8%–10% SDS-PAGE by electrophoresis under reducing conditions and was transferred to NC membrane (Pall Gelman Laboratory, USA). The blotted membrane was then blocked with 5% skim milk or 5% BSA for 1 h at room temperature and was incubated respectively with α-SMA antibodies (1 : 200, Ab-
TO EXPLOR THE ROLE OF LF IN THE PROGRESSION OF LIVER FIBROSIS, WE ESTABLISHED LIVER FIBROSIS MODEL BY ADMINISTERING CCl₄ INTRAPERITONEALLY FOR 6 WEEKS AND HARVESTED TISSUES AFTER THE FINAL LF TREATMENT WITH A DOSAGE OF 5, 20 mg·kg⁻¹ (Fig. 1B). Fig. 1C EXHIBITS A TIME COURSE OF THE BODY WEIGHT GAIN FOR THE TREATED MICE OVER 6 WEEKS. THE CCl₄ GROUP GAINED LESS BODY WEIGHT THAN THE CONTROL GROUP, MORE LIVER WEIGHT THAN THE CONTROL GROUP, AND THE CCl₄ + L-LF GROUP AND CCl₄ + H-LF GROUP RESULTED IN AN INTERMEDIATE AMOUNT OF BODY WEIGHT AND LIVER GAIN (Fig. 1D). COMPARED TO THE CCl₄ GROUP, THE LIVER/BODY WEIGHT RATIO OF THE CCl₄ + H-LF GROUP HAS DECREASED BY ABOUT 16% (Fig. 1D). MOREOVER, THE RESULTS ALSO SHOWED THAT LF HAD NO EFFECT ON HEALTHY MICE.

**LF AMELIORATED CCl₄-INDUCED HEPATIC DYSFUNCTION AND OXIDATIVE INJURY**

ALT AND AST ARE COMMONLY USED BIOMARKERS FOR LIVER DAMAGE. COMPARED TO THE CONTROL GROUP, THE SERUM ALT AND AST ACTIVITIES WERE SIGNIFICANTLY HIGHER, WHILE THE ALB ACTIVITY WAS SIGNIFICANTLY LOWER (P < 0.01) IN THE CCl₄ GROUP. COMPARED TO CCl₄ GROUP, ALT AND AST ACTIVITIES OF THE CCl₄ + H-LF GROUP WERE RESPECTIVELY DOWN-REGULATED ABOUT 81% AND 66%, WHILE THE ALB ACTIVITY OF CCl₄ + H-LF GROUP WAS UP-REGULATED 1.2-FOLD. (FIG. 2A)

COMPARED TO THE CONTROL GROUP, THE GSH-Px CONCENTRATION AND SOD ACTIVITY WERE SIGNIFICANTLY LOWER, AND MDA LEVEL WAS SIGNIFICANTLY HIGHER (P < 0.01) IN THE CCl₄ GROUP. ADDITIONALLY, COMPARED TO THE CCl₄ GROUP, THE RESULTS SHOWED A SIGNIFICANT DECREASE IN LIVER MDA LEVEL IN THE LF HIGH-DOSE
groups ($P < 0.01$). Additionally, liver GSH-Px concentration and liver SOD activity were increased significantly in the LF high-dose groups ($P < 0.05$). Moreover, the results also showed that LF had no effect on healthy mice.

**LF ameliorated CCl$_4$-induced histological damage and liver fibrosis in liver injury**

According to H&E staining (Fig. 3A), the liver tissues of the control group showed normal lobular architecture with central veins and radiating hepatic cords, with no regenerating collagen fibers. In the CCl$_4$ group, extensive degeneration, a dense inflammatory infiltrate, and the disappearance of the normal structure of the lobes were observed. Liver tissues in the CCl$_4$ + H-LF group showed significantly less necrosis of hepatic cells and less collagen deposition than the CCl$_4$ group.

In addition, the results of Masson’s Trichrome staining (Figs. 3B and 3D) showed that collagen fibers area in the CCl$_4$ group was largely enhanced compared with the control group, but was markedly decreased in the CCl$_4$ + H-LF group ($P < 0.01$). The extent of liver fibrosis was also documented by Sirius Red staining. A marked increase in Sirius Red staining area was observed in the livers of the CCl$_4$ group compared with the control group (Figs. 3C and 3E). By contrast, the CCl$_4$-induced increase in Sirius Red staining was markedly reduced in the CCl$_4$ + H-LF group ($P < 0.01$). Hydroxyproline serves as a marker of collagen deposition in liver fibrosis. Compared to the control group, Hyp content in liver tissues was significantly increased in the CCl$_4$ group. Hyp content was decreased significantly in the CCl$_4$ + H-LF group compared with the CCl$_4$ group (Fig. 3F, $P < 0.01$). The extent of liver fibrosis was also documented by Sirius Red staining. A marked increase in Sirius Red staining area was observed in the livers of the CCl$_4$ group compared with the control group (Figs. 3C and 3E). By contrast, the CCl$_4$-induced increase in Sirius Red staining was markedly reduced in the CCl$_4$ + H-LF group ($P < 0.01$). Hydroxyproline serves as a marker of collagen deposition in liver fibrosis. Compared to the control group, Hyp content in liver tissues was significantly increased in the CCl$_4$ group. Hyp content was decreased significantly in the CCl$_4$ + H-LF group compared with the CCl$_4$ group (Fig. 3F, $P < 0.01$). The CCl$_4$-induced liver injury and fibrosis were significantly attenuated in the CCl$_4$ + H-LF group, but the CCl$_4$ + L-LF group had no statistical significance. Moreover, the results also showed that LF treatment in absence of CCl$_4$ had no significant effects on histological changes.

**LF attenuated the expressions of α-SMA and COL 1A1 in CCl$_4$-induced liver fibrosis**

The results of immunohistochemistry showed that the positive expressions of α-SMA and COL 1A1 proteins in CCl$_4$ group was significantly increased, and the positive expressions of α-SMA and COL 1A1 proteins were found in central vein, hepatic sinusoid space, portal area. Compared
with CCl₄ group, the expression of α-SMA and COL 1A1 proteins significantly decreased in CCl₄ + H-LF group (Figs. 4A–4D, P < 0.01). The histopathology, biochemical, and immunohistochemical analyses of high-dose LF treatment with CCl₄-induced liver fibrosis had significant significance. In contrast, the low-dose group did not. Therefore, the

Fig. 4  LF attenuated the expressions of α-SMA and COL 1A1 in CCl₄-induced liver fibrosis. (A) and (B) Representative immunohistochemical staining of α-SMA and COL1A1. The scale bar is 50 μm. (C) and (D) Quantification of histological changes in different treatment groups with positive area using Image J analysis software (n = 6–7). (E) The protein expression of α-SMA, COL1A1, Vimentin and E-cadherin were examined by Western blots. (F), (G), (H) and (I) Quantitative analysis of COL1A1, α-SMA, Vimentin, E-cadherin and β-actin expression (n = 3). All data were presented as means ± SEM. * P < 0.05, ** P < 0.01 vs the CCl₄ group, * P < 0.05, ** P < 0.01 vs control group
following experiments were conducted in the 20 mg·kg⁻¹ dose group (CCl₄ + H-LF). The results of WB further confirmed that the expressions of α-SMA and COL 1A1 proteins in CCl₄ + H-LF group was significantly lower than that of CCl₄ group (P < 0.05). Meanwhile, LF had no effect on the expression of COL 1A1 and α-SMA in the liver of healthy mice (Figs. 4E–4G). In addition, EMT has been regarded as a crucial mechanism in the progress of liver fibrosis. As shown in Fig. 4E, the results of western blot analysis showed that the protein levels of vimentin were significantly higher in the CCl₄ group compared with the control group, and the α-E-cadherin (Figs. 4E and 4H) level was significantly lower than that of the control group. Additionally, vimentin (Figs. 4E and 4I) levels in the CCl₄ + H-LF groups were significantly lower than in the CCl₄ group. E-cadherin levels in the CCl₄ + H-LF groups were significantly higher than in the CCl₄ group, as expected.

**LF reduced the expressions of α-SMA and COL 1A1 in TGF-β₁-stimulated LX-2 cells**

Immunofluorescence analysis’s results showed that the α-SMA and COL 1A1 expressions were significantly higher in the TGF-β₁-stimulated group compared with the control group. Additionally, compared to the TGF-β₁-stimulated group, the α-SMA (Figs. 5A and 5C) and COL 1A1 (Figs. 5B and 5D) expressions were significantly lower (P < 0.01) in the TGF-β₁ + LF group. In accordance with the Immunofluorescence analysis’s results, the upregulation of α-SMA and COL 1A1 by LF were further confirmed with protein levels using the Western blots. LF, when co-treated with TGF-β₁, presented the inhibitory effects on the TGF-β₁-stimulated expression of α-SMA (Figs. 5E and 5F) and COL 1A1 (Figs. 5E and 5G) proteins (P < 0.01) in LX-2 cells compared with the TGF-β₁-stimulated group. Treatment with LF alone did not change the state of hepatic stellate cells.

**LF down-regulated TGF-β/Smad signaling pathway in vitro**

As shown in Figs. 6A and 6B, western blot analysis showed that the protein expressions of Tj/R I, Tj/R II, P-Smad2, P-Smad3 and Smad4 were significantly higher in the TGF-β₁-stimulated group compared with the control group, and this effect in the LF + TGF-β₁ group was significantly lower than in the TGF-β₁-stimulated group, as expected.

**Discussion**

As is well known, liver fibrosis is the last reversible stage in chronic hepatopathy. However, there is lack of ideal drugs such as anti-fibrotic agents for curing hepatic fibrosis at present. Therefore, it is an urgent task to discover some effective drugs for preventing or treating liver fibrosis and investigate the mechanism of action against it. Although it is reported that flavonoid glycosides derived from LLF has anti-inflammatory property, its mechanism for the treatment of liver fibrosis is not clear. In our study, we have reported that LF has therapeutic effects on CCl₄-induced liver fibrosis and have investigated its underlying mechanism of action. The result indicated that LF modulated the TGF-β/Smad signaling pathway in reducing the expressions of hepatic stellate cells specific marker proteins against liver fibrosis during the progress of the disease.

In this study, the classical subcutaneous injection of carbon tetrachloride was used to establish the model of hepatic fibrosis in mice. It has been proved that carbon tetrachloride can directly destroy the hepatic cell membrane and cause hepatic lobular cell necrosis, resulting in hepatic fibrosis in mice [24].

According to our study, the liver of mice was observed, weighed and the organ coefficient was calculated. It is suggested that LF can improve the swelling and edema of liver in mice with hepatic fibrosis (Fig. 1D). AST and ALT are common markers of liver injury. LF can down-regulate the expression of ALT, AST and up-regulate the expression of Alb (Fig. 2A) in mice with hepatic fibrosis. These results confirmed that LF improved the liver function in CCl₄-induced liver fibrosis.

Oxidative stress is recognized as one of liver injury mechanisms related to liver fibrosis, and it accelerates collagen synthesis by activating hepatic stellate cells (HSCs) and ECM deposition through the TGF-β₁ signaling pathway during hepatic fibrosis progression [25–27]. MDA, GSH, and SOD are important indicators of oxidative stress in the liver [28]. According to our experiments, biochemical results indicated that the protective effect of LF on CCl₄-induced liver fibrosis in mice may be achieved by increasing the expression of antioxidants SOD and GSH-Px as well as reducing the level of lipid peroxide MDA. Its mechanism may be that LF contains multiple hydroxyl groups, which can provide electrons to react with free radicals, thus scavenging free radicals and inhibiting lipid peroxidation.

The effect of LF on CCl₄-induced hepatic injury and fibrosis were evaluated by H&E, Masson and Sirius Red staining (Figs. 3A–3E). Hydroxyproline, a major constituent of collagen, is a key marker for ECM accumulation. LF can down-regulate the expression of Hyp (Fig. 3F). It was found that LF could significantly prevent death of hepatocytes and cholangiocytes, decrease a large number of inflammatory cells infiltrated and aggregated in the high-dose group. As a consequence, liver fibrosis is ameliorated based on that LF reduced the area of portal area fibrosis and bridging fibrosis in the high-dose group. Under the continuous stimulation of injury factors, HSCs are transformed from a static vitamin A storage type to α-SMA expressing myofibroblasts, which are the main cells that synthesize and secrete collagen fibers. It mainly secretes type I, type III and type IV collagen, and its ability to synthesize ECM is stronger, which plays an important role in the occurrence and development of hepatic fibrosis. α-SMA is regarded as an important marker of the HSCs activation which are the primary cell type in the liver responsible for excess collagen synthesis during liver fibrosis [29]. The above results suggested that LF can down-regulate the marker of HSCs’ activation (α-SMA and COL 1A1), and this may be the potential mechanism underlying its liver...
fibrosis-mitigating effect in vitro and in vivo.

Recent studies have demonstrated that ECM is not the only factor leading to liver fibrosis, and HSCs can be capable of transferring and expressing typical markers of fibroblasts by EMT and then participate in the formation of liver fibrosis [14]. Therefore, EMT is also a main part of liver fibrosis pathology [30]. In the present study, our results from Western blot analyses showed that LF attenuated the expression of EMT related protein Vimentin and up-regulated the expression of EMT related protein E-cadherin, which indicated that the inhibition of EMT could be one of the mechanisms for LF to exert its effects on liver fibrosis.

Long-term inflammation and oxidative stress caused by liver injury can lead to liver fibrosis. The morphology of HSCs can change and transform into myofibroblasts secreting many fibrosis related factors including TGF-β1 in the process of liver injury. TGF-β1 is one of the most important fibrosis mediators. TGF-β1 strongly stimulates HSCs to produce collagen type I, thereby producing a large number of ECM and inhibiting ECM degradation [31]. TGF-β1 can bind to its receptor on the cell surface and activate the TGF-β receptor signaling pathway, leading to the expression of ECM proteins such as collagen type I. The increase in ECM deposition results in the accumulation of collagen fibers and the formation of fibrous scar tissue, which is the hallmark of liver fibrosis.

Fig. 5  LF reduced the expressions of α-SMA and COL 1A1 in TGF-β1-stimulated LX-2 cells. (A) and (B) Immunofluorescence staining of LX-2 cells with antibodies against α-SMA and COL 1A1. The scale bar is 50 μm. (C) and (D) The positive staining areas were measured by Image J software (n = 5). (E) The protein expression of α-SMA and COL 1A1 were examined by Western blots. (F) and (G) Quantitative analysis of COL 1A1, α-SMA and β-actin expressions (n = 3). All data were presented as means ± SEM. *P < 0.05, **P < 0.01 vs the TGF-β1 treatment; #P < 0.05, ##P < 0.01 vs control group.
Fig. 6  LF down-regulated TGF-β/Smad signaling pathway in vitro. (A) western blot analysis for TβR I, TβR II, P-Smad2, P-Smad3, Smad4 and a loading control of (Smad2, Smad3 and β-actin) protein levels in LX-2 cells. (B) Quantitative analysis of TβR I, TβR II, P-smad2, P-Smad3, Smad4 and β-actin expressions (n = 3). All data were presented as means ± SEM. *P < 0.05, **P < 0.01 vs the TGF-β treatment. #P < 0.05, ##P < 0.01 vs the control group. (C) Schematic diagram of the antifibrotic effect of LF through down-regulating the TGF-β/Smad signaling pathway.
to TGF-β receptor (TβR) on the HSC membrane, in which HSCs receptors include type I and type II receptors. Smads protein family, as the main effector downstream of TGF-β1, can mediate the intracellular signal transduction of TGF-β1 from the receptor to the nucleus. When TGF-β1 binds to the HSCs membrane receptor and activates TβR1, activated TβR1 can phosphorylate activated Smad2 and Smad3 into P-Smad2 and P-Smad3, and form a complex of Smad2/3/4, transferring into nucleus together with Smad4 [32-34]. The transport of this complex into the nucleus enhances the expression of α-SMA and COL 1A1 and promotes fibrosis. Subsequently, we used a human immortal HSC cell line (LX-2) to more thoroughly investigate the protective mechanism. Our results showed that LF treatment was able to down-regulate TGF-β1-induced α-SMA and COL 1A1 expressions in LX-2 cells (Fig. 5). It is due to that LF could inhibit the phosphorylation level of Smad2 and Smad3 induced by TGF-β1 (Figs. 6A and 6B). It inhibits their entry into the nucleus, thus reducing the fibrosis activity of TGF-β1, LF reduced the expression of hepatic stellate cells specific marker proteins, including collagen I and α-SMA, by down-regulating the TGF-β/Smad signaling pathway, so as to alleviate liver fibrosis. This further suggested that inhibition of Smad2/3 activation may be one of mechanisms of beneficial effects of LF on liver fibrosis.

According to previous reports, the pairing of Sophora flavescens and LLF ameliorated CCl4-induced histological damage and hepatic dysfunction, but the research evidence was limited, and the effective components and mechanism were not clear [39]. In our study, it was confirmed that Ligustrofлавоне had anti-fibrotic effects in vivo and in vitro.

We acknowledge several limitations with this study. First, our results showed that LF had a protective effect on CCl4-induced liver fibrosis in mice, but there are other fibrosis models of different mechanisms, such as thioacetamide (TAA)-induced liver fibrosis model and biliary duct ligation (BDL)-induced liver fibrosis model, which need to be further verified the mechanisms of LF effect on anti-fibrosis. Second, some changes of oxidative stress indicators of SOD activity, GSH-Px concentration and MDA level were observed in our study, indicating that the attenuation of oxidative stress also be one of the mechanisms of hepatoprotective effect of LF. Many studies have revealed that ROS is overproduced to promote hepatocyte apoptosis and activate hepatic stellate cells, when oxidative stress occurs in the liver [38]. Subsequently, the occurrence and development of liver fibrosis were regulated by activation of Nrf2, TGF-β, NF-xB and other related cytokines, signal molecules and their downstream signal pathways [39]. However, whether LF affects the occurrence of liver fibrosis through ROS remains to be further studied. Third, the hepatoprotective effect of LF was obvious which LF alleviate CCl4-induced liver fibrosis, but we only preliminarily studied the possible mechanism. Additional works should be required to investigate more details on TGF-β/Smad signaling pathway involved in the anti-fibrogenesis of LF. It points the way to find the target protein directly acted by LF through virtual screening and molecular docking techniques. Furthermore, if the anti-fibrosis mechanism of LF was further confirmed in the primary hepatic stellate cells and related inhibitors, which will be a comprehensive supplement to the existing studies.

**Conclusion**

In conclusion, LF was involved in inhibiting the occurrence of liver fibrosis, and the mechanism may be associated with the following factors: (1) The inhibition of EMT could be one of the mechanisms for LF to exert its effects on liver fibrosis. (2) The hepatoprotective effect of LF might be related to alleviation of CCl4-induced oxidative stress. (3) LF down-regulated TGF-β/Smad signaling pathway in LX-2 based on the protein expression of Smad2/3 phosphorylation decreasing, which resulted in down-regulating the transcription of α-SMA and COL 1A1.

**References**


