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Research Paper

# Potential protective effects of a traditional Chinese herb, *Litsea coreana* Levl., on liver fibrosis in rats

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## Abstract

**Objectives** It was found that total flavonoids from *Litsea coreana* Levl. (TFLC), which is a traditional Chinese medicine, had a preventive effect against hepatic steatosis in our previous study. This study was designed to evaluate whether TFLC could improve liver fibrosis in rats.
**Methods** The liver fibrosis model rats were treated with composite factors of high-fat emulsion (10 ml/kg) via gavage accompanied by a subcutaneous injection of low-dose $CCl_4$. Thirty rats were given composite factors plus TFLC (100, 200, 400 mg/kg), respectively, for 8 weeks.
**Key findings** The results showed that TFLC (200 and 400 mg/kg) treatment significantly reduced the elevation of liver index (liver weight/body weight) and spleen index (spleen weight/body weight), alanine transaminase, aspartate aminotransferase, hyaluronic acid, laminin, procollagen III N-terminal peptide, procollagenase IV and hydroxyproline. In addition, TFLC treatment improved the morphologic changes of hepatic fibrosis, suppressed expression of $\alpha$-smooth muscle actin, collagen I, transforming growth factor (TGF)-$\beta$1 and TGF$\beta$ receptor (TGF$\beta$R)1, and increased peroxisome proliferator-activated receptor-$\gamma$ expression in the liver of hepatic fibrosis rats.
**Conclusions** In conclusion, TFLC is able to ameliorate liver injury and protect rats from liver fibrosis. This process may be related to inhibiting the expression of transforming growth factor-$\beta$1 and increasing the expression of peroxisome proliferator-activated receptor-$\gamma$.
**Keywords** liver fibrosis; peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$); total flavonoids of *Litsea coreana* Levl. (TFLC)

## Introduction

Fibrogenesis occurs, in the context of chronic liver disease, as a result of the persistent activation of the wound healing process. Hepatic stellate cells (HSC) play an essential role in the development of liver fibrosis. Following fibrotic injury, HSCs undergo transdifferentiation from quiescent vitamin-A-storing cells to an activated myofibroblastic phenotype change, including enhanced cell proliferation, expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) and excessive production of fibrillar extracellular matrix (ECM).[1] Activation of HSCs is associated with the sequential expression of several key cytokines and their surface receptors, including transforming growth factor-$\beta$ (TGF-$\beta$), one of the strongest pro-fibrotic cytokines.[2,3] The peroxisome proliferator-activated receptor-$\gamma$ (PPAR$\gamma$) imparts diverse cellular effects in biologic systems, including being a regulator of adipocyte differentiation and expression, dyslipidaemia and cancer. Studies have shown that PPAR$\gamma$ also plays an important role in liver fibrosis.[4,5] Without effective treatment at an early stage, reversible hepatic fibrosis progresses to irreversible cirrhosis.[6] This is a major public health problem, owing to life-threatening complications of portal hypertension, liver failure and increased incidence of hepatocellular carcinoma. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis.

Traditional Chinese medicines (TCMs), treasures of Chinese people, are a range of medical practices used in China for more than four millennia. This system has been

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recognized as a popular complementary and alternative medicine in Western countries because Chinese medicine is generally extracted from natural products without artificial additives, which creates mild healing effects and incurs fewer side effects.[7] *Litsea coreana* Levl. (also called hawk tea), which has been recorded by 'Ben Cao Gang Mu' (the *Compendium of Materia Medica*, a classical traditional Chinese medicine book), is a xylophyta, widely distributed in many countries, especially in the southern part of China. As one of the most important heat-clearing and dampness-drying teas, leaves of this plant have been widely used in the treatment of gastrosis, hepatitis and inflammatory diseases.[8,9] Modern pharmacological and clinical studies have shown that *Litsea coreana* Levl. possesses various biological actions, such as antioxidant, hepatoprotective, hypolipidaemic, anti-inflammatory and immunomodulatory activity.[10,11] Therefore, based on our previous result in the nonalcoholic steatohepatitis rat model, this study was designed to evaluate the preventive effect of total flavonoids of *Litsea coreana* Levl. (TFLC) on composite factor-induced liver fibrosis and its possible mechanisms of action.

## Materials and Methods

### Total flavonoids of *Litsea coreana* Levl. compounds

The dried leaves were obtained from Ningguo Medicinal Materials Company (Anhui Province, China) and authenticated as *Litsea coreana* Levl. based on its microscopic and macroscopic characteristics by Professor Shou-jin Liu who specializes in traditional Chinese herbal medicine at Anhui University of Traditional Chinese Medicine. The voucher specimen of the crude drugs was deposited in the herbarium of the School of Pharmacy, Anhui Medical University and registered under the number LC20080301. The dried leaves of *Litsea coreana* Levl. (4.0 kg) were refluxed with 80% ethanol (48.0 l) for 2 h. After filtration, the ethanol solution was combined and condensed to obtain a syrup. The syrup was suspended in water (4.0 l) and the pH was adjusted to 2 and partitioned between *n*-butanol and water. The water fraction was then adsorbed to D101 macroporous adsorptive resin and eluted with 90% ethanol. The elution and the above butanol fraction were combined and concentrated under reduced pressure to obtain total flavonoids. The concentrated extract was chromatographed over a silica gel column eluted with DAB-6–EtOAc–EtOH and Sephadex LH-20 repeatedly. Quercetin-3-*O*-$\beta$-D-galactopyranoside, kaempferol-3-*O*-$\beta$-D-glucopyranoside, quercetin-3-*O*-$\beta$-D-glucopyranoside and kaempferol-3-*O*-$\beta$-D-galactopyranoside were obtained and their chemical structures are shown in Figure 1a. The compounds were identified by means of physical, compound methods and instrumental analysis such as UV, $^{1}$H NMR, $^{13}$C NMR and MS and the chromatogram of a mixture of standard compounds is shown in Figure 1b.

### High-performance liquid chromatographic analysis of total flavonoids of *Litsea coreana* Levl.

An amount of 0.1 g pulverized TFLC sample was accurately weighed, and dissolved in 50 ml methanol. Before use, all samples were filtered through a 0.45-$\mu$m membrane and 10 $\mu$l was injected onto the HPLC column for analysis. Chromatographic analysis was performed on a Waters 600E liquid chromatography system. Analysis was carried out at 30°C on a Hypersil ODS column (4.6 mm i.d. × 200 mm, 5 $\mu$m). A linear gradient elution of eluents A (acetonitrile) and B (water–glacial acetic acid 2%, v/v, pH 2.6) was used for the separation. The peaks were recorded using DAD absorbance at 350 nm and the solvent flow rate was kept at 1.0 ml/min.

### Animals and treatment

Male Sprague–Dawley rats (200 ± 20 g) were obtained from the Experimental Animal Center, Anhui Medical University. They were housed in plastic cages under standardized conditions of temperature (21–22°C) and humidity (40–60%) with a 12-h light–dark cycle. The study and procedures were approved by the Ethic Committee and the Animal Experimental Committee of Anhui Medical University. All animals received human care in compliance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. The rats were given a normal diet for one week while adapting to vivarium conditions and then randomly divided into five groups ($n = 10$ per group): normal group, model group, and three treatment groups (TFLC 100, 200 and 400 mg/kg). The liver fibrosis model was induced by composite factors as previously reported.[12] Briefly, rats were treated with high-fat emulsion (10 ml/kg) via gavage once per day for eight weeks accompanied by a subcutaneous injection of 400 g/l $CCl_4$ (Xi'an Chemical Factory) ($CCl_4$ : corn oil, 2 : 3), 1 ml/kg, at 8:00 a.m. each day, every three days. In the normal group, which received an injection of peanut oil, high-fat emulsion was replaced by an equal volume of saline. For the treatment groups, 100, 200 or 400 mg/kg of TFLC was given via gavage at 6:00 p.m. each day to the liver fibrotic rats after the first week, respectively, while an equal volume of 0.5% CMC-Na solution was used as substitution in the normal and model group. The high-fat emulsion was prepared as previously reported.[13] The macronutrients present in this emulsion were as follows: corn oil 400 g; saccharose 150 g; total milk powder 80 g; cholesterol 100 g; sodium deoxycholate 10 g; Tween 80 36.4 g; propylene glycol 31.1 g; vitamin mixture 2.5 g; cooking salt 10 g; mineral mixture 1.5 g; distilled water 300 ml. After eight weeks rats were sacrificed. Serum was collected and stored at −20°C for determination. The liver and spleen were immediately removed and weighed to calculate the liver and spleen indexes. Specimens of rat isolated livers were fixed in formaldehyde and stored in liquid nitrogen.

### Biochemical determination

Serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured by routine laboratory methods using a 7170-automatic biochemistry analyser (Tokyo, Japan). Serum hyaluronic acid (HA), laminin (LN), procollagen III N-terminal peptide (PIIINP) and procollagenase IV (CIV) were detected by radioimmunoassay, and the content of hydroxyproline in liver was determined

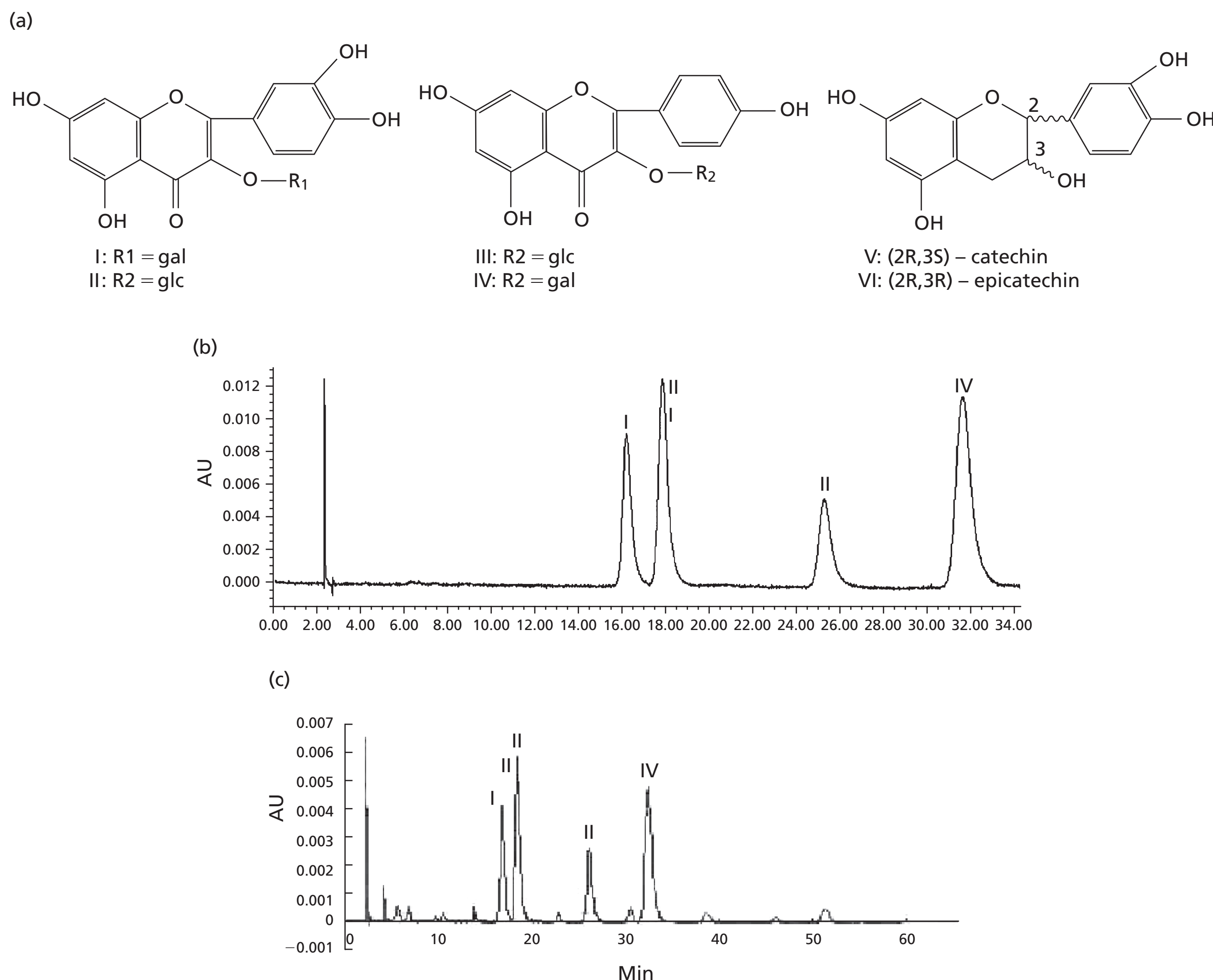


**Figure 1** (a) Chemical structure of six flavonoids of *Litsea coreana* Level. (b) Chromatogram of mixture of flavonoids at 350 nm. (c) Standardized chromatographic fingerprint of TFLC by HPLC-DAD at 350 nm. Based on separation, structure elucidation and HPLC analysis, TFLC mainly consists of six flavonoids, identified as quercetin-3-$\beta$-D-galactoside (I), quercetin-3-$\beta$-D-glucoside (II), kaempferol-3-$\beta$-D-glucoside (III), kaempferol-3-$\beta$-D-galactoside (IV), (2R,3S)-catechin (V) and (2R,3R)-epicatechin (VI). Chromatographic conditions: Hypersil ODS column (4.6 mm i.d. × 200 mm, 5 $\mu$m). A linear gradient elution of eluents A (acetonitrile) and B (water–glacial acetic acid 2%, v/v, pH 2.6) was used for the separation. The elution programme was optimized and conducted as follows: a linear gradient of 5–10% A with the range of 0.0–10.0 min, a linear gradient of 10–13% A with the range of 10.0 – 35.0 min, a linear gradient of 13–15% A with the range of 35.0–60.01 min.

according to the commercial analysis kits (Jiancheng, Nanjing, China).

## Histological examination

The liver specimens were fixed with 10% neutral formalin and embedded in paraffin. Haematoxylin and eosin (HE) staining was performed according to a standard procedure. To quantify liver fibrosis, tissue slides were classified as follows: 0, no fibrosis; 1, pericellular and perivenular fibrosis; 2, focal bridging fibrosis; 3, extensive bridging fibrosis with lobular distortion; and 4, cirrhosis.[14] Areas in sections stained for collagens by Masson trichrome were quantified by image analysis using a Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, USA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, USA) and × 4 objective lens. Pathology was scored in a blinded manner by two independent pathologists with expertise in rodent liver. Fibrosis scores were given after the pathologist had thoroughly examined three different areas of the tissue slide for each rat.

## Reverse transcriptase-polymerase chain reaction analysis of hepatic genes related to liver fibrosis

Expression levels of transcripts of $\alpha$-SMA, collagen I, TGF$\beta$ receptor (TGF$\beta$R)1, PPAR$\gamma$ and $\beta$-actin in the liver were analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) coupled with agarose gel electrophoresis. Briefly, total RNA was isolated from liver using an RNA isolation kit. Sequences and accession numbers of primers used are summarized in Table 1. The resulting single-stranded cDNA

**Table 1** Sequences of the primers used in the PCR measurements

| Target mRNA | Accession No. | Primer | Sequence | Tm (°C) | No. cycles | Product length (bp) |
|---|---|---|---|---|---|---|
| β-actin | NM_031144 | Sense | 5′TGGAATCCTGTGGCATCCATGAAAC3′ | 58 | 35 | 350 |
| | | Antisense | 5′ACGCAGCTCAGTAACAGTCCG3′ | | | |
| α-SMA | NM_031004 | Sense | 5′TGGCCACTGCTGCTTCCTCTTCTT3′ | 51 | 32 | 422 |
| | | Antisense | 5′GGGGCCAGCTTCGTCATACTCCT 3′ | | | |
| Collagen I | Z78279 | Sense | 5′TACAGCACGCTTGTGGATG3′ | 55 | 35 | 259 |
| | | Antisense | 5′TTGAGTTTGGGTTGTTGGTC3′ | | | |
| TGFβR1 | NM_012775 | Sense | 5′ATCCACGAAGACTACCAGTTGCCT3′ | 56 | 35 | 252 |
| | | Antisense | 5′CATTTTGATGCCTTCCTGTTGGCT3′ | | | |
| PPARγ | NM_013124 | Sense | 5′ATTCTGGCCCACCAACTTCGG 3′ | 51 | 35 | 339 |
| | | Antisense | 5′TGGAAGCCTGATGCTTTATCCCCA 3′ | | | |

Nucleotide sequence of primers used for RT-PCR. Position is defined as the 5-nucleotide of the respective primer related to the source sequence.

(5 μl) was denatured at 94°C for 5 min and, after the addition of the polymerase, subjected to 35 cycles of amplification, each consisting of 40 s at 94°C, 40 s at 51°C and 1 min at 72°C, with a 10-min final extension at 72°C during the last cycle. Four or five samples were chosen randomly from each group and the assay of every gene in each sample was replicated three times. The rat β-actin gene was amplified as a loading control. The PCR products were fractionated by electrophoresis on an agarose gel, visualized by ethidium bromide staining and photographed using a Kodak DC120 digital camera, scanned by the Kodak Electrophoresis Documentation and Analysis System (EDAS 120), and quantified using Kodak Image Analysis Software.

### Immunoblot analysis

Liver samples were homogenized in extraction buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% NP-40 and a protease inhibitor fluid. Homogenized proteins (15 μg/lane) were separated by electrophoresis in 4–20% gradient SDS-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) at 130 V over 2 h. The proteins were transferred to a polyvinylidene difluoride membrane using a Transblot apparatus (Bio-Rad Laboratories, Hercules, USA). The membranes were blocked in a buffer containing 5% non-fat milk, 50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h and then incubated with specific antibodies: β-actin (Sigma-Aldrich), type I collagen (Sigma), TGF-β1 and PPARγ (Santa Cruz Biotechnology). Membranes were subsequently washed, incubated with specific secondary horseradish peroxidase-conjugated antibodies and revealed with the enhanced chemiluminescence (ECL) kit by Pierce (Rockford, USA). The band intensity was analysed by scanning densitometry.

### Statistical analysis

Data were expressed as mean ± SD. All comparisons were done by one-way analyses of variance with Dunn's test for post-hoc analysis. The Kruskal–Wallis test was used for the analysis of degree of histopathological grades of fibrosis. Differences were considered significant when $P < 0.05$.

## Results

### The chemical composition of total flavonoids of *Litsea coreana* Levl.

The chemical constituents of TFLC are quercetin-3-*O*-β-D-galactopyranoside, kaempferol-3-*O*-β-D-glucopyranoside, quercetin-3-*O*-β-D-glucopyranoside, kaempferol-3-*O*-β-D-galactopyranoside, catechin and epicatechin, and their chemical structures are shown in Figure 1a. Quercetin-3-*O*-β-D-galactopyranoside and kaempferol-3-*O*-β-D-galactopyranoside were isolated for the first time from *Litsea coreana* Levl. The purity of each compound was determined to be higher than 98% by normalization of the peak area detected by HPLC. Based on separation, structure elucidation and HPLC analysis, TFLC mainly consists of six flavonoids, identified as quercetin-3-β-D-galactoside (I, 2.9%), quercetin-3-β-D-glucoside (II, 3.4%), kaempferol-3-β-D-glucoside (III, 13.4%), kaempferol-3-β-D-galactoside (IV, 4.5%), (2R,3S)-catechin (V, 29.8%) and (2R,3R)-epicatechin (VI, 2.6%). The chromatograms of TFLC are shown in Figure 1b, c and a linear gradient elution of eluents A (acetonitrile) and B (water–glacial acetic acid 2%, v/v, pH 2.6) was used for the chromatographic analysis. The elution programme was optimized and conducted as follows: a linear gradient of 5–10% A with a range of 0.0–10.0 min, a linear gradient of 10–13% A with a range of 10.0–35.0 min, a linear gradient of 13–15% A with a range of 35.0–60.01 min. The content of total flavonoids in *Litsea coreana* Levl. was 59.5%, which indicates that flavonoids are the main component and are probably responsible for its activity. All doses given are the gram-weight of the administered TFLC powder in 0.5% sodium carboxymethylcellulose (CMC-Na) solution.

### Effect of total flavonoids of *Litsea coreana* Levl. on liver index, spleen index and serum levels of alanine transaminase and aspartate aminotransferase

The relative liver index and spleen index were significantly increased compared with the normal control group. In contrast, treatment with TFLC (200 and 400 mg/kg) significantly reduced the liver weights compared with the

**Table 2** Effect of total flavonoids of *Litsea corean*a Levl. on the liver index, spleen index, hyaluronic acid, laminin, procollagen III N-terminal peptide and procollagenase IV and hydroxyproline content in liver fibrosis rats

| Group | Dose (mg/kg) | Liver index (%) | Spleen index (%) | ALT (U/l) | AST (U/l) | HA (ng/ml) | LN (ng/ml) | PIIINP (ng/ml) | CIV (ng/ml) | Hyp ($\mu$g/g liver) |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal | n.a. | 3.8 ± 0.5 | 0.32 ± 0.04 | 12.4 ± 3.6 | 43.2 ± 5.5 | 165.2 ± 38.9 | 75.9 ± 13.1 | 13.6 ± 2.7 | 3.9 ± 2.8 | 7.11 ± 0.7 |
| Model | n.a. | 4.8 ± 0.7** | 0.43 ± 0.06** | 75.1 ± 19.7** | 110.9 ± 27.6** | 558.5 ± 117.1** | 100.7 ± 12.8** | 30.9 ± 6.5** | 16.8 ± 6.9** | 14.8 ± 3.8** |
| TFLC | 100 | 4.6 ± 0.4 | 0.40 ± 0.07 | 67.4 ± 15.1 | 96.2 ± 18.3 | 424.5 ± 112.3# | 91.1 ± 10.4 | 26.7 ± 6.8 | 9.8 ± 2.2# | 10.6 ± 1.9 |
| | 200 | 4.2 ± 0.4# | 0.37 ± 0.07# | 54.7 ± 11.2# | 79.5 ± 12.7# | 308.3 ± 43.8## | 85.4 ± 9.1## | 20.4 ± 5.1## | 7.9 ± 4.9## | 8.53 ± 1.8# |
| | 400 | 4.0 ± 0.3# | 0.35 ± 0.05## | 51.3 ± 9.7## | 68.3 ± 9.9## | 233.7 ± 55.3## | 80.1 ± 8.8## | 18.9 ± 5.0## | 5.1 ± 3.6## | 8.22 ± 0.4## |

Liver index was calculated as liver weight divided by body weight; spleen index was calculated as liver weight divided by body weight. ALT, alanine transaminase; AST, aspartate transaminase; HA, hyaluronic acid; LN, laminin; PIIINP, procollagen III N-terminal peptide; CIV, procollagenase IV; Hyp, hydroxyproline; n.a., not applicable. Each group consists of 10 rats. Values are mean ± SD. **$P < 0.05$ vs normal control group; #$P < 0.05$, ##$P < 0.01$ vs model group.

model group (Table 2). Similarly, the model group had elevated serum ALT and AST levels. Administration of TFLC (200 and 400 mg/kg) attenuated the composite factors-induced increase in ALT and AST activity ($P < 0.05$) (Table 2). TFLC interestingly reverses these changes, suggesting that TFLC has a potential to prevent liver injury.

## Effect of total flavonoids of *Litsea coreana* Levl. on levels of serum hyaluronic acid, laminin, procollagen III N-terminal peptide, procollagenase IV and hepatic hydroxyproline in liver fibrosis rats

The levels of serum HA, LN, PIIINP, CIV and hepatic hydroxyproline were significantly increased in the model group compared with the normal control group ($P < 0.05$). The elevation of those markers of liver fibrosis were significantly lower in TFLC (200 and 400 mg/kg)-treated rats (Table 2). These data suggest that TFLC can reduce fibrogenesis.

## Effect of total flavonoids of *Litsea coreana* Levl. on the pathological changes in hepatic fibrosis rats

Liver tissues were collected to assess the effect of TFLC on liver pathological changes. The morphological analysis shows that no evidence of inflammatory cells and no accumulation of collagen were observed in the normal control group (Figure 2a1, a2). However, in the model group, more fibrous tissues were formed, extending into the hepatic lobules to separate them completely; meanwhile a large number of inflammatory cells infiltrated the intralobular and interlobular regions. We also found that the liver structure was disordered and more necrotic, and fatty degenerated liver cells were seen (Figure 2b1, b2). In the rats administered with TFLC, hepatocyte degeneration, necrosis and infiltration of inflammatory cells were all apparently ameliorated and collagen deposition was also markedly reduced (Figure 2c1, c2). Moreover, TFLC could significantly reduce the histopathological grading (Table 3) and area of stained fibrosis (FI) of liver fibrosis rats (Figure 2d).

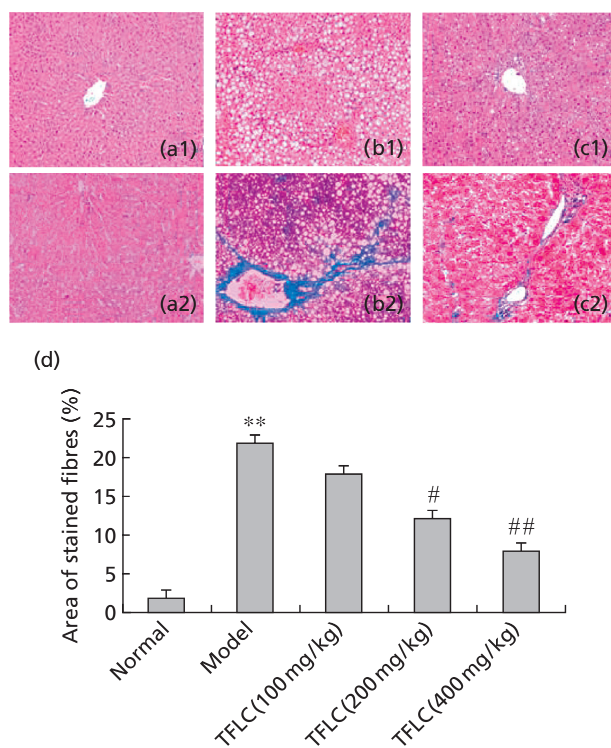


**Figure 2** Light microscopic examination of liver sections taken from the experimental groups. (a1) normal; (b1) model; (c1) TFLC (400 mg/kg): haematoxylin and eosin stain, original magnification ×100. (a2) normal; (b2) model; (c2) TFLC (400 ml/kg): Masson stain, original magnification ×200. The percentage area of stained collagen was further characterized by using image analysis (d). **$P < 0.01$ vs normal control group; #$P < 0.05$, ##$P < 0.01$ vs model group.

## Effect of total flavonoids of *Litsea coreana* Levl. on $\alpha$-smooth muscle actin and collagen I expression in liver

HSC activation is characterized by expression of $\alpha$-SMA and overproduction of ECM components, especially type I collagen. Further experiments were to evaluate the effect of TFLC on the expression of $\alpha$-SMA and collagen I. The expression of $\alpha$-SMA and collagen I in the model group was

**Table 3** Effect of total flavonoids of *Litsea coreana* Levl. on pathological grading of liver in liver fibrosis rats

| Group | Dose (mg/kg) | Pathological grading | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| Normal | n.a. | 10 | 0 | 0 | 0 | 0 | 0 |
| Model | n.a. | 0 | 0 | 0 | 2 | 2 | 6** |
| TFLC | 100 | 0 | 1 | 1 | 2 | 3 | 3 |
| | 200 | 0 | 2 | 4 | 2 | 1 | 1# |
| | 400 | 0 | 4 | 5 | 1 | 0 | 0# |

n.a., not applicable. Each group consists of 10 rats and figures represent number of rats per grade. $^{**}P < 0.05$ vs normal control group; $^{\#}P < 0.05$ vs model group.

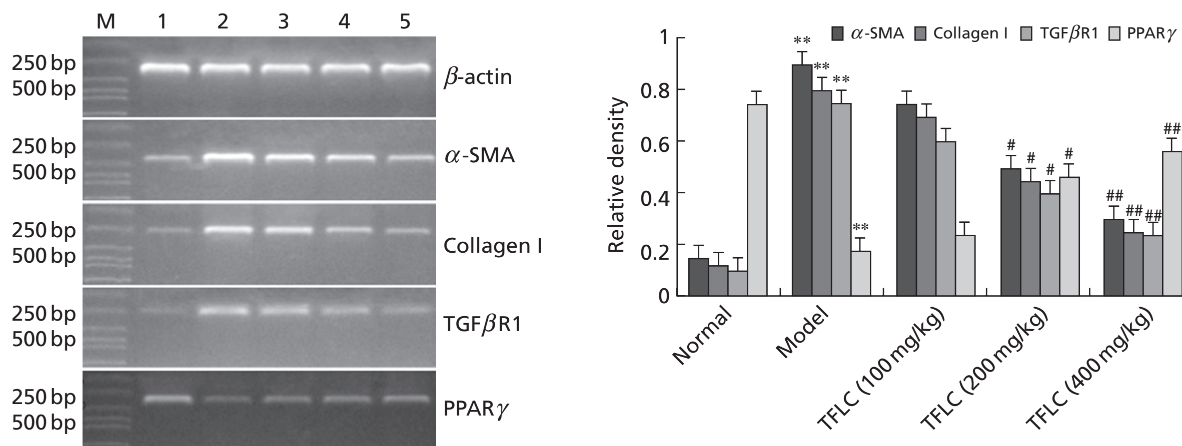


**Figure 3** Effect of total flavonoids of *Litsea coreana Levl.* on α-SMA, collagen I, TGFβR1 and PPARγ in rat liver fibrosis model. The mRNA was corrected by β-actin. 1 = Normal; 2 = model; 3 = TFLC 100 mg/kg; 4 = TFLC 200 mg/kg; 5 = TFLC 400 mg/kg. Values are means ± SD. $^{**}P < 0.01$ vs normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model group.

up-regulated, and decreased effectively when treated with TFLC (400 mg/kg) (Figures 3 and 4).

### Effect of total flavonoids of *Litsea coreana* Levl. on transforming growth factor-β, transforming growth factor-βR1 and peroxisome proliferator-activated receptor γ expression in liver

Additional studies were focused on elucidating the mechanisms by which TFLC inhibited liver fibrosis. As TGF-β1 was reported to affect tissue fibrogenesis and PPARγ, which inhibit HSC proliferation, we also analysed the effect of TFLC on TGF-β1, TGFβR1 and PPARγ expression. As shown in Figures 3 and 4, TFLC (400 mg/kg) treatment reduced the expression of TGF-β1, TGFβR1 and increased the expression of PPARγ significantly.

## Discussion

Liver fibrosis is thought to be a reversible disease, although there has not yet been a satisfactory method in clinical practice to reverse the pathological process.[15] Some traditional Chinese drugs have been found effective in preventing fibrogenesis and other causes of chronic liver injury, and this may help to develop a more hopeful future in controlling liver fibrosis.[16–18] Previous study has shown that TFLC, which is the main component of *Litsea coreana* Levl., has the effect of protecting hepatocytes, inhibiting the inflammation in liver, and anti-oxidating properties. The present study aimed at exploring the potential mechanisms of TFLC in the prevention of liver fibrosis in rats.

In this study, compared with the model group, rats treated with TFLC were in an obvious better condition irrespective of fur colour, activity or diet. We demonstrated that TFLC significantly reduced the elevated liver and spleen index, serum levels of ALT, AST, HA, LN, PIIINP, CIV and hepatic hydroxyproline content in liver fibrosis. Histological examination showed the degree to which histological changes of liver injury and liver fibrosis were also remarkably ameliorated after TFLC treatment. Therefore administration of TFLC had apparently inhibitory effects on hepatic fibrosis.

The development of liver fibrosis is a complex process and includes hepatocyte injury and HSC activation. Since the liver is a major organ and mediates the metabolism of a variety of drugs and chemicals in the body, hepatocytes can be easily damaged by drug or chemical metabolites, which induce oxidative stress in liver cells.[19,20] Oxidative stresses

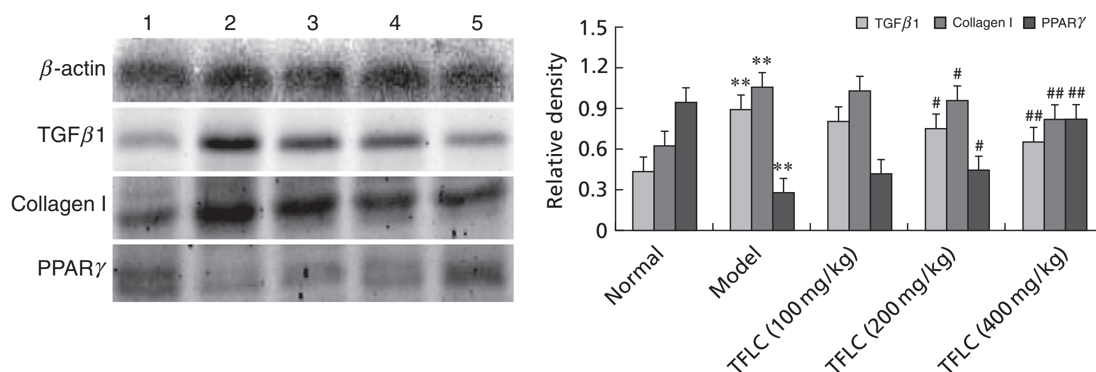


**Figure 4** Effect of total flavonoids of *Litsea coreana* Levl. on TGF$\beta$1, collagen I and PPAR$\gamma$ in rat liver fibrosis model. The protein was corrected by $\beta$-actin. 1 = Normal; 2 = model, 3 = TFLC 100 mg/kg; 4 = TFLC 200 mg/kg; 5 = TFLC 400 mg/kg. Values are means ± SD. $^{**}P < 0.01$ vs normal control group; $^{\#\#}P < 0.01$ vs model group.

have been detected in almost all the clinical and experimental conditions of chronic liver diseases with different aetiology and fibrosis progression in rats often in association with decreased antioxidant defences.[21–23] Previous studies indicated that the attenuation of liver injury and non-alcoholic steatohepatitis using TFLC was due to the reduction of lipid peroxidation and protection of hepatocytes from injury. However, the mechanism of TFLC protection of liver fibrosis remains to be investigated.

It has been reported that hepatocytes undergoing oxidative stress release reactive oxygen species (ROS) that stimulate HSC proliferation and transformation into $\alpha$-SMA-positive myofibroblast-like cells.[19,24] The activated HSCs not only express $\alpha$-SMA as a characteristic cytoskeletal protein, and produce an excess of ECM molecules, but also produce a broad array of cytokines and chemokines. Among the numerous pro-fibrogenic mediators a functional hierarchy might exist; TGF-$\beta$1 has been demonstrated in most research to be an essential factor.[25,26] TGF-$\beta$1 is a major fibrogenic cytokine, regulating the production, degradation and accumulation of ECM proteins in liver fibrogenesis. TGF-$\beta$1 gene expression correlates with the extent of liver fibrosis and an increased production of ROS, such as $H_2O_2$, in fibrotic livers is associated with the up-regulation of TGF-$\beta$1. Data also demonstrate a direct connection between TGF-$\beta$1-mediated accumulation of $H_2O_2$ and the up-regulation of collagen I in HSC.[27] Active TGF$\beta$1 binds to specific high-affinity receptors present on most cells, initiating a signalling cascade that results in biological effects.[2] Our findings suggest that treatment with TFLC leads to a significant decrease in TGF-$\beta$1 and TGF$\beta$R1 expression, causing the potent inhibition of activation of HSC with a significant decrease in both $\alpha$-SMA expression and collagen accumulation compared with the model group. In conclusion, TGF-$\beta$1 may act as a surviving factor for activated rat HSC. TFLC may protect rats from liver fibrosis through suppressing this pathway.

PPAR$\gamma$, a member of the steroid/retinoid nuclear hormone receptor superfamily of ligand-activated transcription factors,[28] has been proposed as a potential molecular target for inhibition of HSC trans-differentiation. In-vitro and in-vivo experiments suggested that PPAR$\gamma$ was expressed in quiescent HSC and its expression and activity decreased during HSC activation.[29] In addition, forced expression of PPAR$\gamma$ in culture-activated HSC by means of adenoviral transfection has been shown to revert their phenotype to that of quiescent cells.[30–32] Furthermore, PPAR$\gamma$ agonists could inhibit TGF-$\beta$1-induced liver fibroblast activation. Our study showed that the expression of PPAR$\gamma$ in the model group liver was lower than that in normal rats, and it was increased by TFLC, suggesting that the regulation of lipid metabolism of TFLC may be associated with the increased expression of PPAR$\gamma$.

## Conclusions

Our investigation showed, for the first time, the preventive effect of TFLC in rats with liver fibrosis and suggested that its anti-fibrosis property may be associated with the regulation of lipid metabolism through the regulation of adipocytokine release and increasing the expression of PPAR$\gamma$, as well as its antioxidant ability. These results have shed some light on the clinical therapeutic potential of TFLC against liver fibrosis. However, as a therapeutic agent, the effect of TFLC on restraining liver fibrosis still needs to be investigated. Further studies are necessary to investigate the upstream and downstream pathways of TGF-$\beta$1 and the relationship of TGF-$\beta$1 and PPAR$\gamma$ to elucidate the underlying molecular mechanisms.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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