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Original Article

Protective effect of tripterine on carbon tetrachloride-induced hepatic fibrosis in immature rats

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Abstract: This study was designed to investigate the hepatoprotective activity of Tripterine (Tri) against carbon tetrachloride (CCl_4) induced hepatic fibrosis in rats by suppressing TGF-β1/Smad signaling. In this study, a total of forty healthy male Wistar rats were randomly divided into five groups, including normal control, CCl_4-treated, Colchicine (Col)-treated (0.1 mg/kg, b.w.), Tri-treated (4, 8 mg/kg, b.w.). After a period of 6 weeks, liver and spleen indexes were calculated. The levels of liver biological activities such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronate (HA), laminin (LN), collagen type IV (COL4), and procollagen III (PCIII) were determined after Tri administration. Histopathological analysis of the liver tissues was also determined. Expression of hepatic gene TGF-β Receptor I (Tβ-RI), Smad3, Smad7, Collagen I and Collagen III, were detected using qRT-PCR. Tri was found to ameliorate liver injury, and hepatic fibrogenesis induced by CCl_4 in rats. Tri could significantly decrease the contents of ALT and AST ($P<0.01$). Compared with the CCl_4-treated group, levels of HA, LN, COL4 and PCIII were significantly increased, and Tri treatment significantly reduced the levels of four biomarkers in CCl_4-induced rats ($P<0.01$). Liver morphological examination showed that Tri ameliorated CCl_4-induced state of fibrotic septa, necrosis, and hepatic steatosis. The results of mRNA expressions studies displayed that Tri (8 mg/kg, b.w.) could inhibit Tβ-RI, Smad3, Smad7, Collagen I and Collagen III expression in the liver tissues ($P<0.01$ or $P<0.05$). This study provides evidence of protective effects of Tri against CCl_4-induced hepatic fibrosis by reducing inflammatory response in the liver.

Keywords: Carbon tetrachloride, hepatic fibrosis, TGF-β1/Smad signaling, tripterine

Introduction

Hepatic fibrosis (HF) is a reversible wound repair response to chronic liver injuries, which is characterized by overproduction and irregular deposition of extracellular matrix (ECM) in the Disse's space [1]. Accumulating evidence suggests that portal hypertension, liver failure and liver cirrhosis related to hepatocellular carcinoma have become common sequelae of HF for young patients [2]. Infant hepatitis syndrome occurs in the infant period and is a series of clinical signs including jaundice and liver function injury. Then the liver goes into the fibrosis stage after liver cells are damaged directly or by immunity due to the continuous effects of pathogenic factors and immune function disorders. It is widely recognized that many signal conduction pathways show a functional disorder in HF, such as TGF-β1/Smad, Wnt/β-catenin and Nuclear factor-κB (NF-κB) signaling [3-5]. Given these findings, it is a suitable strategy to against liver inflammation and inhibit TGF-β1/Smad signaling for the treatment of HF.

Tripterine (Tri, CAS: 34157-83-0, $C_{29}H_{38}O_4$), also known as 'celastrol', is a pentacyclic triterpenoid isolated from the traditional Chinese medicine "Leigongteng", which is used to treat inflammatory and autoimmune diseases [6]. Tri has been shown to possess anti-oxidative and anti-inflammatory properties, and proposed as a potential therapeutic agent for allergic asthma, rheumatoid arthritis, and neurodegenerative disease [7-9]. Moreover, Tri inhibits the progression of HF by inhibiting collagen synthesis and activation in hepatic stellate cells (HSCs) [10]. However, the mechanism of Tri in TGF-β1/Smad signal pathway in the treatment of HF remains unclear.

Therefore this study is of great interest to investigate the efficacy of Tri in HF and explore the underlying mechanisms through the inhibition of the TGF-β1/Smad signaling pathway. The

Table 1. The qRT-PCR gene-specific primers

Genes	Forward primers	Reverse primers
TGFβ-RI	AGG AAG GAT GAC TTA CAG AGG CT	GCG GGT CAG GGA CAA TAA GT
Smad3	GGG GAG ACA TTC CAC GCT T	TGA GGC ACT CTG CAA AGA CC
Smad7	TGG TGC GTG GTG GCA TAC T	CGA TCT TGC TCC TCA CTT TCT G
Collagen I	TGT TCC CCA CTC AGC CCT CT	GAA CCT TCG CTT CCA TAC TCG
Collagen III	GCC ACC CTG AAC TCA AGA GC	GCA CCA GCA TCT GTC CAC CA
GAPDH	GGC TCT CTG CTC CTC CC	CCG TTC ACA CCG ACC TT

fingdings will enhance our understanding of the possible mechanisms of Tri on carbon tetrachloride (CCl_4)-induced HF in Wistar rats.

Material and methods

Animals and Tri administration

In this study, 40 male rats of Wistar strain (40-50 g, aged three weeks) obtained from Shanghai Slac laboratory Animal Co., Ltd. (China). The animals were housed in a quiet and humidity-controlled room (24±2°C and 55± 15% relative humidity) with a 12-hour dark/light cycle, standard food and free drinking water. All experimental procedures were performed upon the approval of the Institutional Animal Care and Use Committee of The First People's Hospital of Wenling, followed the accepted standards of the Guide for the Care and Use of Laboratory Animals.

In the study, rats were randomly separated into five groups (eight animals per group) after a 7-day acclimation. The experimental design of the study was as follows. (I) Normal control group. Rats were intraperitoneally (i.p.) with saline. (II) CCl_4-treated group. Rats were induced with carbon tetrachloride (0.15 ml/kg, i.p.). (III) Colchicine (Col)-treated group. Rats were injected with the same CCl_4 as CCl_4-treated group and administrated colchicine at 0.1 mg/kg. (IV) Tri-treated groups. Rats were injected with the same CCl_4 as CCl_4-treated group and administrated Tri (4, 8 mg/kg, b.w.). Rats in groups II, III and IV were i.p. treated with a mixture of CCl_4 and olive oil [1:1 (v/v)] twice a week for a period of 6 weeks. Colchicine and Tri (4, 8 mg/kg) were dissolved in sterile saline and i.p. injected once daily for 6 weeks, respectively [11].

At the end of experiment, 24 hours after the last Tri treatment, rats in each group were euthanized by an overdose of anesthetic agent. At sacrifice, the liver, spleen specimens and body were weighed at the indicated time. The portal blood was collected for liver function tests and biochemical analysis. Then, the liver samples were excised for histopathological and real time PCR.

Liver and spleen indexes

At the end of the 6 weeks' experimental procedure, the livers and the spleens were weighed. The liver index was refers to the percentage of liver weight to body weight. Similarly, the spleen index was calculated as the percentage of spleen weight divided by body weight (mg/g).

Evaluation of liver biological activities

Serum samples of rats were collected to evaluating liver status, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronate (HA), laminin (LN), collagen type IV (COL4), and procollagen III (PCIII) were determined with a corresponding ELISA kit according to the manufacturer's protocols [12]. The ALT, AST, HA and LN ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The COL4 and PCIII ELISA kits were obtained from Wuhan ColorfulGene Biological Technology (Wuhan, China).

Histopathological analysis

For the histopathological study, the liver tissues from the same liver lobes were fixed in 10% neutral buffered formalin immediately and processed routinely by embedding in paraffin [13]. Then, five micro-meter (5 mm) sections were sliced into 5 μm slices, stained with hematoxylin and eosin (H&E) using standard methods. The extent of hepatic damage were evaluated under light microscope (Motic, Hongkong).

Real-time quantitative PCR

Total RNA was extracted from the liver tissues with the TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was then carried out after spectrophotometric quantification. Gene-specific primers for TGF-β Receptor I (Tβ-RI), Smad3, Smad7, Collagen I and Collagen III (shown in **Table 1**) were used for conventional and qPCR analyses. Expressions of hepatic gene TGF-β Receptor I, Smad3, Smad7, Collagen I and Collagen III, were detected using

Table 2. Effects of Tri on liver and spleen indexes in hepatic fibrosis rats

Group	Dose (mg/kg)	Liver weight (g)	Hepatic index (mg/g)	Spleen weight (g)	Spleen index (mg/g)
Control	-	10.34±0.68	3.14±0.215	1.27±0.56	0.26±0.038
CCl_4	-	10.63±0.53	4.16±0.112**	1.86±0.73	0.36±0.021**
CCl_4 + Col	0.1	10.22±0.61	3.83±0.281##	1.76±0.69	0.31±0.054##
CCl_4 + Tri-I	4	10.47±0.57	3.63±0.166##	1.64±0.57	0.31±0.037##
CCl_4 + Tri-II	8	10.26±0.39	3.54±0.084##	1.61±0.77	0.30±0.025##
F			26.073		3.844
P			0.000		0.005

Data are expressed as mean ± SEM, n = 8 rats per group. Superscript letters represents the statistical significant done by ANOVA followed by Tukey's multiple comparison tests. **P<0.01 when compared with control group; ##P<0.01 when compared with CCl_4 group.

Table 3. Effects of Tri on the levels of serum ALT and AST in CCl_4-induced hepatic fibrosis in rats

Group	Dose (mg/kg)	ALT (IU/L)	AST (IU/L)
Control	-	21.65±3.31	46.17±4.48
CCl_4	-	84.73±10.39**	127.86±14.73**
CCl_4 + Col	0.1	37.68±9.59##	86.14±11.93##
CCl_4 + Tri-I	4	46.73±7.20##	82.85±10.39##
CCl_4 + Tri-II	8	41.03±8.57##	76.94±17.18##
F		19.200	10.290
P		0.000	0.000

Data are expressed as mean ± SEM, n = 8 rats per group. Superscript letters represents the statistical significant done by ANOVA followed by Tukey's multiple comparison tests. **P<0.01 when compared with control group; ##P<0.01 when compared with the CCl_4 group.

the SYBR Green PCR Master Mix assay (Applied Biosystems, MA, USA) with the Lightcycler 2.0 (Roche Diagnostics, Penzberg, Germany). Relative expression levels of genes were normalized to GAPDH reference gene.

Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). Data were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. A value of P<0.05 was considered as statistically significant. All the data was performed using SPSS 17.0 software.

Results

Tri attenuates CCl_4-induced hepatic swelling in rats

The effects of the Tri on the liver and spleen indexes after 6 weeks' treatment are summarized in **Table 2**. In contrast to the control group, the liver index of rats in the model group showed a significantly increased (P<0.01) due to the damage induced by CCl_4. However, the rats that received Col and Tri (4 or 8 mg/kg, b.w.) for 6 weeks induced a significantly lower liver index compared with that of CCl_4-treated group (P<0.01), indicating that Col and doses of Tri had a significant effect against hepatic swelling induced by CCl_4. The same trend of spleen index was seen in Col- and Tri-treated groups compared to CCl_4-treated group (P<0.01).

Effects of Tri on serum ALT, AST, HA, LN, COL4 and PCIII activity levels

Levels of serum ALT and AST in CCl_4-treated group were evaluated significantly compared to normal controls (P<0.01) in **Table 3**. The treatment of rats with Col and Tri at 4 and 8 mg/kg of body weight markedly lowered serum ALT and AST activities (P<0.01) after 6 weeks' treatment, and there was no significant difference in liver function indexes between Col and Tri-treated groups. These results suggest that Tri attenuates HF induced by CCl_4.

To demonstrate the anti-liver fibrosis activity of Tri, the levels of serum HA, LN, COL-IV and PCOL-III were analyzed and are summarized in **Table 4**. Compared to the normal control group, the levels of four biomarkers were elevated in CCl_4-induced rats (P< 0.01). Col and Tri treatment significantly reduced the HA, LN, COL-IV, and PCOL-III activities as compared with model group (P<0.01 or P<0.05).

Table 4. Effects of Tri on the levels of serum HA, LN, COL-IV and PCOL-III in CCl_4-induced hepatic fibrosis in rats

Group	Dose (mg/kg)	HA (ng/mL)	LN (ng/mL)	COL-IV (ng/mL)	PCOL-III (ng/mL)
Control	-	103.34±23.89	45.08±11.47	41.09±4.74	7.18±1.65
CCl_4	-	195.34±43.24**	107.52±24.59**	90.76±10.73**	17.83±2.57**
CCl_4 + Col	0.1	123.68±25.46$^{\#\#}$	51.55±12.80$^{\#\#}$	70.95±13.82$^{\#}$	8.91±2.52$^{\#\#}$
CCl_4 + Tri-I	4	130.19±22.12$^{\#\#}$	57.58±10.77$^{\#\#}$	69.16±14.14$^{\#}$	12.38±2.49$^{\#\#}$
CCl_4 + Tri-II	8	124.76±30.02$^{\#\#}$	58.78±13.89$^{\#\#}$	77.31±7.90$^{\#}$	9.82±2.09$^{\#\#}$
F		7.263	13.726	19.710	16.029
P		0.000	0.000	0.000	0.000

Data are expressed as mean ± SEM, n = 8 rats per group. Superscript letters represents the statistical significant done by ANOVA followed by Tukey's multiple comparison tests. $^{**}P<0.01$ when compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ when compared with the CCl_4 group.

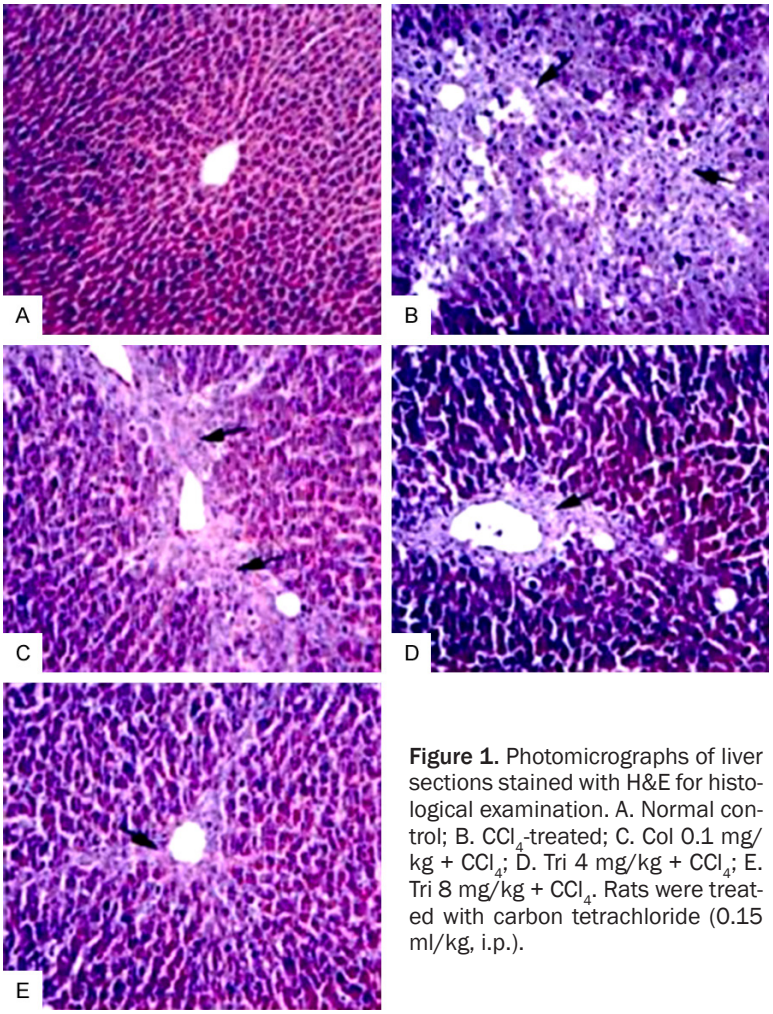

Figure 1. Photomicrographs of liver sections stained with H&E for histological examination. A. Normal control; B. CCl_4-treated; C. Col 0.1 mg/kg + CCl_4; D. Tri 4 mg/kg + CCl_4; E. Tri 8 mg/kg + CCl_4. Rats were treated with carbon tetrachloride (0.15 ml/kg, i.p.).

Tri ameliorates morphological abnormalities during CCl_4-induced hepatic fibrosis

The liver morphological changes pathologically were observed by H&E staining as shown in **Figure 1A-E**. The livers from rats in CCl_4 group swollen with scattered patches compared with the normal liver. However, treatment with Col and Tri (4 or 8 mg/kg, b.w.) ameliorated CCl_4-induced state of fibrotic septa, hepatic steatosis and necrosis in the liver, especially in group of high dose Tri (**Figure 1E**).

Effect of TSA on the mRNA expression CCl_4-induced rat

To determine the underlying mechanisms of Tri on liver fibrosis through the inhibition of the TGF-β1/Smad signaling pathway, we investigated the changes in mRNA of Tβ-RI, Smad3, Smad7, Collagen I and Collagen III in liver using qRT-PCR. The expression of five mRNA were increased in CCl_4-induced rat compared to normal controls, such as Tβ-RI (4.1 times), Smad3 (1.4 times), Smad7 (1.3 times), Collagen I (10.9 times) and Collagen III (5.5 times) (**Figure 2**), respectively. Administration of Col and High-dose of Tri were able to significantly reduce the expressions of Smad3, Collagen I and Collagen III as compared to CCl_4-induced group ($P<0.01$). Furthermore, the expression of Tβ-RI was reduced in Col and Tri-treatment (4 or 8 mg/kg, b.w.) group compared with that of the

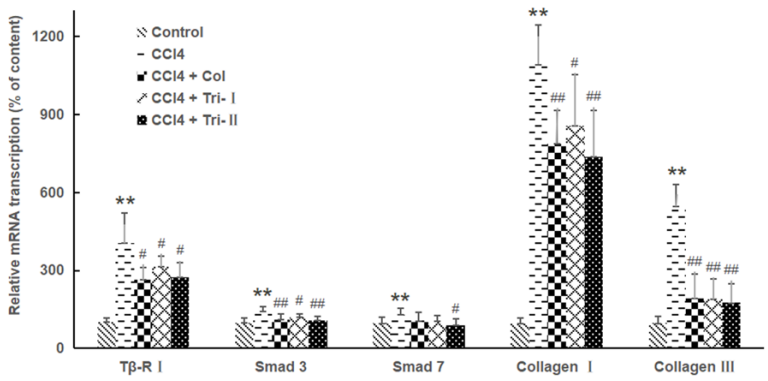

Figure 2. Relative expression ratio of each mRNA calculated after normalization by GAPDH reference gene. Data are expressed as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$ when compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ when compared with the CCl_4 group.

CCl_4-induced group ($P<0.05$). In addition, high-dosage Tri induced a slight increase in Smad7 expression compared with that of the CCl_4-induced group ($P<0.05$). However, there was no difference in mRNA expression of Smad7 in Col and Tri-treatment (4 mg/kg, b.w.) group compared with the CCl_4-induced group.

Discussion

Chronic liver disease is one of the most prevalent causes of human sufferings and death, and liver fibrosis is considered to be the most important pathological feature of chronic liver disease [14]. Many studies have proven that liver fibrosis is a reversible lesion and it is different from cirrhosis [15, 16]. However, synthetic drugs have limited efficacy in the treatment of liver diseases.

Research data suggest that CCl_4 is a potent hepatoxin that results in hepatocyte damage, necrosis, inflammation, and it is widely used for animal models of liver fibrosis [17, 18]. A trichloromethyl peroxy radical, the cleavable product of the carbonchloride bond of CCl_4, has been proven to be involved in the pathogenesis of liver injury [19]. For CCl_4 can induce similar hepatotoxicity caused by variety of hepatotoxinsin in human, so the hepatoprotective activity of drugs were usually screened in experimental model induced by CCl_4.

As important indicators of liver fibrosis, increased ALT and AST activity can show the degrees of liver cell membrane damage and liver mitochondrial damage separately [20]. Therefore, ALT and AST are considered as the effective indexes for evaluating liver cell damage. In the present study, pretreatment of Tri can decrease serum enzyme activities of ALT and AST which were elevated by the injection of CCl_4 in mice. These findings suggest that Tri may effectively protect hepatocytes by repairing liver cell membrane and mitochondrial damage.

HA, LN, COL-IV and PCOL-III, basic components of extracellular matrix, are believed to be bio markers of liver fibrosis [21]. As the important and effective index for liver fibrosis and inflammation change, HA level increases insignificantly because of proliferation of liver stromal cells and fibrocyte. Additionally, LN is also an important indicator of advanced fibrosis, and it is not affected by the influence of liver function. PCOL-III is seen as a serum marker in the early stage of liver fibrosis owing to active synthesis in early liver fibrosis and slowly in later period. Similarly, COL-IV level increase and reflect the degree of liver fibrosis when patients aggravate from chronic hepatitis to cirrhosis of liver. In this study, Tri significantly inhibited serum HA, LN, COL-IV and PCOL-III levels, suggesting that Tri may block or reversing delay the process of liver fibrosis by decreasing contents of biochemical markers in serum.

The TGF-β1/Smad signaling pathway has become a new target in the prevention and treatment of liver fibrosis, and plays crucial roles during the processes of hepatocyte survival/damage, inflammatory cytokine production, and stellate and inflammatory cell activation. These results show that expression of Tβ-RI, Smad3, Smad7, Collagen I and Collagen III was significantly increased in liver tissue from CCl_4-treated rats. However, treatment with 8 mg/kg prevented these changes. Therefore, Tri successfully alleviated CCl_4-induced liver fibrosis through decreasing mRNA levels of TGFβ-RI, Smad3, Smad7, Collagen I and Collagen III in the liver tissues. Therefore, Tri ameliorated HF level via inhibition of the TGF-β1/Smad signaling pathway.

Taken together, Tri protected hepatic function from CCl_4-induced HF by suppressing hepatic inflammation and fibrosis in rats. This protective effect of Tri might be associated with its inhibitory role by downregulating the TGF-β1/Smad signaling pathway. These findings indicate Tri may have a beneficial effect on the treatment of chronic liver diseases, and the molecular mechanisms involved in the therapeutic effects need to be further explored.

Disclosure of conflict of interest

None.

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References

[1] Zhan YY, Wang JH, Tian X, Feng SX, Xue L and Tian LY. Protective effects of seed melon extract on CCl_4-induced hepatic fibrosis in mice. J Ethnopharmacol 2016; 193: 531-537.

[2] Ismail BE and Cabrera R. Management of liver cirrhosis in patients with hepatocellular carcinoma. Chin Clin Oncol 2013; 2: 34.

[3] Li W, Zhu C, Chen X, Li Y, Gao R and Wu Q. Pokeweed antiviral protein downregulates Wnt/beta-catenin signalling to attenuate liver fibrogenesis in vitro and in vivo. Dig Liver Dis 2011; 43: 559-566.

[4] Inokuchi S, Aoyama T, Miura K, Osterreicher CH, Kodama Y, Miyai K, Akira S, Brenner DA and Seki E. Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. Proc Natl Acad Sci U S A 2010; 107: 844-849.

[5] Hu Z, Qin F, Gao S, Zhen Y, Huang D and Dong L. Paeoniflorin exerts protective effect on radiation-induced hepatic fibrosis in rats via TGF-β1/Smad signaling pathway. Am J Transl Res 2018; 10: 1012-1021.

[6] Li W, Zhang T, Ye Y, Zhang X and Wu B. Enhanced bioavailability of tripterine through lipid nanoparticles using broccoli-derived lipids as a carrier material. Int J Pharm 2015; 495: 948-955.

[7] Xiong Y, Yan Y and Li Y. Tripterine alleviates LPS-induced inflammatory injury by up-regulation of miR-146a in HaCaT cells. Biomed Pharmacother 2018; 105: 798-804.

[8] Chen S, Gu C, Xu C, Zhang J, Xu Y, Ren Q, Guo M, Huang S and Chen L. Celastrol prevents cadmium-induced neuronal cell death via targeting JNK and PTEN-Akt/mTOR network. J Neurochem 2014; 128: 256-266.

[9] Li G, Liu D, Zhang Y Qian Y, Zhang H, Guo S, Sunagawa M, Hisamitsu T and Liu Y. Celastrol inhibits lipopolysaccharide-stimulated rheumatoid fibroblast-like synoviocyte invasion through suppression of TLR4/NF-κB-mediated matrix metalloproteinase-9 expression. PLoS One 2013; 8: e68905.

[10] Wei HE, Song SS, Yuan PF, Lu JT and Wei W. Therapeutic effects of celastrol on DEN induced liver fibrosis rats and its mechanisms. Chinese Pharmacological Bulletin 2013; 29: 519-524.

[11] Chang W, He W, Li PP, Song SS, Yuan PF, Lu JT and Wei W. Protective effects of celastrol on diethylnitrosamine-induced hepatocellular carcinoma in rats and its mechanisms. Eur J Pharmacol 2016; 784: 173-180.

[12] Sun H, Chen G, Wen B, Sun J, An H, Pang J, Xu W, Yang X and He S. Oligo-peptide I-C-F-6 inhibits hepatic stellate cell activation and ameliorates CCl_4-induced liver fibrosis by suppressing NF-κB signaling and Wnt/β-catenin signaling. J Pharmacol Sci 2018; 136: 133-141.

[13] Mihailović V, Mihailović M, Uskoković A Arambašić J, Mišić D, Stanković V, Katanić J, Mladenović M, Solujić S and Matić S. Hepatoprotective effects of *Gentiana asclepiadea* L. extracts against carbon tetrachloride induced liver injury in rats. Food Chem Toxicol 2012; 52: 83-90.

[14] Liu Y, Liu Q, Ye G, Khan A, Liu J, Gan F, Zhang X, Kumbhar S and Huang K. Protective effects of selenium-enriched probiotics on carbon tetrachloride-induced liver fibrosis in rats. J Agric Food Chem 2015; 63: 242-249.

[15] Chen X, Ying X, Zhang W, Chen Y, Shi C, Hou Y and Zhang Y. The hepatoprotective effect of fraxetin on carbon tetrachloride induced hepatic fibrosis by antioxidative activities in rats. Int Immunopharmacol 2013; 17: 543-547.

[16] Chen S, Zou L, Li L and Wu T. The protective effect of glycyrrhetinic acid on carbon tetrachloride-induced chronic liver fibrosis in mice via upregulation of Nrf2. PLoS One 2013; 8: e53662.

[17] Lee JI, Lee KS, Paik YH Nyun Park Y, Han KH, Chon CY, Moon YM. Apoptosis of hepatic stellate cells in carbon tetrachloride induced acute liver injury of the rat: analysis of isolated hepatic stellate cells. J Hepatol 2003; 39: 960-966.

[18] Kao YH, Lin YC, Tsai MS, Sun CK, Yuan SS, Chang CY, Jawan B and Lee PH. Involvement of the nuclear high mobility group B1 peptides released from injured hepatocytes in murine hepatic fibrogenesis. Biochim Biophys Acta 2014; 1842: 1720-1732.

[19] Fujii T, Fuchs BC, Yamada S, Lauwers GY, Kulu Y, Goodwin JM, Lanuti M and Tanabe KK. Mouse model of carbon tetrachloride induced liver fibrosis: histopathological changes and expression of cd133 and epidermal growth factor. BMC Gastroenterol 2010; 10: 1-11.

[20] Yang L, Wang CZ, Ye JZ and Li HT. Hepatoprotective effects of polyprenols from *Ginkgo biloba* L. leaves on CCl_4-induced hepatotoxicity in rats. Fitoterapia 2011; 82: 834-840.

[21] Kaneda H, Hashimoto E, Yatsuji S, Tokushige K and Shiratori K. Hyaluronic acid levels can predict severe fibrosis and platelet counts can predict cirrhosis in patients with nonalcoholic fatty liver disease. J Gastroenterol Hepatol 2006; 21: 1459-1465.