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BASIC RESEARCH

Ameliorative effect of *Ganoderma lucidum* on carbon tetrachloride-induced liver fibrosis in rats

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Abstract

AIM: To investigate the effects of *Reishi mushroom*, *Ganoderma lucidum* extract (GLE), on liver fibrosis induced by carbon tetrachloride (CCl_4) in rats.

METHODS: Rat hepatic fibrosis was induced by CCl_4. Forty Wistar rats were divided randomly into 4 groups: control, CCl_4, and two GLE groups. Except for rats in control group, all rats were administered orally with CCl_4 (20%, 0.2 mL/100 g body weight) twice a week for 8 weeks. Rats in GLE groups were treated daily with GLE (1 600 or 600 mg/kg) via gastrogavage throughout the whole experimental period. Liver function parameters, such as ALT, AST, albumin, and albumin/globulin (A/G) ratio, spleen weight and hepatic amounts of protein, malondiladehyde (MDA) and hydroxyproline (HP) were determined. Histochemical staining of Sirius red was performed. Expression of transforming growth factor β1 (TGF-β1), methionine adenosyltransferase (MAT1) 1A and MAT2A mRNA were detected by using RT-PCR.

RESULTS: CCl_4 caused liver fibrosis, featuring increase in plasma transaminases, hepatic MDA and HP contents, and spleen weight; and decrease in plasma albumin, A/G ratio and hepatic protein level. Compared with CCl_4 group, GLE (600, 1 600 mg/kg) treatment significantly increased plasma albumin level and A/G ratio ($P < 0.05$) and reduced the hepatic HP content ($P < 0.01$). GLE (1 600 mg/kg) treatment markedly decreased the activities of transaminases ($P < 0.05$), spleen weight ($P < 0.05$) and hepatic MDA content ($P < 0.05$); but increased hepatic protein level ($P < 0.05$). Liver histology in the GLE (1 600 mg/kg)-treated rats was also improved ($P < 0.01$). RT-PCR analysis showed that GLE treatment decreased the expression of TGF-β1 ($P < 0.05$-0.001) and changed the expression of MAT1A ($P < 0.05$-0.01) and MAT2A ($P < 0.05$-0.001).

CONCLUSION: Oral administration of GLE significantly reduces CCl4-induced hepatic fibrosis in rats, probably by exerting a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

Key words: Ganoderma lucidum; Carbon tetrachloride; Liver fibrosis

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INTRODUCTION

Reishi mushroom, *Ganoderma lucidum* (Fr.) Krast (Polyporaceae), is a well-known Chinese crude drug used clinically in East Asia. The fruit bodies are used for the treatment of neurasthenia, deficiency fatigue, insomnia, bronchial cough in elderly people and carcinoma[1].

A number of animal studies have indicated that water or ethanol extracts of *G. lucidum* showed protective actions against acute hepatitis in rats or mice[2-4]. Other reports had previously indicated that triterpenoids isolated from *G. lucidum* possessed the protective effect against acute hepatitis caused by CCl_4[5,6]. Furthermore, Park et al.[7] demonstrated that, in rats, polysaccharides extracted from *G. lucidum* could antagonize liver fibrosis caused by biliary obstruction. These results demonstrate that *G. lucidum* possesses a protective effect in the liver.

Liver fibrosis is the common end-stage of most chronic liver disease, regardless of etiology, and its progression leads to cirrhosis and liver cancer[8]. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of free radicals and lipid peroxides has attracted considerable attention[9]. It has been found that metabolism of CCl_4 involves the production of free radicals through its activation by drug-metabolizing enzymes located in the endoplasmic reticulum[10]. CCl_4 is capable of causing liver lipid peroxidation, resulting in liver fibrosis[11].

Data from *in vitro* and *in vivo* studies have indicated that *G. lucidium* has potent antioxidative and radical-scavenging effects[3,12-15], which contribute to hepatoprotection[3,15]. Nevertheless, to our knowledge, no reports have recorded the effect of *G. lucidium* on chronic hepatitis. In the present study, we therefore investigated the effect of extracts

of G. lucidum on chronic CCl_4-induced liver fibrosis.

MATERIALS AND METHODS

Preparation of test substance

Crude *G. lucidum* extract (GLE), which also contains cracked spores of *G. lucidum*, was obtained from the Taiwan branch of the American company NuSkin Pharmanex. GLE was suspended in distilled water and administered orally to each rat at a volume of 1 mL/100 g body weight. To guarantee reproducibility of pharmacological experiments, we assayed the total triterpene content of GLE.

Determination of total triterpenes in GLE by HPLC

GLE (100 mg) was extracted with ethyl acetate and then evaporated to dryness under vacuum. The residue was dissolved in methanol and diluted to 2 mL. The sample solutions were filtered through a 0.45-μm filter before HPLC analysis as follows. HPLC instrument: Waters 2690 separation unit plus Waters 996 PDA; column: Phenomenex Lunca C18(2); flow rate: 1.0 mL/min; detection: absorption at 252 nm; gradient solvent system: CH_3CN+0.1% trifluoroacetic acid. The total peak area for a retention time of 8.0-38.0 min was used to calculate total triterpenes. The peak area of ganoderic acid A (Shanghai R&D, Pharmanex) was used as standard. This method showed that the total triterpene content of GLE was over 6%.

Animals

Male Wistar rats were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory diet and tap water *ad libitum*. Experimental animals were housed in an air-conditioned room at 22-25 ºC and a 12 h light/dark cycle. Rats were allowed free access to powdered feed and mains water that was supplied through an automatic watering system. When they reached 250-300 g, forty rats were divided randomly into 4 groups, such as control, model and two GLE treatment groups, according to body weight 1 d before administration of the test substance. All animals received humane care and the study protocols were in compliance with Institutional Guidelines for the use of laboratory animals.

CCl_4-induced liver fibrosis

Liver fibrosis was induced by oral administration of 0.2 mL/100 g body weight of CCl_4 (200 mL/L; diluted in olive oil) twice a week for 8 wk. Animals received CCl_4 only (model group), CCl_4 with GLE (600 or 1 600 mg/kg per day) throughout whole experimental period. During CCl_4 administration, the time interval between CCl_4 and GLE administration was at least 5 h to avoid disturbance in absorption of each substance. At the end of the experimental period, rats were sacrificed under ether anesthesia and blood was withdrawn from the abdominal artery. Liver and spleen were quickly removed, weighed after washing with cold normal saline and removing excess moisture. The largest lobe of liver was divided into four parts, which were then used as follows: (1) submerged in 100 mL/L neutral formalin for the preparation of pathological sections; (2) frozen directly in liquid nitrogen for transcript analysis; (3) after weighing, the liver was completely dried at 100 ºC for the determination of collagen content; and (4) remaining samples were stored at -80 ºC as reserves.

Assessment of liver functions

Whole blood was centrifuged at 4 700 r/min at 4 ºC for 10 min to separate the plasma. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), albumin and total protein were determined spectrophometrically with an automatic analyzer (Cobas Mira; Roche, Rotkreuz, Switzerland) using commercially available kits (Roche Diagnostics).

Assays for hepatic protein, lipid peroxidation and hydroxyproline

Livers were homogenized in 9 vols ice-cold 0.15 mol/L KCl and 1.9 mmol/L ethylenediaminetetraacetic acid. Liver protein concentration was measured according to Lowry *et al*[16] using bovine serum albumin as standard. Lipid peroxidation was measured by the methods of Ohkawa *et al*[17] using 2-thiobarbituric acid. Lipid peroxidation was expressed as the amount of malondialdehyde/mg protein.

Hydroxyproline determination followed a method designed by Neuman and Logan[18]. After hydrolysis, dried liver tissue was oxidized by H_2O_2 and colored by p-dimeth ylaminobenzoaldehyde; and absorbance was determined at 540 nm. The amount of hydroxyproline was expressed as μg/g tissue.

RNA extraction and RT-PCR analysis

Total RNA was isolated from rat livers using the acid guanidum thiocyanate-phenol-chloroform extraction methods, as described by Chomczynski and Sacchi[19]. A total of 5 μg RNA from each liver sample was subjected to reverse transcription (RT) by using MMuLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the reverse transcription mixture were used for amplification by polymerase chain reaction (PCR) of fragments specific to transforming growth factor-β1 (TGF-β1), methionine adenosyltransferase 1A (MAT1A) and MAT2A using the primer pairs listed in (Table 1). The levels of expression of all transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. The primer pairs for TGF-β1 and GAPDH were designed by Wolf *et al*[20]. In addition, the primer pairs for MAT1A and MATA2A were designed using the Primer select program[21]. The identities of the resultant PCR products were confirmed by sequence analysis. The cycling parameters were 30 min at 55 ºC for cDNA first strand synthesis, and 5 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC for 32 cycles in a Perkin Elmer 9700 Gene Amp PCR system. The PCR product was electrophoresed on a 20 g/L agarose gel recorded by Polaroid film, and the bands were quantitated by using densitometry.

Pathological examinations

After formalin fixation, tissue samples were sliced, embedded in a standard manner and stained with Sirius

Table 1 Primer sequences for PCR amplification

mRNA	Primer sequences	Length (bp)
TGF-β1	Sense 5' TAT AGC AAC AAT TCC TGG CG 3'	162
	Antisense 5' TGC TGT CAC AGG AGC AGTG 3'	
MAT1A	Sense 5' AAA TGA AGA GGA TGT TGG TG 3'	264
	Antisense 5' ATT GTG TTG GCA CAG AGA GAT GA 3'	
MAT2A	Sense 5' ATG CTG TCC TTG ATG CAC 3`	400
	Antisense 5' GCG TA A CCA AGG CAA TG 3'	
GAPDH	Sense 5' CTT CAT TGA CCT CAA CTA CAT GGT CTA 3'	
	Antisense 5' GATG ACA AGC TTC CCA TTC TCA G 3'	99

Table 2 Effect of GLE on weight of liver and spleen in CCl_4-treated rats

Group	Dose (mg/kg)	Liver (g)	Spleen (g)
Control	–	15.1 ± 1.8	0.90 ± 0.11
CCl_4+H_2O	–	16.6 ± 3.3	2.58 ± 0.32[b]
CCl_4+GLE	600	16.7 ± 2.2	2.21 ± 0.36
	1600	18.2 ± 2.0	2.09 ± 0.56[a]

[a]P<0.05 *vs* CCl_4 + H_2O group; [b]P<0.001 *vs* control group.

Table 3 Effect of GLE on plasma AST and ALT activity in CCl_4-treated rats

Drugs	Dose (mg/kg)	AST (U/L)	ALT (U/L)
Control	-	71.5 ±15.6	41.9 ± 1.9
CCl_4 + H_2O	-	610.8 ± 149.9[b]	464.7 ± 126.7 [b]
CCl_4 + GLE	600	648.9 ± 153.8	499.9 ± 112.1
	1600	459.3 ± 105.3[a]	324.7 ± 41.3[a]

[a]P < 0.05 *vs* CCl_4 + H_2O group; [b]P < 0.001 *vs* control group.

Table 4 Effect of GLE on plasma albumin concentra-tion and A/G ratio in CCl4-treated rats

Group	Dose (mg/kg)	Albumin (g/dL)	A/G ratio
Control	–	3.35 ± 0.15	1.22 ± 0.07
CCl_4 + H_2O	–	2.33 ± 0.21[b]	0.75 ± 0.04[b]
CCl_4 + GLE	600	2.73 ± 0.18[a]	0.87 ± 0.07[a]
	1600	2.70 ± 0.25[a]	0.88 ± 0.13[a]

[a]P < 0.05 *vs* CCl4 + H2O group; [b]P < 0.001 *vs* control group.

red. Fibrosis was graded according to the method of Ruward *et al*[22] as follows: Grade 0 = normal liver; grade 1 = increase of collagen without formation of septa; grade 2 = formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); grade 3 = complete but thin septa interconnecting with each other, so as to divide the parenchyma into separate fragments; and grade 4 = as grade 3, except with thick septa (complete cirrhosis). To avoid sampling error, all biopsies were obtained from the same lobe and these semi-quantitative grades were performed by the observer without knowledge of sample treatment.

Statistical analysis

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were analyzed by one-way analysis of variance using the Dunnett's test. Liver histopathological examination data were analyzed by the Kruskall-Wallis non-parametric test, followed by a Mann-Whitney *U*-test. A P value < 0.05 was considered statistically significant.

RESULTS

Body weight and weights of liver and spleen

Treatment with CCl_4 caused a significant decrease in the body weight of rats as compared with control rats. There were no differences in the body weight of rats in the CCl_4 alone and CCl_4 + GLE groups. The final body weights for control and CCl_4-treated groups were 474.5 ± 26.7 and 405.4 ± 31.6 g, respectively.

CCl_4 treatment obviously caused splenomegaly in the rats, demonstrating that the weight of spleen in the CCl_4-treated group was about 290% of the control group (Table 2). On contrary, GLE (1 600 mg/kg) significantly reduced the weight of spleen induced by CCl_4 (Table 2). Moreover, CCl_4 did not change liver weights, and no effect of GLE on the liver weight was observed.

Effects of GLE on biochemical parameters

As shown in (Table 3), CCl_4 treatment resulted in a significant increase in plasma AST and ALT activities as compared to the control group. Oral administration of GLE (1 600 mg/kg) significantly reduced the CCl_4-induced increase in AST and ALT activities.

The plasma albumin content and A/G ratio in CCl_4-treated groups were significantly lower than that in the control group. The CCl_4-induced decrease in plasma albumin concentration and A/G ratio were significantly increased following the administration of GLE (600 and 1 600 mg/kg; Table 4).

Hepatic protein, malondialdehyde and hydroxyproline concentrations

CCl_4-induced liver fibrosis in rats resulted in a significant decrease in hepatic protein content compared to the control group. GLE (1 600 mg/kg) attenuated the decrease of hepatic protein level induced by CCl_4 (Table 5).

CCl_4 induced liver fibrosis in the rats, accompanied by a marked elevation of malondialdehyde and hydroxyproline concentrations. GLE (600 and/or 1 600 mg/kg) could lower the increase in hepatic malondialdehyde and hydroxyproline content (Table 5).

TGF-β1, MAT1A and MAT2A mRNA expression

Fragments specific to TGF-β1, MAT1A and MAT2A were amplified by using RT-PCR (Figure 1). Values from densitometric analysis, after normalization against the corresponding GAPDH transcript, were expressed as the TGF-β1:GAPDH, MAT1A:GAPDH and MAT2A:GAPDH ratios (Table 6). CCl_4 treatment could significantly increase the levels of both TGF-β1 and MAT2A. The administration of GLE (600 or 1600 mg/kg) significantly decreased the expression of TGF-β1 and

Table 5 Effect of GLE on hepatic protein, malondialdehyde and hydroxyproline content in CCl_4-treated rats

Group	Dose (mg/kg)	Protein (mg/g tissue)	Malondialdehyde (nmol/mg protein)	Hydroxyproline (μg/g tissue)
Control	–	186.3 ± 26.4	2.6 ± 0.5	550.0 ± 42.5
CCl_4 + H_2O	–	108.7 ± 4.8 [d]	4.4 ± 1.1[d]	1201.2 ± 151.7[d]
CCl_4 + GLE	600	124.4 ± 10.2	3.8 ± 0.4	1050.4 ± 187.2[b]
	1600	128.2 ± 13.5[a]	3.4 ± 0.5[a]	877.5 ± 137.9[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* CCl_4 + H_2O group; [d]$P < 0.001$ *vs* control group.

Table 6 Effect of GLE on the mRNA expression of hepatic TGF-β1, MAT1A and MAT2A

Group	Dose (mg/kg)	TGF-β1/GAPDH ratio	MAT1A/GAPDH ratio	MAT2A/GAPDH ratio
Control	–	0.18 ± 0.03	4.34 ± 0.0.81	0.29 ± 0.02
CCl_4 + H_2O	–	22.17 ± 7.20[f]	1.75 ± 0.46[f]	4.95 ± 0.21[f]
CCl_4 + GLE	600	16.99 ± 3.26[a]	3.07 ± 1.15[a]	1.68 ± 0.14[d]
	1600	4.83 ± 0.48[d]	3.83 ± 1.35[b]	0.56 ± 0.03[d]

[a]$P<0.05$, [b]$P<0.01$, [d]$P<0.001$ *vs* CCl_4 + H_2O group; [f]$P<0.001$ *vs* control group.

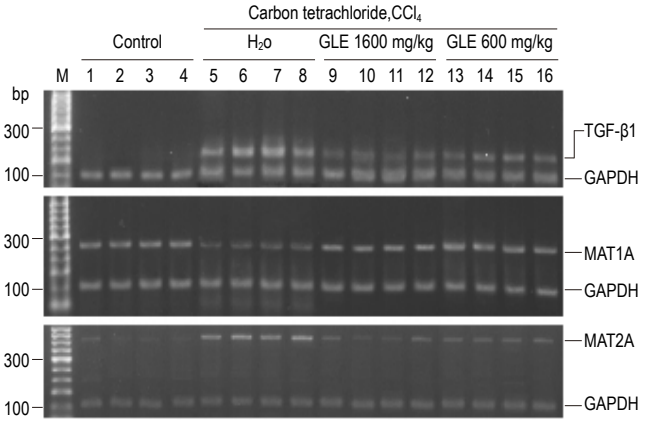

Figure 1 Effect of GLE on mRNA expression of *TGF-β1*, *MAT1A* and *MAT2A* in hepatic tissue. M: DNA marker

MAT2A mRNA. In contrast, the level of MAT1A mRNA was significantly decreased by CCl_4 treatment. However, treatment with GLE significantly increased the level of MAT1A mRNA.

Pathological changes

As shown in Figure 2, CCl_4 induced liver lesions in rats. Sirius red stain showed clear nodular fibrosis (Figure 2B). Treatment with GLE (1 600 mg/kg) showed a marked improvement in the pathological changes to these tissues (Figure 2C and Table 7).

DISCUSSION

The present study revealed the beneficial effect of GLE in prevention of liver fibrosis induced by CCl_4 treatment. An improvement brought about by GLE was also seen in plasma biochemical parameters.

CCl_4 treatment caused hepatocellular damage in rats, as indicated by a drastic increase in both plasma ALT and AST levels after CCl_4 administration. Rats treated with GLE showed a protection against CCl_4-induced hepatotoxicity, with the levels of both plasma AST and ALT being reduced.

It is well known that adenosylmethionine-dependent methylation is central to many biological processes[23]. Methionine adenosyltransferase (MAT) is a key enzyme for liver methionine metabolism, which catalyzes the synthesis of S-adenosylmethionine[24]. In mammalian tissue, three different forms of MAT (MAT I/III and MAT II) have been identified, which are the product of two different genes (MAT1A and MAT2A). MAT1A is primarily restricted to adult liver[25]. MAT2A is high in fetal liver, decays at birth to negligible levels and, in the adult liver, increases during regeneration after partial hepatectomy[26–28]. Thus, in response to liver injury, MAT1A expression is switched off and MAT2A expression is switched on. Consistent with this, the expression of MAT1A was found to be reduced in the livers of rats with chronic CCl_4 injury, whereas the expression of MAT2A increased. In this study, we also found that the changes in MAT expression in chronic CCl_4-injured rats were reduced by GLE treatment. These results further support the fact that GLE possesses a hepatoprotective effect.

The liver synthesizes not only the protein it needs, but also produces numerous export proteins. Among the latter, plasma albumin is the most important[29]. Export proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum of the hepatocytes. In contrast, protein destined for intracellular use is synthesized on free polyribosomes rather than bound polyribosomes[29]. In this experiment, CCl_4 induced liver fibrosis in rats and it appeared to cause a decrease in both hepatic protein and plasma albumin contents. GLE clearly reduced the decrease in protein content in the liver and albumin content in the plasma; thus it was shown to ameliorate the decline in liver synthesis function caused by CCl_4-induced fibrosis.

Immunoglobulin is synthesized by immunocytes and hyperglobulinemia is found in hepatocellular disorders, appearing as an inflammatory reaction of liver[30]. In the present experiments, we observed CCl_4-induced chronic liver lesions in rats and also a decrease in A/G ratio. GLE could clearly lessen the decrease in the A/G ratio caused by CCl_4, thereby exhibiting suppressive actions on liver

Table 7 Effect of GLE on CCl_4-induced liver fibrosis in rats

Group	Dose (mg/kg)	Score of hepatic fibrosis					Average
		0	1	2	3	4	
Control	–	10	0	0	0	0	0
$CCl_4 + H_2O$	–	0	0	1	9	0	2.9±0.3
CCl_4 + GLE	600	0	0	3	7	0	2.7±0.3
	1600	0	0	6	4	0	2.4±0.6[b]

[b]$P<0.01$ *vs* CCl_4 group.

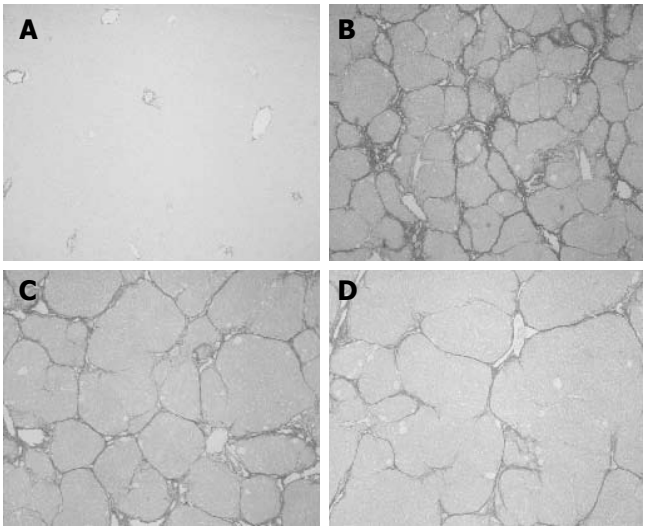

Figure 2 Sirius red staining of rat liver sections. **A**: Control; **B**: $CCl_4 + H_2O$, showing micronodular formation and complete septa interconnection with each other; **C**: CCl_4 + GLE (600 mg/kg); **D**: CCl_4 + GLE (1 600 mg/kg), showing a marked reduction in fiber deposition.

inflammation caused by CCl_4.

It is well known that liver fibrosis is a result of increased collagen synthesis[31]; hydroxyproline is the characteristic component in collagen[32]. The amount of collagen can be reflected by determining hydroxyproline concentration and can be used to express the extent of fibrosis[32]. In this experiment, when CCl_4 was administered to induce liver fibrosis, the hydroxyproline contents in liver obviously increased. Interestingly, GLE could reduce hydroxyproline concentration, indicating that it could lessen the actions of hepatic fibrosis caused by CCl_4, which was further proved by histopathological inspection.

When the liver is damaged, it can initiate regenerative actions[33], thus increasing the weight of liver. If it was heavily damaged, however, liver fibrosis and cirrhosis appear resulting in liver atrophy[34]. Therefore, the change in weight of liver can not directly predict the pathological process in chronic liver injuries.

Liver fibrosis leads to blockage of blood flow into the liver and causes portal hypertension. It also influences blood flow to the spleen and gives rise to splenomegaly[35]. In this study, CCl_4 induced chronic hepatic fibrosis and splenomegaly, but GLE could improve splenomegaly, indicating that GLE possesses actions in ameliorating fibrosis.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl_4 hepatoxicity[10]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl_4 administration[11]. Our results also confirmed these findings that hepatic lipid peroxidation increases during hepatic fibrogenesis. Moreover, we observed that GLE inhibited CCl_4-induced hepatic lipid peroxidation. These results indicated that GLE might inhibit lipid peroxidation and consequently attenuate the development of liver fibrosis. Numerous studies have indicated that G. lucidum extracts are good free-radical scavengers[12–14], suggesting that the ameliorative effects of GLE on liver fibrosis induced by CCl_4 are due, at least in part, to its free-radical scavenging ability.

TGF-β1 is a profibrogenic cytokine as it directly stimulates extracellular matrix production by both endothelial and stellate cells[36,37]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[38,39]. Blockade of TGF-β1 synthesis or signaling is a primary target for the development of antifibrotic approaches, and modern hepatology has facilitated the design of drugs removing this causative agent[40]. In this study, CCl_4 treatment increased while GLE treatment significantly reduced TGF-β1 mRNA expression, suggesting that GLE might ameliorate liver fibrosis via reducing TGF-β1 secretion.

In conclusion, oral administration of GLE is effective in the reduction of chronic liver injury, probably via a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

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