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BASIC RESEARCH

Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To investigate the effects of filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) on liver fibrosis induced by carbon tetrachloride (CCl_4) in rats.

METHODS: Forty Wistar rats were divided randomly into control group and model group. All model rats were given 200 mL/L CCl_4 (2 mL/Kg, po) twice a week for 8 wk. Four weeks after CCl_4 treatment, thirty model rats were further divided randomly into 3 subgroups: CCl_4 and two FMAC subgroups. Rats in CCl_4 and 2 FMAC subgroups were treated with FMAC 0, 0.5 and 1.0 g/kg, daily via gastrogavage beginning at the fifth week and the end of the eighth week. Spleen weight, blood synthetic markers (albumin and prothrombin time) and hepatic malondialdehyde (MDA) and hydroxyproline (HP) concentrations were determined. Expression of collagen I, tissue inhibitor of metalloproteinases (TIMP)-1 and transforming growth factor β1 (TGF-β1) mRNA were detected by RT-PCR. Histochemical staining of Masson's trichrome was performed.

RESULTS: CCl_4 caused liver fibrosis, featuring increased prothrombin time, hepatic MDA and HP contents, and spleen weight and decreased plasma albumin level. Compared with CCl_4 subgroup, FMAC subgroup (1 g/kg) significantly decreased the prothrombin time (36.7 ± 7.2 and 25.1 ± 10.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$) and increased plasma albumin concentration (22.7 ± 1.0 and 30.7 ± 2.5 in CCl_4 and FMAC groups, respectively, $P < 0.05$). Spleen weight was significantly lower in rats treated with CCl_4 and FMAC (1 g/kg) compared to CCl_4 treated rats only (2.7 ± 0.1 and 2.4 ± 0.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$). The amounts of hepatic MDA and HP in CCl_4 ± FAMC (1 g/kg) subgroup were also lower than those in CCl_4 subgroup (MDA: 3.9 ± 0.1 and 2.4 ± 0.6 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; HP: 1730.7 ± 258.0 and 1311.5 ± 238.8 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$). Histologic examinations showed that CCl_4 + FMAC subgroups had thinner or less fibrotic septa than CCl_4 group. RT-PCR analysis indicated that FMAC (1 g/kg) reduced mRNA levels of collagen I, TIMP-1 and TGF-β1 (collagen I: 5.63 ± 2.08 and 1.78 ± 0.48 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TIMP-1: 1.70 ± 0.82 and 0.34 ± 0.02 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TGF-β1:38.03 ± 11.9 and 4.26 ± 2.17 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$) in the CCl_4-treated liver.

CONCLUSION: It demonstrates that FMAC can retard the progression of liver fibrosis induced by CCl_4 in rats.

Key words: *Antrodia camphorata*; Liver fibrosis; Carbon tetrachloride

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INTRODUCTION

Antrodia camphorata is a new species of the genus *Antrodia* (*Polyporaceae*) parasitic in the inner cavity of the endemic species *Cinnamomum kanehirai* Hay[1]. Traditionally, it has been used as a remedy for food-, alcohol-, drug-intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer among Chinese. The growth rate of natural *A. camphorata* in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged culture method to obtain useful cellular materials, or to produce effective substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies. In Taiwan, several biotechnology companies have developed the submerged culture method for *A. camphorata*. In the market of Taiwan, the yield of mycelia or culture filtrate of fermented mycelia

is dependent on the different biotechnology companies. Preliminary pharmacological studies revealed that the antioxidant abilities of the culture filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) were correlated with their total polyphenols content based on the evaluation of different antioxidant system[2].

Liver fibrosis is the common end stage of most chronic liver disease regardless of the etiology[3], and its progression leads to cirrhosis and liver cancer. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of the free radical and lipid peroxides has attracted considerable attention[4]. It has been found that the metabolism of CCl_4 involves the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum[5]. CCl_4 is capable of causing liver lipid peroxidation, resulting in liver fibrosis[6]. Hsiao *et al*[7] reported that *A. camphorata* extract exerted protection against chronic chemical-induced hepatic injury in mice. In addition, Song *et al*[8] showed that FMAC possessed a protective activity against acute liver injury induced by CCl_4. However, the effect of FMAC in chronic liver disease is still unknown. In the present study, we attempted to assess the effect of FMAC on chronic CCl_4-induced liver fibrosis in rats.

MATERIALS AND METHODS

Preparation of test substance

FMAC was provided by Food Industry Research & Development Institute, Hsinchu, Taiwan. Culture of *Antrodia camphorata* BCRC 930032 was inoculated onto potato dextrose agar (PDA) and incubated at 30 ℃ for 15 to 20 d. The whole colony was then cut and put into the bottle with 50 mL sterile water. After homogenization, the fragmentated mycelia suspension was used as inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 r/min with aeration rate of 0.2 vvm and temperature of 30 ℃. A 5-d culture of 15 L mycelia inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation condition was the same as the seed fermentation but operating with an aeration rate of 0.075 vvm. The deep red culture filtrate was separated from the broth harvested at the 331st hour and poured through the non-woven fabric on a 20-mesh sieve. FMAC was concentrated about 20 fold (450 g/L) under reduced pressure at 50 ℃, and stored at -30 ℃ until use. FMAC was suspended in distilled water and administered orally to each rat at a volume of 10 mL/kg body weight.

Since antioxidant and anti-radical properties of plant extracts have been attributed to most phenolic compounds, it is expected that the effectiveness of the extracts is related to their phenolic content[9]. To guarantee the reproducibility of pharmacological experiments, the phenolic compounds in FMAC were determined by a modification of the method of Barness *et al*[10] using catechin as the standard. The concentration of phenolic groups in FMAC was 39.71 μg/mg.

Animals

Male Wistar rats were obtained from the National Laboratory of Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory chow and tap water *ad libitum*. The experimental animals were housed in air-conditioned room of 21-24 ℃ with 12 h of light. The rats were allowed free access to powdered feed, and main water that was supplied through an automatic watering system. When they reached 250-300 g, the rats were used for experiments. Rats were divided randomly into control and model groups according to the body weight in proper range one day before administration of the test substance. All animals received humane care and the study protocols were in compliance with our institution's guidelines for use of laboratory animals.

CCl_4-induced liver fibrosis

Fibrosis was induced in thirty rats by an oral administration of 2 mL/kg body weight of 200 mL/L CCl_4 (diluted in olive oil) twice a week for 8 wk. At the end of 4 th wk after CCl_4 treatment, the CCl_4-treated rats were further divided into 3 subgroups based on the plasma alanine aminotransferase (ALT) level, since the plasma ALT is the major parameter for liver injury. The plasma ALT levels for normal control and 3 CCl_4-treated subgroups were 675 ± 62, 8856 ± 1321, 9005 ± 1659 and 8208 ± 1324 (nkat/L), respectively. The animals received CCl_4 with distilled water or FMAC (0.5, 1.0 g/kg; *po*, daily) which was added at the last four wk of the treatment. The time interval between CCl_4 and FMAC administrations were 5 h to avoid the disturbance of absorption of each other. After blood was drawn from rats at the eighth week, the animals were sacrificed at the same time and the liver and spleen were quickly taken off. They were then weighed after being clearly washed with cold normal saline and sucked up of the moisture. The largest lobe of liver was divided into four parts, and the same parts were 1) submerged in 40 g/L neutral formaldehyde for the preparation of pathological section; 2) after weighed, the liver was completely dried at 100 ℃ for the determination of collagen content; 3) the samples for RT-PCR analysis were kept in liquid nitrogen; 4) other sample was stored at -80 ℃ until assay.

Assessment of liver functions

The blood was centrifuged at 4700 r/min (Jouan BR4i, France) at 4 ℃ for 15 min to separate the plasma. The levels of plasma ALT and albumin were assayed using clinical test kits (Roche Diagnostics) spectrophotometrically (Cobas Mira; Roche, Rotkreuz, Switzerland). Prothrombin time was measured using a coagulation analyzer (Sysmex-CA1000) and reagent (Dade thromboplastin C plus).

Assays of hepatic lipid peroxidation and hydroxyproline

Livers were homogenized in nine volumes of ice-cold 0.15 mol/L KCl, 1.9 mmol/L ethylenediaminetetraacetic acid. The homogenate was used for the determination of lipid peroxidation. Lipid peroxidation was measured by the methods of Ohkawa *et al*[11] using 2-thiobarbituric acid. The lipid peroxidation was expressed as malondialdehyde (MDA) μmol/g protein. Protein was measured by the method of Lowry *et al*[12] using bovine serum albumin as the standard. Hydroxyproline (HP) determination fol-

Table 1 Primer sequences for PCR amplification

mRNA		Primer sequence	Length (bp)
Collagen I	Sense	5' CGA CTA AGT TGG AGG GAA CGG TC 3'	182
	Antisense	5' TGG CAT GTT GCT AGG CAC GAC 3'	
TIMP-1	Sense	5' TCC CTT GCA AAC TGG AGA GT 3'	140
	Antisense	5' GTC ATC GAG ACC CCA AGG TA 3'	
TGF-b1	Sense	5' TAT AGC AAC AAT TCC TGC CG 3'	162
	Antisense	5' TGC TGT CAC AGG AGC AGTG 3'	
GAPDH	Sense	5' CTT CAT TGA CCT CAA CTA CAT GGT CTA 3'	99
	Antisense	5' GATG ACA AGC TTC CCA TTC TCA G 3'	

Table 2 Effect of FMAC on plasma albumin concentration and prothrombin time in CCl_4-treated rats

Group	Dose (g/kg per d)	Albumin (g/L)	Prothrombin time (sec)
Control	–	36.0 ± 1.3	17.7 ± 0.9
CCl_4 + H_2O	–	22.7 ± 1.0[b]	36.7 ± 7.2[b]
CCl_4 + FMAC	0.5	23.1 ± 5.1	28.5 ± 9.9
	1	30 .7± 2.5[a]	25.1 ± 10.2[a]

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

Table 3 Effect of FMAC on spleen weight, hepatic malondialdehyde and hydroxyproline contents in CCl_4-treated rats

Group	Dose (g/kg per d)	Spleen (g)	Malondialdehyde (μmol/g protein)	Hydroxyproline (μg/g tissue)
Control	–	1.1 ± 0.1	1.9 ± 0.1	645.0 ± 64.5
CCl_4 + H_2O	–	2.7 ± 0.1[d]	3.9 ± 0.1[d]	1730.7 ± 258.0[d]
CCl_4 + FMAC	0.5	2.8 ± 0.2	2.7 ± 0.1	1741.5 ± 257.1
	1	2.4 ± 0.2[a]	2.4 ± 0.6[b]	1311.5 ± 238.8[b]

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

lowed a method designed by Neuman *et al*[13]. Dried liver tissue after hydrolysis was oxidized by H_2O_2 and colored by p-dimethylaminobenzoaldehyde and absorbance was determined at 540 nm. The amount of HP is expressed in mg/g wet tissue.

RNA extraction and RT-PCR analysis

Total RNA was isolated from livers of the rats using the acid guanidum thiocyanate-phenol-chloroform extraction methods as described by Chomczynski *et al*[14]. Five micrograms of total RNA from each liver sample were subjected to reverse transcription (RT) by MMuLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the reverse transcription mix were used for amplification by polymerase chain reaction (PCR) of fragments specific to collagen I, transforming growth factor (TGF)-β1 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 using the primer pairs listed in Table 1. The levels of expression of all the transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. PCR product was run on a 20 g/L agarose gel recorded by polarid film; bands were quantitated by densitometer.

Pathological examinations

For histopathological examination, the formalin-fixed liver was embedded in paraffin, cut into 4-5 μm thick sections, stained with Masson's trichrome. Fibrosis was graded according to the method of Ruwart *et al*[15], 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; 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 without knowledge of sample treatment.

Statistical analysis

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were treated 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. The significance level was set at $P < 0.05$.

RESULTS

Concentrations of plasma albumin and prothrombin time

The plasma albumin concentrations were lower in rats given CCl_4 than that in the control group (Table 2). While in the rats treated by FMAC (1 g/kg), the levels of plasma albumin was markedly higher than that in the CCl_4 model group. The prothrombin time in the CCl_4 model group was much longer than that in control group. FMAC (1 g/kg) significantly shortened the prothrombin time.

Weights of spleen

Marked splenomegaly was caused by CCl_4 treatment; the weight of spleen in the CCl_4-treated group was about 245% of the control group (Table 3). The increase of spleen weight by CCl_4 treatment was significantly reduced by FMAC (1 g/kg).

Liver MDA and HP contents

CCl_4 induced liver fibrosis to the rats resulting in a marked increase of hepatic MDA and HP contents (Table 3). FMAC (1 g/kg) treatment significantly reduced the increase of hepatic MDA and HP contents caused by CCl_4.

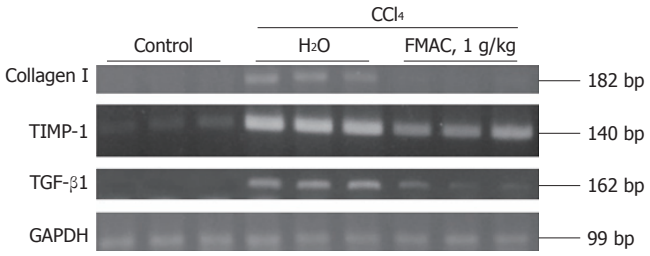

Figure 1 Effect of FMAC on the hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats.

Table 4 Effect of FMAC on hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats

Group	Dose (g/kg per d)	Collagen I: GAPDH ratio	TGF-β1: GAPDH ratio	TIMP-1: GAPDH ratio
Control	–	0.68 ± 0.55	0.38 ± 0.18	0.14 ± 0.08
CCl_4 + H_2O	–	5.63 ± 2.08[b]	38.03 ± 11.9[b]	1.70 ± 0.82[b]
CCl_4 + FMAC	0.5	3.94 ± 1.18	28.90 ± 7.22	0.58 ± 0.02
	1	1.78 ± 0.48[d]	4.26 ± 2.17[d]	0.34 ± 0.02[d]

[b]$P < 0.001$ *vs* Control group; [d]$P < 0.01$ *vs* CCl_4 + H_2O group.

RT-PCR analysis of liver tissue

Fragments specific to collagen I, TIMP-1 and TGF-β1 were amplified by RT-PCR (Figure 1). The values from densitometric analysis, after normalization against the corresponding GAPDH transcript were expressed as the collagen I/GAPDH, TIMP-1/GAPDH and TGF-β1/GAPDH ratios. The levels of collagen I, TIMP-1 and TGF-β1 mRNA in rat liver were significantly increased by CCl_4 treatment (Table 4), while the administration of FMAC (1 g/kg) significantly decreased the levels of collagen I, TIMP-1 and TGF-β1 mRNA.

Pathological examination

CCl_4 induced liver damage of the rats. Masson's stain showed clear nodular fibrosis at the central vein and the portal vein area (Figure 2B). Treatment of FMAC (1 g/kg) showed marked improvement of these pathological changes of the tissues (Figure 2C and Table 5).

DISCUSSION

The results of the present study indicate that even after the initiation of hepatic fibrosis in a rat model of CCl_4-induced liver damage, FMAC administration reduced liver fibrosis, as demonstrated by smaller increases in hepatic collagen and lower mRNA expression of collagen I compared with CCl_4 model group. These effects were mainly observed when FMAC was administered from wk 5 to wk 8 of CCl_4 treatment. Both plasma albumin and blood clotting factors were mainly synthesized in the liver. When the chronic liver damage led to fibrosis, the albumin contents dropped and prothrombin time prolonged[16,17]. In this experiment, CCl_4 induced chronic liver lesions in rats and there appeared a decrease of plasma albumin and an increase of prothrombin time. FMAC clearly counteracted both the decrease of albumin content in the plasma and the prolongation of prothrombin time. These results showed that FMAC ameliorated the decline of liver synthetic functions caused by chronic liver injuries.

Liver fibrosis or cirrhosis leads to blockage of blood flow into the liver and causes portal hypertension and it also influences the blood flow of spleen and gives rise to splenomegalia[18]. CCl_4 in this experiment induced chronic

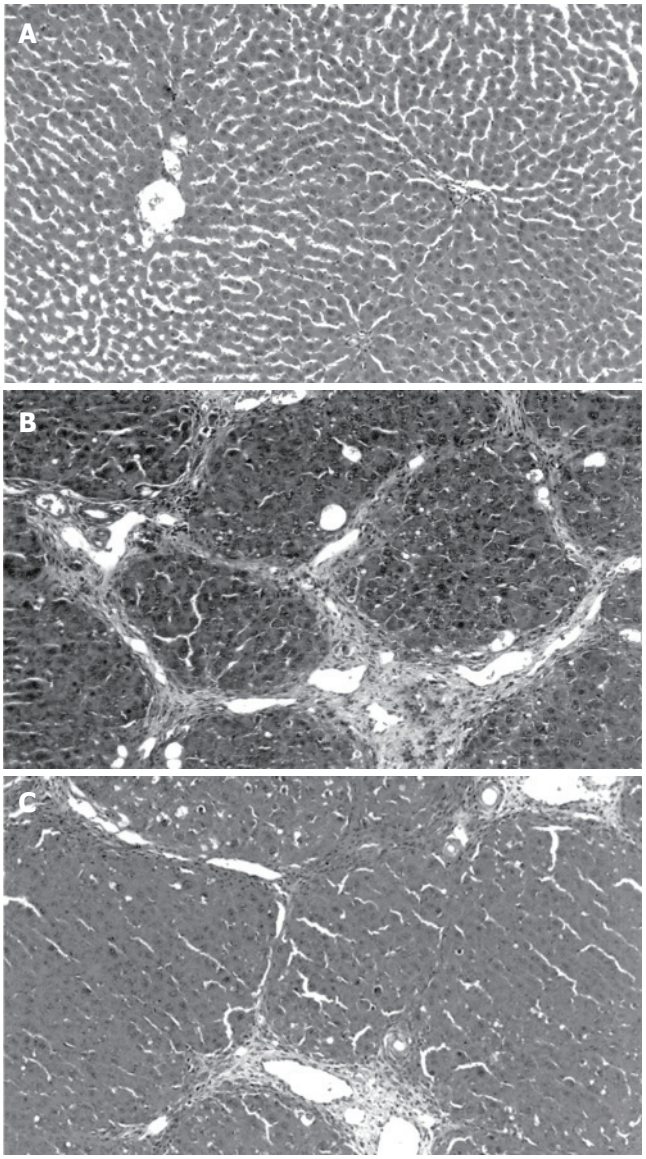

Figure 2 Liver histopathology of rats (Masson's stain). **A**: control group; **B**: CCl_4 + H_2O group, showing micronodular formation and complete septa interconnection with each other; **C**: CCl_4 + FMAC (1 g/kg) group, showing a marked reduction in fiber deposition. Scale bar = 50 μm.

Table 5 Effect of FMAC on CCl4-induced liver fibrosis in rats

Group	Dose (g/kg per d)	Score of hepatic fibrosis 0	1	2	3	4	Average
Control	–	10	0	0	0	0	0
CCl_4 + H_2O	–	0	0	3	6	1	2.8 ± 0.6
CCl_4 + FMAC	0.5	0	1	3	5	1	2.7 ± 0.8
	1.0	0	4	5	1	0	1.7 ± 0.7[b]

[b]$P < 0.01$ *vs* CCl_4 + H_2O group.

hepatic fibrosis as well as splenomegalia. FMAC could improve splenomegalia, indicating that it might ameliorate portal hypertension.

It is well known that liver fibrosis is a result of increased collagen synthesis[19], and HP is the unique component in collagen[19]. The amount of collagen can be reflected by the contents of HP and can be used to express the extent of fibrosis[19]. When CCl_4 was applied in this experiment to induce liver fibrosis, the content of HP in liver obviously increased. FMAC could reduce the content of HP, which was confirmed by the histopathological examinations. Many studies have shown that level of collagen I increases during liver fibrosis[20]. Therefore, we also investigated the effect of FMAC on the mRNA expression of collagen I. Treatment with FMAC was effective in reducing the amount of collagen I mRNA expression. This result further confirmed that FMAC could remit hepatic fibrosis.

Regardless of the etiologic factors, gross remodeling of extracellular matrix in the fibrotic liver is regulated by a balance of synthesis and enzymatic degradation of extracellular matrix[21]. Matrix degradation is catalyzed by the activity of matrix metalloproteinases. The activities of matrix metalloproteinases are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The expression of TIMPs drastically increased or decreased with time during liver fibrogenesis and fibrosis resolution, respectively[22]. Four members of the TIMP family have been characterized so far and designated as TIMP-1 to TIMP-4[23]. It has been suggested that TIMP-1 plays an important role in the pathogenesis of liver fibrosis[24]. Consistent with previously published work[25], we observed elevated levels of TIMP-1 upon treatment with CCl_4. Treatment with FMAC was effective in reducing the level of TIMP-1 expression, indicating liver fibrosis resolution might be enhanced. This result supported that FMAC could suppress liver fibrotic progression caused by CCl_4.

TGF-β1 is a profibrogenic cytokine, because it directly stimulates extracellular matrix production by both Kupffer cells and stellate cells[26,27]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[28,29]. 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[30]. In this experiment, CCl_4 treatment increased, while FMAC significantly reduced TGF-β1 mRNA expression. This result suggested that FMAC ameliorated liver fibrosis perhaps by reducing TGF-β1 secretion.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl_4 hepatotoxicity[5]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl_4 administration[6]. Our results confirmed these findings that hepatic lipid peroxidation is increased during hepatic fibrogenesis. We also found that FMAC inhibited CCl_4-induced hepatic lipid peroxidation. These results indicated that FMAC might inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis. A large number of studies indicated that FMAC is a good free radical scavenger[3, 31].

In conclusion, the present study has demonstrated that FMAC retards the progression of liver fibrosis in CCl_4-treated rats possibly by scavenging free radicals formed in the liver. It may be expected that FMAC has preventive potentials in liver fibrosis.

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