

Full Paper

Magnolia officinalis Reverses Alcoholic Fatty Liver by Inhibiting the Maturation of Sterol Regulatory Element–Binding Protein-1cHu-Quan Yin¹, Young-Tae Je¹, Young-Chul Kim¹, Young-Kee Shin¹, SangHyun Sung¹, KiYong Lee¹, Gil-Saeng Jeong², Youn-Chul Kim², and Byung-Hoon Lee^{1,*}¹College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea²College of Pharmacy, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

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Abstract. The generally accepted hypothesis for the pathogenesis of alcoholic liver disease (ALD) is the two-hit model, which proposes that fat accumulation in the liver increases the sensitivity of the liver to a second hit that leads to inflammatory liver cell damage. In this study we evaluated the effects of *Magnolia officinalis* (MO), which contains honokiol and magnolol as the primary pharmacological components, to eradicate fatty liver in rats fed an ethanol diet. In vitro studies showed that MO was able to protect RAW 264.7 cells from ethanol-induced production of tumor necrosis factor- α , reactive oxygen species, and superoxide anion radicals; the activation of NADPH oxidase; and subsequent cell death. We also investigated the therapeutic effects of MO on alcoholic fatty liver in Lieber-DeCarli ethanol diet-fed rats. MO treatment of the rats for the last 2 weeks of ethanol feeding completely reversed all the serum, hepatic parameters, and fatty liver changes. The increased maturation of sterol regulatory element-binding protein-1c in the liver by ethanol treatment was completely inhibited by treatment with MO. Therefore, MO may be a promising candidate for development as a therapeutic agent for ALD.

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.08182FP>]

Keywords: *Magnolia officinalis*, alcoholic fatty liver, tumor necrosis factor- α (TNF- α), reactive oxygen species, sterol regulatory element-binding protein (SREBP)

Introduction

Excessive consumption of alcohol is recognized worldwide as a leading cause of disease, disability, and death (1). Alcoholic liver disease (ALD) is a collective term for the pathophysiological changes caused by chronic alcohol consumption. These include progressive liver damage due to fatty infiltration, inflammation, fibrosis, and finally cirrhosis. Although the mechanisms are not completely understood, the widely accepted hypothesis for the pathogenesis of ALD is the two-hit model, which proposes that fat accumulation in the liver increases the sensitivity of the liver to a second hit that leads to inflammatory liver cell damage (2–4).

Alcoholic fatty liver, or steatosis, is the earliest and mildest response of the liver to chronic exposure to ethanol. Clinical studies have shown that alcoholic fatty liver, which is widely assumed to be benign, may lead to cirrhosis if not treated properly (5, 6).

The activation of sterol regulatory element-binding protein-1c (SREBP-1c) in the liver has been reported in rats chronically fed ethanol. SREBP-1c, a membrane-bound transcription factor, translocates to the nucleus upon maturation and regulates lipid homeostasis by controlling the expression of lipogenic genes such as ATP citrate lyase (*Acl*), fatty acid synthase (*Fas*), and stearyl-CoA desaturase-1 (*Scd-1*). The role of SREBP-1c in alcoholic steatosis was first reported by You et al. (7), and this finding was later corroborated using SREBP-1c-null mice (8). Additionally, two recent studies have demonstrated that the modulation of SREBP-1c maturation was effective at reversing and preventing experi-

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mental alcoholic steatosis (9, 10).

Cytokines are thought to be the targets of the second hit in the pathogenesis of ALD. Studies have shown that Kupffer cell activation by endotoxin increases intracellular calcium, which is accompanied by the release of cytotoxic mediators (11, 12). The agents that cause the second hit include reactive oxygen species (ROS), endotoxin, hypoxia, and an immune response to protein adducts that arise during ethanol metabolism and are either released from necrotic hepatocytes or expressed on the surface of hepatocytes undergoing apoptosis (13). Oxidative stress is a trigger for cellular immune responses in patients with ALD (14). Accordingly, the suppression of Kupffer cell sensitization and tumor necrosis factor- α (TNF- α) production can prevent alcoholic liver injury (15–17).

The stem bark of *Magnolia officinalis* REHD. et WOLS. (Magnoliaceae) is a traditional Chinese herbal medicine that has been used for the treatment of gastrointestinal disorders, cough, and phlegm (18). Honokiol and magnolol are biphenolic compounds contained in the stem bark of *M. officinalis*. These two compounds are structural isomers and are responsible for the main pharmacological effects of the bark, which demonstrates antioxidant and anti-inflammatory properties (19, 20). In the present study, we describe the effects of *M. officinalis* on ethanol-induced hepatotoxicity in terms of cytokine production and oxidative stress in murine macrophage cells as well as the effects on fat accumulation in rats fed the Lieber-DeCarli ethanol diet.

Materials and Methods

Cell culture

The mouse leukemia macrophage cell line RAW 264.7 was maintained in the logarithmic phase of growth in RPMI1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with heat inactivated 10% fetal bovine serum (GIBCO BRL) at 37°C in a 5% CO₂–95% air humidified incubator.

Preparation of standardized fraction of *M. officinalis* (MO)

The stem bark of *M. officinalis* was purchased in March, 2004 at the University Oriental Herbal Drugstore, Iksan, Korea; and its identity was verified by Dr. Y.C. Kim, a committee member of the Botanical Garden, Wonkwang University. A voucher specimen (No. WP04-084) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Dried and pulverized stem bark of *M. officinalis* (1.2 kg) was extracted with hot ethanol for 2 h and filtered with filter paper. The filtrate was evaporated *in vacuo* to produce

Table 1. Representative HPLC chromatogram of authentic honokiol, magnolol, and the standardized fraction of *M. officinalis* (MO)

Sample	Retention time (min)
Honokiol	14.22
Magnolol	16.32
MO	14.22; 16.39

an ethanol extract (48.3 g). The extract was dissolved in ethanol and suspended with distilled water. Acidification of the mixture to pH 4.0 resulted in a precipitate, which was dissolved again with ethanol. Evaporation of the solvent gave MO (19.3 g). MO was standardized based on the contents of honokiol and magnolol determined using reverse-phase high-performance liquid chromatography–mass spectrometry. Separation was carried out by using a Waters XTerra™ RP18 column (5 μ m; 4.6 mm \times 150 mm; Waters, Milford, MA, USA) under the following conditions: acetonitrile: 0.03% formic acid (65:35, v/v) as the mobile phase at a flowrate of 0.3 mL/min. The detector wavelength was set at 254 nm. Honokiol and magnolol were found in the MO at a level of 1.16 \pm 0.03% and 1.71 \pm 0.04%, respectively (Table 1) (Supplementary Fig. 1: available in the online version only).

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. Briefly, 1 mg/mL MTT solution was added to each well of the plate and incubated at 37°C under 5% CO₂ for 3 h. The formazan product was dissolved in dimethyl sulfoxide and the optical density was measured at 540 nm.

Measurement of TNF- α

TNF- α levels were detected from the cell culture media by enzyme-linked immunosorbent assay using a commercially available kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measurement of ROS and superoxide anion

ROS level was determined by the 6-carboxy-2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescence method. Cells were incubated with ethanol in the presence or absence of MO for 24 h. After the addition of 10 μ M DCF-DA, fluorescence intensity was analyzed after 30 min with an excitation wavelength at 485 nm and emission at 530 nm. Superoxide anion was determined by the modified cytochrome c reduction method (21). Briefly, ferricytochrome c (50 μ M) was added to the cell culture media with or without superoxide dismutase and incubated for 1 h. The amount of

ferricytochrome c decreased by superoxide was measured by spectrophotometer at 550 nm.

Measurement of NADPH oxidase activity

NAD(P)H oxidase activity was measured by the lucigenin-enhanced chemiluminescence technique as described previously (22). Cell lysates were mixed with lucigenin and the reaction was initiated by the addition of NADPH to the mixture; then the luminescence intensity was measured every 5 s for a total 1 h. The reaction was stopped by diphenylene iodonium, an NAD(P)H oxidase inhibitor.

Animal treatments

Specific pathogen-free male Wistar rats (180–200 g) were obtained from Jung-Ang Laboratory Animal Co. (Seoul, Korea). They were kept in temperature-controlled and filter-sterilized animal quarters with 12-h light and dark cycles and allowed free access to standard chow and tap water during the acclimation period. The use of animals was in compliance with the guidelines established by the Animal Care Committee of our institute. The rats were randomly assigned to the groups specified and given free access to Lieber-DeCarli liquid diet of which 36% of the total calories were supplied from ethanol (ethanol group) or maltodextran (control group) for 4 weeks (23). During the last 2 weeks of diet feeding, the rats were daily treated with MO (15 or 45 mg/kg per day) by gavage. *S*-Adenosyl-L-methionine (SAM) in the stable form of sulfate-*p*-toluenesulfonate (25 mg/kg per day, s.c.) was used as a positive control (24).

Serum biochemistry and pathological evaluation

Serum levels of alanine aminotransferase (ALT) were measured using an Automated Chemistry Analyzer (Prestige 24I; Tokyo Boeki Medical System, Tokyo). Total liver lipids were extracted from the homogenate prepared from 100 mg of rat liver using a chloroform/methanol mixture (2:1, v/v) (25), and triglyceride content was measured by a commercially available kit (Sigma-Aldrich, St. Louis, MO, USA). A cross section of the left lateral lobe of the liver was fixed in 10% neutral buffered formalin for 24 h. The liver tissues were dehydrated, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (H&E). For Oil Red O staining, liver tissues were embedded in OCT compound (Tissue-tek; Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen. Ten-micron cryosections of the tissue were stained with Oil Red O (Sigma-Aldrich). Histopathologic examinations of the liver sections were conducted by a pathologist and were peer-reviewed.

Quantification of hepatic glutathione (GSH) and SAM concentration

Liver was homogenized in a four-fold volume of ice-cold 1 M perchloric acid. After the denatured protein was removed by centrifugation at $10,000 \times g$ for 10 min, the supernatant was assayed for the total GSH concentration using the HPLC separation/fluorometric detection method of Neuschwander-Tetri and Roll (26). The method of She et al. (27) was employed to determine SAM concentrations using an HPLC system with a UV-975 detector (Jasco Co., Tokyo).

Western blot analysis

Nuclear extracts from the liver homogenate were prepared using a commercially available kit (BioVison, Mountain View, CA, USA) according to the manufacturer's instructions. A 40- μ g sample of the protein were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and then immunoblotted with rabbit polyclonal SREBP-1 antibody (Abcam, Cambridge, MA, USA). Detection was performed by enhanced chemiluminescence Western Blotting Detection Reagents (Amersham, Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using the Easy-blue™ Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea), and single-stranded cDNA was synthesized from the RNA by using oligo (dT) primer and the Superscript III reverse transcriptase supplied with the Superscript First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). PCR was performed with gene-specific primers designed using Oligo 6.0 software (Molecular Biology Insights Inc., Cascade, CO, USA). Primer pairs used in PCR are as follows: Acl γ (sense, 5'-CAAGAGGGAT TACGAGTGA, antisense, 5'-GGTATGTCGGCTGAA GAG); FAS (sense, 5'- CCGAGTGGCTGGGTATTC TTTT, antisense, 5'-AGGGAGCTGTGGATGATGT TGA); SCD1 (sense, 5'-CCTCATCATTGCCAACAC CAT; antisense, 5'-AGCCAACCCACGTGAGAGAA); GAPDH (sense, 5'-AACATCATCCCTGCATCCAC; antisense, 5'-CTGCTTCACCACCTTCTTG).

Statistical analyses

All data were expressed as the mean \pm S.D. (for in vitro experiments) or the mean \pm S.E.M. (for in vivo experiments). Statistical analyses were performed by Student's *t*-test (for in vitro experiments) or the one-way analysis of variance (ANOVA) test (for in vivo experiments) followed by the Newman-Keuls multiple ranges test. Differences between groups were considered to be

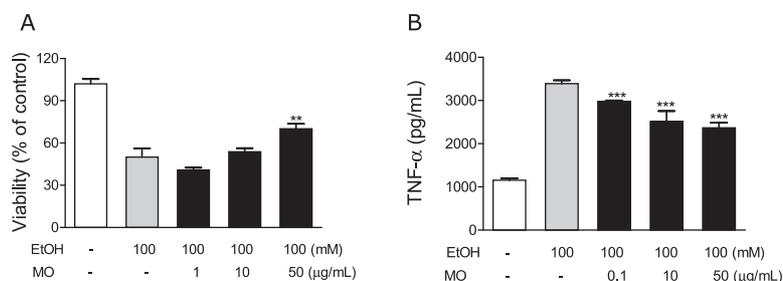


Fig. 1. Inhibition of ethanol-induced cell death (A) and TNF- α production (B) by MO. RAW 264.7 cells were incubated with ethanol in the presence or absence of various concentrations of MO for 24 h. Cell viability was determined by the MTT reduction assay, and the TNF- α level in the cell culture medium was measured by a commercially available enzyme-linked immunosorbent assay kit. Data are each the mean \pm S.D. of more than three independent experiments. ** P <0.01, *** P <0.001, compared with the ethanol control.

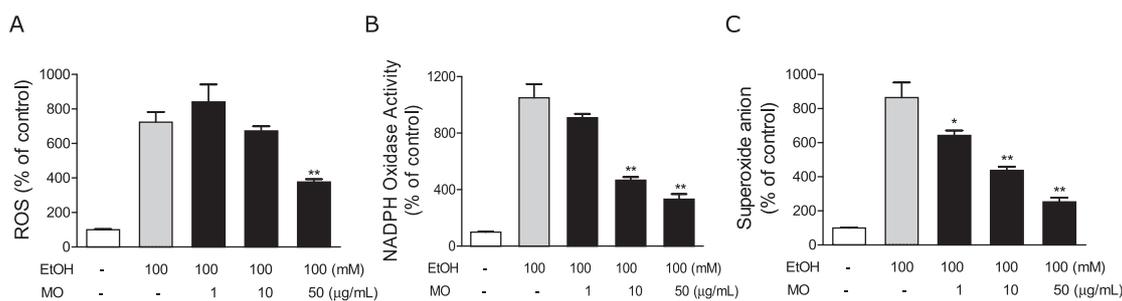


Fig. 2. Inhibition of ethanol-induced ROS production (A), NADPH oxidase activity (B), and superoxide anion formation (C) by MO. RAW 264.7 cells were incubated with ethanol (100 mM) in the presence or absence of various concentrations of MO for 24 h. The ROS level was measured by DCF-DA fluorescence. The activity of NADPH oxidase was measured by the lucigenin-enhanced chemiluminescence method as described in the Materials and Methods section. Superoxide anion was measured by the reduction of ferricytochrome C. Data are each the mean \pm S.D. of more than three independent experiments. * P <0.05, ** P <0.01, compared with the ethanol control.

statistically significant at P <0.05.

Results

MO protects cells from ethanol-induced cytotoxicity, TNF- α production, and oxidative stress in vitro

The viability of cells in culture was measured for RAW 264.7 mouse leukemia macrophages using the MTT assay. A 24-h incubation of RAW 264.7 cells with ethanol (100 mM) decreased cell viability by $51.5 \pm 6.7\%$ compared with the vehicle control. Treatment of the cells with MO (1–50 $\mu\text{g}/\text{mL}$) dose-dependently and significantly reduced the cell death caused by ethanol (Fig. 1A). Ethanol induced approximately 3.5-fold increases in TNF- α production in RAW 264.7 cells, and this was significantly inhibited by the addition of MO (Fig. 1B).

To investigate the effects of MO on ethanol-induced oxidative stress, the levels of total ROS and superoxide anion were analyzed in RAW 264.7 cells. MO dose-dependently and significantly reduced the production of ethanol-induced ROS (Fig. 2A). Ethanol-stimulated activation of NADPH oxidase and the formation of superoxide anions were also significantly inhibited by MO treatment in a dose-dependent manner (Fig. 2: B and C).

In the experiments using a low concentration of

ethanol (25 mM), TNF- α production and ROS formation were also inhibited by the treatment with a high dose of MO (Fig. 3).

MO inhibited ethanol-induced fatty liver in vivo

Based on the in vitro data, we tested the therapeutic effects of MO on ethanol-induced fatty liver in rats fed the standard Lieber-DeCarli ethanol diet for 4 weeks. During the last 2 weeks of the diet, rats were treated daily with MO (15 or 45 mg/kg per day) by gavage. Our previous study had shown that 2 weeks of ethanol consumption induced a fatty liver (28). At the end of the treatment, serum ALT, hepatic triglyceride, SAM, and GSH levels were measured. MO treatment at doses of 15 and 45 mg/kg significantly reduced the levels of serum ALT and hepatic triglyceride (Fig. 4: A and B). The ethanol-induced decreases in the levels of hepatic SAM and GSH were completely restored by MO treatment (Fig. 4: C and D). The elevated level of serum TNF- α was decreased by the MO treatment but not differ significantly from that of the ethanol treatment group (Fig. 4E). To verify the therapeutic effects of MO on alcoholic steatosis, histopathological examinations of the liver samples were performed by H&E staining. In contrast to the control and MO-treated rats, ethanol-treated rats exhibited moderate to severe micro- and macrovesicular steatosis (Fig. 5A: a–c). However, MO

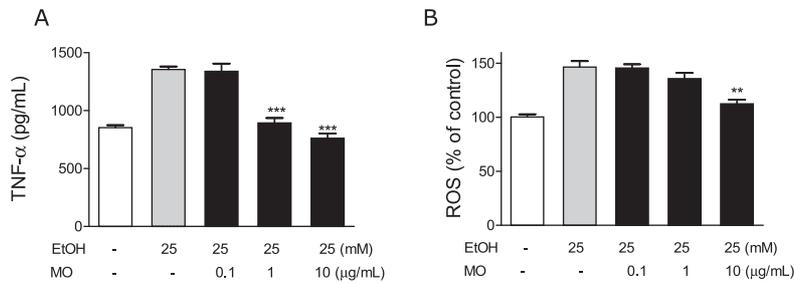


Fig. 3. Inhibition of ethanol-induced TNF- α production (A), ROS production (B) by MO. RAW264.7 cells were incubated with a low dose of ethanol (25 mM) in the presence or absence of various concentrations of MO for 24 h. The TNF- α level in the cell culture medium and ROS level were measured by the same method as that used for the experiments shown in Figs. 1 and 2. Data are each the mean \pm S.D. of more than three independent experiments. ** P <0.01, *** P <0.001, compared with the ethanol control.

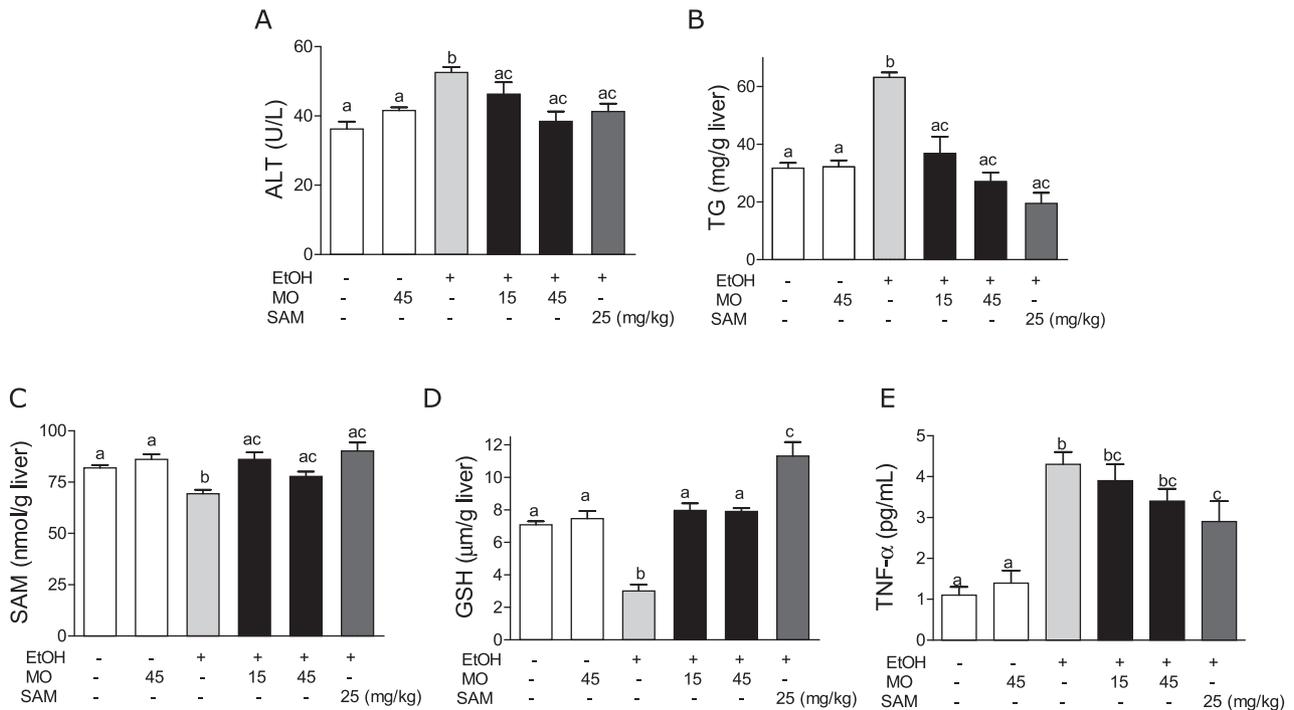


Fig. 4. Restoration of serum and hepatic parameters by MO in rats fed an ethanol diet. Rats were fed a standard Lieber-DeCarli ethanol diet for 4 weeks. During the last 2 weeks of the diet, the rats were treated daily with MO, SAM, or vehicle. At the end of the treatment, serum ALT (A), hepatic triglyceride (B), SAM (C), GSH (D), and serum TNF- α level (E) were measured as described in the Materials and Methods section. The data each represent the mean \pm S.D. from five rats. Values that do not share a common subscript letter are significantly different (P <0.05).

treatment completely reversed the alcohol-induced steatosis (Fig. 5A: d and e). The histological evaluation scores of the livers are presented in Table 2. Oil Red O staining confirmed the histopathological results by demonstrating that the intensive staining of neutral triglycerides and lipids in the liver samples from ethanol-fed rat was diminished in the MO-treated rats. Treatment with SAM as a positive control decreased ethanol-induced fat accumulation in the liver (Fig. 5B: a–e). These data clearly indicate that MO can completely reverse alcoholic steatosis.

MO inhibited the maturation of SREBP-1c and the expression of genes involved in fatty acid synthesis

Based on the evidence that SREBP-1c is involved in hepatic lipid homeostasis, we tested the effects of MO treatment on the maturation and nuclear translocation of SREBP-1c in H4IIEC3 cells. Incubation of H4IIEC3 cells with ethanol (100 mM) for 24 h markedly increased the levels of SREBP-1c in the nuclear fractions isolated from the total cell lysates, and cotreatment with MO markedly decreased the level of nSREBP-1c in a dose-dependent manner (Fig. 6A). The effects of MO treatment on the maturation of SREBP-1c and the expression of SREBP-1c-responsive genes were also tested in the animal model. Treatment of both ethanol- and pair-fed

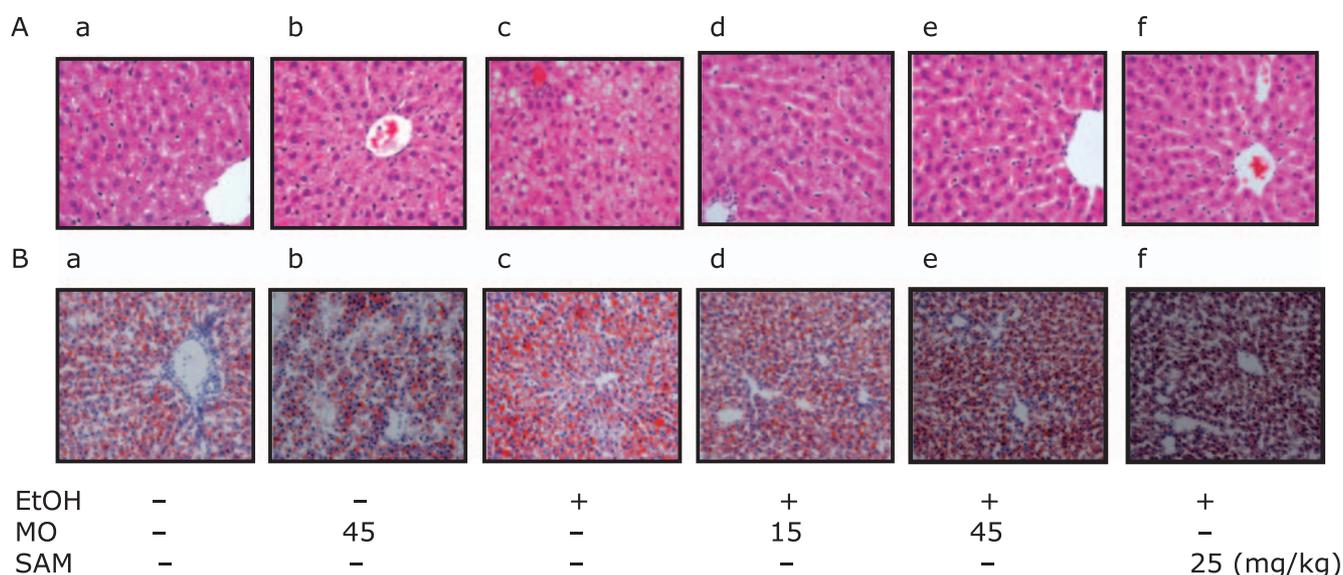


Fig. 5. Reversion of fatty liver by MO in rats fed an ethanol diet. Rats were fed a standard Lieber-DeCarli ethanol diet for 4 weeks. During the last 2 weeks of the diet, the rats were treated daily with MO, SAM, or vehicle. A cross section of the left lateral lobe of the liver was fixed in 10% neutral buffered formalin. The liver tissues were dehydrated, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (A). Frozen liver sections were sectioned at 10 μ m and stained with Oil Red O (B). Histology pictures are shown at original magnification $\times 400$. a, control diet; b, control diet with MO (45 mg/kg); c, ethanol diet; d, ethanol diet with MO (15 mg/kg); e, ethanol diet with MO (45 mg/kg); and f, ethanol diet with SAM (25 mg/kg).

Table 2. Histological evaluations of livers from rats fed a standard Lieber-DeCarli ethanol diet for 4 weeks

	a	b	c	d	e	f
Microvesicular fatty change	- (5)	- (5)	+++ (5)	- (1); + (4)	- (3); + (2)	- (3); + (1)
Macrovesicular fatty change	- (5)	- (5)	+++ (5)	- (1); + (4)	- (3); + (2)	- (3); + (1)
Spotty necrosis	- (5)	- (5)	- (5)	- (5)	- (5)	- (4)
Portal inflammation	- (5)	- (5)	- (5)	- (5)	- (5)	- (4)
Fibrosis	- (5)	- (5)	- (5)	- (5)	- (5)	- (4)

Absent (-), mild (+), mild to moderate (++), and moderate (+++). The number of experiments was presented in parentheses. a, control diet; b, control diet with MO (45 mg/kg); c, ethanol diet; d, ethanol diet with MO (15mg/kg); e, ethanol diet with MO (45 mg/kg); and f, ethanol diet with SAM (25 mg/kg).

rats with MO significantly reduced the nuclear translocation of SREBP-1c protein (nSREBP-1c) (Fig. 6B). To determine whether the change in the nSREBP-1c protein level was associated with a corresponding change in the expression of genes known to be regulated by SREBP-1c, RT-PCR was performed using gene-specific primers for *Acy*, *Fas*, and *Scd-1*. The change in the nSREBP-1c level corresponded well with the expression of the genes in ethanol- and MO-treated rats (Fig. 6: C–E). These results demonstrate that the inhibition of both the maturation of SREBP-1c and the expression of its target genes may contribute to the anti-steatosis effects of MO in ethanol-fed rats.

Discussion

In this study, we evaluated the effect of a standardized fraction of *M. officinalis* on ethanol-induced fatty liver in rats fed an ethanol diet for 4 weeks. In vitro studies showed that MO is able to protect RAW 264.7 cells from ethanol-induced production of TNF- α , ROS, and superoxide anion radicals; activation of NADPH oxidase; and subsequent cell death. In the ethanol-induced fatty liver model, rats treated with MO showed decreases in serum ALT and hepatic TG and increases in hepatic GSH and SAM, returning the levels of all four to control levels. Alcohol-induced fatty liver was also completely reversed by MO, and this observation was verified by H&E staining and Oil Red O staining. These data clearly indicate that MO can completely reverse alcoholic

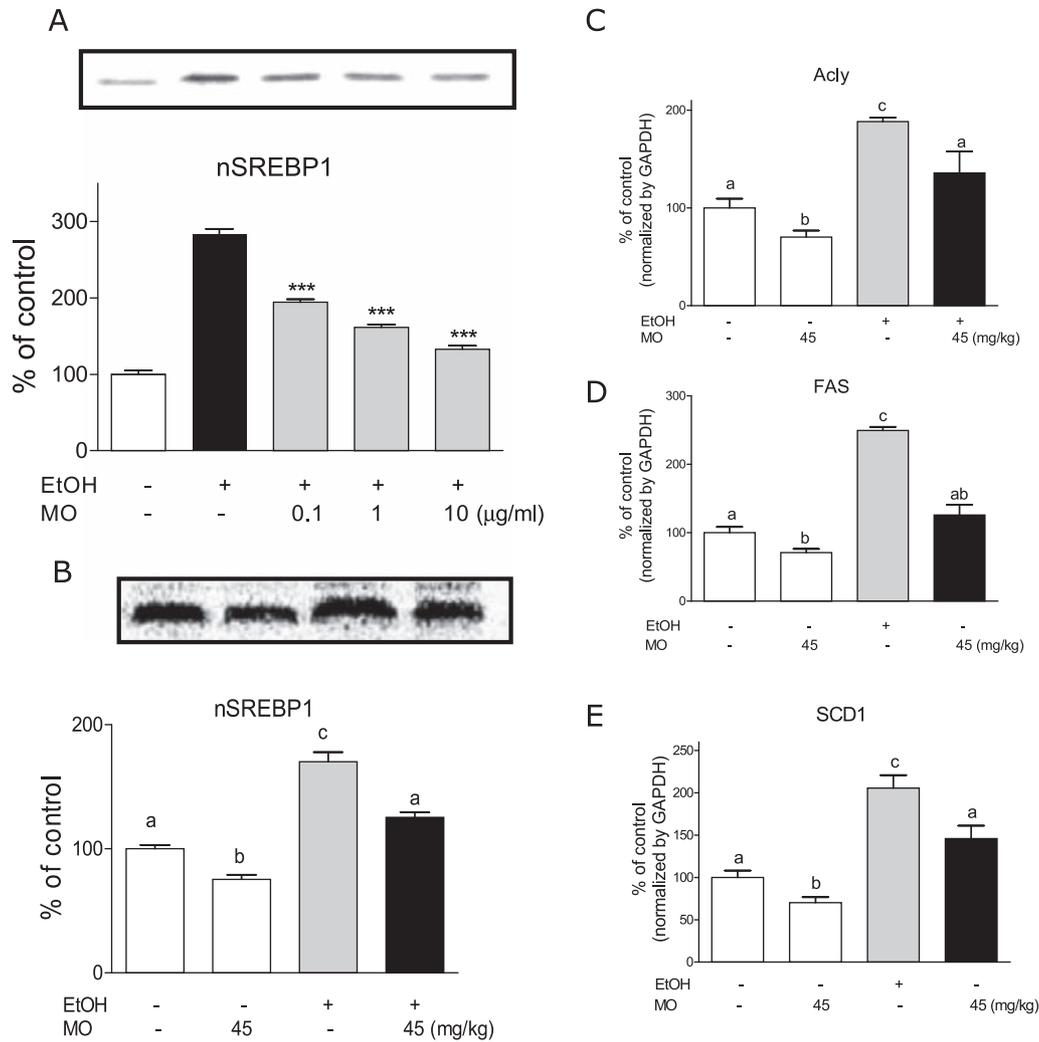


Fig. 6. Inhibition of the maturation of SREBP-1c in H4IIEC3 cells (A), inhibition of the maturation of SREBP-1c in livers (B), and the expression of its target genes (C–E) by MO in rats fed an ethanol diet. H4IIEC3 cells were incubated with ethanol (100 mM) in the presence or absence of various concentrations of MO for 24 h. Rats were fed a standard Lieber-DeCarli ethanol diet for 4 weeks. During the last 2 weeks of the diet, the rats were treated daily with MO or vehicle, by gavage. Aliquots of nuclear extracts from the liver homogenate were electrophoresed in a 10% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with rabbit polyclonal SREBP-1 antibody. Single-stranded cDNA was synthesized from the RNA, and RT-PCR was performed with gene-specific primers designed using Oligo 6.0 software. In vitro experiment data are each the mean \pm S.D. of more than three independent experiments. *** $P < 0.001$, compared with the ethanol control. In the case of the in vivo data, each bar represents the mean intensity \pm S.D. based on independent experiments with five rats per group. Values that do not share a common subscript letter are significantly different ($P < 0.05$).

steatosis in rats.

Alcohol abuse is a leading cause of morbidity and mortality worldwide. The earliest and mildest hepatic disorder following chronic ethanol consumption is fatty liver, which is defined by the American Liver Foundation as the build-up of excess fat in liver cells, especially when fat accounts for more than 10% of the liver weight [American Liver Foundation, 2007]. A fatty liver does not always result in damage to the liver, but excess fat can lead to steatohepatitis. Fatty liver is asymptomatic on its own, which makes it difficult to detect at an early

phase and to perceive the condition as a potentially serious health problem. However, recent clinical studies have shown that fatty liver is an important pathogenic step in the development of end-stage liver disease (6). Therefore, the development of efficacious therapeutics to prevent or treat alcoholic fatty liver is urgently needed. Many attempts have been made to develop effective therapeutics for fatty liver disease using drugs, chemicals, and natural products derived from medicinal plants. Although the mechanisms by which ethanol causes fatty liver appear to be complex and multi-

factorial, enhanced lipogenesis has been proposed as an important biochemical mechanism underlying the development of alcoholic fatty liver (29).

Kupffer cell activation by gut-derived lipopolysaccharide (LPS) produces pro-inflammatory cytokines and ROS, which are critical components in the initiation and development of ALD. The pivotal role of TNF- α in ALD was supported by experiments involving anti-TNF- α antibody and TNF- α receptor-knockout mice that were resistant to liver damage, including steatosis, caused by long-term exposure to ethanol (30, 31). Therefore, regulating the TNF- α production in Kupffer cells is one possible approach for the treatment of ALD. In fact, thalidomide, a well-known inhibitor of TNF- α production by macrophages and other cell types, has been shown to reduce the LPS-induced TNF- α production by Kupffer cells by decreasing TNF- α mRNA. Thalidomide prevented liver damage caused by chronic ethanol exposure by suppressing the TNF- α production and eliminating the sensitization of Kupffer cells to LPS (14). Similar results were observed when L-carnitine was administered to rats chronically treated with ethanol via a liquid diet (32). In addition to the anti-inflammatory effects, the suppression of TNF- α production can also affect peripheral and hepatic lipid metabolism, indicating the possible role of this cytokine in alcoholic fatty liver (33, 34). In the present study, we showed that MO dose-dependently suppresses the production of TNF- α in ethanol-treated RAW264.7 cells. Given the important role of TNF- α in ALD, this effect of MO suggests that it may be a promising agent for development as an anti-steatosis drug.

Alcohol consumption shifts the balance between pro- and antioxidants, favoring pro-oxidants, which can lead to liver damage. Among the pro-oxidants formed in vivo, superoxide anion plays the most important role in ALD (35). The fact that levels of superoxide dismutase correlated inversely with pathological changes in the liver caused by ethanol supports the role of superoxide anions in ALD (36). The mechanisms of pro-oxidant-induced fatty liver or, inversely, antioxidant-induced amelioration of alcoholic fatty liver are not completely understood. Based on recent data, some convincing explanations include the impairment of mitochondrial lipid oxidation (37) or the impairment of mitochondrial β -oxidation of fatty acid owing to the deletion of mtDNA (38). Pan et al. (39) recently reported that lipid peroxidation reduces lipoprotein secretion by enhancing the intrahepatic degradation of newly synthesized ApoB100, leading to the accumulation of lipid in the liver. Kupffer cells express the NADPH oxidase complex as a major oxidant-generating enzyme. NADPH oxidase is a membrane-bound enzyme complex that is

found in monocytes. When activated, it generates superoxide anion by catalyzing the reduction of O₂ to O₂⁻. Therefore, the regulation of NADPH oxidase activity and thus the level of superoxide anion, which ultimately regulates the inflammatory and metabolic signals associated with lipid metabolism, is the key step in preventing the pathogenesis of early alcohol-induced liver injury (35). In this context, MO possesses properties that can contribute positively to the suppression of the disease.

Acute ethanol treatment increases the concentration of mature SREBP-1c protein in rat liver nuclear extract and the binding to SRE or related sequences of lipogenic target genes (40). The maturation of SREBP-1c has also been observed in animals exposed chronically to ethanol (41). SREBPs are membrane-bound transcription factors belonging to the basic helix-loop-helix leucine zipper family (42). Upon maturation, SREBP translocates to the nucleus and exerts transcriptional regulation of lipogenic genes such as acetyl CoA carboxylase, fatty acid synthase, and stearyl CoA desaturase-1 (43). Transgenic mice that over-express the NH₂-terminal domain of SREBP-1a developed massively enlarged livers owing to increased expression of the gene for lipogenesis (44). In contrast, mice that are deficient for leptin and SREBP-1 [*ob/ob* \times *Srebp-1*^(-/-)] had markedly attenuated fatty livers (45). In the present study, feeding rats a standard Lieber-DeCarli diet supplemented with ethanol for 4 weeks increased the maturation of SREBP1 as well as the expression of the target genes involved in hepatic lipogenesis. However, the treatment of rats with MO for the last 2 weeks of the total period of the ethanol diet completely reversed the level of nSREBP protein in the nucleus and the expression of transcripts for genes known to be regulated by SREBP-1c. MO does not change the activity of cytochrome P450 2E1 (Supplementary Fig. 2: available in the online version only), indicating that the effect of MO on ethanol-induced steatosis was not due to the decreased ethanol oxidation.

In view of the importance of SREBP-1c for the development of alcoholic steatosis, the suppressive effects on the maturation of this protein and subsequent expression of genes may contribute to the overall therapeutic effects of MO in ALD.

In summary, the results presented in this paper demonstrate that MO reverses fatty liver induced by the chronic administration of ethanol. The effects of MO may result from the suppression of TNF- α and superoxide anion production and the inhibition of SREBP-1c maturation. An important issue in the management of ALD is controlling the progression of simple steatosis to steatohepatitis. In this context, MO may be a promising

candidate for development as a therapeutic agent for alcoholic fatty liver.

Acknowledgments

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