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Effects of Mangosteen on α-SMA Expression in HSC-T6 Cells and Liver Fibrosis in Rats

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Liver fibrosis is always proceeded by inflammation and oxidative stress. Traditionally Mangosteen is well known for its anti-inflammatory, antioxidant and free radical scavenging effects. This study investigated the effect of α-mangostin(α-MG) in immortalized rat hepatic stellate cell line (HSC-T6) and the anti-fibrotic effects of MG on carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.

This investigation indicates that α-MG reduced all fibrosis stimulators (PDGF, TNF-α, TGF-β1, and LPS) induced smooth muscle actin (α-SMA) secretion, especially that stimulated by LPS. *In vivo* study showed the prophylactic administration of 100 mg/kg MG significantly inhibited the increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and significantly reduced the liver fibrosis scores in Sprague–Dawley rats. These results reveal that the administration of α-MG effectively attenuated inflammation-induced fibrosis. The mechanism of the treatment of hepatic fibrosis by MG may involve anti-fibrotic, anti-inflammatory activity and the inhibition of the function of HSC-T6 cells.

Key words: α-Mangostin, liver fibrosis, rat hepatic stellate cell, α-smooth muscle actin, carbon tetrachloride

Introduction

Cirrhosis is a chronic liver disease that is caused by alcoholism, hepatitis B and C, and fatty liver disease, among other causes. In liver cirrhosis, liver tissue is replaced by fibrosis scar tissue and regenerative nodules, leading to loss of liver function. The development of fibrosis in the liver is the result of a multicellular process, in which are involved various cells.[1] Fibrosis mainly damages hepatocytes

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and generates several mediators, such as reactive oxygen species and fibrogenic cytokines (PDGF-BB, TNF-α, TGF-β_1, and LPS) that initiate the activation and proliferation of hepatic stellate cells (HSC) and other fibrogenic cells and the production of excess extracellular matrix (ECM), including collagens.[2] The activated HSC phenotype is characterized by the loss of lipid content, enhanced proliferation and migration, the expression of α-smooth muscle actin (α-SMA), the production of excess scar proteins (mostly type 1 collagen), and contractile and immune capability.[3] Liver injury leads to the recruitment of immune cells into the liver and their activation of local Kupffer cells, which can further promote the fibrotic process via the secretion of inflammatory and fibrogenic cytokines. The most effective anti-fibrotic therapies are treating, or eliminating possible stimuli of fibrogenesis. Furthermore, several therapies eliminate fibrogenesis by targeting specific steps in the fibrogenic response. These may be anti-inflammatory or inhibit cellular injury or stellate cell activation.Current methods for treating liver fibrosis include (1) removing the injurious agent(eradication of hepatitis B virus), (2) the use of anti-inflammatory agents (corticosteroids in autoimmune hepatitis), (3)the use of antioxidants (polyenylphosphatidylcholine in alcoholic hepatitis), (4) the use of cytoprotective agents (ursodeoxycholic acid), (5) inhibition of stellate cell activation (Interferon-γ), and (6) inhibition of stellate cell-activating phenotypes (colchicine).[4] Purple mangosteen (*Garcinia mangostana*) is commonly referred to as the "queen of fruits" and it is cultivated in the tropical rainforests of some Southeast Asian countries, including Thailand, Malaysia, and the Philippines. In those countries, the pericarp (peel, rind, hull or ripe) of mangosteen is used as a traditional medicine in the treatment of abdominal pain, diarrhea, dysentery, infected wounds, suppuration, and chronic ulcers.

α-MG is a main constituent of the fruit hull of the mangosteen. Previous studies have demonstrated that α-MG has pharmacological effects, such as antioxidant, antitumor, anti-inflammatory, antiallergic, antibacterial, antifungal and antiviral effects[5]. Xanthones have been isolated from mangosteen pericarp, whole fruit, heartwood, and leaves. The most investigated xanthones are α-, β-, and γ-mangostins, garcinone E, 8-deoxygartanin, and gartanin [6]. In Japan, MG (α-MG rich mangosteen extract: which contains about 82.1% α-MG and 9.0% γ-mangostin) has been commercialized and is used as a dietary supplement.[7]

In our previous studies, the inhibitory effects of α-MG on inflammatory cytokines were evaluated by measuring the amounts of secreted TNF-α and IL-4 in LPS-stimulated U937 cells after treatment with α-MG.[8] In an *in vivo* study, MG and α-MG also inhibited TNF-α and IL-6 secretion in mouse plasma that had been stimulated with LPS.[9] This investigation studies the effect of α-MG on α-SMA expression in immortalized rat hepatic stellate cell line (HSC-T6), and furthermore the anti-fibrotic effects of MG on carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.

Materials and Methods

Chemicals

LPS (from *Escherichia coli*), RPMI 1640 medium, 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (MTT), phosphate-buffered saline (PBS), antibiotics, L-glutamine and trypsin-EDTA were purchased from Gibco BRL (USA). Fetal bovine serum was purchased from Hyclone Laboratories Inc. (USA).

Waymouth's MB752/1 was purchased from Invitrogen Corporation(USA) Enzyme-linked immunosorbent assay (ELISA) test kits for α-SMA were obtained from Uscn Life Science Inc. (UK). Silymarin, Sirius Red and Fast Green were purchased from Sigma-Aldrich (USA). Picric acid (2, 4, 6-trinitrophenol) and carbon tetrachloride were obtained from Showa (Japan). All other chemicals were purchased from Sigma-Aldrich.

Extraction of mangosteen

The α-mangostin rich mangosteen extract(MG) was prepared as described elsewhere.[7] Briefly, the fresh plant materials were washed with water at 95-100°C (to remove water-solvable resin, pigment and mucilage, among other substances). Then, 50% alcohol was added to the washed pericarp at 80-85°C for 1 hour with mechanical stirring to extract the pericarp. After filtration, the solvent was slowly cooled to room temperature. The crude mangosteen precipitate were filtered and reprecipitated to yield the MG (which contained 82.1% α-MG and 9.0% γ-mangostin).

Cell culture

The HSC-T6 cell line, a generous gift of Prof. S.L. Friedman (Mount Sinai School of Medicine, New York, NY, USA) and Prof. Y.T. Huang (National Research Institute of Chinese Medicine, Taiwan, R.O.C). HSC-T6 cell line is immortalized rat HSCs , which are transfected by the large T-antigen of SV40 vector, which contains a Rous sarcoma virus promoter. HSC-T6 cells were cultured in Waymouth's medium containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cytotoxicity assay

Cytotoxicity assays were performed using the MTT method. HSC-T6 cells were seeded onto 24-well plates and incubated for 24 hours at 37°C in Waymouth's MB752/1 medium (serum-free) with or without α-MG at different concentrations. Then, HSC-T6 cells were incubated with 100 μL of 1 mg/mL MTT for 1 hour at 37°C under 5% CO_2. After incubation, the medium was aspirated, and 100 μL of DMSO was added to the wells to solubilize the formazan dye. Absorbance was read at 560 nm by ELISA reader (Spectrafluor Plus, Tecan, Switzerland), and the surviving cell fraction was calculated. Finally, inhibition of cell viability was calculated through means of the following formula:

% inhibition= (1-absorbancy of treated cells/absorbancy of untreated cells)x100.

α-smooth muscle actin (α- SMA) assay

HSC-T6 cells (2×10^5 cells/well) were cultured in 24-well plates, for studies that are described in detail below [10-13]. (1) PDGF: After 24 hours of incubation, HSC-T6 cells were rinsed with PBS and then subjected to serum-free starvation for 24 hours. HSC-T6 cells were exposed to PDGF (10 ng/ml) in the absence or presence of α-MG at different concentrations in serum-free medium. Cells were washed with PBS and immediately lysed. Samples were detected by α-SMA ELISA Kit, according to the manufacturer's instructions. (2) TNF-α: HSC-T6 cells were co-treated with TNF-α (10 ng/ml) and α-MG for 24 hours in serum-free medium. Cells were washed with PBS and immediately lysed. Samples were detected by α-SMA ELISA Kit. (3) TGF-β_1: HSC-T6 cells were pretreated with or without α-MG for 1 hour, and were then incubated with TGF-β_1 (1 ng/ml) for another 24 hours in serum-free medium. Cells were washed with PBS and immediately lysed. Samples were analyzed using

an α-SMA ELISA Kit. (4) LPS：HSC-T6 cells were co-treated with LPS (1 μg/ml) and α-MG for 24 hours in serum-free medium. Cells were washed with PBS and immediately lysed. Samples were analyzed using an α-SMA ELISA Kit.

Animals

Male Sprague–Dawley rats, eight weeks-old and weighing around 250 g were purchased from BioLASCO Taiwan Co., Ltd., (Taipei, Taiwan). Animals were acclimatized for one week and housed in a temperature-and-humidity-controlled environment (at a room temperature of 23±2°C and a relative humidity of 40-70%) with unrestricted access to food and water under 12 hours light-dark cycles. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biomedical Technology and Device Research Laboratories, ITRI.

Carbon tetrachloride-induced liver fibrosis model and treatment

Rats were randomly divided into three treatment groups (n = 8) and a naïve group (n = 3). The rats, with the exception of those in the naïve group, were injected intraperitoneally with 0.4 ml/kg of CCl_4 twice a week for eight weeks. The rates in each of the three treatment groups were also administered daily by gavage 200 mg/kg Silymarin, 100 mg/kg MG or a vehicle, respectively. Blood samples were collected from the tail vein before treatment with CCl_4 and at the second, fourth, sixth and eighth weeks for a biochemical assay. At the end of the experiment, the animals were sacrificed and the left lobe of the liver was fixed in 3.7% formalin for paraffin-embedded sectioning and collagen staining.

Histopathological observations

The left lobe of liver was fixed in 3.7% formalin for two to three days, and the liver tissues were dehydrated using a graded alcohol series (30, 50, 70, 95 and 99.5%), embedded in paraffin, and sectioned to a thickness of 5 μm. For histopathological examination, the paraffin sections were de-waxed, hydrated, stained with 0.1% Sirius Red and 0.1% Fast Green for one hour. After staining, the tissues were dehydrated using a graded alcohol series. They were finally cleaned in xylene and mounted in a resinous medium. Liver fibrosis was graded using the Metavir system. The fibrosis was scored on a five-point scale from (zero to four): 0, normal; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; and 4, cirrhosis.[14]

Assay of serum AST and ALT activities

Blood samples were allowed to coagulate at room temperature for 60 min. Serum was then separated by centrifugation at 25°C and 6000 rpm for 10 min [15-16]. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using an auto-biochemistry detector (Kodak EKTACHEM DT60 II, New York, USA).

Statistical analysis

Quantitative data were presented as mean ± S.D. and compared using Student's t-test.The Mann–Whitney rank-sum test was used to determine the histopathological fibrosis scores. $P < 0.05$ was regarded as statistically significant.

Results

Effects of α-MG on PDGF, TNF-α, TGF-β_1, and LPS induced α-SMA production in HSC-T6 cells

Fibrosis stimulators (PDGF, TNF-α, TGF-β_1, and LPS)induced the production of an amount of α-SMA in HSC-T6 cells. The inhibitory effects of α-MG on the stimulators were evaluated by measuring the amount of secreted α-SMA in HSC-T6 cells. All of the stimulators stimulated α-SMA secretion in HSC-T6 cells (Fig. 1). α-MG (1, 3, 9, and 27 μM) reduced PDGF-stimulated α-SMA secretion (to 99.5 ± 4.3%, 94.0 ± 94.0%, 71.9 ± 6.7%, and 63.2 ± 4.3%, respectively, of the PDGF level) (Fig. 1A). α-MG (1, 3 , 9, and 27 μM) reduced TNF-α-stimulated α-SMA secretion (to 89.0 ± 6.2% , 87.8 ± 4.4%, 75.3 ±4.0%, and 62.3 ± 4.3%, respectively, of the TNF-α level) (Fig. 1B). α-MG (1, 3, 9, and 27 μM) reduced TGF-β1-stimulated α-SMA secretion (99.0 ± 4.8%, 81.0 ± 8.1%, 78.8 ± 4.6%, and 70.6 ± 4.5%, respectively, of the TGF-β1 level) (Fig. 1C). α-MG (1, 3, 9, and 27 μM) reduced LPS -stimulated α-SMA secretion (to 100.0 ± 4.7%, 94.5 ± 9.0%, 65.8 ± 4.1%, and 57.2 ± 7.3%, respectively, of the LPS level) (Fig. 1D). α-MG inhibited the production of α-SMA in a dose-dependent manner.

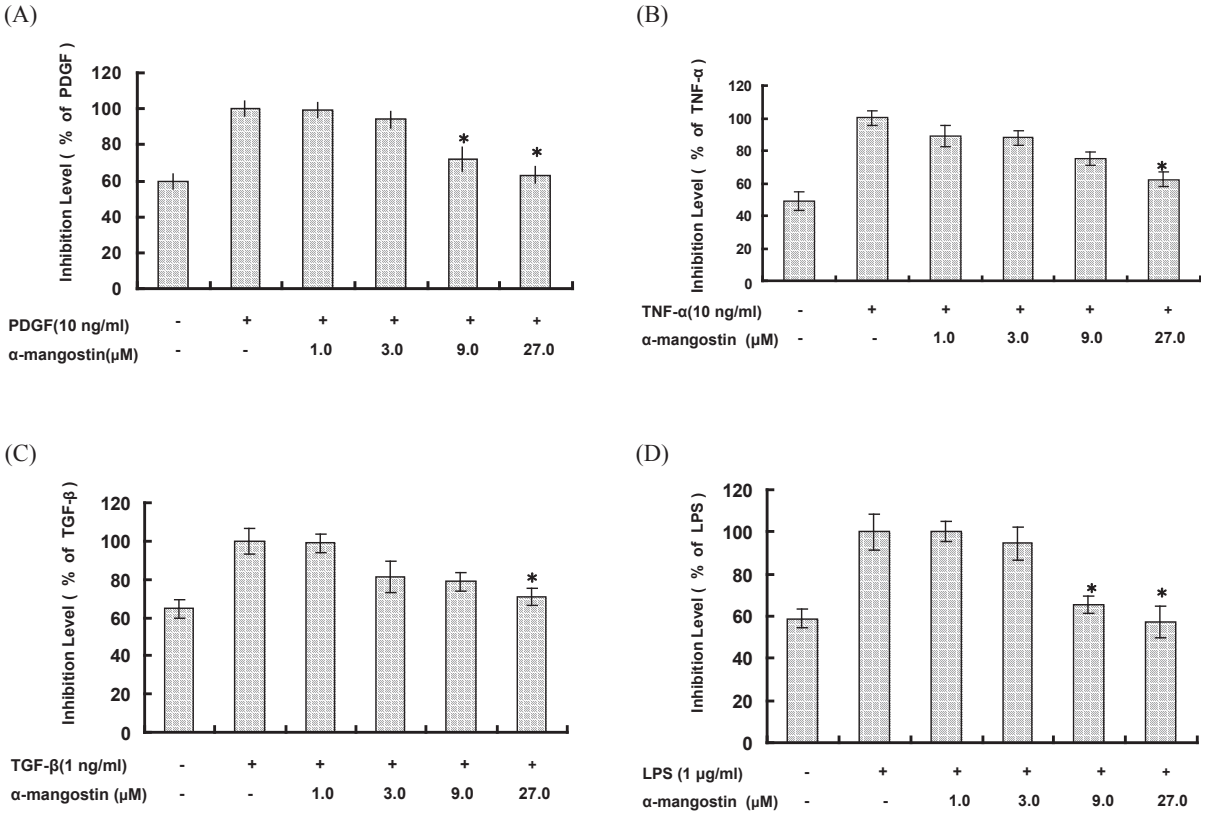


Fig. 1 Effects of α-mangostin on stimulator-induced α-SMA secretion in HSC-T6 cells.

Serum-free-passaged HSC-T6 cells were exposed to stimulators in absence or presence of α-MG (1.0– 27.0 nM) and incubated for 24 hours to analyze α-SMA content using ELISA kit. Data are expressed as mean ± SEM from three independent experiments. * $p < 0.05$ indicates significant difference from stimulators-only group. (A) PDGF, (B) TNF-α, (C) TGF-β_1, (D) LPS

The anti-fibrosis effects of α-MG could be caused by the inhibition of α-SMA production or a reduction in the number of HSC-T6 cells because of cytotoxicity. The latter possibility was excluded by comparing the numbers of cells that were cultured with the different concentrations of α-MG: no significant decreases in cell viability were observed when the concentration was below 27.0 μM (Fig. 2). The results thus obtained showed that α-MG reduced all stimulators-stimulated α-SMA secretion, especially that stimulated by LPS.

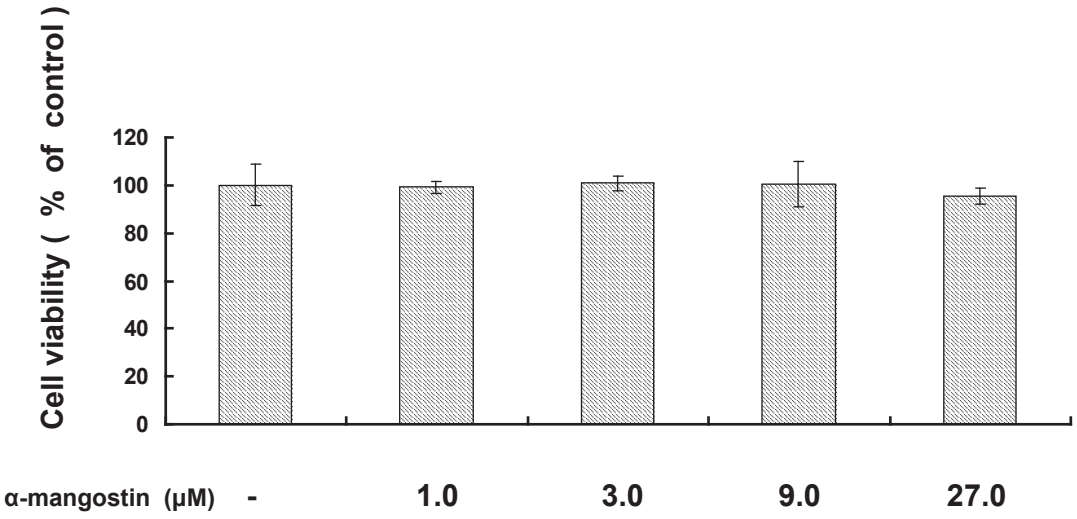


Fig. 2 Cell viability of HSC-T6 cells that were exposed to α-mangostin.

Serum-free-passaged HSC-T6 were exposed to α-MG (1.0 - 27.0 μM) and incubated for 24 h. MTT assay was used to determine number of viable cells. Data are expressed as mean ± SEM from three independent experiments. * $p < 0.05$ indicates significant difference from control.

Rat liver fibrosis induced by intraperitoneal injection of CCl_4

Circulating liver function enzymes, serum AST and ALT, were used as biochemical markers in the monitoring of hepatic injury following CCl_4 treatment and herbal therapy. Approximately 0.3 ml of blood was collected from the tail vein before treatment with CCl_4 and at the second, fourth, sixth and eighth weeks thereafter. Figure 3 indicates that the liver serum AST and ALT activities were significantly higher in the CCl_4-treated group than in the normal control group (naïve group). MG significantly reduced the activities of AST and ALT in serum. Statistical analysis revealed that 100 mg/kg MG significantly inhibited the increase in both AST and ALT in the sixth week, relative to those of the solvent (vehicle) control group; in contrast, the 200 mg/kg Silymarin group exhibited a significantly increased AST value in the fourth week (Fig. 3).

Effect of MG on CCl_4-induced liver fibrosis in rats

In this investigation, rat liver fibrosis was induced by the intraperitoneal injection of CCl_4 in olive oil, to produce generally extensive liver fibrosis, as evidenced

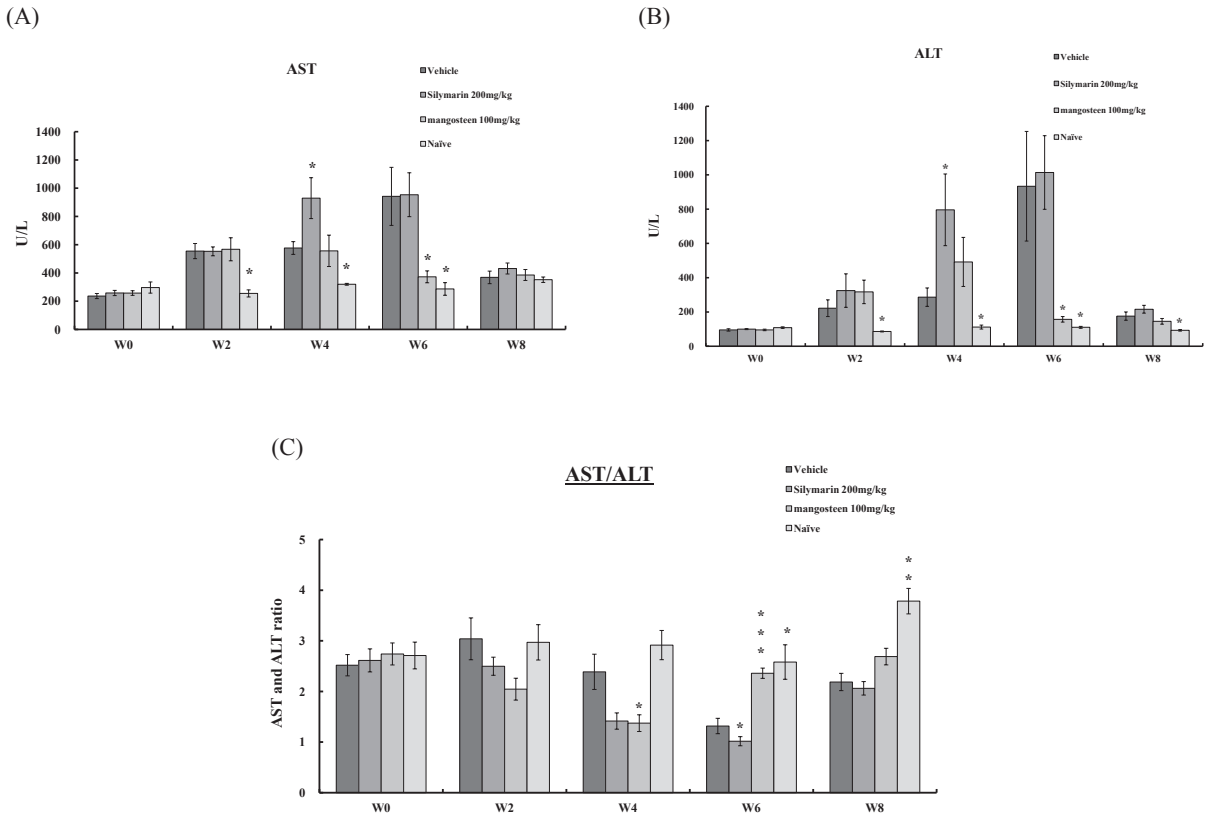


Fig. 3 Biochemical levels of α-MG in CCl_4-induced liver fibrosis in rats.

(A) Effect of α-MG on plasma alanine aminotransferase (AST) levels. (B) Effect of α- MG on plasma aspartate aminotransferase (ALT) levels. (C) Effect of α-MG on plasma AST/ALT ratio. Data are presented as mean ± SD (n = 8). Comparisons are made with vehicle group: ***p<0.001, *p<0.05.

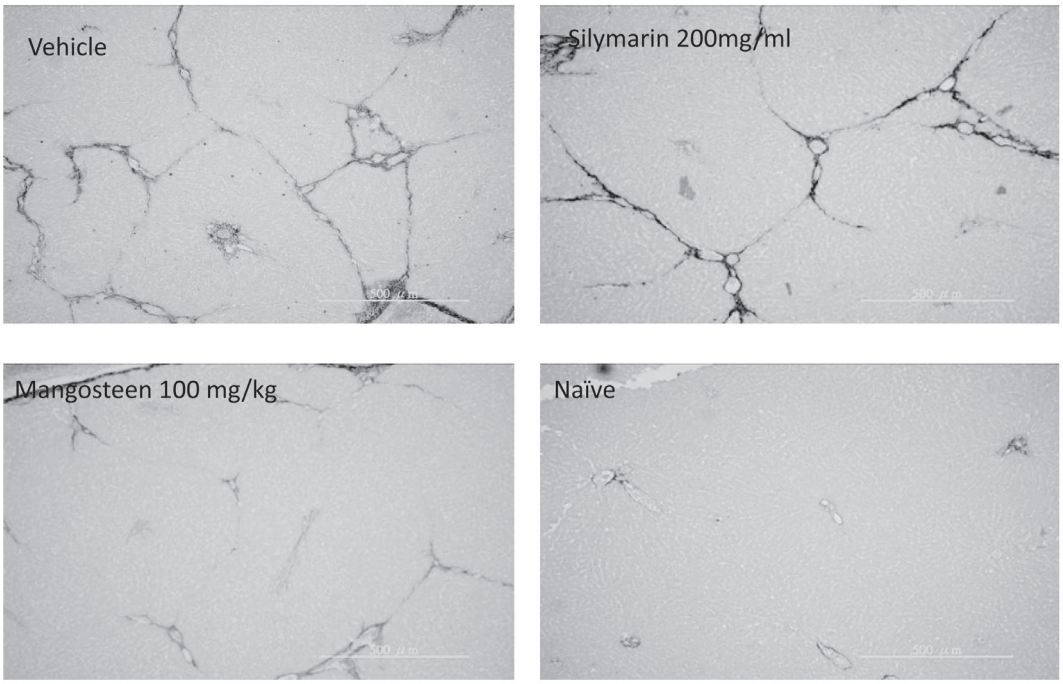
by both qualitative and quantitative histopathological examinations. Figure 4A shows representative photographs of liver morphology.

CCl_4-induced fibrosis was evidenced by extension of fibers, *pseudo* lobe separation and collagen accumulation, identified by comparison with normal rat liver morphology. The treatment of CCl_4-intoxcated rats with MG significantly reduced CCl_4-induced fibrosis and collagen accumulation below those exhibited following treatment with silymarin or vehicle. The MG-treated group of CCl_4-treated rats had a lower liver fibrosis scores at the eighth week (Fig. 4B) than the CCl_4 and the group that was treated with 200 mg/kg silymarin.

Discussion

The antifibrotic targets in liver fibrosis include (1)reduction of inflammation and tissue injury (Hepatoprotectants); (2)prevention of HSC activation and proliferation; (3)reduction of fibrogenesis (by inhibiting angiotensin system, reducing the extent of TGF-β synthesis); (4)stimulating HSC-MFB apoptosis, and (5)promoting ECM degradation.[3] Liver fibrosis is always precede by inflammation and oxidative stress. Therefore, many agents that attenuate or

(A)



(B)

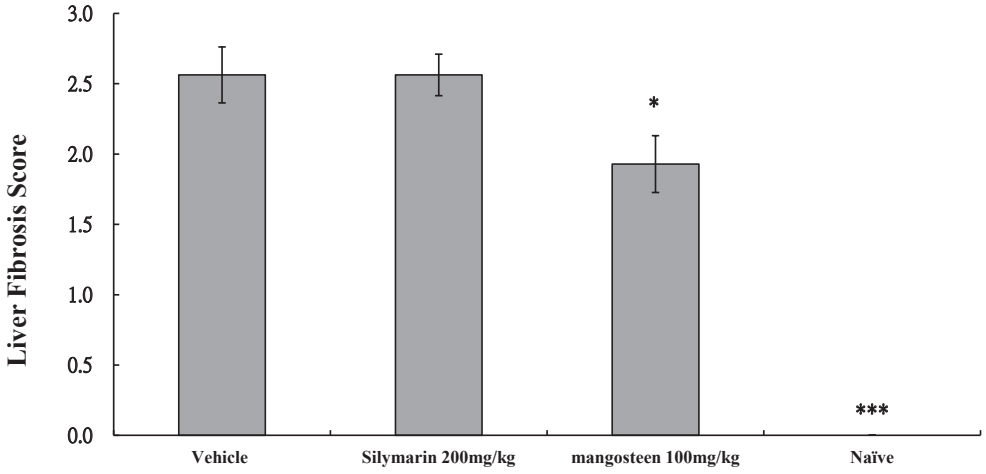


Fig. 4 Liver fibrosis prevention effects of MG in CCL_4 animal models.

Rats were treated with CCl_4 (0.4 ml/kg) twice weekly for eight weeks.

(A)Histological analysis of liver sections. Liver tissues were stained with 0.1% Sirius Red and 0.1% Fast Green for one hour. (B) Liver Fibrosis Score. Data represent the number of rats that had a given level of hepatic fibrosis. Liver fibrosis was graded using the method of Metavir. The grades are as follows: 0, normal; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; and 4, cirrhosis. *$p < 0.05$, ***$p < 0.001$: indicates significant difference from the vehicle.

neutralize upstream inflammatory responses, HSC activation, have been investigated *in vitro* and *in vivo*.[3] Some complementary medicinal agents, such as silymarin (from milk thistle), curcumin (from turmeric), resveratrol (from red wine), and coffee, have antioxidant effects, are generally safe, and are widely consumed, although controlled trials in humans are inadequate.[3,11-13]

Mangosteen is traditionally well known for its anti-inflammatory properties, it is used in the treatment of skin infections and wounds. Two xanthones, α- and γ-mangostins, were isolated from the fruit hull of mangosteen, and both significantly markedly inhibited the production of nitric oxide (NO) and prostaglandin 2 (PGE2) in LPS-stimulated RAW264.7 cells.[17-20] Prenylated xanthones that were isolated from mangosteen also exhibited antioxidant and free radical scavenging effects.[21]

In our earlier studies the inhibitory effects of α-MG on inflammatory cytokines were evaluated by measuring the amounts of secreted TNF-α and IL-4 in LPS-stimulated U937 cells that had been treated with α-MG. We demonstrated that α-MG attenuates the LPS-mediated activation of MAPK, STAT1, c-Fos, c-Jun and EIK-1, inhibiting the production of TNF-α and IL-4 in U937 cells. α-MG reduced gene expressions in oncostatin M (OSM) signaling via mitogen-activated protein kinase (MAPK) pathways, that involve extracellular signal-regulated kinases, c-Jun N-terminal kinase , and p38.[8] However, the OSM-R knockout mice exhibit delayed hepatocyte proliferation, persistent liver necrosis, and increased tissue destruction following CCl_4 treatment. Additionally, OSM reduces CCl_4-induced acute liver failure in wild type mice.[22] These results suggest that OSM, like HGF, has an important role in liver regeneration.

α- and γ-mangostin (antifibrotic constituents from *Garcinia mangostana*) inhibited HSC-T6 viability and significantly reduced collagen content.[23] In this study, α-MG was found to attenuate the PDGF, TNF-α, TGF-β_1, and LPS-stimulated expressions of α-SMA protein in HSC-T6 cells. In the *in vivo* investigation, the 8-week treatment of MG that contained about 82.1% α-MG and 9.0% γ-mangostin reduced hepatic fibrosis scores and plasma AST and ALT levels associated with hepatic injury. Taken together, the results herein suggest that α-MG and γ-mangostin, the two major phenolic compounds that were extracted from mangosteen, reduced hepatic fibrosis that was induced by CCl_4 in a rat model. These results suggest that the mechanism of action may involve anti-inflammatory activity and inhibition of the function of HSC. These results provide a basis for the use of MG to treat hepatic fibrosis or as a prevent nutrient.

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Disclosure of Interest

The authors declare that they have no conflicts of interest concerning this article.

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List of Abbreviations

ALT	serum alanine aminotransferase
AST	aspartate aminotransferase
α-MG	α-mangostin
MG	mangoteen extract (α-mangostin rich extract)
α-SMA	α--smooth muscle actin
TGF-β_1	transforming growth factor-β_1
LPS	lipopolysaccharide
PDGF	platelet-derived growth factor
TNF-α	tumor necrosis factor
ELISA	Enzyme-linked immunosorbent assay
Elk-1	Ets-like molecule 1
ERK1/2	Extracellular signal-regulated kinases 1 and 2
IKK	IκB kinase
IL	Interleukin; iNOS, Inducible NOS
MAPK	Mitogen activated protein kinase
OSM	Oncostatin M
PGE2	Prostaglandin E2
STAT1	Signal transducers and activators of transcription-1

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山竹素對老鼠星狀細胞生成 α-SMA 之抑制及其對老鼠肝纖維化之預防作用

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肝發炎及氧化之壓力常為肝纖維化之導因。文獻顯示山竹素具有抗發炎、抗氧化及自由基清除作用。本研究探討山竹萃取物主要活性成份 α- 山竹素在老鼠肝星狀細胞 (HSC-T6) 的作用，進而評估山竹提取物在四氯化碳誘發老鼠肝纖維化之作用。

結果顯示，α- 山竹素可以降低所有纖維化刺激因子（包括：血小板原性生長因子、腫瘤壞死因子 -α、轉變增長因子 -β_1 和脂多醣）在 HSC-T6 細胞的刺激所導致 α-SMA 的生成，尤其是 LPS。在老鼠四氯化碳誘發肝纖維化動物試驗上，經口先期投與 100 mg/kg 山竹提取物可以明顯抑制血清 ALT、AST 的增加並可以顯著降低纖維化的程度。此結果看出 α- 山竹素可以降低發炎所引起的肝纖維化。

山竹提取物降低肝纖維化的機制應該是由山竹素之抑制纖維生成過程、抗發炎和抑制 HSC-T6 細胞系功能之綜合結果。

關鍵字：α- 山竹素、肝纖維化、老鼠肝星狀細胞、α- 平滑肌肌動細胞、四氯化碳

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