**Graptopetalum Paraguayense Ameliorates Chemical-Induced Rat Hepatic Fibrosis In Vivo and Inactivates Stellate Cells and Kupffer Cells In Vitro**

Li-Jen Su1, Chia-Chuan Chang2, Chih-Hsueh Yang3, Shur-Jong Hsieh3, Yi-Chin Wu3, Jin-Mei Lai4, Tzu-Ling Tseng5, Chi-Ying F. Huang6, Shih-Lan Hsu3*

1 Institute of Systems Biology and Bioinformatics, National Central University, Jhongli City, Taiwan, 2 Division of Medicinal Chemistry, National Research Institute of Chinese Medicine, Taipei City, Taiwan, 3 Department of Education and Research, Taichung Veterans General Hospital, Taichung City, Taiwan, 4 Department of Life Science, Fu Jen Catholic University, Taipei County, Taiwan, 5 Biomarker Technology Development Division, Biomedical Technology and Device Research Labs, Industrial Technology Research Institute, Hsinchu, Taiwan, 6 Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan

**Abstract**

**Background:** Graptopetalum paraguayense (GP) is a folk herbal medicine with hepatoprotective effects that is used in Taiwan. The aim of this study was to evaluate the hepatoprotective and antifibrotic effects of GP on experimental hepatic fibrosis in both dimethylnitrosamine (DMN)- and carbon tetrachloride (CCl4)-induced liver injury rats.

**Methods:** Hepatic fibrosis-induced rats were fed with the methanolic extract of GP (MGP) by oral administration every day. Immunohistochemistry, biochemical assays, and Western blot analysis were performed. The effects of MGP on the expression of fibrotic markers and cytokines in the primary cultured hepatic stellate cells (HSCs) and Kupffer cells, respectively, were evaluated.

**Results:** Oral administration of MGP significantly alleviated DMN- or CCl4-induced liver inflammation and fibrosis. High levels of alanine transaminase, aspartate transaminase, bilirubin, prothrombin activity and mortality rates also decreased in rats treated with MGP. There were significantly decreased hydroxyproline levels in therapeutic rats compared with those of the liver-damaged rats. Collagen I and alpha smooth muscle actin (α-SMA) expression were all reduced by incubation with MGP in primary cultured rat HSCs. Furthermore, MGP induced apoptotic cell death in activated HSCs. MGP also suppressed lipopolysaccharide-stimulated rat Kupffer cell activation by decreasing nitric oxide, tumor necrosis factor-α and interleukin-6 production, and increasing interleukin-10 expression.

**Conclusions:** The results show that the administration of MGP attenuated toxin-induced hepatic damage and fibrosis in vivo and inhibited HSC and Kupffer cell activation in vitro, suggesting that MGP might be a promising complementary or alternative therapeutic agent for liver inflammation and fibrosis.

**Introduction**

Hepatic fibrosis, a precursor of cirrhosis, is characterized by an excessive generation of extracellular matrix constituents (particularly collagens) that impair normal function with progression of liver disease to cirrhosis [1]. Liver fibrosis can result in the development of liver carcinoma and the mortality of patients with liver fibrosis is gradually increasing [2]. Numerous agents, including corticosteroids, penicillamine, methotrexate, silymarin and colchicines, have been used in the therapy of hepatic fibrosis, but there is no definitive treatment [3,4,5,6]. An effective therapy for treatment of patients with hepatic fibrosis is thus urgently needed.

Herbal medicines that have been used in China for thousands of years are now being manufactured in China as drugs with standardized quality and quantity of ingredients. Over the past several decades, there has been a growing trend in Western countries to use herbal medicines to treat a wide range of diseases, including obesity, insomnia, eczema, arthritis, immunodeficiency syndrome, inflammatory diseases, and chronic liver diseases [7]. *Graptopetalum paraguayense* (GP) is a Crassulacean acid metabolism plant with antioxidative and antiproliferative activities [8,9] and is commonly used as a health food in Taiwan. It is considered to have potentially beneficial effects in hypertension, diabetes, hyperuricemia, and chronic liver diseases [10]. However, there
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is no definitive experimental or clinical evidence for the efficacy of GP in the treatment of these diseases.

Our previous study indicated that a concentration of 70% methanolic extract of GP (MGP) was the most effective for liver protection. The present study was initiated to examine the effects of MGP on hepatic fibrosis in vivo through induction by dimethylnitrosamine (DMN) and carbon tetrachloride (CCl₄) in rats. The effects of MGP on the myofibroblast transformation of rat hepatic stellate cells (HSCs) and Kupffer cell activation in primary culture were also investigated. The results indicate that the oral administration of MGP attenuated collagen deposition in toxin-induced liver fibrosis in rats. MGP also inhibited the proliferation of activated HSCs and reduced collagen and alpha smooth muscle actin (α-SMA) expression. Moreover, MGP was shown to be capable of modulating lipopolysaccharide (LPS)-stimulated tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), and nitric oxide (NO) production in Kupffer cells.

Our findings indicate that the protective effects of MGP against liver injury likely involve multiple mechanisms, including an anti-inflammatory effect through decreased TNF-α, IL-6 and NO production in LPS-stimulated conditions as well as an antifibrogenic effect that is mediated by inhibiting the activation of cell transformation and by the induction of apoptosis in HSCs. These observations strongly suggest that MGP might have therapeutic potential for treatment of liver fibrosis and chronic liver disorders.

Materials and Methods

Ethics Statement
All animal work has been conducted according to relevant national and international guidelines. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Taichung Veterans General Hospital. IACUC Approval Number is La-97475. Period of Protocol was valid from: 08/01/2008 to 07/31/2009. The Principle Investigator (PI) is Shih-Lan Hsu.

Preparation of MGP
The leaves of GP were purchased from a local herb farm in Taiwan and were washed with distilled water, air-dried overnight, and then freeze-dried at −50°C with a freeze dryer and ground to a powder (100 mesh). Lyophilized GP powder was stored at 4°C until use. The method for preparing MGP was modified based on the procedure described by Wang et al. [11] MGP extract was prepared as follows: lyophilized GP powder (100 g) was dissolved in 70% methanol (1000 ml). After centrifugation at 1400 g for 20 min, the resulting precipitates were discarded and the supernatant was filtered through a 0.22 μm filter. The filtrate was evaporated to dryness on a rotary evaporator and then lyophilized. The dried methanolic extract powder of GP was stored at 4°C until use.

Preparation of Liver Injury and Fibrosis Model Rats by DMN and CCl₄
Male Sprague-Dawley rats, weighing 250 to 300 g, were purchased from the National Laboratory Animal Breeding and Research Center, National Science Council, Taiwan. All experiments were performed in accordance with The National Laboratory Animal Breeding and Research Center's guidelines. Liver injury and fibrosis model rats were produced by the administration of DMN (Sigma, USA) as previously reported [12] with minor modification. For the DMN-induced injury model, rats were intraperitoneally (i.p.) injected with DMN diluted in phosphate-buffered saline (PBS) (7 mg/kg per day) on the first 3 days each week for 3 weeks to cause liver injury and fibrosis. The control groups were injected with PBS alone. Fifty-four rats were randomized into three experimental groups (n = 18 in each group) as follows: (1) Control (injection of PBS i.p. and oral administration of water); (2) DMN (injection of DMN i.p. and oral administration of water); (3) DMN+MGP (injection of DMN i.p. and oral administration of MGP). MGP was suspended in water and administered orally once every day at a dose of 400 mg/kg for 6 weeks, starting on the 6th day after the first injection of DMN. The 6th day means that there was a 7-day period after the first DMN injection and on the following day, i.e., the 6th day, MGP was given. Control rats received distilled water alone.

For the CCl₄-induced liver injury model, rats were randomly sorted into three groups (n = 20; control, model group (CCl₄), and CCl₄+MGP group). Each group, except the control group, received an oral dose of CCl₄ (1 ml/kg body weight) (Panreac Quimica SAU, Spain) twice a week (40%, diluted in olive oil) for 10 weeks [13]. MGP was suspended in water and was administered orally once per day at a dose of 400 mg/kg for 9 weeks, starting on the 8th day after the first treatment of CCl₄. Control rats received only distilled water. At the end of the in vivo experiments, blood samples were taken to determine biochemical indicators. The livers were excised, weighed and fixed in formaldehyde for histopathological examination. Protocols for these studies were reviewed by the Ethics Committee on Animal Experimentation of Taichung Veterans General Hospital.

Histopathological and Biochemical Examination of the Liver
MGP was orally administered to toxin-treated rats and the effects on liver fibrosis were evaluated by histopathological examinations of hepatic fibrosis with hematoxylin/eosin staining, Sirius red-Fast green staining, and computerized score of hepatic fibrosis. Liver specimens were fixed with phosphate-buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. The fibrosis scoring system was modified from the Histology Activity Index (HAI) [14,15], includes necroinflammatory and fibrosis as previously described [16]. Three representative images of each histology sample section (at 100× magnification) of each rat have been selected randomly and scored. The differential staining of collagenous and noncollagenous proteins was performed with 0.1% Sirius red and 0.1% Fast green as a counterstain in saturated picric acid. In this procedure, collagen is stained red [17]. Liver tissue sections were photographed using AXIO, Imager. A1 microscope (ZEISS, Germany). The content of collagen fibers was estimated by Image-Pro Plus 5.0. Biochemical determinations of hydroxyproline content in liver and serum biochemical markers including alanine transaminase (AST), aspartate transaminase (ALT), bilirubin, albumin, prothrombin time, and platelet number were also performed using commercial kits (Wako Inc., Japan).
Immunocytochemical Staining for Collagen I and α-SMA

To detect collagen I and α-SMA in cultured HSCs, immunocytochemical staining was performed using anti-rat collagen I and α-SMA antibodies (Calbiochem-Merck, USA) and a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, USA). Photomicrographs were taken using a fluorescence microscope.

Immunohistochemical Staining for TNF-α and IL-6

Hepatic staining for TNF-α and IL-6 was examined by immunohistochemical procedure. Briefly, the sections were deparaffinized and treated with 3% hydrogen peroxide to inactivate endogenous peroxidases. Epitope unmasking was performed by immersing sections in antigen retrieval solution A (BD Pharmingen, USA) and heating to 120°C for 20 min. After cooling, blocking with 10% fetal bovine serum at room temperature for 30 min followed by the sequential application of mouse anti-rat TNF-α and IL-6 antibodies, biotin-conjugated goat anti-mouse immunoglobulin G, and streptavidin-conjugated horseradish peroxidase for 30 min. Finally, the sections were incubated in diamobenzidine according to the manufacturer’s instructions (Vector Laboratories, USA) and counter-stained with hematoxylin.

Determination of Hydroxyproline Content

Livers were collected at the moment of sacrifice, and 150 mg of liver tissue was subjected to acid hydrolysis to determine the amount of hydroxyproline according to a procedure described elsewhere [18] with some modifications. Briefly, 2 ml of homogenized liver tissue was hydrolyzed by adding 3 ml of 10 N HCl and then incubated at 110°C for 16 h. After cooling, the hydrolysate was filtered through a 0.45 μm filter. A mixture of 1 ml of 6 N NaOH and 1 ml of citrate buffer was added to 1 ml hydrolysate and mixed well, and then, 1 ml of 0.05 M chloramine-T in citrate-acetate buffer (pH 6.0–6.5) in the presence of n-propanol (1.32 M) was added to the mixture. The solution was thoroughly mixed and incubated for 20 min at room temperature. The reaction was then stopped by adding 1 ml perchloric acid/p-dimethylaminobenzaldehyde solution in n-propanol (32.5 ml 70% perchloric acid, 18.75 g dimethylaminobenzaldehyde, 75 ml n-propanol) and then incubated at 60°C for 30 min. After cooling, the absorbance was determined by a spectrophotometer at 550 nm. The concentration of hydroxyproline was calculated using a standard curve. Further purification of the cells was achieved by attachment to the plastic plates for 2–3 h. Medium was renewed after 3 h and on the following day. Cells were then used on the third day. Kupffer cells were identified by their ability to phagocytose 1 μm latex particles and by their staining with ED2. Cells isolated in this way were 90% Kupffer cells and less than 95% viable (Trypan blue exclusion).

TUNEL Assay

The TUNEL assay was performed according to the manufacturer’s protocol (Boehringer Mannheim, Roche). Briefly, rat activated HSCs were treated without or with MGP (400 μg/ml) for 72 h. After incubation, cells were fixed with 4% paraformaldehyde, incubated in reaction buffer (34 mU/ml terminal transferase, 280 pmol of dATP, 90 pmol of fluorescein-11-dUTP, 30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM CaCl2, pH = 7.2) at 37°C for 60 min in the dark, and then rinsed twice with PBS. Cells were visualized using a fluorescence microscope. TUNEL positive cells were counted as apoptotic.

Caspase Activity Assay

Caspase-2, caspase-3, caspase-8 and caspase-9 activities were estimated according to the manufacturer’s protocol. Briefly, cell lysates (100 μg total protein) were added to reaction mixtures (final volume 50 μL) containing fluorogenic peptide substrate specific for each caspase. The reaction was performed at 37°C for 2 h. Fluorescence intensity was measured with a fluorescence microplate reader (Thermo Labsystem, Finland) (excitation wavelength 400 nm, emission wavelength 505 nm). Caspase-2 inhibitor (Z-VDVAD-fmk), caspase-3 inhibitor (Z-DEVD-fmk), caspase-8 inhibitor (Z-IETD-fmk), and caspase-9 inhibitor (Z-LEHD-fmk) were obtained from Kamya (Thousand Oaks, USA).
Protein Preparation and Western Blot Analysis

HSCs were treated without or with 400 μg/ml MGP for 48 hrs. After treatment, cells were lysed, and total protein was isolated. The protein content was determined by the Bradford method. For Western blot analysis, equal amounts of total protein were loaded onto SDS-polyacrylamide gels, and the proteins were electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA). The protein expression was detected by each immunoblotting with the corresponding specific primary antibodies (against collagen I, α-SMA, B-cell CLL/lymphoma 2 (Bcl-2), myeloid cell leukemia sequence 1 (Mcl-1), Bcl2-associated X protein (Bax), and β-actin) at room temperature for 4 hrs. After washing three times with TBST, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 hr. The membrane

Figure 1. Effects of MGP on liver fibrosis in rats following DMN administration. (A) Schematic illustration for in vivo experiments. Each Sprague-Dawley rat was either injected with DMN three times per week for three consecutive weeks (triangle) or injected with normal saline as a control under the same regime. Subsequently, MGP (400 μg/ml) was suspended in water and administered orally from day 8 until day 49 (black column). Rats were weighed and sacrificed 7 weeks after first DMN injection. (B) Survival analysis. The death of rats was recorded every week and used to calculate the survival rate. Livers were excised and fixed in formaldehyde for (C) histopathological examinations. The rat liver sections were stained with hematoxylin-eosin and Sirius red-Fast green. The original magnification was 40×. (D) Collagen content. The collagen areas were calculated in eight fields independently, and the extent of fibrosis in each field was expressed as the area of collagen as a percentage of the total area of the field. There was a significant difference between the DMN and the DMN+MGP groups. (E) Hydroxyproline content. The level of hydroxyproline in the liver tissue was measured as described in the Materials and Methods section. Significance under p-values<0.05, 0.001 and 0.0001 are marked with *, ** and ***, respectively.
doi:10.1371/journal.pone.0053988.g001
was washed, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA).

**TNF-α and IL-6 Measurement**

The concentrations of TNF-α and IL-6 in the medium were determined by ELISA kits OptEIA Set (BD Biosciences, USA) that were specific against rat cytokines. Assays were performed according to the manufacturer’s instructions.

**Nitrite Measurement**

The nitrite concentration in the medium was measured using the Griess reaction, and the calculated concentration was taken as an indicator of NO production. The medium of cell cultures was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in water). The optical density at 550 nm (A_{550}) was measured and calculated against a sodium nitrite standard curve.

**Statistical Analysis**

All data are shown as the mean±S.D. from at least 3 independent experiments. Figures were obtained from at least three independent experiments with similar patterns. The biochemical experimental data were analyzed by two-way ANOVA. When the interaction was significant, Tukey’s multiple-comparison test was performed. Survival analyses were conducted using the Kaplan-Meier method. Comparisons were made using one-way ANOVA followed by the Mann-Whitney test. All analyses were performed with SPSS software version 13.0 (SPSS Inc., USA).

**Results**

**Effects of GP Methanolic Extract on Liver Fibrosis in Rats given DMN**

Repeated administration of DMN or CCl₄ to rats resulted in a reproducible animal model of liver fibrosis [21,22] and enabled us to examine the hepatoprotective effects of MGP on DMN- and CCl₄-induced chronic liver injury in rats. The schematic illustration for in vivo DMN-induced hepatic fibrosis experiments is shown in Fig. 1A. After 6 weeks of treatment, the liver damage and fibrosis-improving effects of MGP were assessed according to changes in body, liver and spleen weights, the levels of ALT, AST, bilirubin, and albumin, prothrombin activity, and platelet number, as well as liver hydroxyproline content and fibrosis area. As shown in Table 1, treatment with DMN caused a significant decrease in rat body and liver weights, and increased spleen weight compared with those of the control rats. By comparison, oral feeding of MGP significantly increased body and liver weights and decreased spleen weight. Moreover, the survival rates in the DMN-treated and DMN-MGP-treated groups were 61.1% (11 of 18 rats survived) and 94.4% (17 of 18 rats survived), respectively. No rats died in the control group (Fig. 1B).

Serum biochemical marker determinations showed that the serum AST, ALT and bilirubin levels and the prothrombin time increased, whereas the serum albumin and platelet levels decreased in DMN-treated rats compared with those of the normal controls (Table 2). However, oral feeding with MGP effectively reduced DMN-induced elevation of AST, ALT, bilirubin, and prothrombin time. DMN-mediated decline in serum albumin and platelet number was increased by MGP administration.

The effect of MGP on liver fibrogenesis was investigated by hematoxylin-eosin staining. We found that administration of DMN induced centrilobular and periportal deposition of fibers, and continuous fibrotic septa were observed between the central and portal veins (Fig. 1C, upper panel). Treatment with MGP thus obviously suppressed fibroid deposition. Data from Fast green-Sirius red staining exhibited a gradual and marked increase in collagen fiber content in the livers of DMN-treated rats, displaying bundles of collagen (stained in red) surrounding the lobules. This resulted in large fibrous septa, indicating the onset of severe fibrosis (Fig. 1C, lower panel). However, the thickening of these collagen bundles was reduced drastically in the DMN+MGP group. Furthermore, there were very low levels of collagen deposition in the livers of control rats (lower panel of Fig. 1C).

The content of collagen in liver tissue was measured by computer-assisted morphometric analysis of liver sections. The results indicated that DMN-induced fibrotic livers had higher levels of collagen than those of the control rat livers. In rats fed with MGP after administration of DMN, collagen levels were decreased significantly (Fig. 1D). Next, hepatic collagen was also quantified by the measurement of hepatic hydroxyproline content, which parallels the extent of fibrosis. Similar results were found in the DMN group which had significantly higher hydroxyproline content than that of the DMN+MGP group (DMN, 276±67 μg/g vs. DMN+MGP, 60±26 μg/g, p<0.01) and the control group (29±5 μg/g, p<0.001) (Fig. 1E).

**Effects of GP Methanolic Extract on CCl₄-induced Liver Fibrosis in Rats**

The CCl₄-induced liver injury and fibrosis experimental schedule is presented in Fig. 2A, where the CCl₄ was repeatedly administered (1 ml/kg, 2 times per week) and orally fed to the rats with or without MGP for 9 weeks. All of the rats in the control group survived during the experimental period. The survival rate of the CCl₄-treated group and the CCl₄-MGP-treated group was 80% (16 of 20 rats survived) and 90% (18 of 20 rats survived), respectively (Fig. 2B). The influences of CCl₄ and MGP on rat body, liver, and spleen weights are depicted in Table 3. As can be seen, the body weight of CCl₄-administered rats significantly decreased, but treatment with MGP exhibited a significant increase in body weight when compared with that of the CCl₄-administered group. MGP also reversed CCl₄-induced spleno-megaly.

As shown in Table 4, the serum AST, ALT and bilirubin levels as well as the prothrombin time were increased, whereas the albumin and platelet levels decreased in CCl₄-treated rats. Oral administration of MGP significantly reduced the serum levels of AST, ALT, and bilirubin while the serum level of albumin increased. In addition, treatment with CCl₄ drastically reduced

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**Table 1. Body, liver, and spleen weights in DMN- and/or MGP-treated rats.**

| Group       | n | Body | Liver | Spleen |
|-------------|---|------|-------|--------|
| Control     | 18| 543.0±47.4 | 26.6±1.2 | 1.0±0.0  |
| DMN         | 11| 473.8±39.0 | 17.8±3.2 | 2.6±0.4  |
| DMN+MGP     | 17| 513.1±39.1 | 22.2±2.2 | 1.2±0.2  |

*a* number of surviving rats.

Data expressed as mean±S.D.

Values in the same column with different superscripts are significantly different (p<0.05).

doi:10.1371/journal.pone.0053988.t001
serum platelet number and prolonged the prothrombin time. Co-administration of MGP dramatically reversed the CCl4-induced platelet number decrease and prothrombin time elongation.

Representative photographs of liver morphology examined by hematoxylin-eosin staining are shown in Fig. 2C. Compared with normal rat liver morphology, CCl4-treated rat livers showed diffuse fatty tissue and micronodular fibrosis along the central vein and portal area. However, feeding CCl4-injured rats with MGP significantly ameliorated CCl4-induced fibrosis and collagen accumulation. In addition, the degree of CCl4-induced fatty liver and other pathological changes were also mitigated (Fig. 2C, upper panel). Fast green-Sirius red staining results demonstrated that little red staining was observed in the pericentral area in the control rat livers. In contrast, the livers injured by chronic CCl4 treatment displayed an excessive deposition of collagen fibrils. However, MGP treatment clearly suppressed collagen accumulation (Fig. 2C, lower panel). Quantitative analysis of Fast green-Sirius red staining photographs by image analysis showed that hepatic collagen was increased 11-fold as a result of CCl4 exposure and that MGP treatment decreased CCl4-mediated elevation of hepatic collagen by approximately 80% (Fig. 2D).

The biochemical determination of hydroxyproline strongly correlated with the diminished CCl4-induced fibrosis by MGP, as measured by image morphometric analysis (Fig. 2D and 2E). These data suggest that MGP could suppress hepatic fibrosis in chronic liver injury. Collectively, our findings indicate that MGP effectively alleviated fibrogenic progression in the liver following toxic chemical (both DMN and CCl4) administration.

Data from HAI pathological fibrosis scoring analysis indicated that treatment with MGP could significantly reduce DMN- and CCl4-induced hepatic fibrosis (Table 5). Based on our results, DMN indeed caused more severe liver damage than CCl4. DMN caused cirrhosis whereas CCl4 just caused fibrosis. We also found that DMN induced an irreversible liver fibrosis and cirrhosis model while CCl4 induced a reversible liver fibrosis model. Collectively, our findings indicate that MGP treatment decreased CCl4-mediated elevation of hepatic collagen by approximately 80% (Fig. 2D).

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Table 2. Effect of administration of MGP on serum biochemical indicators in DMN-induced liver injury rats.

| Group | AST (U/L) | ALT (U/L) | PT (sec) | PLT (10^9/uL) | Bilirubin (μmole/L) | Albumin (g/dL) |
|-------|-----------|-----------|---------|-------------|------------------|--------------|
| Control | 94.2±15.6a | 39.1±6.5a | 13.0±1.2a | 894.9±157.4d | 0.1±0.0d | 4.4±0.3b |
| DMN   | 520.8±198.8b | 224.7±47.8b | 18.3±6.1b | 269.6±183.4c | 1.0±0.4a | 2.8±0.9b |
| DMN+MGP | 129.1±43.5a | 67.2±18.4b | 13.4±1.0a | 785.9±96.0b | 0.1±0.1a | 4.3±0.5b |

*Data expressed as mean±S.D.
1Values in the same column with different superscripts are significantly different (p<0.05).
2AST: aspartate transaminase; ALT: alanine transaminase; PT: prothrombin time; PLT: number of platelets.

To test whether MGP reversed the myofibroblastic-like activation of transformed HSCs, activated HSCs (over 5 passages) were treated without or with 200 μg/ml MGP. The expression of myogenic markers α-SMA and collagen I was then examined. Data from Western blotting showed that a large amount of α-SMA and collagen I were expressed in the activated HSCs while a significantly reduced expression of these two activated HSC markers was observed after exposure to MGP (Fig. 3C). Moreover, the immunoreactivity of both α-SMA and collagen I was strong in activated HSCs, but treatment with 200 μg/ml MGP for 3 days largely inhibited the expression of these two markers. These observations indicate that MGP suppressed activation of HSC transformation and inhibited α-SMA and collagen I production in primary cultured HSCs.

MGP Induces Apoptosis of Activated HSCs

We also assayed the effects of MGP on the viability of activated HSCs (over 5 passages) and primary cultured hepatocytes. As shown in Fig. 4A, MGP caused a dose-dependent reduction in viability of activated HSCs within 72 h. Conversely, the viability of primary cultured rat hepatocytes did not alter after 72 h of MGP (400 μg/ml) incubation. Morphological investigation of MGP-treated HSCs showed apoptotic features (Fig. 4B). We therefore determined whether apoptosis was a mechanism in MGP-mediated reduced viability of HSCs. The TUNEL assay confirmed substantial genomic DNA fragmentation upon incubation with MGP (400 μg/ml) for 48 h (Fig. 4C). However, at the same dose of MGP, no TUNEL positive cells could be detected in hepatocyte cultures.

To determine the role of caspases in MGP-induced apoptosis, the activities of caspases were measured in control and MGP-treated activated HSCs. As depicted in Fig. 4D, MGP-induced apoptosis of activated HSCs was associated with increased activities of caspase-2, caspase-3, caspase-8, and caspase-9. Moreover, selective inhibitors of caspase-2 (Z-VDVAD-fmk), caspase-3 (Z-DEVD-fmk), caspase-8 (Z-IETD-fmk), and caspase-9 (Z-LEHD-fmk) could effectively attenuate MGP-triggered apoptosis of activated HSCs (Fig. 4E), suggesting that MGP induced caspase-dependent apoptotic cell death in activated HSCs. Since MGP induced both intrinsic and extrinsic caspase activation, the regulation of the Bcl-2 family and molecules in the death receptor family by MGP was examined. As shown in Fig. 4F, treatment with MGP significantly decreased the levels of anti-
apoptotic proteins, Bcl-2 and Mcl-1. In contrast, the proapoptotic molecules, Bax and Fas, were increased in MGP-treated HSCs.

**MGP Suppresses LPS-stimulated Cytokine Production in Kupffer Cells**

Accumulating evidence indicates that the bulk release of inflammatory mediators by Kupffer cells is critical during the early stages of liver inflammation and fibrosis [23]. Both experimental animal studies and clinical investigations have suggested that the endogenous gut-derived endotoxin LPS is an important cofactor in the pathogenesis of liver injury and fibrosis [24,25,26,27]. Therefore, the effects of MGP on LPS-stimulated cytokines and NO production in primary cultured rat Kupffer cells were characterized. The basic levels of TNF-α, IL-6, IL-10, and NO production in rat Kupffer cells were low. However, following 0.1 μg/ml LPS administration, the production of these cytokines in Kupffer cells significantly increased. Treatment with MGP decreased LPS-stimulated NO (Fig. 5A), TNF-α (Fig. 5B), and IL-
We thus hypothesized that MGP may suppress the induced apoptosis in activated HSCs. Moreover, LPS-stimulated HSCs. In addition, MGP treatment inhibited cell growth and a study, MGP prevented activation of transformation and proliferation of early-stage fibroblasts in vitro. These results suggest that MGP might suppress liver inflammation and fibrogenesis partly by inhibiting Kupffer cell activation.

MGP Attenuates the CCl4-induced Acute Hepatic Damage and Inflammation in vivo

The inflammation usually appears to improve fibrosis formation, so we further examined histopathology or immunohistochemistry in a CCl4-induced acute hepatotoxic model. We found that intraperitoneal administration of CCl4 resulted in markedly greater histological evidence of necrosis and inflammation compared to control group. In contrast, oral feeding with MGP blunted Kupffer cell and leukocyte accumulation in necrotic areas (Fig. 6A). Moreover, MGP also effectively reduced the CCl4-stimulated TNF-α and IL-6 expression (Fig. 6B).

Discussion

Chinese herbal medicines have been used over thousands of years on the basis of clinical experience and practice, but the underlying mechanisms in most of these medications have not been characterized. We demonstrated here that the methanolic extract of GP, a hepatoprotective herbal medicine that is used in Taiwan, ameliorated injury and fibrosis in liver caused by toxic chemical (DMN and CCl4) administration in rats. In cellular studies, MGP prevented activation of transformation and suppressed α-SMA and collagen I expression in primary cultured HSCs. In addition, MGP treatment inhibited cell growth and induced apoptosis in activated HSCs. Moreover, LPS-stimulated inflammatory cytokine production in Kupffer cells was attenuated by MGP. We thus hypothesized that MGP may suppress the activation of HSCs and Kupffer cells during the course of liver inflammation and fibrosis, alleviating subsequent liver injury and fibrosis progression.

Accumulating evidence indicates that activated HSC plays a crucial role in the development and resolution of liver fibrosis [28]. Therefore inhibiting activation of HSCs and stimulating their apoptosis could be an effective treatment for liver fibrosis. Here, we found that MGP effectively inhibited the myofibroblast-like activation of isolated rat HSCs and reduced the expression of α-SMA and collagen I in vitro. In addition, treatment with MGP caused a mitochondrial-dependent apoptosis in activated HSCs. Therefore, suppression of myofibroblast-like transformation and induction of apoptotic death in activated HSCs upon MGP treatment may help alleviate chemical (both DMN and CCl4)-induced liver fibrosis in vivo. Inflammation is essential for host defense during liver injury, but an uncontrolled inflammatory response is the common mechanism involved in the majority of clinical liver pathologies, resulting in tissue damage, fibrosis, and cirrhosis [29]. Kupffer cells, the resident hepatic macrophages, play a crucial role in liver inflammation and fibrogenesis [30]. In acute or chronic liver diseases, Kupffer cells and infiltrating monocyte-derived macrophages are well known to promote inflammatory cascades by releasing these pro-inflammatory mediators with consequences such as T cell attraction, induction of hepatocyte apoptosis, and activation of fibrogenic HSCs [29,31]. Therefore, depletion of Kupffer cell function significantly alleviates thioacetamide-induced hepatotoxicity [32]. In addition, selective inhibition of Kupffer cells by administration of either gadolinium chloride or methyl palmitate results in abrogation of liver injury [30,33,34]. Therefore, the factors that control the Kupffer cell response are clearly critical in the progression of liver fibrosis. In this study, MGP exhibited strong anti-inflammatory activity by reducing the production of TNF-α, IL-6 and NO in LPS-stimulated Kupffer cells (Figs. 6A, 6B, and 6C), suggesting that inhibition of proinflammatory cytokines (TNF-α and IL-6) and no production by kupffer cells may contribute to MGP-mediated liver protection.

Increases in anti-inflammatory mediators also may contribute to an anti-inflammatory state in the liver and ameliorate injury. For example, IL-10, an immunomodulatory cytokine with potent anti-inflammatory and immunosuppressive properties, decreases production of pro-inflammatory cytokines, including TNF-α and IL-1β [35]. The ability of IL-10 to modulate the inflammatory response and to limit hepatotoxicity has been shown in several models of liver injury [36,37,38]. Previous reports have indicated that IL-10 has a role in the remodeling of the extracellular matrix. In vivo, IL-10 downregulates collagen I and upregulates metalloproteinase gene expression [39]. IL-10 might also have antifibrogenic properties through downregula-

| Table 3. Body, liver, and spleen weights in CCl4- and/or MGP-treated rats. |
|-----------------|---------------|---------------|---------------|
| Group           | n  | Body (g) | Liver (g) | Spleen (g) |
| Control         | 20 | 691±50    | 31±6.0     | 1.1±0.5*    |
| CCl4            | 16 | 651±71*   | 30±7.7     | 1.4±0.5*    |
| CCl4+MGP        | 18 | 679±64*   | 29±5.0     | 1.0±0.3*    |

*p* number of surviving rats.

Values in the same column with different superscripts are significantly different (*p*<0.05).

doi:10.1371/journal.pone.0053988.t003

| Table 4. Effect of administration of MGP on serum biochemical indicators in CCl4-induced liver injury rats. |
|-----------------|-----------------|---------------|---------------|-----------------|
| Group           | AST (U/L)       | ALT (U/L)     | PT (sec)      | PLT (10⁴/uL)    | Bilirubin (µmole/L) | Albumin (g/dL) |
| Control         | 94.9±27*        | 45.9±18*      | 13.6±0.7*     | 912±167*       | 0.1±0.01*           | 5.0±0.5*        |
| CCl4            | 314±46*         | 282±53*       | 15.3±0.9b     | 767±141*       | 1.1±0.50*           | 3.8±0.3*        |
| CCl4+MGP        | 180±59*         | 132±10*       | 13.0±0.7*     | 1047±203*      | 0.1±0.05*           | 4.9±0.1b* |

*Data expressed as mean±S.D.

Values in the same column with different superscripts are significantly different (*p*<0.05).

AST: aspartate transaminase; ALT: alanine transaminase; PT: prothrombin time; PLT: number of platelets.

doi:10.1371/journal.pone.0053988.t004
of profibrogenic cytokines, like TGF-β1 [40]. Increased endogenous IL-10 expression ameliorates acute inflammatory burst and subsequent liver fibrosis after repeated stimulations with CCl₄ [41]. Endogenous IL-10 synthesized during the course of liver inflammation and fibrosis may modulate Kupffer cell actions, and influence the subsequent progression of fibrosis [42]. Our results also showed that MGP effectively increased IL-10 generation in isolated rat Kupffer cells. Taken together, it is likely that MGP suppressed the activation of Kupffer cells and modulated the expression of cytokines during DMN or CCl₄ intoxication, thereby decreasing inflammation and the subsequent fibrogenic response in the liver.

In this study, we found that MGP was safe and nontoxic for in vitro and in vivo experimental use. Cellular studies demonstrated that MGP had no cytotoxic effect on primary cultured hepatocytes, as examined by morphological investigation and viable cell counting. In addition, our pilot study for MGP toxicity indicated that daily oral administration of a high dose of MGP (1.6 g/kg body weight) for nine months did not cause any apparent adverse side-effects in rats, and there was no evidence of mortality or

| Fibrosis score | DMN model (6th week) | CCl₄ model (9th week) |
|---------------|----------------------|----------------------|
|               | Control n (%)        | DMN n (%)            | DMN+MGP n (%)        |
|               |                      | Control n (%)        | CCl₄ n (%)           | CCl₄+MGP n (%)        |
| 0–1           | 18 (100)             | 3 (19)               | 12 (67)              | 20 (100)             | 7 (35)               | 16 (80)              |
| 2–3           | 0 (0)                | 13 (81)              | 6 (33)               | 0 (0)                | 13 (65)              | 4 (20)               |

Fibrosis was graded as (0–1) for observations ranging from normal tissue to fibrous expansion of portal tracts and (2–3) for bridge fibrosis to frequent bridging fibrosis with nodule formation. The number of rats was counted and used to calculate the percentage of each histopathological level at each time point.

doi:10.1371/journal.pone.0053988.t005

Figure 3. MGP inhibits transformed activation of HSCs. Isolated HSCs were incubated in the presence or absence of 200 μg of MGP for 1, 3, and 7 days. After treatment, (A) the cell morphology was investigated by phase-contrast microscopy, and (B) the expression levels of α-SMA and collagen I after 7 days incubation were examined by immunostaining. (C) MGP suppressed α-SMA and collagen I expression in activated HSCs. The activated HSCs (5 passages) were incubated without or with 200 μg of MGP for 1 and 3 days. After incubation, the expression levels of α-SMA and collagen I were examined by immunostaining. (D) The expression of α-SMA and collagen I after 3 days incubation was investigated by immunostaining.

doi:10.1371/journal.pone.0053988.g003
Figure 4. MGP induces apoptotic death in activated HSCs. (A) Cytotoxic effect of MGP. Activated HSCs were treated with various concentrations of MGP (100, 200, and 400 μg/ml) for 24, 48, and 72 h. Viable cells were measured by the trypan blue dye exclusion method. (B) Morphological changes. HSCs and hepatocytes were incubated with MGP (400 μg/ml) for 72 h. Cell morphology was investigated under an Olympus IX70 phase-contrast microscope. (C) Induction of apoptosis by MGP. The HSCs were maintained in the presence or absence of MGP (400 μg/ml) for 48 h. After treatment, the TUNEL assay was performed as described in the Materials and Methods. The morphology of HSCs was again investigated under a phase-contrast microscope. TUNEL positive cells were considered apoptotic cells. The original magnification was 200×. (D) Activation of caspases by MGP. Activated HSCs were treated without or with MGP (400 μg/ml) for 48 h and the activity of each caspase was then assessed. (E) Inhibitors of caspases protected against MGP-induced apoptosis. Activated HSCs were pretreated with 100 μg/ml caspase inhibitor for 1 h and then treated with MGP (400 μg/ml) for another 72 h. After treatment, apoptotic cell count was assessed by the TUNEL assay. Significance for p-value<0.0001 is marked with ***. (F) Regulation of anti-apoptotic and proapoptotic molecules by MGP. Activated HSCs were treated without or with MGP (400 μg/ml) for 48 h. The levels of Bcl-2, Mcl-1, and Bax were determined by Western blot analysis.

doi:10.1371/journal.pone.0053988.g004
hepatic or renal injury in histological sections of liver and kidney (data not shown). Moreover, these MGP-fed rats did not exhibit any significant changes in body weight, liver weight, or serum ALT, AST, bilirubin, albumin, BUN, and creatinine levels in comparison with the control group (data not shown), suggesting that MGP had no liver or renal toxicity at the tested doses. In Taiwan, the daily intake amount suggested by folk medicine for treatment of chronic liver disorder patients is 200 g/day of fresh GP, this dosage is equal to 55 mg/kg/day of MGP. In our animal study, DMN or CCl₄-injured rats were fed with 400 mg/kg/day of MGP. We translated this dosage from rat to human by the Meeh-Rubner formula and obtained an equivalent human intake amount of 60 mg/kg/day of MGP. These data demonstrate that the daily intake amount suggested by folk medicine is extremely consistent with the translated amount of our animal study (55 mg/kg/day vs. 60 mg/kg/day). Further in vivo and in vitro studies, including the use of different cell types in the liver (e.g., HSCs, Kupffer cells and sinusoidal endothelial cells), will be required to determine the exact molecular pathways affected by MGP, which may be useful in the development of novel treatments for hepatic injury and fibrosis.

In conclusion, GP is a folk herbal medicine with hepatoprotective effects that is used in Taiwan. Our findings showed that the 70% methanol extract of GP prevented fibrogenesis responses induced by toxic chemicals (DMN and CCl₄) in vivo. In cellular studies, GP extract exhibited anti-inflammatory activity via suppressing LPS-stimulated pro-inflammatory cytokines and mediator production in liver macrophage lineage Kupffer cells. Moreover, GP extract effectively suppressed activation of HSC transformation, inhibited cell growth, and induced apoptosis in activated HSCs. Our findings confirm that GP extract has hepatoprotective and anti-fibrotic activities and that it has the

![Figure 5. MGP reduced LPS-stimulated inflammatory cytokines and NO production.](image-url)
potential to become a novel and promising antifibrotic medicine for liver fibrosis. The modes of action and the active components of GP extract require further elucidation.

Acknowledgments
The authors thank the technical supports provided by Core Facilities for High Throughput Experimental Analysis, Institute of Systems Biology and Bioinformatics, College of Science, National Central University.

Author Contributions
Conceived and designed the experiments: LJS CYH SLH. Performed the experiments: CHY SJH. Analyzed the data: CCC YCW. Contributed reagents/materials/analysis tools: JML TLT. Wrote the paper: LJS SLH.

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