A traditional formula, Chunggan extract, attenuates thioacetamide-induced hepatofibrosis via GSH system in rats

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Abstract

Chunggan extract (CGX) is a hepatotherapeutic herbal formula which has been traditionally used for patients suffering from various hepatic disorders. This study aimed to elucidate antifibrotic effect and mechanisms of CGX in thioacetamide (TAA) model. Hepatic fibrosis was induced in 45 Sprague-Dawley rats by TAA (200 mg kg^{-1}, intraperitoneally [ip]) on twice per week for 12 weeks. CGX (100 or 200 mg kg^{-1}, per oral [po]) was administrated once a day throughout the experiment. CGX treatment ameliorated serum biomarkers. CGX administration significantly attenuated distortion of histopathologic finding, and accumulation of hydroxyproline and malondialdehyde (MDA). CGX treatment significantly decreased transforming growth factor-beta (TGF-β) concentrations and inactivated hepatic stellate cells (HSCs). CGX treatment drastically restored glutathione (GSH) system, while inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) significantly down-regulated in liver tissue. CGX showed antifibrotic effect in thioacetamide-induced chronic liver injury model. Its corresponding mechanisms may be mediated via anti-oxidative stress property sustaining GSH system and inhibition of ROS production.

Keywords

liver fibrosis, glutathione system, oxidative stress, hepatic satellite cells, thioacetamide

Introduction

The process of hepatic fibrosis is a critical step deciding clinical outcome of chronic liver diseases. Liver cirrhosis, end-stage of fibrosis, contribute to leading cause of deaths worldwide, 1.3% of total deaths in 2004 and predicted to be 1.2% in 2015.[1] Any chronic liver injuries including alcoholic disorder, viral hepatitis, biliary obstruction, autoimmune-related biliary disorders, and hemochromatosis consequently can lead to fibrosis.[2,3] The progress of liver fibrosis and cirrhosis is evidently accompanied with abnormal liver architecture resulting in severe alternation of intra-/extra-hepatic hemodynamic and finally failure of liver function.[4]

It has been commonly accepted that liver cirrhosis is an irreversible disease and none of therapeutic drug has been established to date.[5] So, realistic strategies are the eradication of the underlying etiology and inhibition of fibrotic progress.[6] Recently, many interests in herbal medicine have been noticed on hepatoprotective or antifibrotics.[7-9]

Chunggan extract (CGX) is a herbal drug with 13 medicinal plants, originated from a traditional formula, Chunggan meaning 'cleaning liver.' It is prescribed for patients with various chronic liver diseases such as alcoholic liver disorders, chronic viral hepatitis, and liver cirrhosis.[10] Its hepatoprotective effect has been demonstrated in carbon tetrachloride

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(CCl4)-induced acute injury model.[11] We also reported antifibrotic effects of CGX using 4-week dimethylnitrosamine (DMN) model.[12,13]

DMN-induced hepatic fibrosis is a well-accepted pre-clinical model, owing to quick development of rigorous fibrosis, but failed to mimic the pathogenesis in human body.[14] On the contrary, thioacetamide (TAA) model is believed to closely follow human characteristic in morphological and biochemical aspects.[15]

Therefore, herein, a long-term TAA-induced fibrosis model (12 weeks) was chosen to confirm the antifibrotic effects of CGX and its potential mechanisms.

Materials and methods
Reagents and chemicals

Thioacetamide (TAA) and other reagents, including silymarin, hydroxyproline, *p*-dimethylaminobenzaldehyde, 1,1,3,3-tetraethoxypropane (TEP), chloramines-T, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione, glutathione reductase (GSH-red), glutathione peroxidase (GSH-px), and β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), were purchased from Sigma (St. Louis, Missouri, USA); perchloric acid was obtained from GFS Chemical Co. (Columbus, Ohio, USA); and thiobarbituric acid (TBA) was purchased from Lancaster Co. (Lancashire, England, UK).

Preparation of CGX

The CGX consists of 13 herbs including 5 g each of Artemisia capillaris Herba, Trionycis Carapax, Raphani Semen; 3 g each of Atractylodis Macrocephalae Rhizoma, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Salviae Miltiorrhizae Radix; 2 g each of Polyporus, Amomi Fructus, Aurantii Fructus, and 1 g of Glycyrrhizae Radix and Helenii Radix. All the herbs used in this formulation are fulfilled with the Korean Pharmacopoeia standards. CGX was manufactured by Samik Pharmacy (Seoul, Korea) using the approved Good Manufacturing Practice (GMP) of the Korea Food and Drug Administration (KFDA) according to over-the-counter Korean monographs. Briefly, 120 kg of CGX was boiled in 1200 L of distilled water for 4 hours at 100°C, and then filtered using a 300-mesh filter (50 μm). Some part was filtered through filter paper (Advantec, Toyo Roshi Kaisha, Tokyo, Japan) and lyophilized in our laboratory for this study. The final extraction gave a yield of 10.71%, and the batch used

in this experiment was stored for future use (voucher specimen No: CGX-2007-09-WE-SI). The final CGX extract satisfied the criteria of the Korean Pharmaceutical Codex, including the quantity of each herb, contamination by heavy metals, general bacteria, fungi and specific pathogens, and the quantity of ingredients.
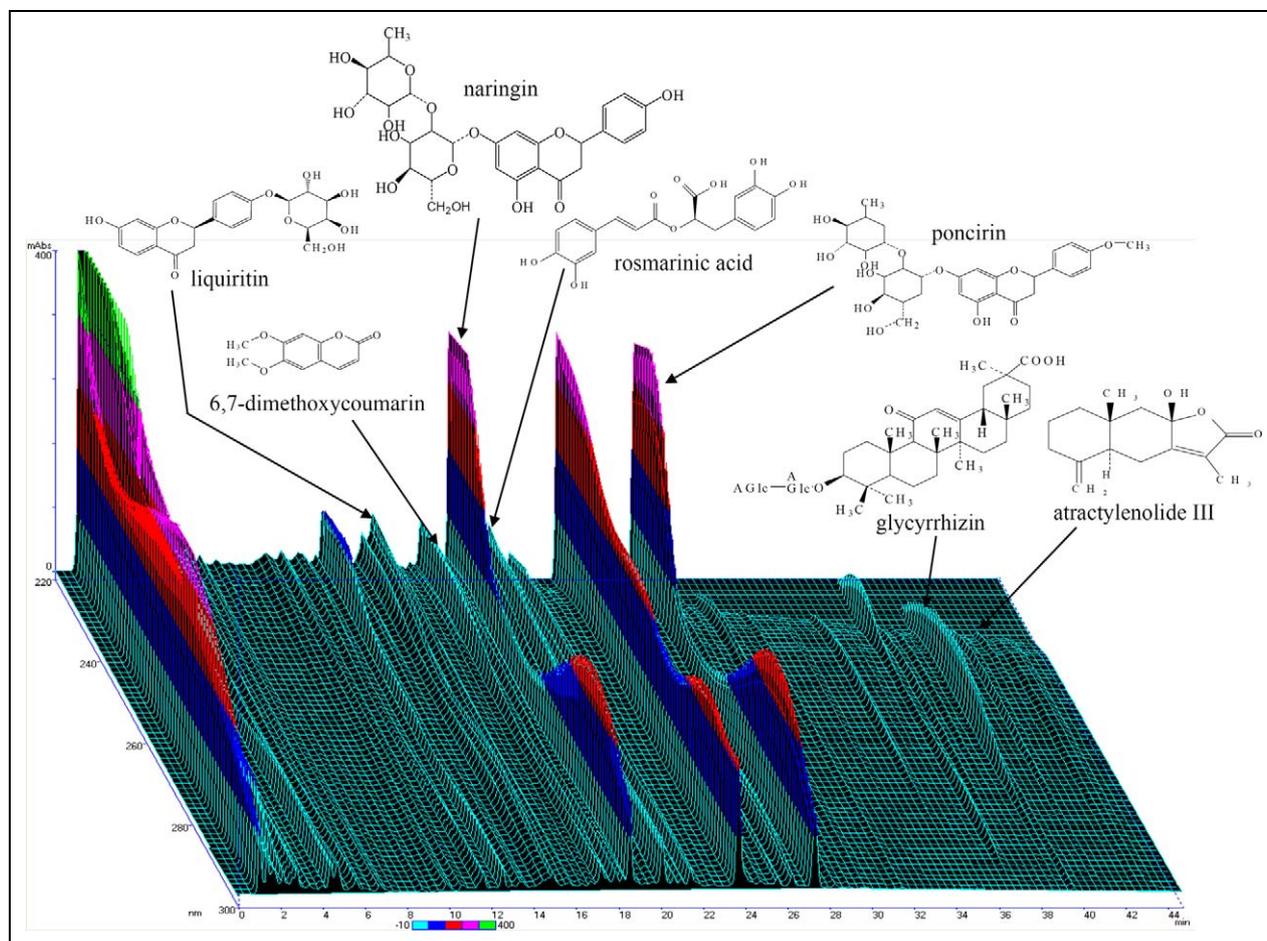
Fingerprint of CGX

Fingerprint for CGX was developed using three-dimensional high-performance liquid chromatography (3D-HPLC) profile of five major compositional herbs and their major compounds: Glycyrrhizae Radix, Artemisiae Capillaris Herba, Atractylodis Macrocephalae Rhizoma, Salvia Miltiorrhizae Radix and Ponciri Fructus vs liquiritin and glycyrrhizin, 6,7-dimethoxycoumarin, atractylenolide III, rosmarinic acid, naringin, and poncirin, respectively. Briefly, after dissolution (20 mg of CGX and 0.01 mg of seven standards in 1 mL of water or 50% methanol) and filtration, these drugs were subjected to HPLC analysis. The HPLC system consisted of SCL-10A system controller, LC-10AD pump, SPD-10MVP diode array detector, and CTO-10AS column temperature controller (Shimadzu, Kyoto, Japan). A phenomenex prodigy C18 (2.0 × 150 mm) column was eluted with solvents A (10% acetonitrile in water containing 0.05% formic acid) and B (90% acetonitrile in water) at flow rate of 0.4 mL min⁻¹. Solutions 100% A and 0% B changing over 30 min to 25% B, 60 min to 75% B were used. The chromatogram were obtained using wavelength of 220 to 300 nm. Standard components of five herbs were detected and three main peaks and several minor peaks were observed (Figure 1).

Animals and experimental schedule

Forty-five, 6-weeks-old Sprague Dawley (SD) male rats were procured from a commercial animal breeder (Orient Bio, Gyeonggi-do, Korea). After 1 week of acclimation in an environmentally controlled room at 22 ± 2°C with a 12/12-hour light/dark cycle with commercial pellets (Orient Bio) and tap water ad libitum, the rats were divided randomly into five groups of nine animals each: Normal, TAA (TAA only), CGX 100 (TAA with 100 mg kg⁻¹ CGX), CGX 200 (TAA with 200 mg kg⁻¹ CGX), and positive control (TAA with 50 mg kg⁻¹ silymarin).

To induce liver fibrosis, TAA (200 mg kg⁻¹) was intraperitoneally (ip) injected twice a week for 12 weeks, to four groups except normal group (normal saline, ip). CGX (100 or 200 mg kg⁻¹), silymarin

Figure 1. Finger print of Chunggan extract (CGX) using Liquid chromatography/Diode array detector (LC/DAD). Dissolution of CGX and six standards were filtrated and subjected to high-performance liquid chromatography (HPLC) analysis. Phenomenex Prodigy C18 column was eluted with solvents A (10% acetonitrile in water containing 0.05% formic acid) and B (90% acetonitrile in water) at flow rate of 0.4 mL min^{-1}. Solutions 100% A and 0% B changing over 30 min to 25% B, 60 min to 75% B were used.

(50 mg kg^{-1}), or distilled water was given by gastric gavage six times per week throughout the experimental period. Body weight was recorded once a week. After last drug administration, animals were fasted for 18 hours, and then blood was collected from abdominal aorta under ether anesthesia. Liver and spleen were removed to determine absolute and relative weight. A portion of liver and spleen tissue stored at −70°C separately were used for hydroxyproline, lipid peroxidation, and antioxidant enzymes determination. Liver tissue fixed in Bouin's solution was processed for histomorphological finding and immunohistochemistry. A small portion of liver tissue fixed in RNAlater solution was stored at −20°C for gene expression studies.

This animal experiment was approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUARB2009-010) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH).[16]

Serum biochemical analysis

Serum was prepared following blood clotting. The serum levels of total protein, albumin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) were determined using an Autoanalyzer (Chiron, Emeryville, California, USA).

Histomorphology and immunohistochemistry for α-smooth muscle actin

After general processing of tissue treatment, paraplast-embedded liver tissues were sectioned (4 μm thick) and stained with hematoxylin and eosin

(H&E) or Masson's trichrome dye for histopathological evaluation.

Immunohistochemistry for α-smooth muscle actin (α-SMA) was also performed to examine the activation of hepatic stellate cells (HSCs). Briefly, liver tissue sections were deparaffinized, hydrated and heated in citrate buffer at 100°C for 15 min, and then treated with normal serum for 30 min. Next, the slides were treated with an anti-α-SMA mouse monoclonal antibody (1:200; Abcam, Cambridge, UK) overnight. After washing three times with PBS, samples were stained with the secondary antibody, N-Histofine Simple Stain MAX PO (Nichirei Biosciences, Tokyo, Japan), and its substrate, diaminobenzidine (BioGenex, San Ramon, California, USA). After counterstaining with Mayer's hematoxylin (Wako Pure Chemical Industries), the slides were examined under an optical microscope (Leica Microsystems, Wetzlar, Germany).

Determination of hydroxyproline in liver tissues

Hydroxyproline determination was performed with a slight modification of a method described previously.[12] Briefly, liver tissues (200 mg) stored at −70°C were homogenized in 2 mL of 6 N HCl and incubated overnight at 110°C. After filtering the acid hydrolyzates using filter paper (Toyo Roshi Kaisha, Tokyo, Japan), 50 µL samples or hydroxyproline standards in 6 N HCl were incubated at 60°C to dry. The dried samples were dissolved with methanol (50 µL); then 1.2 mL of 50% isopropanol and 200 µL of chloramine-T solution were added to each sample, followed by incubation at room temperature for 10 min. Ehrlich's solution (1.3 mL) was added, and the samples were incubated at 50°C for 90 min. The optical density of the reaction product was read at 558 nm using a spectrophotometer. A standard curve was constructed using serial twofold dilutions of 1 mg solution of hydroxyproline.

Determination of malondialdehyde in liver tissues

Lipid peroxidation levels in the liver tissue were determined using the method of TBA reactive substances (TBARS), as described previously.[17] The concentration of TBARS was expressed as µmol per gram tissue using TEP as a standard. Briefly, 200 mg of liver tissue was homogenized in 2 mL of ice-cold 1.15% KCl, and 0.13 mL of homogenate was mixed

with 0.08 mL of 1% phosphoric acid and 0.26 mL of 0.67% TBA. After heating the mixture for 45 min at 100°C, 1.03 mL of n-butanol was added, followed by vigorous vortexing and centrifugation at 3000 rpm for 15 min. The absorbance of the upper organic layer was measured at 535 and 520 nm with a spectrophotometer (Cary 50; Varian, Palo Alto, California, USA) and compared to the value from freshly prepared TEP as a standard.

Determination of catalase, superoxide dismutase, GSH-reductase and GSH-px activity, and total glutathione (GSH) content in liver tissues

Briefly, 100 mg liver tissue was homogenized with RIPA buffer and centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatant fraction was transferred into a clean tube, and used to determine the antioxidant enzymes and protein.

Catalase activity in the liver tissue was determined using the method of Beers and Siezer.[18] The supernatant or standard solution (100 µL) were mixed with 2.9 mL of substrate solution (0.0036% hydrogen peroxide), followed by measurement of the absorbance at 240 nm after 5 min.

Superoxide dismutase (SOD) activity in the liver tissue was determined using a SOD assay kit (Dojindo Laboratories, Kumamoto, Japan). Bovine erythrocyte SOD (Sigma) was diluted serially from 100 to 0.001 U mL^{-1} and used as a standard.

GSH-red and GSH-px activity in liver tissues were determined with GSH-red assay kit (Sigma), and GSH-px cellular activity assay kit (Sigma), respectively.

Total GSH content was determined according to the method of Ellman.[19] Briefly, duplicate 50 µL aliquots of the supernatant (or GSH standard) were combined with 80 µL of a previously prepared DTNB/NADPH mixture (10 µL 4 mM DTNB and 70 µL 0.3 mM NADPH) in a 96-well plate. Finally, 20 µL (0.06 U) of GSH-red solution was added to each well and the absorbance was measured at 405 nm after 5 min.

Determination the level of transforming growth factor-beta (TGF-β), platelet-derived growth factor-beta, connective tissue growth factor, tissue inhibitor of matrix metalloprotease-1 in liver tissues

One hundred milligram liver tissue was homogenized with RIPA buffer and centrifuged at $10,000 \times g$ for 15

min at 4°C. The supernatant fraction was used to determine the level of fibrosis-related cytokines. Transforming growth factor-beta (TGF-β) level was measured using ELISA assay kit (Biosource, Camarillo, California), and platelet-derived growth factor-beta (PDGF-β) and tissue inhibitor of matrix metalloprotease-1 (TIMP-1) level were also determined by ELISA assay kit (R&D system, Minneapolis, Minnesota).

Quantitation of tissue connective tissue growth factor (CTGF) was performed using a modification of sandwich ELISA method described previously.[20] Briefly, a 96-well ELISA plate was coated with 100 μL of goat polyclonal anti-rat antibody (Santa Cruz Biotechnology, Germany) at a concentration of 10 μg/mL in PBS and 0.02% sodium azide overnight. After incubation with blocking buffer (PBS, 0.02% sodium azide and 1% bovine serum albumin) and washing (four times), 50 μL of sample or recombinant human CTGF standard was added for 1 hour. Then, 100 μL of rabbit polyclonal anti-goat antibody (2 μg/mL, Santa Cruz Biotechnology, Germany) as first antibody and 50 μL of donkey anti-rabbit IgG-HRP antibody (as 1:2000 dilution, Santa Cruz Biotechnology, Germany) as second antibody were attached. CTGF was quantified by measuring the absorbance at 405 nm after mix substrate solution and stop solution (2 N H_2SO_4).

Reverse transcription-polymerase chain reaction (RT-PCR) for analyzing gene expression in liver tissue

Liver tissue samples in RNAlater (Ambion, Austin, Texas, USA) were homogenized in TRI reagent (Molecular Research Center, Cincinnati, Ohio, USA), and total RNA was extracted using an RNA Easy column (QIAGEN, Valencia, California). The same amounts of RNA from each sample were mixed and used for complementary DNA (cDNA) synthesis. PCR was performed for 14 genes including the β-actin. The primers sequences were used as follows (forward and reverse, respectively): for β-actin, GTGGGGCGCCCCAGG-CACCA and CTCCTTAATGTCACGCACGATTTC; for α-SMA, CATCAGGAACCTCGAGAAGC and TCGGATACTTCAGGGTCAGG; for cytochrome P450 2E1 (CYP2E1), CATGGCTACAAGGCTGT-CAA, and TGGCCTTTGGTCTTTTTGAG; for hepatocyte growth factor (HGF), ACACATCTGTGGG GGATCAT and TGGTGCTGACTGCATTTCTC; for inducible nitric oxide synthase (iNOS), TGGTGGTGA-CAAGCACATTT and TGTTGCGTTGGAAGTGTA

GC; for PDGF-β, CTGCCTCTCTGCTGCTACCT and GATGAGCTTTCCGACTCGAC; for transforming growth factor beta (TGF-β), CTCCCAGGTTCTCTT-CAAGG and TGGAAGACTCCTCCCAGGTA; for connective tissue growth factor (CTGF), ATGGAGA-CATGGCGTAAAGC and TTGCATGACAATGACA-CACG; for tumor necrosis factor alpha (TNF-α), CTCCCAGGTTCTCTTCAAGG and TGGAAGACT C CTCCCAGGTA; for glutathione peroxidase type 1 (GSH-px 1), GAGGCACTTGGTCATTCCAT and AGCCGAGCAGCAGACATACT; for TIMP-1, CGG ACCTGGTTATAAGGGCT and ACTCTCCAGTTT GCAAGGGA; for SOD-1, CAGGACAGATTACAG-GATTAACTGAAGG and GCCACATTGCCCAGGT CTCC; for SOD-2, GGCCAAGGGAGATGTTACAA and GAACCTTGGACTCCCACAGA; for SOD-3, CGTTCTTGGGGAGAGCTTGTC and CCGTTGTTTT CCTAGCTCCA.

Statistical analysis

The results are expressed as the mean $\pm$ standard deviation ($n = 9$). Statistical analysis was carried out using Student's *t*-test. Difference at the level of $p < 0.05$ and $p < 0.01$ was regarded as statistically significant.

Results

Body and organ weights

TAA-treatment considerably inhibited gain of body weight by 50% comparing to normal group. However, CGX treatment significantly attenuated this weight loss when compared with TAA group ($p < 0.01$ for both 100 and 200 mg of CGX). The relative (but not absolute) weight of liver and spleen was increased by TAA treatment, and 100 mg of CGX treatment significantly reduced the change of relative weight of liver ($P < 0.05$, Table 1). The above-mentioned observations were similar in silymarin group.

Serum biochemistry

TAA treatment elevated serum levels of AST, ALT, ALP, and total bilirubin by two or three folds than normal group. These elevations were significantly attenuated by CGX administration: serum ALT ($p < 0.05$, CGX 200), ALP ($p < 0.05$, CGX 100 and 200), bilirubin ($p < 0.05$, CGX 200). For AST, the statistical significance was not observed. Total protein was lowered by TAA treatment, but this abnormality was significantly recovered in CGX 200 group ($p <$

Table 1. Organ weights and serum biochemistry

Groups	Normal	TAA only	TAA + CGX100	TAA + CGX200	TAA + silymarin
Body mass (g)	545.7 ± 64.8	364.7 ± 16.3[a]	403.2 ± 24.3[b]	396.1 ± 19.9[b]	391.8 ± 30.5[b]
Liver mass (g)	14.58 ± 2.92	14.02 ± 1.29	14.25 ± 1.51	13.98 ± 1.88	13.97 ± 1.65
Liver relative weight (%)	2.7 ± 0.25	3.8 ± 0.29[a]	3.5 ± 0.23[c]	3.5 ± 0.47	3.5 ± 0.29[c]
Spleen mass (g)	0.92 ± 0.15	0.94 ± 0.28	0.96 ± 0.11	0.95 ± 0.21	1.04 ± 0.14
Spleen relative weight (%)	0.17 ± 0.03	0.26 ± 0.08[a]	0.24 ± 0.02	0.24 ± 0.02	0.23 ± 0.10
AST (IU L^{-1})	131.2 ± 17.6	299.0 ± 150.0[a]	242.0 ± 114.4	219.1 ± 64.9	247.4 ± 74.1
ALT (IU L^{-1})	49.1 ± 8.7	114.5 ± 45.6[a]	93.3 ± 55.6	72.0 ± 24.4[c]	83.0 ± 40.3
ALP (IU L^{-1})	217.9 ± 35.5	638.0 ± 202.4[a]	419.4 ± 119.6[c]	421.4 ± 119.6[c]	445.9 ± 146.5[c]
Bilirubin (g dL^{-1})	0.11 ± 0.02	0.38 ± 0.18[a]	0.23 ± 0.12	0.20 ± 0.10[c]	0.21 ± 0.11[c]
Total protein (g dL^{-1})	6.32 ± 0.17	5.47 ± 0.19[a]	5.73 ± 0.34	5.99 ± 0.44[b]	5.91 ± 0.28[b]
Albumin (g dL^{-1})	2.36 ± 0.11	2.38 ± 0.11	2.46 ± 0.17	2.46 ± 0.11	2.50 ± 0.15
A/G ratio	0.59 ± 0.03	0.78 ± 0.07[a]	0.76 ± 0.05	0.69 ± 0.08[c]	0.73 ± 0.05

Abbreviations: TAA: thioacetamide, AST: aspartate transaminase, ALP: alkaline phosphatase, ALT: alanine transaminase.
[a] $p < 0.01$, compared with the normal group ($n = 9$).
[b] $p < 0.01$, compared with the TAA group. ($n = 9$).
[c] $p < 0.05$, compared with the TAA group. ($n = 9$).

0.01, Table 1). Those improvement patterns were observed partially in silymarin group.

Histophathological findings

By visual observation, a severe nodule feature on liver surface was observed in TAA group, whereas it was mild in CGX and silymarin group (data not shown). In TAA group, liver showed features of moderate inflammation, whereas the CGX treatment significantly ameliorated these changes (Figure 2A). In addition, severe hepatic collagen deposition and large septa was observed in TAA group by Masson's trichrome stain, and then the CGX treatment reduced it significantly (Figure 2B). Immunostain revealed positive signal for α-SMA antibody around collagen septa region in TAA group; however, it was markedly weak in CGX-treated group (Figure 2C). Silymarin treatment showed a similar pattern with CGX group.

Hydroxyproline and malondialdehyde content in liver tissue

The concentration of hydroxyproline, as a constituent of collagen, was markedly higher, approximately threefold in TAA group than normal group, CGX treatment significantly reduced it in a dose-dependent manner ($p < 0.01$; Figure 3A). In addition, CGX treatment significantly reduced malondialdehyde (MDA) that was increased in TAA group ($p < 0.05$, Figure 3B). Silymarin treatment showed similar effects with 100 mg of CGX treatment.

## Antioxidant enzymes and GSH content in liver tissue

TAA treatment notably depleted activity of catalase, SOD, GSH-red, and GSH-px in liver tissue, CGX administration ameliorated especially GSH-red and GSH-px with statistical significance ($p < 0.05$ for CGX 200, Figure 3D−F). Compared with TAA group, GSH content was also significantly normalized by CGX treatment (but not by Silymarin; $p < 0.05$). Silymarin treatment improved only GSH-px activity.

TGF-β, PDGF-BB, CTGF and TIMP-1 levels of liver tissue

In analysis for three main profibrotic cytokines, TAA treatment increased protein level of TGF-β and PDGF-BB. CGX treatment significantly reduced TGF-β comparing to TAA group ($p < 0.05$, Figure 4A), while no change was observed for PDGF-BB (Figure 4B). CTGF was not changed by either TAA or CGX treatment (Figure 4C). TIMP-1 level in tissue was increased by threefold, and this alteration was significantly reduced by CGX treatment ($p < 0.01$, Figure 4D). Silymarin treatment showed very similar pattern of effects with CGX treatment.

Gene expression associated with liver fibrosis

The pattern of gene expression associated with liver fibrosis was analyzed. TAA treatment remarkably up-regulated gene expressions of α-SMA, iNOS, PDGF-β, TGF-β, CTGF, TNF-α and TIMP-1 while
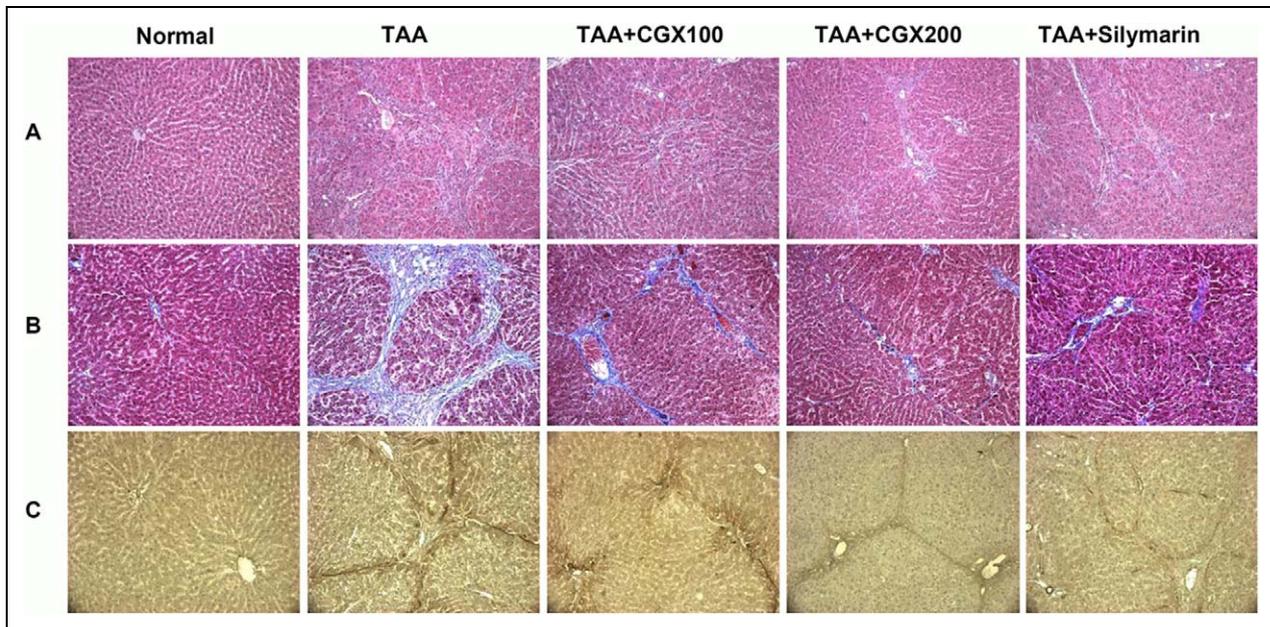
Figure 2. Histopathological examination. Nine male Sprague Dawley (SD) rats per group were treated with thioacetamide (TAA) together with CGX (100, 200 mg kg^{-1}), silymarin (50 mg kg^{-1}), or distilled water. On the final day of the experiment, all of the livers in each group were removed and then fixed in Bouin's solution. After staining with hematoxylin and eosin (A) or Masson's trichome (B) and immunohistochemistry for α-smooth muscle actin (α-SMA; C), histological examinations were performed at 200× magnification under light microscopy.

it significantly down-regulated the gene expression of GSH-px 1. Then, CGX treatment almost completely normalized gene expression of iNOS. CGX treatment also moderately suppressed gene expression of PDGF-β, TGF-β, CTGF and TNF-α. The gene expression of GSH-px 1 was down-regulated by TAA treatment, while it was slightly recovered by administration of CGX. No change was observed for the gene expression of CYP2E1, HGF, SOD-1, SOD-2 and SOD-3 by TAA, or CGX treatment (Figure 5).

Discussion

In this study, 12 weeks repeated injection with TAA induced severe histopathologic deterioration including accumulation of extracellular matrix (ECM) in liver tissues, while CGX treatment significantly ameliorated those histopathologic findings (Figure 2A and B). These results were in accordance with serum parameters (Table 1) and hydroxyproline level in liver tissues (Figure 3A). In particular, immunohistochemistry for α-SMA showed a significant inactivation of hepatic stellate cells (HSCs; Figure 2C). It is well known that activated HSCs are corresponding cell to secrete ECM including collagen types I and III.[21]

To explain the mechanisms of CGX effect on antifibrosis in liver tissues, we investigated the fibrogenesis-specific cytokines and oxidative stress-associated proteins in liver tissues. CGX treatment suppressed the protein levels of TGF-β and TIMP-1 (Figure 4A and D). TGF-β is a major key cytokine leading to activation of HSCs to secrete ECM.[22] TIMP-1 is an endogenous inhibitor of metallo-matrix proteinase degrading ECM. Previous reports showed that high expression of TGF-β and TIMP-1 led to substantial accumulation of ECM and liver fibrosis.[23,24] Other two cytokines, PDGF-β and CTGF, were not affected by CGX administration, and these findings were also correlated with gene expression pattern (Figure 5).

Most of pathologic condition, for example alcohol over-consumption or virus infection, and imbalance of prooxidant-antioxidant homeostasis due to administration of toxic agents, including TAA and DMN, are well known.[14,15] Oxidative stress causes alteration of DNA, protein, and lipids and activates HSCs directly or indirectly; nevertheless, it can be overwhelmed by activation of antioxidant enzymes.[25,26] Lipid peroxide represents holistic cellular oxidative stress, antioxidant effect of CGX was confirmed by quantification of the end products of lipid peroxidation, MDA (Figure 3B). Liver tissue has extensive antioxidant enzymes such as SOD and catalase as well as GSH system.[27] TAA treatment drastically exhausted
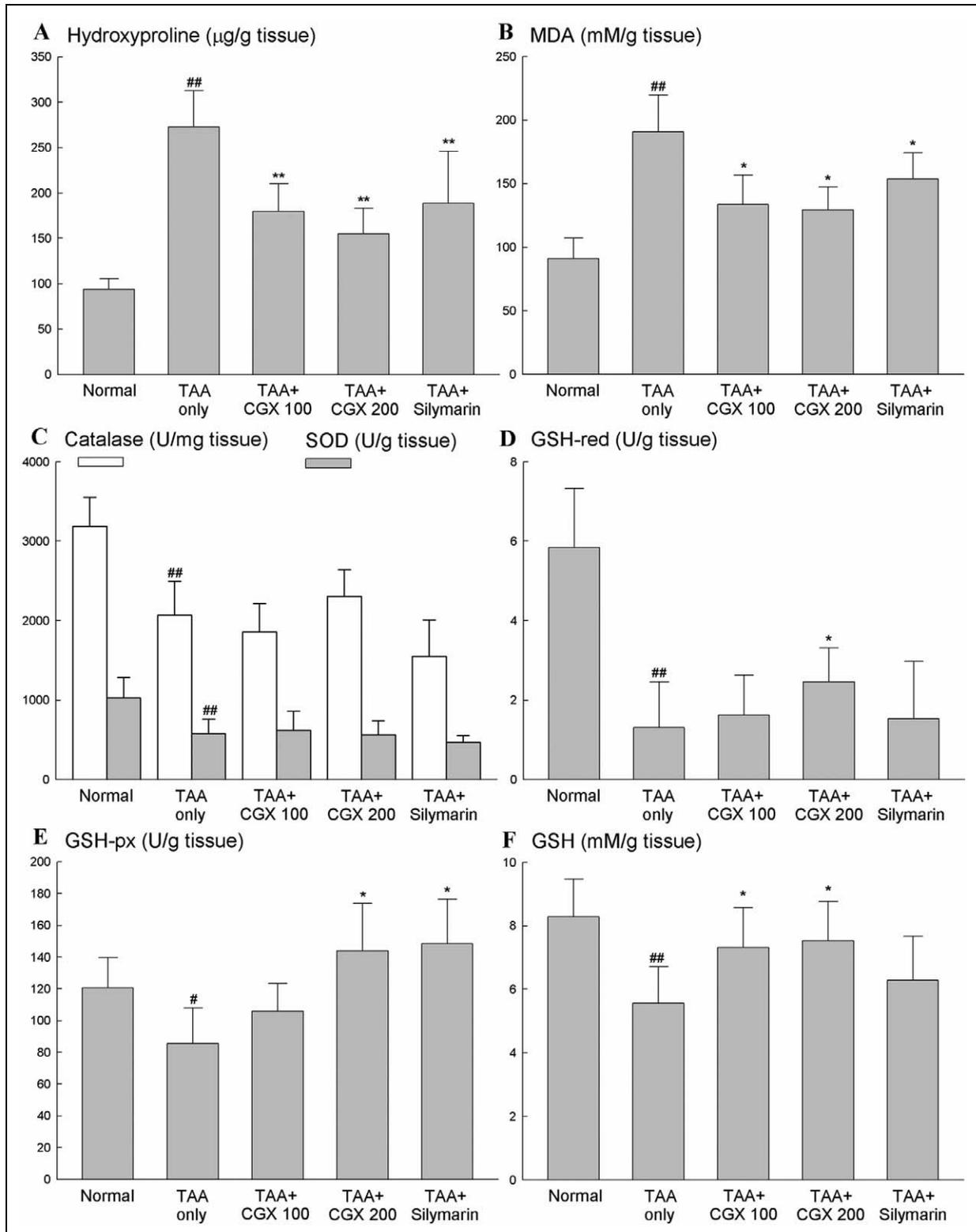
Figure 3. Hydroxyproline, malondialdehyde (MDA) and antioxidant enzyme activities in liver tissues. Nine male Sprague Dawley (SD) rats per group were treated with TAA together with CGX (100, 200 mg kg^{-1}), silymarin (50 mg kg^{-1}), or distilled water. At the end of the experiment, hydroxyproline, MDA, superoxide dismutase (SOD), catalase, glutathione reductase (GSH-red), glutathione peroxidase (GSH-px) and GSH were determined in the liver tissues. $^{#}p < 0.05$, $^{##}p < 0.01$, compared with the normal group, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the thioacetamide (TAA) group.
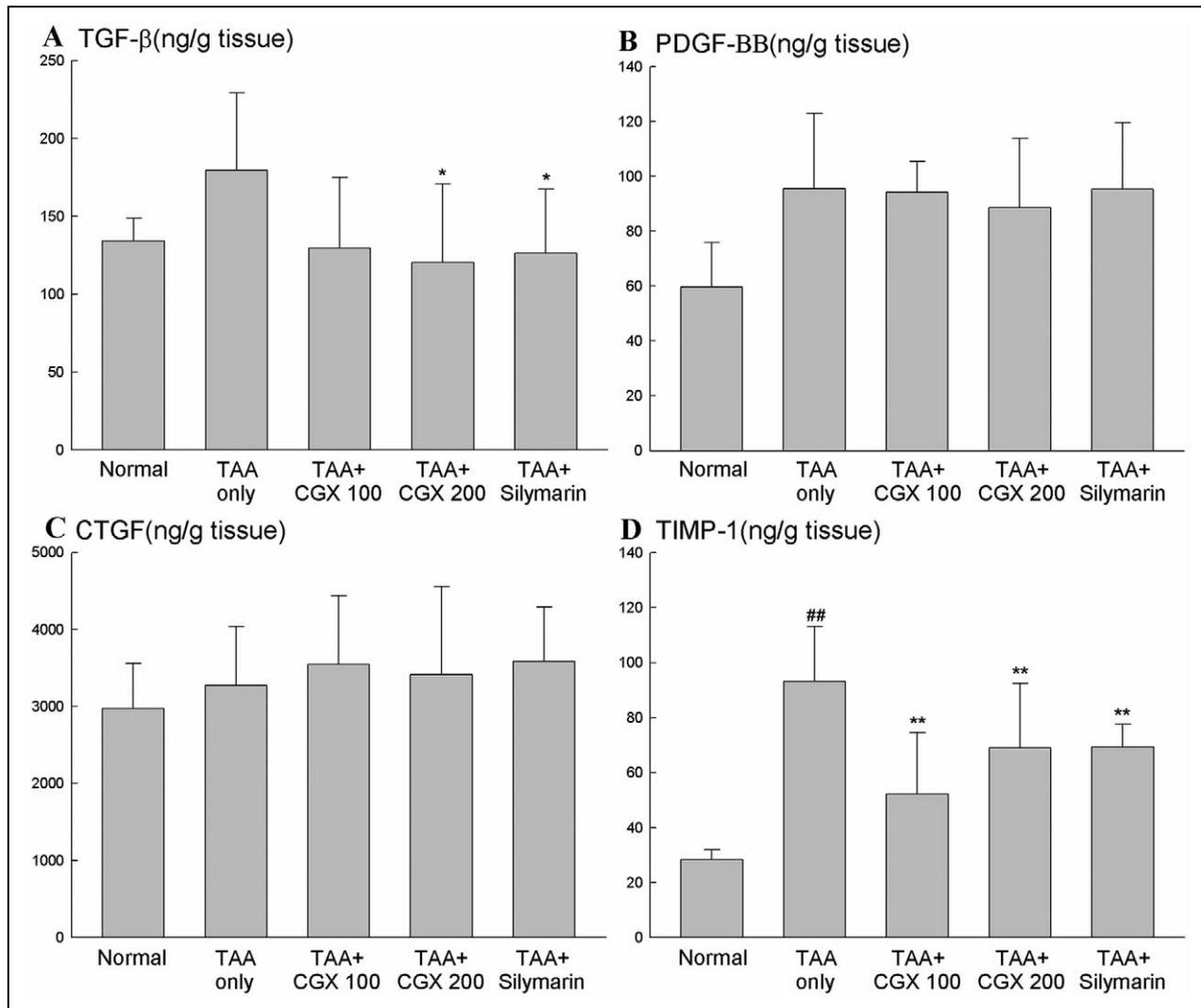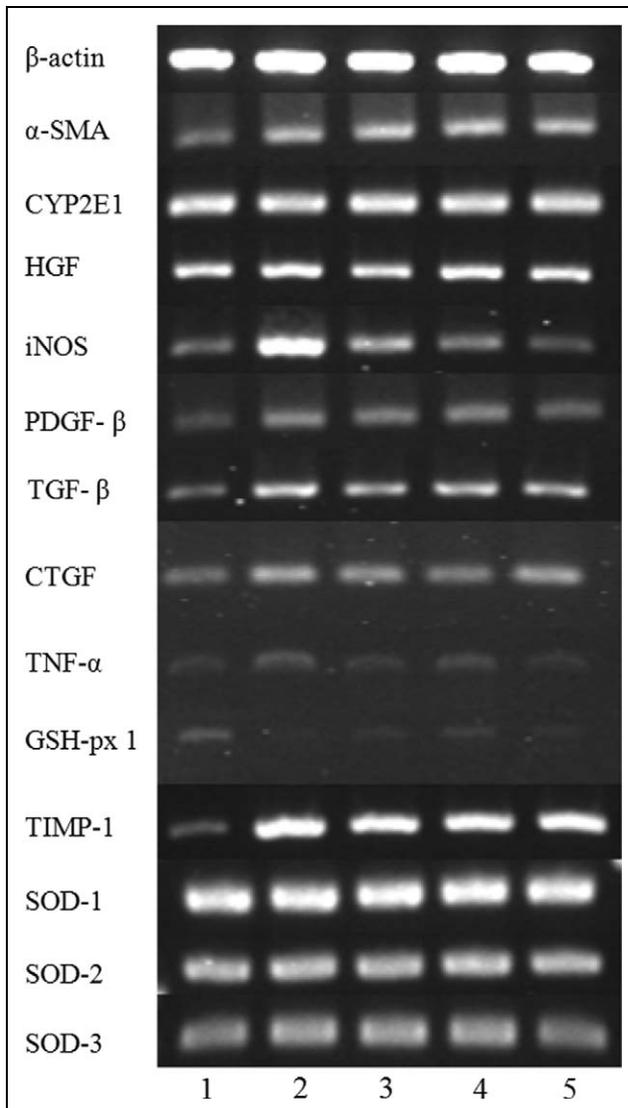
Figure 4. Profibrotic cytokines in liver tissue. Fibrosis-related cytokines (platelet-derived growth factor-beta [PDGF-β], transforming growth factor-beta [TGF-β], connective tissue growth factor [CTGF], and tissue inhibitor of matrix metalloprotease-1 [TIMP-1]) were determined in the liver lysates using ELISA method. $^{##}p < 0.01$, compared with the normal group, $*p < 0.05$, $**p < 0.01$, compared with the thioacetamide (TAA) group.

activities of those enzymes. In particular, CGX administration significantly restored GSH system including GSH-red and GSH-px enzymes and total GSH content in this experiment (Figure 3D−F). Interestingly, the first defense of antioxidant enzymes SOD and catalase activity was not affected by CGX (Figure 3C). The pharmacological capacities of CGX in reducing oxidative stress were supported by dramatic downregulation of iNOS and TNF-α gene expression representing inhibition of ROS production and inflammation, respectively (Figure 5).

Chronic hepatic injury commonly leads to liver fibrosis, resulting in development of liver cirrhosis and consequently failure of hepatic function.[28] So, prevention or treatment of hepatic fibrosis is the main

target in patients with chronic hepatic disorders.[3] In spite of poor clinical evidence or conflicting data, many plant-derived drugs such as phyllanthus, silybum marianum (milk thistle), glycyrrhizin (licorice root extract), Sho-saiko-to and LIV-52 are reported to have antifibrotic property.[29-33]

CGX is a traditional herbal remedy that has been used for patients with chronic hepatic disorders since 1991. Our previous studies have demonstrated the safety of CGX and its hepatoprotective and antifibrotic properties in different animal models.[11-13,34-36] Previously, we have shown that ip administration of DMN resulted in a drastically shrunken liver, decreased liver weight, and spleen enlargement.[12,13] However, 12 weeks repeated ip injection with TAA

Figure 5. Expression of fibrosis-related genes. Total RNA was extracted from a portion of each removed liver, followed by complementary DNA (cDNA) synthesis and PCR amplification according to standard protocols. Amplified products for the genes that encode α-smooth muscle actin (α-SMA), cytochrome P450 2E1 (CYP2E1), hepatocyte growth factor (HGF), nitric oxide synthase (iNOS), platelet-derived growth factor-beta (PDGF-β), transforming growth factor-beta (TGF-β), connective tissue growth factor (CTGF), tumor necrosis factor-alpha (TNF-α), glutathione peroxidase type 1 (GSH-px 1), tissue inhibitor of matrix metalloprotease-1 (TIMP-1), SOD 1, SOD 2, and SOD 3, including β-actin, were electrophoresed in a 1% agarose gel and visualized by UV illumination. 1, Normal; 2, TAA only; 3, TAA + CGX100; 4, TAA + CGX200; 5, TAA + Silymarin.

did not show those changes. The progression of human liver fibrosis or cirrhosis is generally very slow, so more suitable for clarifying the effects of

drug targeting hepatofibrosis is thought to be TAA model.[15] Our results strongly emphasize the antifibrotic effect of CGX and its probable mechanism of action.

In summary, CGX has hepatoprotective effects evidenced by inhibition of hepatic inflammation, and exhibits antifibrotic property through inactivation of HSCs in TAA-induced chronic liver fibrosis model. The responsible mechanism may involve reduction of oxidative stress via inhibition of ROS production and maintaining GSH system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Reference

1. WHO. *World health statistics 2008*. Switzerland: WHO press, 2008.
2. Albanis E and Friedman SL. Antifibrotic agents for liver disease. *Am J Transplant* 2006; 6: 12–19.
3. Bataller R and Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115: 209–218.
4. Kumar V, Abbas AK, and Fausto N. *Robbins and Cotran pathologic basis of disease*. Philadelphia, PA: Elsevier Saunders, 2004.
5. Hammel P, Couvelard A, O'Toole D, et al. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* 2001; 344: 418–423.
6. Gines P and Cardenas A. The management of ascites and hyponatremia in cirrhosis. *Semin Liver Dis*. 2008; 28: 43–58.
7. Levy C, Seeff LD, and Lindor KD. Use of herbal supplements for chronic liver disease. *Clin Gastroenterol Hepatol* 2004; 2: 947–956.
8. Rino Y, Tarao K, Morinaga S, et al. Reduction therapy of alanine aminotransferase levels prevent HCC development in patients with HCV-associated cirrhosis. *Anticancer Res* 2006; 26: 2221–2226.
9. Yen MH, Weng TC, Liu SY, Chai CY, and Lin CC. The hepatoprotective effect of Bupleurum kaoi, an endemic plant to Taiwan, against dimethylnitrosamine-induced hepatic fibrosis in rats. *Biol Pharm Bull*. 2005; 28: 442–448.

10. Cho JH, Lee YY, Seo SH, et al. A clinical report about 57 patients with chronic liver disease. *Korean J Orient Med* 2001; 21: 112–121.

11. Hu XP, Shin JW, Wang JH, et al. Antioxidative and hepatoprotective effect of CGX, an herbal medicine, against toxic acute injury in mice. *J Ethnopharmacol* 2008; 120: 51–55.

12. Shin JW, Son JY, Oh SM, et al. An herbal formula, CGX, exerts hepatotherapeutic effects on dimethylnitrosamine-induced chronic liver injury model in rats. *World J Gastroenterol* 2006; 12: 6142–6148.

13. Wang JH, Shin JW, Son JY, Cho JH, and Son CG. Antifibrotic effects of CGX, a traditional herbal formula, and its mechanisms in rats. *J Ethnopharmacol* 2010; 127: 534–542.

14. George J, Rao KR, Stern R, and Chandrakasan G. Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* 2001; 156: 129–138.

15. Muller A, Machnik F, Zimmermann T, and Schubert H. Thioacetamide-induced cirrhosis-like liver lesions in rats–usefulness and reliability of this animal model. *Exp Pathol* 1988; 34: 229–236.

16. *Guide for the care and use of laboratory animals*. Washington, DC: National Academy Press, 1996.

17. Mihara M and Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978; 86: 271–278.

18. Beers RF Jr and Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 1952; 195: 133–140.

19. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70–77.

20. Esson DW, Neelakantan A, Iyer SA, et al. Expression of connective tissue growth factor after glaucoma filtration surgery in a rabbit model. *Invest Ophthalmol Vis Sci* 2004; 45: 485–491.

21. Bataller R and Brenner DA. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Semin Liver Dis* 2001; 21: 437–451.

22. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; 134: 1655–1669.

23. Benyon RC, Iredale JP, Goddard S, Winwood PJ, and Arthur MJ. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. *Gastroenterology* 1996; 110: 821–831.

24. Rippe RA and Brenner DA. From quiescence to activation: gene regulation in hepatic stellate cells. *Gastroenterology* 2004; 127: 1260–1262.

25. Drotman RB and Lawhorn GT. Serum enzymes as indicators of chemically induced liver damage. *Drug Chem Toxicol* 1978; 1: 163–171.

26. Nieto N, Friedman SL, and Cederbaum AI. Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. *J Biol Chem* 2002; 277: 9853–9864.

27. Parola M and Robino G. Oxidative stress-related molecules and liver fibrosis. *J Hepatol* 2001; 35: 297–306.

28. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007; 117: 539–548.

29. Wang J, Guo J, and Liu S. [Inhibitory effect of glycyrrhizin on NF-kappa B binding activity in CCl4 plus ethanol induced liver cirrhosis in rats]. *Zhonghua Gan Zang Bing Za Zhi* 1999; 7: 42–43.

30. Wang BE. Treatment of chronic liver diseases with traditional Chinese medicine. *J Gastroenterol Hepatol* 2000; 15: E67–E70.

31. Tsai JH, Liu JY, Wu TT, et al. Effects of silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in rats. *J Viral Hepat* 2008; 15: 508–514.

32. Ono M, Miyamura M, Kyotani S, Saibara T, Ohnishi S, and Nishioka Y. Effects of Sho-saiko-to extract on liver fibrosis in relation to the changes in hydroxyproline and retinoid levels of the liver in rats. *J Pharm Pharmacol* 1999; 51: 1079–1084.

33. Huseini HF, Alavian SM, Heshmat R, Heydari MR, and Abolmaali K. The efficacy of Liv-52 on liver cirrhotic patients: a randomized, double-blind, placebo-controlled first approach. *Phytomedicine* 2005; 12: 619–624.

34. Choi WJ, Shin JW, Son JY, et al. Toxicological study of the hepatotherapeutic herbal formula, Chunggan extract, in beagle dogs. *World J Gastroenterol* 2006; 12: 7497–7502.

35. Shin JW, Kim HG, Park HJ, Sung NW, and Son CG. Safety of the traditional Korean herbal medicine CGX: a 6-month repeated-dose study in rats. *J Ethnopharmacol* 2010; 128: 221–229.

36. Shin JW, Park HJ, Kwon M, and Son CG. Scientific evaluation of the chronic toxicity of the herbal medicine CGX in beagle dogs. *Food Chem Toxicol* 2010; 48: 743–749.