



Artemisia capillaris extract protects against bile duct ligation-induced liver fibrosis in rats

Jong-Min Han^a, Hyeong-Geug Kim^a, Min-Kyung Choi^a, Jong-Suk Lee^b, Jin-Suk Lee^a, Jing-Hua Wang^c, Hye-Jung Park^a, Seung-Wan Son^d, Seock-Yeon Hwang^e, Chang-Gue Son^{a,*}

^a Liver and Immunology Research Center, Institute of Traditional Medicine and Bioscience of Daejeon University, 22-5 Daeheung-dong, Jung-gu, Daejeon 301-724, Republic of Korea

^b GyeongGi Bio-Center, GSTEP, 864-1 Iui-dong, Yeongtong-gu, Suwon, Republic of Korea

^c Key Laboratory of Xia'an Medicine, Ministry of Education, Anhui Province Key Laboratory of R&D of TCM, Hefei, Anhui Province, People's Republic of China

^d Department of Biomedical Engineering, College of Health Science, Korea University, Seongbuk-Gu, Seoul 136-703, Republic of Korea

^e Department of Biomedical Laboratory Science College of Applied Science and Industry, Daejeon University, Daejeon 300-716, Republic of Korea

ARTICLE INFO

Article history:

Received 1 September 2012

Accepted 6 December 2012

Keywords:

Artemisia capillaris Thunb

Cholestasis

Bile duct ligation

Liver fibrosis

ABSTRACT

Artemisia capillaris has been widely used as a traditional herbal medicine in the treatment of liver diseases. However, no previous study has investigated whether *A. capillaris* alone is effective in treating pathological conditions associated with cholestatic liver injury. In the present study, we evaluated the anti-hepatofibrotic effects of *A. capillaris* (aqueous extract, WAC) in a bile duct ligation (BDL)-induced cholestatic fibrosis model. After BDL, rats were given WAC (25 or 50 mg/kg) or urosodeoxycholic acid (UDCA, 25 mg/kg) orally for 2 weeks (once per day). The serum cholestatic markers, malondialdehyde, and liver hydroxyproline levels were drastically increased in the BDL group, while administering WAC significantly reduced these alterations. Administering WAC also restored the BDL-induced depletion of glutathione content and glutathione peroxidase activity. Cholestatic liver injury and collagen deposition were markedly attenuated by WAC treatment, and these changes were paralleled by the significantly suppressed expression of fibrogenic factors, including hepatic alpha-smooth muscle actin (α -SMA), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF- β). The beneficial effects of WAC administration are associated with antifibrotic properties *via* both upregulation of antioxidant activities and downregulation of ECM protein production in the rat BDL model.

© 2012 Elsevier GmbH. All rights reserved.

1. Introduction

Artemisia is a diverse genus of plants with 400 species belonging to the family *Asteraceae*, with most herbs having strong aromas and bitter tastes arising from the presence of terpenoids and sesquiterpene lactones (Herrmann et al., 2011). The representative species of the *Artemisia* are *Artemisia annua* Linne and *Artemisia capillaris* Thunberg. *A. capillaris* has been used as a traditional medicine to treat liver diseases including hepatitis, jaundice, and fatty liver in Asian countries (Jang, 1975). *A. capillaris* and its major constituents such as phenolic acids, coumarins, flavonoids, and 4-hydroxyacetophenone have been known to have pharmacological activity against viral infection (Seo et al., 2010), oxidative stress (Hong and Lee, 2009), and obesity (Hong et al., 2009). *A. capillaris*

in particular has shown dramatic hepatoprotection in chemical-induced liver injury models (Lee et al., 2007; Wang et al., 2012).

Liver fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) such as type I and type IV collagen during most chronic injuries such as viral, alcoholic, metabolic, and biliary disorders (Haber et al., 2008; Pinzani and Rombouts, 2004). In particular, cholestatic liver diseases exacerbate the retention and accumulation of directly or indirectly toxic hydrophobic bile salts in the liver by the abnormal flux of bile acids and bilirubin (Faubion et al., 1999). Cholestasis is known to cause inflammatory reactions, excessive oxidative stress (Chen et al., 2009), and finally, periductular fibrosis (Webster and Anwer, 1998). The number of patients exhibiting metabolic disorders with hepatic cholestasis and cholestatic liver fibrosis is increasing (Mormone et al., 2011). Therapeutics for cholestatic diseases and hepatic fibrosis are therefore urgently required; however, a potent drug has not yet been developed. To date, experimental evidence and clinical experiences have supported the hepatoprotective ability of *A. capillaris* and herbal formulae containing the plant (Lee et al., 2007; Wang et al., 2010), but no studies have explored whether *A. capillaris* is effective in treating the pathological conditions associated with cholestatic

* Corresponding author at: Liver and Immunology Research Center, Daejeon Oriental Hospital of Daejeon University, 22-5 Daehung-dong, Jung-gu, Daejeon 301-724, Republic of Korea. Tel.: +82 42 229 6484; fax: +82 42 257 6398.

E-mail address: ckson@dju.ac.kr (C.-G. Son).

liver injury. Bile duct ligation (BDL) is a suitable model for research to evaluate therapeutic efficacy in animal cholestatic liver injury (Liu et al., 2001).

This study aimed to elucidate the efficacy of *A. capillaris* against BDL-induced cholestatic liver injury using an aqueous extract (WAC) and the possible mechanisms corresponding to its effects.

2. Materials and methods

2.1. Preparation of WAC and UHPL-MS analysis

Dried *A. capillaris* was obtained from the Jeong-Seoug Oriental Medicine Company (Daejeon, Korea). 100 g of fully dried *A. capillaris* was boiled in 1 L of distilled water for 30 min and concentrated at 60 °C for 120 min. After filtration and lyophilization, the final yield was 3.2% (w/w). The extract was dissolved in distilled water before use; the remainder was stored at –70 °C for future use. To verify the reproducibility of WAC and quantity analysis of main components of *A. capillaris*, ultra high-performance liquid chromatography (UHPLC) using scoparone, chlorogenic acids (3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid), and quercetin was performed. Briefly, 2.5 mg of WAC were dissolved in 1 ml of 50% methanol, and the samples were filtered and analyzed by LTQ-Orbitrap XL linear ion trap mass spectrometer system (Thermo Scientific Co., PA) equipped with an electrospray ionization source. The UHPLC separation was performed with an Accela UHPLC, using an Acquity BEH C18 (1.7 μm, 100 mm × 2.1 mm, Waters) column. Mobile phase A was water and B was acetonitrile, and both phases contained 0.1% formic acid. The gradient elution was performed at a flow rate of 0.3 ml/min with the following conditions: 0–2 min 5% B (isocratic), 2–20 min 5–40% B (linear gradient).

2.2. Animals

Thirty-seven Sprague-Dawley rats (6-week-old male, 230–250 g) were purchased from a commercial animal breeder (Koatech, Korea). Rats were acclimated to an environmentally controlled room at 22 ± 2 °C, 55% ± 10% relative humidity, and a 12 h light/dark cycle. Rats were fed commercial pellets (Koatech) and tap water *ad libitum* for 1 week. This animal experiment was approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUAR2011-19-2) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Bethesda, MD).

2.3. Bile duct ligation (BDL) operation and experimental schedule

After a midline laparotomy was performed under anesthesia with ketamine (100 mg/kg body weight), the common bile duct was ligated at two points (just below the junction of the hepatic duct and before the entrance of the pancreatic duct) with 4-0 nylon sutures (AILEE Co., Ltd., Korea). Then, the midpoint of the bile duct was cut, followed by careful suturing of the peritoneum and muscle layers as well as the skin wound as described previously (Gross et al., 1987). In sham-treated rats, an abdominal incision was made without ligation of the common bile duct.

BDL- and sham-operated rats were maintained on a standard rat pellet diet and tap water *ad libitum* for 3 days. The rats were randomly divided into five experimental groups: (1) sham group (6, without BDL and treated with distilled water), (2) BDL group (9, with BDL and treated with distilled water), (3) WAC 25 group (7, with BDL and oral administration of WAC at 25 mg/kg), (4) WAC 50 group (9, with BDL and oral administration of WAC at 50 mg/kg), and (5) ursodeoxycholic acid (UDCA) group (6, with

BDL and administration of UDCA at 25 mg/kg). Distilled water, WAC, or UDCA was given by gastric gavage once daily for 14 days. Dose of WAC used as in our previous study (Wang et al., 2012), experimental duration was based on Georgiev et al. (2008). Body weight was recorded once a week. After the last drug administration, the animals fasted for 18 h, and blood collection from the abdominal aorta and liver removal were performed under ether anesthesia.

2.4. Complete blood count and serum biochemical analysis

Complete blood counts were performed with a blood cell counter (Hemavet; CDC Technologies Inc., Oxford, CT) using small amounts of blood mixed in EDTA. Serum was prepared following blood clotting. The serum levels of total protein, albumin, total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total cholesterol, high-density lipoprotein (HDL), and triglyceride were determined using an auto-analyzer (Chiron, Emeryville, CA).

2.5. Histopathological and immunohistochemical analyses

For the histopathological evaluation, liver tissues were fixed in 10% formalin or Bouin's solution and embedded with paraffin. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were performed according to standard procedures. For immunohistochemistry, sections were incubated with α-smooth muscle actin (α-SMA) or 4-hydroxynonenal (4-HNE) primary antibody (1:200; Abcam, Cambridge, UK) and biotinylated secondary antibody (Nichirei Biosciences, Tokyo, Japan), followed by the avidin-biotin-peroxidase complex. The immunoreactive signal was developed by their substrates, DAB (3,3'-diaminobenzidine) and AEC (3-amino-9-ethylcarbazole) (Abcam). The slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) and examined under an optical microscope (Leica Microsystems, Wetzlar, Germany).

2.6. Determination of hydroxyproline and malondialdehyde (MDA)

Ten percent (w/v) homogenate of liver was prepared using 0.15 M KCl, followed by centrifugation at 3000 rpm for 15 min. Liver collagen was estimated by measuring hydroxyproline content as described previously (Fujita et al., 2003). Malondialdehyde (MDA) levels in the liver tissues and serum were determined by the thiobarbituric acid reactive substances (TBARS) assay as described previously (Mihara and Uchiyama, 1978).

2.7. Determination of total glutathione (GSH) and GSH-peroxidase (GSH-px), GSH-reductase (GSH-rd), superoxide dismutase (SOD), and catalase activities in liver tissues

Radioimmunoprecipitation assay (RIPA) buffer-based liver tissue homogenates were centrifuged at 10,000 × g for 15 min. Total GSH content was determined according to the Ellman method (Ellman, 1959). GSH-px, GSH-rd, and SOD activities were measured with a GSH-px cellular activity assay kit (Sigma-Aldrich), a GSH-rd assay kit (Sigma-Aldrich), and a SOD assay kit (Dojindo Laboratories, Kumamoto, Japan), respectively, according to each manufacturer's protocol. Catalase activity was determined using a previously described method (Beers and Sizer, 1952).

2.8. Isolation of RNA and gene expression analysis using real-time PCR

Total RNA was extracted from liver tissue samples with Trizol reagent (Molecular Research Center, Cincinnati, OH). The cDNA was synthesized from RNA (2 μ g) in a 20 μ L reaction using a High-Capacity cDNA reverse transcription kit (Ambion, Austin, TX). The primers for α -SMA, NADPH oxidase 1 (NOX-1), platelet-derived growth factor (PDGF)- β , transforming growth factor (TGF)- β , collagen type 1 alpha 1 and 2 (Col1A1 and Col1A2), tissue inhibitor of metalloprotease 1 and 2 (TIMP-1 and TIMP-2), and β -actin were as follows (5' \rightarrow 3', forward and reverse): α -SMA (NM.031004), AACACGGCATCATACCAACT and TTCTCCCGTTGGCCTTA; NOX-1 (NM.053683), ACTCCCTTGCTTCCTTCTGA and GCACCCGTCTCTACAAATCC; PDGF- β (NM.031524), ACCACTCCATCCGCT-CCTTT and TGTGCTCGGGTCAT-GTTCAA; TGF- β (NM.021578), AGGAGACGGA-ATACAGGGCTTT and AGCAGGAAGGGTCGGTTCAT; Col1A1 (NM.053304), CCCAGCGGTGTTATGACTT and GCTGCGGATGTTCTCAATCTG; Col1A2 (NM.053356), CC-CAGAGTGAAGAGCGATT and GCTGCGGATGTTCTCAATCTG; TIMP-1 (NM.053819), CGACGCTGTGGGAAATGC and CTCCTCGCTGCGGTTCTG; TIMP-2 (NM.021989), CCCA-GAAGAAGAGCCTAAACCA and GTCCATCCAGAGGCACTCA-TC; and β -actin (NM.031144), CTAAGCCAACCGTAAAAAGAT and GACCAGAGGCATACAGGGA-CAA. Reactions were performed with 12.5 μ L of iQ SYBR Green Supermix, 1 μ L of 10 pmol/l primer pair, 10.5 μ L of distilled water, and 1 μ L of cDNA. Each PCR run was performed under the following conditions: initial denaturation at 95 $^{\circ}$ C for 5 min and 40 amplification cycles of denaturation at 95 $^{\circ}$ C for 1 min, annealing at 58 $^{\circ}$ C for 40 s, and elongation at 72 $^{\circ}$ C for 40 s, followed by a single fluorescence measurement.

2.9. Statistical analysis

The results are expressed as the means \pm standard deviation (SD). Statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA), followed by Student's unpaired *t*-test. In all analyses, $P < 0.05$ or $P < 0.01$ indicated statistical significance.

3. Results

3.1. Identification of WAC constituents

We performed a comparative analysis of the levels of its constituents using UPLC-MS analysis. Each peak in the UPLC trace was verified by comparison of retention time (RT) to the standard. As shown in Fig. 1, three isomers of chlorogenic acid (3-*O*-caffeoylquinic acid, RT 3.57 min; 5-*O*-caffeoylquinic acid, RT 5.95 min; 4-*O*-caffeoylquinic acid, RT 6.50 min) and quercetin (RT, 10.07 min) were detected in the UPLC chromatogram. We found a compound at RT 12.21 similar to the scoparone standard (RT 12.11 min), but it was determined to be a different compound from scoparone by mass spectrum analysis.

3.2. Effects on hematological parameters and organ weights

The complete blockage of the common bile duct induced a significant inflammatory reaction and an enlargement of the liver and the spleen after 14 days. WAC treatment produced a slight reduction in hepatomegaly and splenomegaly, but these reductions were not statistically significant ($P > 0.05$, Table 1). UDCA did not show any effect on the hematological parameters or organ weight. The peripheral white blood cell (WBC) count and platelet number were drastically increased by 4-fold and 1.5-fold, respectively, and the differential count (neutrophils, lymphocytes, monocytes,

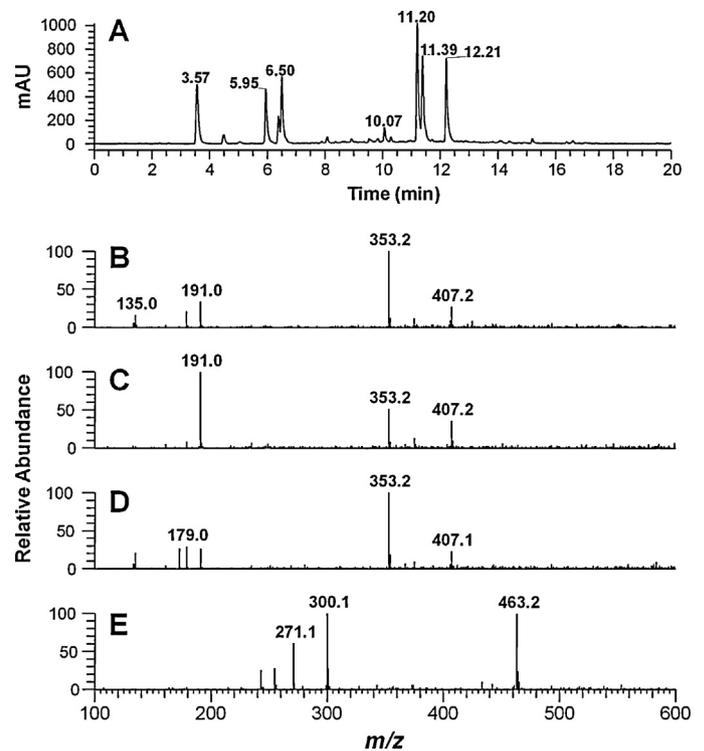


Fig. 1. UPLC-MS analysis results of 4 identified compounds of *Artemisia capillaris* water extract (WAC). (A) UPLC chromatogram at 325 nm. Mass spectrum of 3-*O*-caffeoylquinic acid at RT 3.57 min (B), 5-*O*-caffeoylquinic acid at RT 5.95 min (C), 4-*O*-caffeoylquinic acid at RT 6.50 min (D), and quercetin at RT 10.07 min (E).

eosinophils, and basophils) was predominantly shifted toward neutrophils (from 18.0% to 60.6%). WAC administration (50 mg/kg) significantly ameliorated these alterations in total WBC, differential count, and platelet number, compared to those of the BDL group ($P < 0.05$).

3.3. Effects on serum biochemistry and hepatic antioxidative parameters

The BDL procedure completely blocked bile flow as evidenced by the radical 70-fold elevation of serum total bilirubin. The serum AST and ALT, two key biomarkers of hepatocyte injury, drastically increased by approximately 8- and 5-fold, respectively, and the serum levels of total cholesterol and triglycerides also increased by approximately 2-fold and 5-fold, respectively. No significant change in the HDL level was observed after BDL or WAC administration. However, WAC administration significantly inhibited the hepatocyte damage as shown by the reduction of both AST ($P < 0.05$ by 50 mg/kg) and ALT ($P < 0.05$ by 25 mg/kg). In addition to, WAC administration (50 mg/kg) significantly reduced the elevation of total bilirubin ($P < 0.05$), total cholesterol ($P < 0.05$), and triglycerides ($P < 0.01$). UDCA (25 mg/kg) also significantly reduced the increase in both total cholesterol and triglycerides ($P < 0.01$ for each), but no such effect was observed on total bilirubin, AST, or ALT (Table 2). 14 days of BDL significantly distorted the GSH content and the GSH-px/rd activities compared to the sham group, while WAC treatment significantly reduced the alteration of total GSH content and GSH system enzyme activities ($P < 0.05$ or < 0.01 , Table 2). SOD and catalase activities were slightly increased by BDL, and both were significantly augmented by WAC administration ($P < 0.01$ in each group). The UDCA treatment showed positive effects similar to WAC treatment, except SOD activity (Table 2).

Table 1
Organ weights and hematological parameters.

Groups	Sham (n=6)	BDL (n=7)	WAC 25 (n=5)	WAC 50 (n=8)	UDCA 25 (n=4)
Body mass (g)	292.1 ± 12.5	265.2 ± 16.1	260.8 ± 9.5	262.6 ± 22.6	250.6 ± 24.5
Liver mass (g)	8.3 ± 0.8	17.9 ± 1.0 ^{##}	16.6 ± 2.1	15.7 ± 3.4	18.5 ± 3.5
Liver/body mass (%)	2.9 ± 0.3	7.0 ± 0.9 ^{##}	6.4 ± 0.9	6.1 ± 1.3	6.9 ± 0.4
Spleen mass (g)	0.8 ± 0.1	1.9 ± 0.4 ^{##}	1.6 ± 0.1	1.7 ± 0.5	1.6 ± 0.4
WBCs (×10 ³ /μL)	6.9 ± 1.7	28.4 ± 3.3 ^{##}	26.37 ± 4.7	22.3 ± 6.5 [*]	24.8 ± 2.2
Neutrophils (%)	18.0 ± 2.1	60.6 ± 3.9 ^{##}	52.8 ± 5.2	49.9 ± 14.1 [*]	56.0 ± 3.7
Lymphocytes (%)	76.2 ± 2.6	34.7 ± 3.6 ^{##}	38.1 ± 5.0 [*]	39.9 ± 13.6	38.5 ± 2.9
Monocytes (%)	5.8 ± 0.7	5.5 ± 1.8	8.6 ± 0.4 ^{**}	7.7 ± 3.0	7.0 ± 2.0
Eosinophils (%)	0.1 ± 0.1	0.5 ± 0.1 ^{##}	0.2 ± 0.1 ^{**}	0.3 ± 0.2 [*]	0.4 ± 0.1
Basophils (%)	0.02 ± 0.03	0.07 ± 0.03 [#]	0.05 ± 0.03	0.05 ± 0.03	0.09 ± 0.07
RBCs (×10 ⁶ /μL)	8.5 ± 0.3	8.8 ± 1.4	8.0 ± 0.6	7.8 ± 0.9	8.2 ± 0.7
PLTs (×10 ⁴ /μL)	109.1 ± 9.6	160.6 ± 25.5 ^{##}	159.0 ± 7.3	120.5 ± 32.2 [*]	156.0 ± 14.2

After BDL, rats were pretreated with WAC (25 or 50 mg/kg), UDCA (25 mg/kg), or distilled water once a day for 2 weeks. Data were expressed as the mean ± SD.

[#] P < 0.05 compared with the sham group.

^{##} P < 0.01 compared with the sham group.

^{*} P < 0.05 compared with the BDL group.

^{**} P < 0.01 compared with the BDL group.

Table 2
Serum biochemistry and hepatic antioxidative parameters.

Groups	Sham (n=6)	BDL (n=7)	WAC 25 (n=5)	WAC 50 (n=8)	UDCA 25 (n=4)
T. bilirubin (mg/dL)	0.1 ± 0.0	7.0 ± 0.9 ^{##}	6.5 ± 0.7	4.9 ± 2.5 [*]	6.2 ± 1.1
AST (IU/L)	69.5 ± 6.8	519.6 ± 77.8 ^{##}	356.6 ± 191.0	369.0 ± 103.1 [*]	492.3 ± 99.0
ALT (IU/L)	18.5 ± 3.2	108.3 ± 18.7 ^{##}	70.0 ± 33.4 [*]	91.6 ± 53.1	91.0 ± 28.4
ALP (IU/L)	77.8 ± 17.5	315.4 ± 42.1 ^{##}	283.2 ± 137.6	347.0 ± 144.8	295.3 ± 46.0
TC (mg/dL)	54.3 ± 10.2	99.2 ± 6.3 ^{##}	96.6 ± 14.4	80.6 ± 15.5 [*]	79.7 ± 4.5 ^{**}
HDL (mg/dL)	35.4 ± 4.9	31.1 ± 5.4 ^{##}	35.8 ± 9.4	38.9 ± 7.2	41.2 ± 13.4
TG (mg/dL)	26.0 ± 5.2	126.1 ± 6.7 ^{##}	120 ± 13.4	94.2 ± 16.4 ^{**}	101.2 ± 9.8 ^{**}
Total GSH (mM/mg proteins)	13.0 ± 2.1	9.0 ± 1.2 ^{##}	8.7 ± 3.5	10.5 ± 3.1 [*]	12.1 ± 1.2 [*]
GSH-px (U/mg proteins)	48.9 ± 15.5	32.1 ± 6.5 [#]	93.1 ± 37.6 [*]	143.1 ± 61.9 ^{**}	55.7 ± 11.1 [*]
GSH-rd (U/mg proteins)	22.2 ± 2.6	18.4 ± 1.2 [#]	20.7 ± 2.1	23.8 ± 3.7 ^{**}	22.1 ± 4.3 [*]
SOD (U/mg proteins)	102.7 ± 11.4	146.9 ± 10.5 [#]	157.3 ± 5.5	183.9 ± 17.7 ^{**}	155.6 ± 28.2
Catalase (U/mg tissue)	2114 ± 604	2696 ± 316	2980 ± 258 ^{**}	3337 ± 419 ^{**}	3833 ± 384 ^{**}

After BDL, rats were pretreated with WAC (25 or 50 mg/kg), UDCA (25 mg/kg), or distilled water once a day for 2 weeks. Data were expressed as the means ± SD. TC: total cholesterol; HDL: high-density lipoprotein; AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase; T. bilirubin: total bilirubin; GSH: glutathione; GSH-px: glutathione peroxidase; GSH-rd: glutathione reductase; SOD: superoxide dismutase.

[#] P < 0.05 compared with the sham group.

^{##} P < 0.01 compared with the sham group.

^{*} P < 0.05 compared with the BDL group.

^{**} P < 0.01 compared with the BDL group.

3.4. Effects on MDA content and antioxidant enzymes in liver tissue

To explore the mechanisms corresponding to the hepatoprotective and anti-hepatofibrotic effects of WAC, we determined the parameters for oxidative stress. Immunohistochemistry staining against 4-HNE demonstrated the lipid peroxidation inside cells, and BDL treatment strongly enhanced the 4-HNE expression compared to the sham group (Fig. 2A and B). BDL after 14 days induced oxidative stress, as demonstrated by remarkably large increases of serum NO concentration and lipid peroxidation (measured using MDA) in serum and hepatic tissues by approximately 2-fold (Fig. 2F and G). However, WAC or UDCA administration showed a significant inhibition of 4-HNE staining (Fig. 2C and D), NO production, hepatic lipid peroxidation (P < 0.05, Fig. 2G).

3.5. Effects on hepatic fibrosis

WAC has hepatoprotective activity against the cholestatic condition, in accordance to histopathological findings by H & E staining. An increase in abnormal hepatic activity, including cellular destruction and infiltration of lymphocytes, was shown in BDL group while WAC administration markedly reduced these histological distortions (Fig. 3A). Masson's trichrome staining showed the development of moderate hepatic fibrosis, which was likely ECM

accumulation around the Glisson's sheath, and area of periportal and portal tracts (Fig. 3B). By immunohistochemical staining, BDL increased a strong positive signal for α-SMA around area of periportal and portal tracts compared with the sham group. This signal was obviously diminished by WAC or UDCA administration (Fig. 3C).

3.6. Effects on the expression of fibrogenic mediators in liver tissue

BDL drastically increased the hydroxyproline content of the liver compared with the content in the sham livers (P < 0.01). However, WAC administration significantly attenuated the elevation of hydroxyproline, in a dose dependent manner (P < 0.05 in WAC 50 group) (Fig. 4A). Real-time PCR analysis revealed that BDL drastically upregulated the fibrogenic cytokines (α-SMA, PDGF-β, TGF-β, Col1A1, Col1A2, TIMP1, and TIMP2) and NOX-1 in hepatic tissues (Fig. 4). These mRNA levels were significantly normalized by WAC administration. Especially, BDL-induced α-SMA expression was markedly attenuated by WAC administration as shown in histological findings (P < 0.01 by 50 mg/kg). Two fibrogenic cytokines, PDGF-β and TGF-β, were significantly increased by 7- and 4.2-fold in the BDL group (Fig. 4D). However, WAC and UDCA administration significantly attenuated the production of both fibrogenic cytokines compared with the levels in the BDL group (P < 0.01 in WAC 50 group).

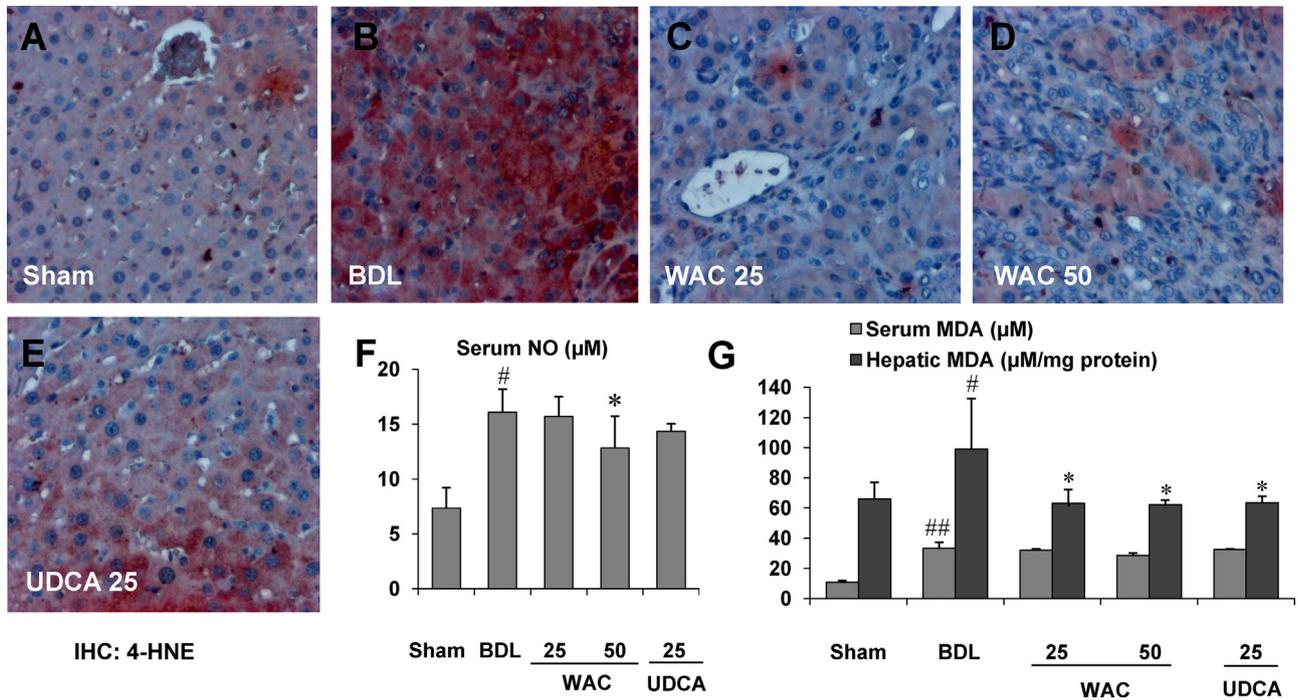


Fig. 2. Determination of oxidative stress markers in the BDL model. Two weeks after BDL, hepatic tissues were evaluated by immunohistochemistry for 4-HNE (A–E). All photos are at 400× magnification. Determination of NO (F) and MDA (G) in serum and liver was performed as described under Section 2. ^{##}*P*<0.01 compared with sham group; ^{*}*P*<0.05 compared with BDL group.

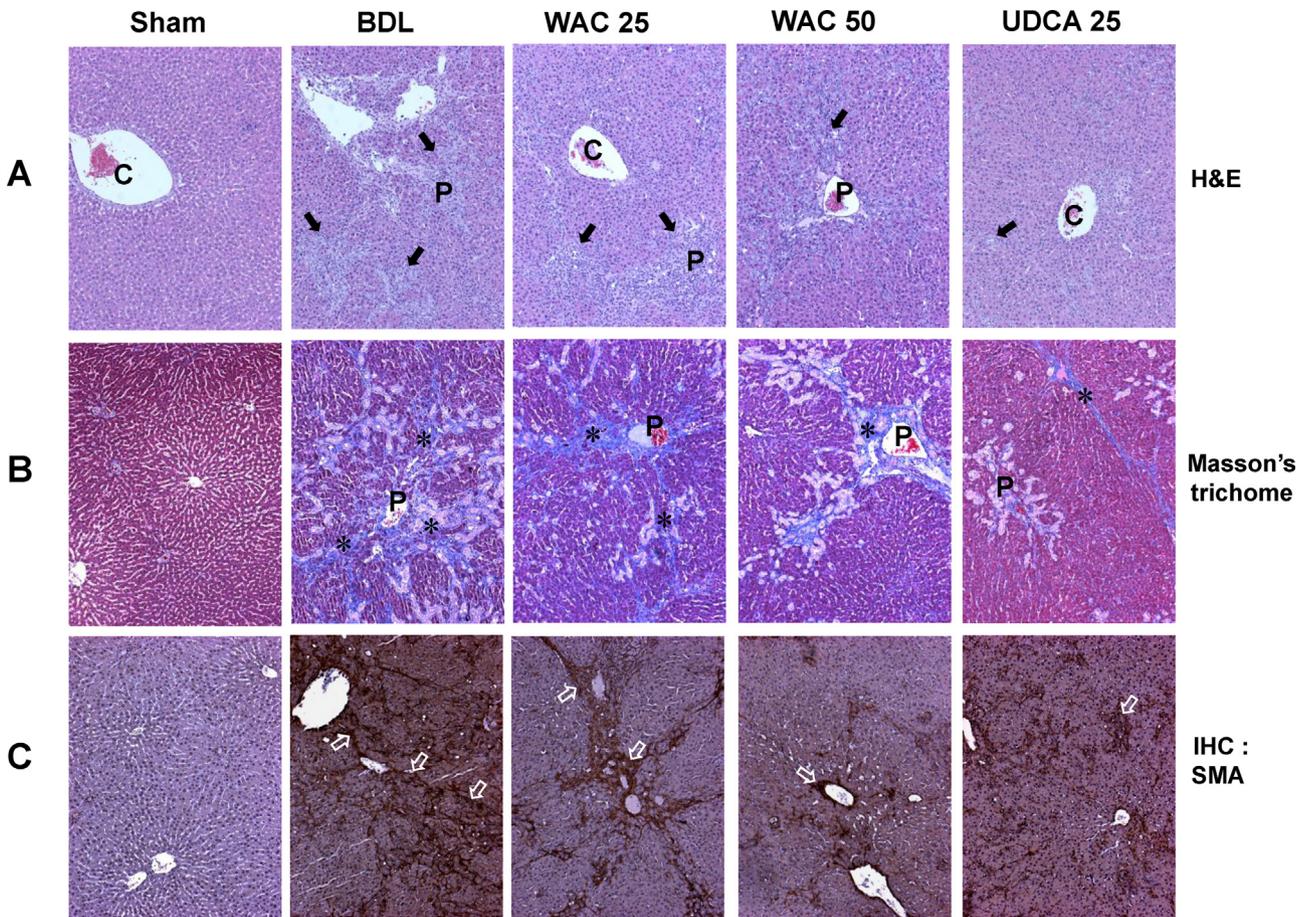


Fig. 3. Histopathological findings of liver tissue in the BDL model. Two weeks after BDL, hepatic tissues were evaluated by staining of H&E (A) and Masson's trichrome (B), and by immunohistochemistry for α-SMA (C). All photos are at 100× magnification. Arrow: liver injury; asterisk: collagen deposition; open arrow: α-SMA expression; C: central vein; P: area of periportal and portal tracts.

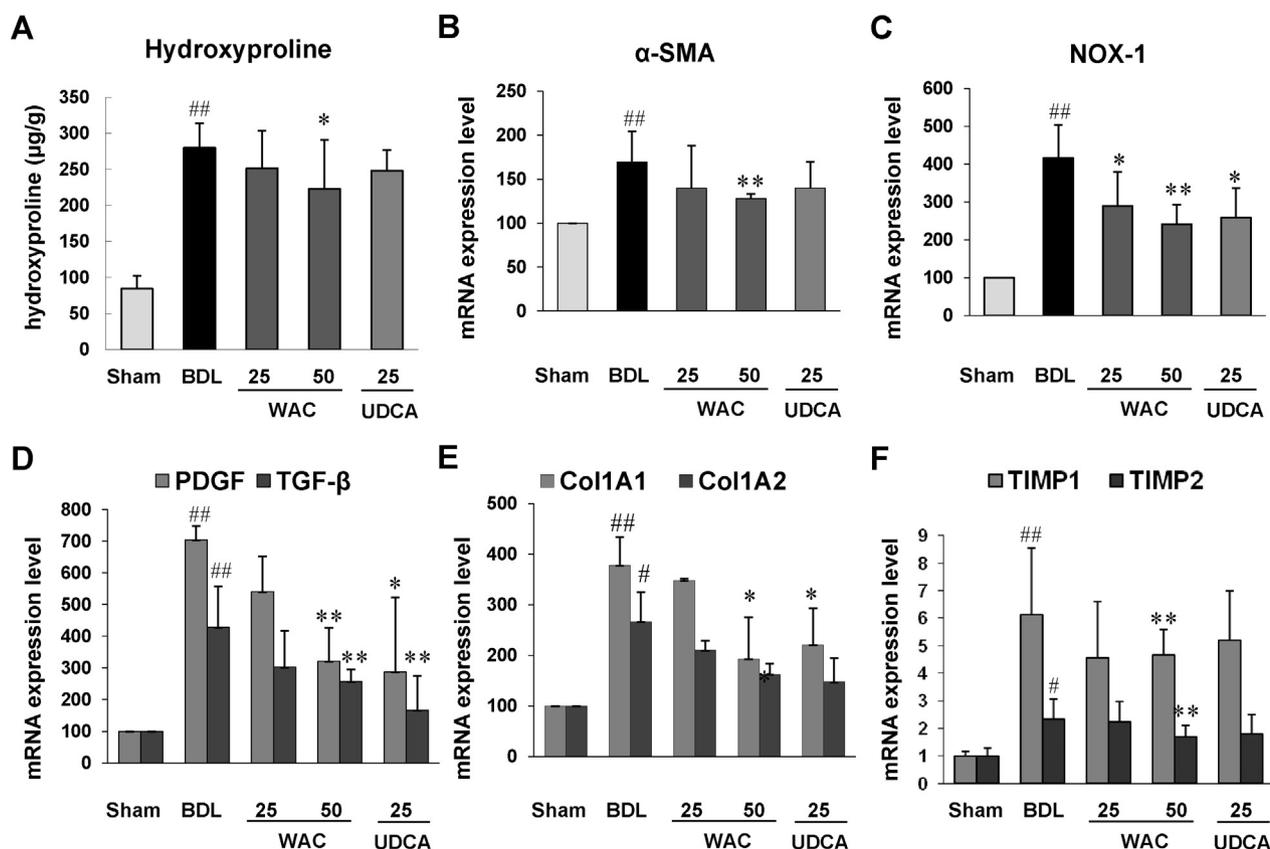


Fig. 4. Hydroxyproline and mRNA expression levels of fibrotic cytokines. Two weeks after BDL, the protein levels of hydroxyproline (A) in hepatic tissues were analyzed by ELISA. Hepatic mRNA expression levels of α -SMA (B), NOX-1 (C), TGF- β /PDGF- β (D), collagen type IA1/2 (E), and TIMP-1/2 (F) were determined by quantitative RT-PCR. Data are expressed as averages \pm SD (fold change relative to sham group). ^{##} $P < 0.01$ compared with sham group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with BDL group.

4. Discussion

Recently, cholestatic fibrosis has been increasingly recognized as a cause of high morbidity and mortality in humans (Haber et al., 2008). Therefore, many pharmacological approaches for cholestatic disorders and hepatic fibrosis have been investigated (Dhiman and Chawla, 2005). For thousands of years, *A. capillaris* has been traditionally used in the treatment of various liver diseases. An aqueous extract of *A. capillaris* inhibits ethanol-induced cytokine secretion and cytokine-induced cytotoxicity/apoptosis of hepatocytes (Koo et al., 2002). *A. capillaris* contains a variety of active compounds, including flavonoids (capillarisin and quercetin), flavonoid glycosides (hyperoside), phenolic compounds (chlorogenic acid), and coumarins (esculetin and scoparone) (Choi et al., 2011; Jang et al., 2006; Mase et al., 2010). *A. capillaris* and its main constituents have been shown to have antioxidative, anti-inflammatory, and hepatoprotective properties in chemical-induced chronic liver damage (Lee et al., 2007, 2008; Mase et al., 2010). In the present study, we found three isomers of chlorogenic acid (3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid) and quercetin. However, scoparone, a well-known constituent of *A. capillaris*, was not detected in our chromatogram analysis. To identify the specific compounds at RT 11.20–12.21, additional studies using nuclear magnetic resonance (NMR) are recommended.

To provide support of *A. capillaris* extract as a potential drug candidate for cholestatic hepatofibrosis, the present study adopted a BDL model in rats. After 14 days, the BDL group showed marked increases of 2- to 70-fold in serum AST, ALT, total cholesterol, triglycerides, and total bilirubin. The retention of bile salts in the liver resulted in hepatocyte damage arising from a rapid inflammatory response including the activation of Kupffer cells

and the accumulation of leukocytes (Saito and Maher, 2000; Schmucker et al., 1990). Our result demonstrated that serum markers of hepatic tissue injury were significantly attenuated in the WAC or UDCA groups compared with the BDL group. UDCA, a positive control in our study, has been widely used for patients with cholestatic disease or gallstones (Paumgartner et al., 1994). Therefore, these results suggest that WAC has hepatoprotective activity against the cholestatic condition.

The BDL model was known to cause notable oxidative stress, which is one of main contributors in the pathogenesis of liver injury and fibrotic progression (Alptekin et al., 1997; Desmet et al., 1995). In the present study, BDL promoted oxidative stress, as evidenced by elevated serum NO concentration and lipid peroxidation (MDA) in serum and hepatic tissues. However WAC administration significantly reduced these changes. MDA is a biomarker for lipid peroxidation generated by ROS, which are critically controlled by NOX, the reduced form of NADPH oxidase (Cui et al., 2011; Levitan et al., 2010). NO is a typical indicator of reactive oxygen species (ROS), and lipid peroxidation is an indicator of oxidative stress in the BDL model (Alptekin et al., 1997; Desmet et al., 1995; Fujita et al., 2003). Immunohistochemistry staining against 4-HNE demonstrated the lipid peroxidation inside cells (Poli and Schaur, 2000), and BDL treatment strongly enhanced the 4-HNE expression compared to the sham group. Inflammatory cells such as neutrophils release ROS, which induce collagen production (Casini et al., 1997); WAC administration significantly inhibited these inflammatory cells. The remarkable upregulation of NOX-1 expression from BDL by up to 4-fold and the suppressive action of WAC on NOX-1 gene expression in hepatic tissue strongly supports the pharmacological action of WAC. NOX-1 plays an important role in the control of free radical production as a catalytic subunit of the

superoxide-generating NADPH (nicotinamide adenine dinucleotide phosphate) oxidase of phagocytes in liver tissue (Cui et al., 2011; Levitan et al., 2010). Liver is susceptible to oxidative stress, and thus hepatic tissue equips well-organized non-enzymatic and enzymatic defense systems against oxidative stress (Sies, 1997). BDL significantly depleted the GSH content and the GSH-px and GSH-rd activities; however, WAC treatment significantly restored the levels. SOD and catalase activities were slightly increased in BDL groups, and both were significantly augmented by WAC administration. This phenomenon might be a compensative response to the BDL-induced oxidative stress and the depletion of the GSH defense system (Abraham et al., 2012).

It is well known that most chronic injury of hepatic tissue ultimately leads to liver fibrosis (Iredale, 2001). We found that BDL markedly induced the development of moderate hepatic fibrosis, which was likely ECM accumulation surrounding the hepatic vein and in the small connection between the veins, as determined by Masson's trichrome staining. The severity of fibrotic change was very similar with other studies (Poli, 2000). In addition, BDL-induced hepatic fibrosis was demonstrated based on the activation of hepatic stellate cells (HSCs) and a drastically high level of hydroxyproline content of up to approximately 3.5 times normal. The hydroxyproline level usually reflects the amount of connective tissue such as collagen in the liver, and the cellular characteristic of liver fibrosis is the activation of HSCs, represented by an immunohistochemical response against α -SMA (Bataller and Brenner, 2005).

In general, liver fibrosis is believed to be an unbalanced consequence of dynamic production and degradation of ECM by HSCs, and it is critically influenced by pro-fibrogenic and anti-fibrogenic cytokines such as PDGF- β , TGF- β , and matrix metalloproteinases (MMPs) (Bataller and Brenner, 2005; Dai and Jiang, 2001). In our study, real-time PCR analysis revealed that BDL drastically upregulated the fibrogenic cytokines including PDGF- β , TGF- β , Col1A1, Col1A2, TIMP1, and TIMP2 in hepatic tissues. PDGF- β and TGF- β are the most well-known fibrogenic cytokines to activate quiescent HSCs to produce collagens inside liver (De Minicis et al., 2007). TGF- β is also known to suppress the GSH synthesis in HSCs and primary hepatocytes (De Bleser et al., 1999; Sanchez et al., 1997), which is in accordance to the reduction of the GSH system in this experiment. The genes Col1A1 and Col1A2 encode the major component of type I collagen, which is an essential component of the accumulated ECM in hepatic fibrosis, whereas TIMP1 and TIMP2 are inhibitors of MMPs, working as anti-fibrotic cytokines by resolving the ECM (Bellayr et al., 2009; Friedman, 2008). WAC and UDCA treatment significantly attenuated abnormal gene expressions. These effects were in accordance with previous results of herbal formulae containing *A. capillaris* (Lee et al., 2007).

Taken together, we conclude that *A. capillaris* can be used as an anti-hepatofibrotic remedy, especially in cholestatic liver disorders, and the responsible mechanisms may involve the regulation of oxidative stress-associated enzymes and fibrogenic cytokines.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology, Republic of Korea (No. 2009-0087375).

References

- Abraham S, Hermes E, Szabo A, Ferencz A, Jancso Z, Duda E, et al. Effects of Kupffer cell blockade on the hepatic expression of metallothionein and heme oxygenase genes in endotoxemic rats with obstructive jaundice. *Life Sciences* 2012;90:140–6.
- Alptekin N, Mehmetcik G, Uysal M, Aykac-toker G. Evidence for oxidative stress in the hepatic mitochondria of bile duct ligated rats. *Pharmacological Research* 1997;36:243–7.
- Bataller R, Brenner DA. Liver fibrosis. *Journal of Clinical Investigation* 2005;115:209–18.
- Beers Jr RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* 1952;195:133–40.
- Bellayr IH, Mu X, Li Y. Biochemical insights into the role of matrix metalloproteinases in regeneration: challenges and recent developments. *Future Medicinal Chemistry* 2009;1:1095–111.
- Casini A, Ceni E, Salzano R, Biondi P, Parola M, Galli A, et al. Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide. *Hepatology* 1997;25:361–7.
- Chen WY, Chen CJ, Liao JW, Mao FC. Chromium attenuates hepatic damage in a rat model of chronic cholestasis. *Life Sciences* 2009;84:606–14.
- Choi JH, Kim DW, Yun N, Choi JS, Islam MN, Kim YS, et al. Protective effects of hyperoside against carbon tetrachloride-induced liver damage in mice. *Journal of Natural Products* 2011;74:1055–60.
- Cui W, Matsuno K, Iwata K, Ibi M, Matsumoto M, Zhang J, et al. NOX1/nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase promotes proliferation of stellate cells and aggravates liver fibrosis induced by bile duct ligation. *Hepatology* 2011;54:949–58.
- Dai WJ, Jiang HC. Advances in gene therapy of liver cirrhosis: a review. *World Journal of Gastroenterology* 2001;7:1–8.
- De Bleser PJ, Xu G, Rombouts K, Rogiers V, Geerts A. Glutathione levels discriminate between oxidative stress and transforming growth factor- β signaling in activated rat hepatic stellate cells. *Journal of Biological Chemistry* 1999;274:33881–7.
- De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, et al. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. *Gastroenterology* 2007;132:1937–46.
- Desmet V, Roskams T, Van Eyken P. Ductular reaction in the liver. *Pathology, Research and Practice* 1995;191:513–24.
- Dhiman RK, Chawla YK. Herbal medicines for liver diseases. *Digestive Diseases and Sciences* 2005;50:1807–12.
- Ellman GL. Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* 1959;82:70–7.
- Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, et al. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *Journal of Clinical Investigation* 1999;103:137–45.
- Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;134:1655–69.
- Fujita M, Shannon JM, Morikawa O, Gauldie J, Hara N, Mason RJ. Overexpression of tumor necrosis factor- α diminishes pulmonary fibrosis induced by bleomycin or transforming growth factor- β . *American Journal of Respiratory Cell and Molecular Biology* 2003;29:669–76.
- Georgiev P, Jochum W, Heinrich S, Jang JH, Nocito A, Dahm F, et al. Characterization of time-related changes after experimental bile duct ligation. *British Journal of Surgery* 2008;95:646–56.
- Gross Jr JB, Reichen J, Zeltner TB, Zimmermann A. The evolution of changes in quantitative liver function tests in a rat model of biliary cirrhosis: correlation with morphometric measurement of hepatocyte mass. *Hepatology* 1987;7:457–63.
- Haber B, Ferreira CT, Aw M, Bezerra J, Sturm E, Thompson R, et al. Cholestasis: current issues and plan for the future. *Journal of Pediatric Gastroenterology and Nutrition* 2008;47:220–4.
- Herrmann F, Romero MR, Blazquez AG, Kaufmann D, Ashour ML, Kahl S, et al. Diversity of pharmacological properties in Chinese and European medicinal plants: cytotoxicity, antiviral and antitrypanosomal screening of 82 herbal drugs. *Diversity* 2011;3:547–80.
- Hong JH, Hwang EY, Kim HJ, Jeong YJ, Lee IS. *Artemisia capillaris* inhibits lipid accumulation in 3T3-L1 adipocytes and obesity in C57BL/6J mice fed a high fat diet. *Journal of Medicinal Food* 2009;12:736–45.
- Hong JH, Lee IS. Effects of *Artemisia capillaris* ethyl acetate fraction on oxidative stress and antioxidant enzyme in high-fat diet induced obese mice. *Chemico-Biological Interactions* 2009;179:88–93.
- Iredale JP. Hepatic stellate cell behavior during resolution of liver injury. *Seminars in Liver Disease* 2001;21:427–36.
- Jang G. *Jung gyeong jeon seo*. Seoul: Hollym Corp. Publishers; 1975.
- Jang SI, Kim YJ, Kim HJ, Lee JC, Kim HY, Kim YC, et al. Scoparone inhibits PMA-induced IL-8 and MCP-1 production through suppression of NF- κ B activation in U937 cells. *Life Sciences* 2006;78:2937–43.
- Koo HN, Hong SH, Jeong HJ, Lee EH, Kim NG, Choi SD, et al. Inhibitory effect of *Artemisia capillaris* on ethanol-induced cytokines (TNF- α , IL-1 α) secretion in Hep G2 cells. *Immunopharmacology and Immunotoxicology* 2002;24:441–53.
- Lee HS, Kim HH, Ku SK. Hepatoprotective effects of *Artemisia capillaris* herba and *Picrorrhiza rhizoma* combinations on carbon tetrachloride-induced subacute liver damage in rats. *Nutrition Research* 2008;28:270–7.

- Lee TY, Chang HH, Chen JH, Hsueh ML, Kuo JJ. Herb medicine Yin-Chen-Hao-Tang ameliorates hepatic fibrosis in bile duct ligation rats. *Journal of Ethnopharmacology* 2007;109:318–24.
- Levitan I, Volkov S, Subbaiah PV. Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxidants and Redox Signalling* 2010;13:39–75.
- Liu TZ, Lee KT, Chern CL, Cheng JT, Stern A, Tsai LY. Free radical-triggered hepatic injury of experimental obstructive jaundice of rats involves overproduction of proinflammatory cytokines and enhanced activation of nuclear factor kappaB. *Annals of Clinical and Laboratory Science* 2001;31:383–90.
- Mase A, Makino B, Tsuchiya N, Yamamoto M, Kase Y, Takeda S, et al. Active ingredients of traditional Japanese (kampo) medicine, inchinkoto, in murine concanavalin A-induced hepatitis. *Journal of Ethnopharmacology* 2010;127:742–9.
- Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry* 1978;86:271–8.
- Mormone E, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chemico-Biological Interactions* 2011;193:225–31.
- Paumgartner G, Pauletzki J, Sackmann M. Ursodeoxycholic acid treatment of cholesterol gallstone disease. *Scandinavian Journal of Gastroenterology Supplement* 1994;204:27–31.
- Pinzani M, Rombouts K. Liver fibrosis: from the bench to clinical targets. *Digestive and Liver Disease* 2004;36:231–42.
- Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. *Molecular Aspects of Medicine* 2000;21:49–98.
- Poli G, Schaur RJ. 4-Hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life* 2000;50:315–21.
- Saito JM, Maher JJ. Bile duct ligation in rats induces biliary expression of cytokine-induced neutrophil chemoattractant. *Gastroenterology* 2000;118:1157–68.
- Sanchez A, Alvarez AM, Benito M, Fabregat I. Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor beta in fetal rat hepatocytes in primary culture. *Hepatology* 1997;26:935–43.
- Schmucker DL, Ohta M, Kanai S, Sato Y, Kitani K. Hepatic injury induced by bile salts: correlation between biochemical and morphological events. *Hepatology* 1990;12:1216–21.
- Seo KS, Jeong HJ, Yun KW. Antimicrobial activity and chemical components of two plants, *Artemisia capillaris* and *Artemisia iwayomogi*, used as Korean herbal Injin. *Journal of Ecology and Field Biology* 2010;33:141–7.
- Sies H. Oxidative stress: oxidants and antioxidants. *Experimental Physiology* 1997;82:291–5.
- Wang JH, Choi MK, Shin JW, Hwang SY, Son CG. Antifibrotic effects of *Artemisia capillaris* and *Artemisia iwayomogi* in a carbon tetrachloride-induced chronic hepatic fibrosis animal model. *Journal of Ethnopharmacology* 2012;140:179–85.
- Wang JH, Shin JW, Son JY, Cho JH, Son CG. Antifibrotic effects of CGX, a traditional herbal formula, and its mechanisms in rats. *Journal of Ethnopharmacology* 2010;127:534–42.
- Webster CR, Anwer MS. Cyclic adenosine monophosphate-mediated protection against bile acid-induced apoptosis in cultured rat hepatocytes. *Hepatology* 1998;27:1324–31.