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Isorhamnetin-3-*O*-galactoside Protects against CCl_4-Induced Hepatic Injury in Mice

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Abstract

This study was performed to examine the hepatoprotective effect of isorhamnetin-3-*O*-galactoside, a flavonoid glycoside isolated from *Artemisia capillaris* Thunberg (Compositae), against carbon tetrachloride (CCl_4)-induced hepatic injury. Mice were treated intraperitoneally with vehicle or isorhamnetin-3-*O*-galactoside (50, 100, and 200 mg/kg) 30 min before and 2 h after CCl_4 (20 μl/kg) injection. Serum aminotransferase activities and hepatic level of malondialdehyde were significantly higher after CCl_4 treatment, and these increases were attenuated by isorhamnetin-3-*O*-galactoside. CCl_4 markedly increased serum tumor necrosis factor-α level, which was reduced by isorhamnetin-3-*O*-galactoside. The levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and heme oxygenase-1 (HO-1) protein and their mRNA expression levels were significantly increased after CCl_4 injection. The levels of HO-1 protein and mRNA expression levels were augmented by isorhamnetin-3-*O*-galactoside, while isorhamnetin-3-*O*-galactoside attenuated the increases in iNOS and COX-2 protein and mRNA expression levels. CCl_4 increased the level of phosphorylated c-Jun N-terminal kinase, extracellular signal-regulated kinase and p38, and isorhamnetin-3-*O*-galactoside reduced these increases. The nuclear translocation of nuclear factor kappa B (NF-κB), activating protein-1, and nuclear factor erythroid 2-related factor 2 (Nrf2) were significantly increased after CCl_4 administration. Isorhamnetin-3-*O*-galactoside attenuated the increases of NF-κB and c-Jun nuclear translocation, while it augmented the nuclear level of Nrf2. These results suggest that isorhamnetin-3-*O*-galactoside ameliorates CCl_4-induced hepatic damage by enhancing the anti-oxidative defense system and reducing the inflammatory signaling pathways.

Key Words: Carbon tetrachloride, Heme oxygenase-1, Hepatotoxicity, Inflammation, Isorhamnetin-3-*O*-galactoside, Oxidative stress

INTRODUCTION

Acute and chronic liver diseases constitute a global concern and the concern is worsened by the lack of reliable liver protective drugs, despite the increasing need for agents to protect the liver from damage. Therefore, complementary and alternative medicines for the treatment of liver diseases have been receiving considerable interest (Seeff *et al.*, 2001). Therapeutically effective agents from natural products may reduce the risk of clinical toxicity.

Carbon tetrachloride (CCl_4) has long been known as a toxicant and has been widely used in many *in vitro* and *in vivo* toxicology studies. CCl_4 causes liver toxicity, resulting in cellular necrosis, fatty degeneration, fibrosis and cirrhosis (Taïeb *et al.*, 2005). Administered CCl_4 is metabolized by cytochrome P450 (CYP), primarily CYP2E1 and results in the formation of trichloromethyl radical ($\cdot CCl_3$), which initiates lipid peroxidation and protein oxidation leading to hepatocellular damage (Manibusan *et al.*, 2007).

Artemisia capillaris Thunb. (Compositae) is one of the oldest and most commonly prescribed herbs in Eastern traditional medicine, and has been used as an analgesic, antimicrobial agent and a remedy for the treatment of hepatitis and bilious disorders (Chang and But, 1987). An aqueous extract of *Artemisia capillaris* was shown to inhibit interleukin (IL)-1 receptor- and tumor necrosis factor (TNF) receptor-induced cytotoxicity and ethanol-induced apoptosis of HepG2 cells (Koo *et al.*, 2002). A previous study reported that *Artemisia capillaris* reduced the lipopolysaccharide-induced inflammatory response in a human hepatoma cell line and in the rat liver (Hong *et al.*, 2004), and prevented 2,2'-azobis(2-amidinopropane) dihydrochloride-induced liver damage in rats (Han *et*

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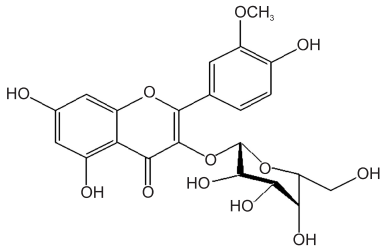

Fig. 1. The structure of isorhamnetin-3-*O*-galactoside.

al., 2006). Isorhamnetin-3-*O*-galactoside (Fig. 1), one of the flavonoid constituents from *Artemisia capillaris*, exerted antiinflammatory activity by inhibiting the production of 5-lipoxygenase-induced leukotriene (Kwon *et al.*, 2011).

Therefore, this study was designed to investigate the protective effects of isorhamnetin-3-*O*-galactoside against CCl_4-induced acute hepatic injury, with particular attention to the oxidative stress and inflammatory pathways.

MATERIALS AND METHODS

Isolation of isorhamnetin-3-*O*-galactoside from an *Artemisia capillaris*

The whole plant of *Artemisia capillaris* Thunb. was dried and grinded to powder. The dried powder (9.5 kg) was then extracted with hot MeOH (50.0 l×3 times) for 3 h. After filtration, total filtrate was concentrated to dryness in vacuo at 400°C to obtain the MeOH extract (900 g). Following this, the MeOH extract was suspended in distilled water:MeOH (9:1) and successively partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), n-butanol (n-BuOH) to yield CH_2Cl_2 (354 g), EtOAc (141 g), n-BuOH (196 g) fractions, respectively, as well as the H_2O residue (218 g). n-BuOH fractions were chromatographed over HP-20 diaion using the H_2O:MeOH; (1:0→6:4→4:6→0:1) solvent system to afford 4 major subfractions H_2O (132 g), 40% MeOH (30.4 g), 60% MeOH (21 g) and MeOH (6.8 g), respectively. 60% MeOH subfraction was chromatographed in a silica gel column using CH_2Cl_2:MeOH (20:1) with a gradual increase in MeOH in order to obtain 17 subfractions (6F-1 to 6F-17). Decantation of subfraction 6F-3 yielded isorhamnetin-3-*O*-galactoside (270 mg).

^{1}H-NMR (400 MHz, DMSO-d_6) δ : 12.63 (^{1}H, s, -OH), 10.91 (^{1}H, brs, -OH), 9.83 (^{1}H, brs, -OH), 8.02 (^{1}H, d, J = 2.0 Hz, H-2'), 7.49 (^{1}H, dd, J = 2.0 & 8.0 Hz, H-6'), 6.90 (^{1}H, d, J = 8.0 Hz, H-5'), 6.45 (^{1}H, d, J = 2.0 Hz, H-8), 6.31 (^{1}H, d, J = 2.0 Hz, H-6), 5.52 (^{1}H, d, J = 8.0 Hz, H-1'), 5.20 (^{1}H, brd), 4.91 (^{1}H, brs, -OH), 4.55 (^{1}H, brd, -OH), 4.50 (^{1}H, brs, -OH), 3.85 (^{3}H, s, -OCH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ : 177.4 (C-4), 164.2(C-7), 161.2 (C-5), 156.4 (C-9), 156.3 (C-2), 149.4 (C-3'), 146.99 (C-4'), 133.1 (C-3), 121.9 (C-6'), 121.1 (C-1'), 115.1 (C-5'), 113.5 (C-2'), 104.0 (C-10), 101.6 (Gal-1), 98.7 (C-6), 93.7 (C-8), 75.9 (Gal-2), 73.1 (Gal-3), 71.3 (Gal-5), 67.96 (Gal-4), 60.3 (Gal-6), 55.99 (-OCH_3).

Treatment of animals

Male ICR mice weighing 25-30 g (Daehan Biolink Co., Eum-seong, Korea) were fasted overnight but given tap water *ad libitum*. All animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines. The animals were randomly assigned to 6 groups comprising 8-10 animals per group. The mice in group 1 (control) intraperitoneally received only olive oil (10 ml/kg). In groups 2 to 6, CCl_4 dissolved in olive oil (1:499, v/v) was administered intraperitoneally (final concentration of 20 μl/kg). Groups 1 and 2 (vehicle) were treated intraperitoneally with Tween-80 in saline (1:9, v/v). The animals in groups 3 to 5 were treated intraperitoneally with isorhamnetin-3-*O*-galactoside (50, 100, and 200 mg/kg), and group 6 was intraperitoneally treated with silymarin (positive control, 800 mg/kg), 30 min before and 2 h after CCl_4 injection. The timing of the isorhamnetin-3-*O*-galactoside treatment was selected based on the previous report (Kim *et al.*, 2010). Blood was collected 24 h after CCl_4 administration. Each liver was isolated and stored at -75°C for analysis, except for the left lobe, which was used for histological studies.

Serum aminotransferases activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard spectrophotometric procedures using the ChemiLab ALT and AST assay kits (IVDLab Co., Uiwang, Korea), respectively.

Histological analysis

Liver tissues were removed from a portion of the left lobe, and fixed immediately in 10% neutral buffered formalin, embedded in paraffin, and then sectioned at 5 μm thickness. Serial sections were stained with hematoxylin and eosin for evaluation of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration. The sections were examined in a blind manner under an Olympus CKX 41 microscope (Olympus optical Co. Ltd., Tokyo, Japan).

Lipid peroxidation

The steady-state level of malondialdehyde (MDA), which is the end product of lipid peroxidation, was analyzed in liver homogenates by spectrophotometric measurement of the levels of thiobarbituric acid reactive substances at 535 nm, as described by Buege and Aust (1978), using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, USA) as the standard.

Serum TNF-α level

Serum concentration of TNF-α was quantified using a commercial TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences Co., CA, USA).

Western blot immunoassay

Freshly isolated liver tissue was homogenized in lysis buffer for the preparation of whole protein extracts. NE-PER® (Pierce Biotechnology, Rockford, IL, USA) was used for the extraction of nuclear proteins according to the manufacturer's instructions. The BCA Protein Assay kit (Pierce Biotechnology) was used to determine protein concentrations. Protein samples were loaded on 10-15% polyacrylamide gels, separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). After the transfer, the membranes were washed with 0.1% Tween-20 in 1×Tris Buffered Saline (TBS/T) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS/T. The blots were then incubated overnight at 4°C with primary antibodies. The

next day, the blots were incubated in appropriate secondary antibodies and were detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Korea), according to the manufacturer's instructions. ImageQuant™TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) was used for the densitometric evaluation of visualized immuno-reactive bands. Primary antibodies against inducible nitric oxide synthase (iNOS) (Transduction Laboratories, San Jose, CA, USA; 1:1,000 dilution), cyclooxygenase-2 (COX-2) (Cayman, Ann Arbor, MI, USA; 1:1,000 dilution), phoshoprylated (p)-p38, p-c-Jun N-terminal kinase (JNK), total p38, and total JNK (Cell Signaling Technology Inc., Beverly, MA, USA; 1:1,000), p-extracellular signal-regulated kinase (ERK) and total ERK (Cell Signaling Technology Inc.; 1:2,000), nuclear factor (NF)-κB/p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000) and c-Jun p39 phosphorylated on serine-63 (Santa Cruz Biotechnology; 1:500), heme oxygenase-1 (HO-1) (Stressgen Bioreagents Corp., Ann Arbor, MI, USA; 1:2,500) and finally nuclear factor erythroid 2-related factor 2 (Nrf2) (Santa Cruz Biotechnology; 1:1,000) were used and the signals were normalized to that of β-actin (Sigma; 1:2,500 dilution) or lamin B1 (Abcam, Cambridge, UK; 1:2,500).

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted and the first strand of cDNA was synthesized by reverse transcription of total RNA using oligo(dT)$_{12-18}$ primer and SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen Tech-LineTM, Carlsbad, CA, USA). PCR reaction was carried out in a 20 μl reaction volume with a diluted cDNA sample. Final reaction concentrations were as follows: sense and antisense primers, 10 pM; dNTP mix, 250 μM; ×10 PCR buffer, and Ex Taq DNA polymerase, 0.5 U/reaction. PCR was carried out with an initial denaturation step at 94°C for 5 min, and a final extension step at 72°C for 7 min in the GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). The primers used in this study are demonstrated in Table 1. Amplification cycling conditions were as follows: 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s for *iNOS*; 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for *COX-2*; 30 cycles of 94°C (30 s), 56°C (30 s), and 72°C (30 s) for *HO-1*; 35 cycles of 94°C (30 s), 62°C (30 s) and 72°C (60 s) for β-*actin*. Following RT-PCR, 10 μl samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. The intensity of each PCR product was analyzed semi-quantitatively using a digital camera (DC120, Eastman Kodak, New Haven, CT, USA).

Table 1. PCR primers used in this study and the amplified product length

Gene (accession number)	Primer sequences (5`-3`)	Product length (bp)
TNF-α (M11731)	Sense: AGCCCACGTCGTAGCAAACCACCAA	446
	Antisense: AACACCCATTCCCTTCACAGAGCAAT	
iNOS (NM_010927)	Sense: AAGCTGCATGTGACATCGACCCGT	598
	Antisense: GCATCTGGTAGCCAGCGTACCGG	
COX-2 (NM_011198)	Sense: ACTCACTCAGTTTGTTGAGTCATTC	582
	Antisense: TTTGATTAGTACTGTAGGGTTAATG	
HO-1 (NM_010442)	Sense: AACAAGCAGAACCCAGTCT	374
	Antisense: TGTCATCTCCAGAGTGTTC	
β-*Actin* (X03672)	Sense: TGGAATCCTGTGGCATCCATGAAA	348
	Antisense: TAAAACGCAGCTCAGTAACAGTCCG	

Table 2. Effect of isorhamnetin-3-*O*-galactoside on serum aminotransferase activities and lipid peroxidation

Groups		ALT (U/l)	AST (U/l)	MDA (nmol/mg protein)
Control		40.0 ± 5.0	32.0 ± 3.3	0.5 ± 0.04
CCl_4				
Vehicle		13,001 ± 636*	10,090 ± 730*	3.2 ± 0.5*
Isorhamnetin-3-*O*-galactoside	50 mg/kg	12,769 ± 542*	9,743 ± 430*	2.6 ± 0.1*
	100 mg/kg	9,501 ± 666*,#	6,345 ± 669*,#	1.5 ± 0.1*,#
	200 mg/kg	9,362 ± 540*,#	6,979 ± 755*,#	1.4 ± 0.1*,#
Sylymarin 800 mg/kg		9,429 ± 607*,#	6,946 ± 765*,#	1.5 ± 0.2*,#

Isorhamnetin-3-*O*-galactoside (50, 100, and 200 mg/kg) or silymarin (800 mg/kg) was administered intraperitoneally 30 min before and 2 h after CCl_4 injection. Liver damage was assessed by measurement of circulating serum ALT and AST activities. The values are presented as mean ± SEM for 8-10 mice per group. *Significantly different (p<0.01) from the control group. #Significantly different (p<0.01) from the vehicle-treated CCl_4 group.

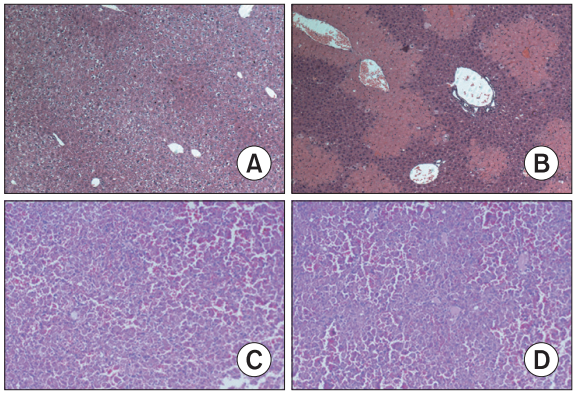

Fig. 2. Effect of isorhamnetin-3-*O*-galactoside on the histological changes in the liver (original magnification ×100). (A) Control group, showing normal lobular architecture and cell structure (B) vehicle-treated CCl_4 group, showing extensive hepatocellular damage with the presence of portal inflammation, centrizonal necrosis, and Kupffer cell hyperplasia (C) isorhamnetin-3-*O*-galactoside (100 mg/kg)-treated CCl_4 group, showing mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia (D) silymarin (800 mg/kg)-treated CCl_4 group, showing mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia.

Statistical analysis

All results are presented as mean ± SEM. The overall significance of the data was examined by one-way analysis of variance. Differences between the groups were considered significant at $p<0.05$ with the appropriate Bonferroni correction made for multiple comparisons.

RESULTS

Effect of isorhamnetin-3-*O*-galactoside on hepatocellular damage

As shown in Table 2, serum ALT and AST activities were 40 ± 5 U/l and 32 ± 3 U/l in the control group. The vehicle-treated CCl_4 group showed increases in serum ALT and AST activities at 24 h after CCl_4 injection (13,001 ± 636 U/l and 10,090 ± 730 U/l, respectively). Isorhamnetin-3-*O*-galactoside at doses of 100 and 200 mg/kg significantly attenuated the increases in ALT activity to 9,501 ± 666 U/l and 9,362 ± 540 U/l, respectively. Consistent with the ALT data, the serum AST levels were significantly attenuated to 6,345 ± 669 U/l and 6,979 ± 755 U/l by 100 and 200 mg/kg of isorhamnetin-3-*O*-galactoside, respectively. Silymarin at 800 mg/kg also significantly attenuated the increases in ALT and AST activities to 9429 ± 607 U/l and 6,946 ± 765 U/l, respectively. However, 50 mg/kg of isorhamnetin-3-*O*-galactoside did not affect the serum ALT and AST activities.

The histological features shown in Fig. 2A demonstrate a normal liver lobular architecture and cell structure in the control group. In contrast, hepatocyte ballooning and necrosis were observed in the vehicle-treated CCl_4 group with multiple areas of portal inflammation as well as a moderate increase in inflammatory cell infiltration (Fig. 2B). These pathological changes were attenuated by 100 mg/kg of isorhamnetin-3-*O*-galactoside (Fig. 2C) and 800 mg/kg of silymarin (Fig. 2D).

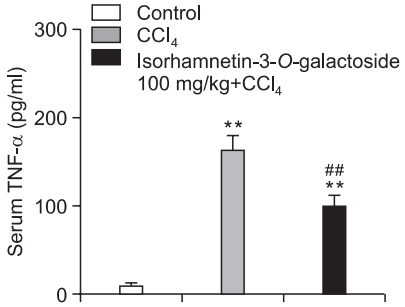

Fig. 3. Effect of isorhamnetin-3-*O*-galactoside (100 mg/kg) on the serum TNF-α level after CCl_4 administration. Serum level of TNF-α was measured using a commercially available ELISA kit. Values are presented as mean ± SEM of 8-10 mice per group. **Significantly different ($p<0.01$) from the control group. ##Significantly different ($p<0.01$) from the vehicle-treated CCl_4 group.

Isorhamnetin-3-*O*-galactoside alone did not affect serum aminotransferase activities (data not shown).

Effect of isorhamnetin-3-*O*-galactoside on lipid peroxidation

In the control group, the level of MDA was 0.5 ± 0.04 nmol/mg protein. Following CCl_4 injection, the level of MDA increased to 3.2 ± 0.5 nmol/mg protein. 100 and 200 mg/kg of isorhamnetin-3-*O*-galactoside significantly attenuated this increase in the MDA level (1.5 ± 0.1 nmol/mg protein and 1.4 ± 0.1 nmol/mg protein, respectively), while 50 mg/kg of isorhamnetin-3-*O*-galactoside did not affect the level of MDA (Table 2).

Effect of isorhamnetin-3-*O*-galactoside on TNF-α, iNOS, COX-2, HO-1 and Nrf2 protein expression

The serum level of TNF-α in the control group was 9.0 ± 2.6 pg/ml. After CCl_4 treatment, the serum level of TNF-α increased to 162.6 ± 17.9 pg/ml and this increase was significantly attenuated (98.3 ± 14.0 pg/ml) by 100 mg/kg of isorhamnetin-3-*O*-galactoside (Fig. 3). Compared with the control group, the levels of iNOS, COX-2 and HO-1 protein expression were 2.2-, 1.7- and 1.7-fold higher in the CCl_4 group, respectively. 100 mg/kg of isorhamnetin-3-*O*-galactoside significantly attenuated the increases in iNOS and COX-2 protein expression levels, while augmenting HO-1 protein expression.

To investigate the mechanism of HO-1 upregulation by isorhamnetin-3-*O*-galactoside, we assessed the nuclear translocation of Nrf2, a well-established transcription factor that regulates HO-1 expression. At 24 h after CCl_4 injection, the level of nuclear Nrf2 protein expression was increased and 100 mg/kg of isorhamnetin-3-*O*-galactoside was found to enhance this increase (Fig. 4).

Effect of isorhamnetin-3-*O*-galactoside on *iNOS*, *COX-2* and *HO-1* mRNA expression

Compared with the control group, the levels of *iNOS*, *COX-2* and *HO-1* mRNA expression increased to 1.7-, 1.9- and 1.5-fold in the CCl_4 group, respectively. 100 mg/kg of isorhamnetin-3-*O*-galactoside significantly attenuated the increase of *iNOS* mRNA expression, while augmenting *HO-1* mRNA expression (Fig. 5).

Effect of isorhamnetin-3-*O*-galactoside on phosphorylation of mitogen-activated protein kinases (MAPKs) and nuclear translocation of NF-κB and activating protein (AP)-1

After CCl_4 injection, phosphorylation of JNK, ERK, and p38 increased to 2.6 times, 1.5 times, and 1.4 times the control value, respectively. 100 mg/kg of isorhamnetin-3-*O*-galactoside attenuated the phosphorylation of JNK, ERK and p38. Moreover, nuclear levels of p 65, a subunit of NF-κB, and phosphorylated c-Jun (p-c-Jun) showed a marked increase in the CCl_4 group compared with the control group, respectively. Isorhamnetin-3-*O*-galactoside at 100 mg/kg significantly attenuated these increases (Fig. 6, 7).

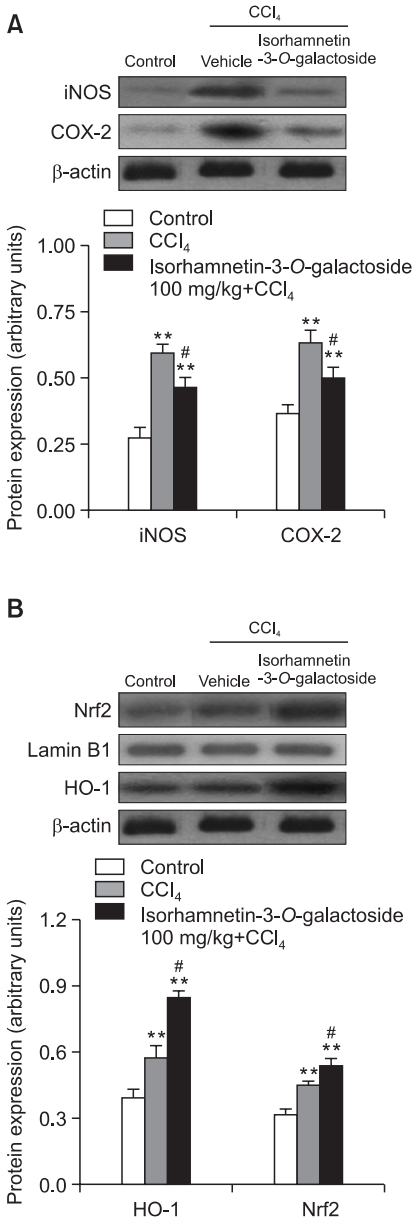

Fig. 4. Effect of isorhamnetin-3-*O*-galactoside (100 mg/kg) on (A) iNOS and COX-2, and (B) HO-1 and Nrf2 protein expression after CCl_4 administration. Western blot analysis was performed to measure iNOS, COX-2 and HO-1 protein expression levels in liver tissue. Nrf2 was measured by western blot analysis using nuclear extracts from the liver. Values are presented as mean ± SEM of 8-10 mice per group. **Significantly different (p<0.01) from the control group. #Significantly different (p<0.05) from the vehicle-treated CCl_4 group.

DISCUSSION

CCl_4-induced hepatic injury is a widely used experimental model for hepatoprotective drug screening. Recently, we screened the 70% ethanol and water extracts of *Artemisia capillaris* and its active components for hepatoprotective agents. Among them, hyperoside and isorhamnetin-3-*O*-galactoside were shown to inhibit CCl_4-induced hepatotoxicity in primary hepatocyte cultures (data not shown). In this study, we investigated the hepatoprotective effects of isorhamnetin-3-*O*-galactoside against CCl_4-induced liver injury *in vivo*.

In the vehicle-treated CCl_4 group, the ALT and AST activities were dramatically increased, indicating severe hepatocellular damage. In contrast, isorhamnetin-3-*O*-galactoside markedly decreased the release of ALT and AST. The hepatoprotective effect of isorhamnetin-3-*O*-galactoside appeared to be similar to that of silymarin, a potent hepatoprotective agent. The histological examination of the liver samples strongly supports the protective effect of isorhamnetin-3-*O*-galactoside. CCl_4 caused centrizonal necrosis, portal inflammation, and Kupffer cell hyperplasia. These alterations were significantly attenuated by isorhamnetin-3-*O*-galactoside. These results suggest that isorhamnetin-3-*O*-galactoside may be appropriate for clinical applications to treat liver disorders.

CCl_4 is responsible for oxidative stress and lipid peroxida-

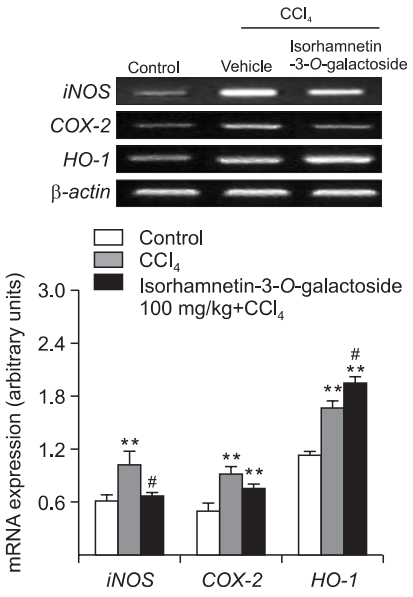

Fig. 5. Effect of isorhamnetin-3-*O*-galactoside (100 mg/kg) on iNOS, COX-2 and HO-1 mRNA expression after CCl_4 administration. RT-PCR was performed to measure iNOS, COX-2 and HO-1 mRNA expression levels in liver tissue. Values are presented as mean ± SEM of 8-10 mice per group. **Significantly different (p<0.01) from the control group. #Significantly different (p<0.05) from the vehicle-treated CCl_4 group.

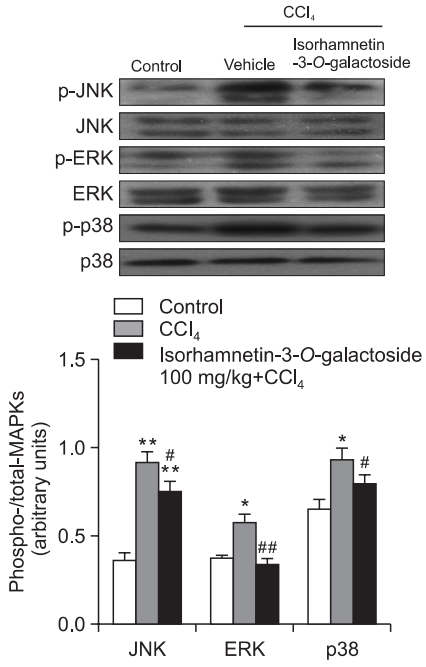

Fig. 6. Effect of isorhamnetin-3-*O*-galactoside (100 mg/kg) on JNK, ERK and p38 protein expression after CCl_4 administration. Western blot analysis was performed to measure the phosphorylated and total forms of JNK, ERK and p38 in liver tissue. Values are presented as mean SEM of 8-10 mice per group. *, **Significantly different (p<0.05, p<0.01) from the control group. #, ##Significantly different (p<0.05, p<0.01) from the vehicle-treated CCl_4 group.

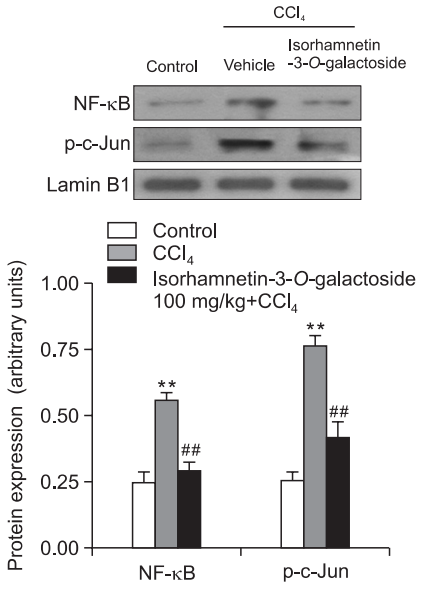

Fig. 7. Effect of isorhamnetin-3-*O*-galactoside (100 mg/kg) on NF-κB and p-c-Jun protein expression after CCl_4 administration. NF-κB and p-c-Jun were measured by western blot analysis using nuclear extracts from liver. Values are presented as mean ± SEM of 8-10 mice per group. **Significantly different (p<0.01) from the control group. ##Significantly different (p<0.01) from the vehicle-treated CCl_4 group.

tion through the CYP2E1-mediated generation of the highly reactive $\cdot CCl_3$, leading to eventual cellular damage characterized by hepatocellular necrosis (Taïeb *et al.*, 2005). In this study, isorhamnetin-3-*O*-galactoside exhibited protective effects by suppressing CCl_4-mediated oxidative damage through decreased production of free radical derivatives, as evidenced by the decreased MDA level at doses of 100 mg/kg and 200 mg/kg. Stressors such as excessive oxidative stress readily induce upregulation of HO-1 (Otterbein and Choi, 2000). HO-1 is an endogenous and cytoprotective enzyme with the main activity catabolizing the oxidative degradation of heme into carbon monoxide (CO), free iron, and biliverdin. Bilirubin/biliverdin is a potent antioxidant that scavenges peroxyradicals, and CO exerts a powerful antiinflammatory effect (Ryter *et al.*, 2007). Several dietary phytochemicals such as glycyrrhizin and hyperoside were shown to induce HO-1 protein and gene expression in our previous studies (Lee *et al.*, 2007; Choi *et al.*, 2011). Nrf2 is a transcription factor that induces expression of various cytoprotective enzymes possessing the antioxidant response element (ARE) in the promoter region and targets genes including glutathione peroxidase, glutathione S-transferase, glutamate cysteine ligase, and HO-1 (Kobayashi and Yamamoto, 2006). In our study, hepatic Nrf2 and HO-1 protein and gene expression levels were markedly increased after CCl_4 treatment. Furthermore, treatment with isorhamnetin-3-*O*-galactoside markedly augmented Nrf2 translocation and HO-1 protein and mRNA expression, suggesting that a strong induction of HO-1 via Nrf2 by isorhamnetin-3-*O*-galactoside protects liver cells from CCl_4-induced oxidative cellular injuries.

Kupffer cells release a number of proinflammatory mediators which are believed to aggravate CCl_4-induced hepatic injury (Badger *et al.*, 1996). TNF-α, a proinflammatory cytokine, is rapidly produced by macrophages in response to tissue damage (Brouckaert and Fiers, 1996). DeCicco *et al.*, (1998) have reported the stimulation of TNF-α production in both serum and the liver following CCl_4 administration, and suggested that $\cdot CCl_3$ activates Kupffer cells to release TNF-α. TNF-α also stimulates the release of cytokines from macrophages and induces the phagocytic oxidative metabolism and NO production (Morio *et al.*, 2001). Nitric oxide (NO) is produced through the action of iNOS, and is involved in various processes including vasodilation and neurotransmission, and the nonspecific host defense system. NO can also react with reactive oxygen species to form cytotoxic oxidants such as peroxynitrite (Rodenas *et al.*, 1995).

Previous studies reported that the induction of COX in the inflammatory response is the secondary effect of CCl_4-induced hepatotoxicity (Basu, 1999). COX-2 is induced by proinflammatory stimuli to form prostaglandins from arachidonic acid (Planagumá *et al.*, 2005). The results of this study showed a significant increase in the serum TNF-α level and iNOS and COX-2 protein and mRNA expression levels in the liver after CCl_4 administration. These increases were attenuated by treatment with isorhamnetin-3-*O*-galactoside treatment, suggesting suppression of inflammatory responses.

The two transcription factors, NF-κB and AP-1, are sensitive to the redox status in abnormal physiological conditions such as CCl_4-induced acute liver injury (Morio *et al.*, 2001). NF-κB is an early response transcription factor and the nuclear translocation of NF-κB leads to gene expression of proinflammatory cytokines. AP-1 is a dimer consisting of JUN, FOS, ATF and

MAF; and the combination in the AP-1 complex affects its DNA binding activity. The MAPK family plays important roles in the regulation of cell proliferation and death in response to various cellular stresses. During CCl_4 challenge, oxidative stress and inflammatory cytokines activate MAPK kinases, leading to phosphorylation of JNK and p38 (Iida *et al.*, 2007). The major target of JNK and p38 is AP-1 and activation of AP-1 mediates ROS-induced hepatocellular death (Czaja, 2003). On the other hand, ERK is involved in survival signals by regulating cell proliferation after partial hepatectomy or CCl_4 intoxication (Taniguchi *et al.*, 2004). In this study, isorhamnetin-3-*O*-galactoside attenuated the increases in MAPK and the nuclear levels of NF-κB and AP-1.

These results provide evidence for the hepatoprotective effect of isorhamnetin-3-*O*-galactoside in CCl_4-induced hepatotoxicity through enhancement of the antioxidative defense system and downregulation of the proinflammatory pathway.

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