

## Protective Effects of Hyperoside against Carbon Tetrachloride-Induced Liver Damage in Mice

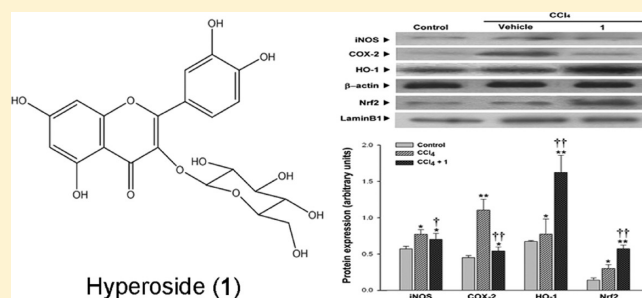
Jun-Ho Choi,<sup>†</sup> Dong-Wook Kim,<sup>†</sup> Nari Yun,<sup>†</sup> Jae-Sue Choi,<sup>‡</sup> Md. Nurul Islam,<sup>‡</sup> Yeong-Shik Kim,<sup>§</sup> and Sun-Mee Lee<sup>\*†</sup>

<sup>†</sup>School of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do, 440-746, Korea

<sup>‡</sup>Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Korea

<sup>§</sup>College of Pharmacy, Seoul National University, Seoul 151-742, Korea

**ABSTRACT:** In this study, the hepatoprotective effects of hyperoside (**1**), a flavonoid glycoside isolated from *Artemisia capillaris*, have been examined against carbon tetrachloride (CCl<sub>4</sub>)-induced liver injury. Mice were treated intraperitoneally with vehicle or **1** (50, 100, and 200 mg · kg<sup>-1</sup>) 30 min before and 2 h after CCl<sub>4</sub> (20 μL · kg<sup>-1</sup>) injection. Levels of serum aminotransferases were increased 24 h after CCl<sub>4</sub> injection, and these increases were attenuated by **1**. Histological analysis showed that **1** prevented portal inflammation, centrilobular necrosis, and Kupffer cell hyperplasia. Lipid peroxidation was increased and hepatic glutathione content was decreased significantly after CCl<sub>4</sub> treatment, and these changes were reduced by administration of **1**. Protein and mRNA expression of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and heme oxygenase-1 (HO-1) and nuclear protein expression of nuclear factor erythroid 2-related factor 2 (Nrf2) significantly increased after CCl<sub>4</sub> injection. Compound **1** suppressed TNF-α, iNOS, and COX-2 protein and mRNA expression and augmented HO-1 protein and mRNA expression and Nrf2 nuclear protein expression. These results suggest that **1** has protective effects against CCl<sub>4</sub>-induced acute liver injury, and this protection is likely due to enhancement of the antioxidative defense system and suppression of the inflammatory response.



Acute and chronic liver diseases are a global concern. However, medical treatment for these diseases is often difficult to administer and has limited efficacy. Therefore, there have been considerable efforts to develop useful herbal medicines for the treatment of liver diseases. Therapeutically effective agents from natural products may reduce the risk of hepatotoxicity when the drug is used clinically.<sup>1</sup>

Carbon tetrachloride (CCl<sub>4</sub>), a potent hepatotoxic chemical, has been used widely in the induction of acute hepatic injury in experimental animal models. Hepatic necrosis caused by CCl<sub>4</sub> has been suggested to involve bioactivation by a microsomal cytochrome P450-dependent monooxygenase system, resulting in the formation of a trichloromethyl radical (CCl<sub>3</sub>·) and reactive oxygen species (ROS). In the initial phase, these can bind covalently to cellular molecules and cause membrane lipid peroxidation, which ultimately leads to apoptosis and necrosis.<sup>2</sup> Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor, which binds to antioxidant response elements (ARE) located in the promoter region of genes encoding phase 2 detoxifying or antioxidant enzymes such as glutathione (GSH) peroxidase (GPx), GSH S-transferase (GST), and heme oxygenase-1 (HO-1).<sup>3</sup> In addition to protective effects against ROS, recent studies have demonstrated that

Nrf2 responds to pro-inflammatory stimuli and rescues cells or tissues from inflammatory injuries.<sup>4</sup>

*Artemisia capillaris* Thunb. (Asteraceae) has been used widely as a remedy for liver diseases, such as hepatitis, jaundice, and fatty liver.<sup>5</sup> An aqueous extract of *A. capillaris* inhibited ethanol-, interleukin (IL)-1α-, and tumor necrosis factor-α (TNF-α)-induced cytotoxicity and ethanol-induced apoptosis of HepG2 cells.<sup>6</sup> Hyperoside (**1**; quercetin 3-O-galactoside), one of the flavonoid glycosides from *A. capillaris*, scavenges ROS and has an anti-inflammatory activity.<sup>7</sup> Compound **1** possesses various biological functions against ROS-induced damage, such as an antidepressant effect by inhibition of nitric oxide synthase in rat blood and cerebral homogenates<sup>8</sup> and the partial uncoupling effect of oxidative phosphorylation in cardiac mitochondria.<sup>9</sup> Hyperoside also has an antihepatitis B virus activity in HepG2 cells transfected with hepatitis B virus genome by suppression of hepatitis B antigen secretion.<sup>10</sup> However, there is little information available on the in vivo hepatoprotective effect of **1**.

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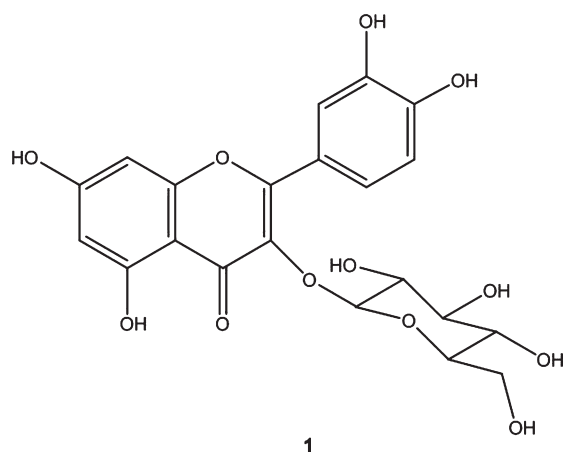
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**Table 1. Effects of Hyperoside (1) on Serum Aminotransferase Activities, Lipid Peroxidation, and Hepatic Glutathione Content (means  $\pm$  SEM,  $n = 10$ )<sup>a</sup>**

group	dose (mg·kg <sup>-1</sup> )	ALT (U·L <sup>-1</sup> )	AST (U·L <sup>-1</sup> )	MDA (nmol·mg protein <sup>-1</sup> )	GSH ( $\mu$ mol·g liver <sup>-1</sup> )	
control		35 $\pm$ 6	39 $\pm$ 7	0.4 $\pm$ 0.04	6.8 $\pm$ 0.4	
CCl <sub>4</sub>	vehicle	10229 $\pm$ 918 <sup>c</sup>	7518 $\pm$ 426 <sup>c</sup>	3.2 $\pm$ 0.5 <sup>c</sup>	4.8 $\pm$ 0.3 <sup>c</sup>	
	1	50	10740 $\pm$ 780 <sup>c</sup>	6820 $\pm$ 646 <sup>c</sup>	2.4 $\pm$ 0.1 <sup>c</sup>	6.1 $\pm$ 0.8 <sup>e</sup>
		100	7134 $\pm$ 603 <sup>c,e</sup>	5488 $\pm$ 375 <sup>c,e</sup>	1.5 $\pm$ 0.1 <sup>c,e</sup>	5.2 $\pm$ 0.4 <sup>b,d</sup>
		200	7055 $\pm$ 316 <sup>c,e</sup>	5382 $\pm$ 343 <sup>c,e</sup>	1.4 $\pm$ 0.2 <sup>c,e</sup>	6.0 $\pm$ 0.5 <sup>b,e</sup>
	silymarin	800	4058 $\pm$ 550 <sup>c,e</sup>	2408 $\pm$ 312 <sup>c,e</sup>	1.8 $\pm$ 0.2 <sup>c,e</sup>	5.6 $\pm$ 0.4 <sup>b,d</sup>

<sup>a</sup> Compound **1** (50, 100, and 200 mg·kg<sup>-1</sup>) or silymarin (positive control, 800 mg·kg<sup>-1</sup>) was administered intraperitoneally 30 min before and 2 h after CCl<sub>4</sub> injection. <sup>b</sup> Significantly different ( $p < 0.05$ ) from the control group. <sup>c</sup> Significantly different ( $p < 0.01$ ) from the control group. <sup>d</sup> Significantly different ( $p < 0.05$ ) from the vehicle-treated CCl<sub>4</sub> group. <sup>e</sup> Significantly different ( $p < 0.01$ ) from the vehicle-treated CCl<sub>4</sub> group.

This study examined the cytoprotective properties of hyperoside (**1**) against CCl<sub>4</sub>-induced hepatic injury, particularly on the extent of oxidative stress and inflammation.



## RESULTS AND DISCUSSION

**Serum Aminotransferase Activities and Histological Analysis.** CCl<sub>4</sub>-induced liver injury in a range of laboratory animals is considered to be analogous to liver damage caused by various hepatotoxins in humans.<sup>11</sup> As a result of hepatic injury, the altered permeability of the membrane causes the enzymes from the cells to be released into the circulation,<sup>12</sup> which damages hepatic cells, as shown by the abnormally high level of serum hepatospecific enzymes.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the control group were 34.5  $\pm$  5.9 and 39.4  $\pm$  6.8 U·L<sup>-1</sup>, respectively. Serum ALT and AST activities showed a marked increase to 10229.3  $\pm$  918.2 and 7518.3  $\pm$  426.4 U·L<sup>-1</sup> 24 h after CCl<sub>4</sub> injection. These increases were attenuated by 100 and 200 mg·kg<sup>-1</sup> treatment with **1** (Table 1). Indeed, histological examination clearly showed that CCl<sub>4</sub>-induced centrilobular necrosis, portal inflammation, and Kupffer cell hyperplasia were lowered dramatically by treatment with **1** (Figure 1).

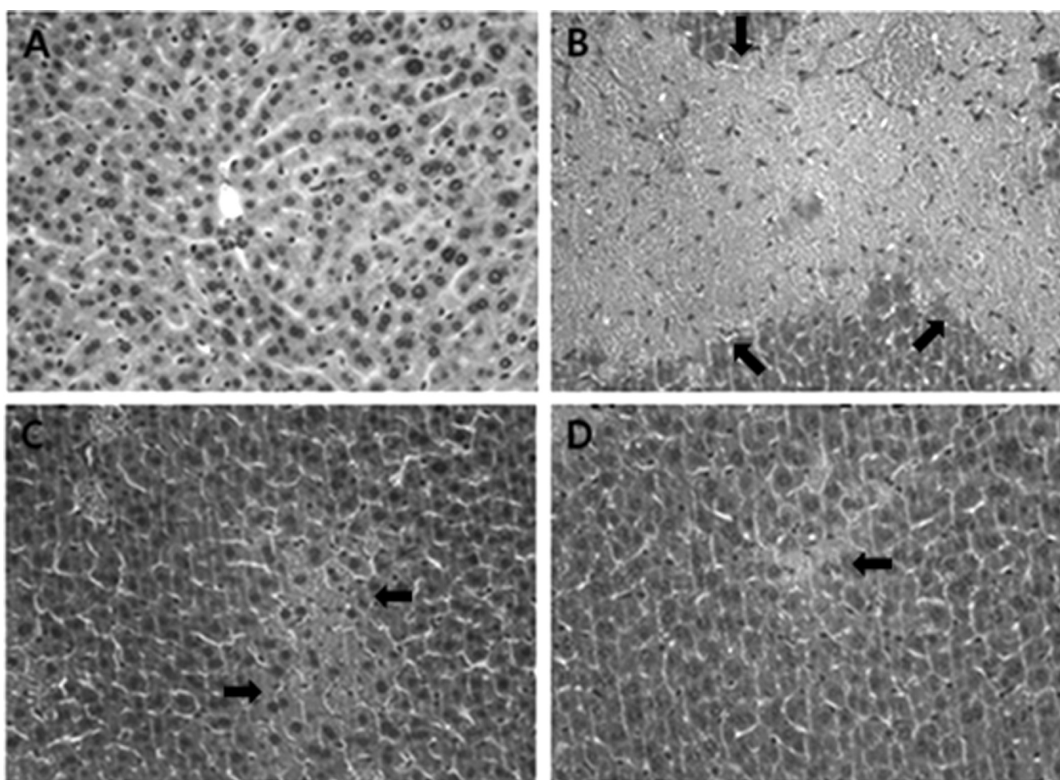
**Hepatic Lipid Peroxidation and GSH Content.** CCl<sub>4</sub>-induced hepatotoxicity is thought to involve two consecutive stages, namely, phase 1 and 2 reactions. During the phase 1 reaction, CCl<sub>4</sub> is metabolized to highly reactive CCl<sub>3</sub>· by cytochrome P450 2E1 and subsequent chloromethylation, saturation, or peroxidation, resulting in progressive destruction of unsaturated fatty acids of membrane phospholipids.<sup>13</sup> Products of the phase 1 reaction are often electrophilic and highly reactive,

thereby resulting in the harmful modification of DNA and proteins. Reactions catalyzed by phase 2 enzymes promote the conjugation of phase 1 products with various hydrophilic moieties such as GSH and glucuronic acid.<sup>14</sup> The GSH system acts as a major antioxidant defense mechanism against free-radical damage in the body. Previous studies of the mechanism of CCl<sub>4</sub>-induced hepatotoxicity have indicated that GSH plays a key role in detoxification of the reactive toxic metabolites of CCl<sub>4</sub> and that hepatic necrosis begins when the GSH pool is depleted.<sup>15</sup> According to previous reports, **1** has been shown to increase indirectly phase 2 enzyme activities including GPx against H<sub>2</sub>O<sub>2</sub>-induced lung fibroblast damage.<sup>16</sup>

As shown in Table 1, the level of malondialdehyde (MDA) increased by 8-fold compared with that of the control group after CCl<sub>4</sub> injection. In the 100 and 200 mg·kg<sup>-1</sup> **1**- and silymarin (positive control)-treated CCl<sub>4</sub> groups, MDA levels decreased significantly to 46.9%, 43.8%, and 56.3% of that of the vehicle-treated CCl<sub>4</sub> group, respectively. The hepatic GSH concentration in the vehicle-treated CCl<sub>4</sub> group decreased to 70% of that of the control group. However, this decrease was attenuated by 50, 100, and 200 mg·kg<sup>-1</sup> **1** and 800 mg·kg<sup>-1</sup> silymarin. The increase in the hepatic GSH level in the **1**-treated CCl<sub>4</sub> group could be due to either its effect on de novo synthesis of GSH, its regeneration, or both. These results suggest that the antioxidant properties observed may be one mechanism through which **1** protects against liver damage induced by CCl<sub>4</sub>.

**HO-1 Protein and mRNA Expression.** HO-1 is an endogenous, cytoprotective enzyme that plays an important protective role against oxidative injury. Hepatic HO-1 expression is up-regulated after CCl<sub>4</sub> administration and confers protection against oxidative injury in the tissues.<sup>17</sup> Several dietary phytochemicals used for medicinal purposes, such as glycyrrhizin and baicalin, were shown to induce HO-1 gene expression in our previous studies.<sup>18,19</sup> In the present investigation, levels of HO-1 protein and gene expression showed a marked increase at 24 h after CCl<sub>4</sub> injection. Treatment with **1** augmented the increase in the levels of HO-1 protein and gene expression (Figures 3 and 4). When animals were pretreated with zinc-protoporphyrin IX for inhibition of HO-1 expression, the histology of baicalin-treated CCl<sub>4</sub>-exposed liver tissue did not markedly differ from that of the CCl<sub>4</sub>-exposed ones (data not shown). This finding suggests that the protective mechanism of **1** against CCl<sub>4</sub>-induced hepatic injury might be closely associated with overexpression of HO-1.

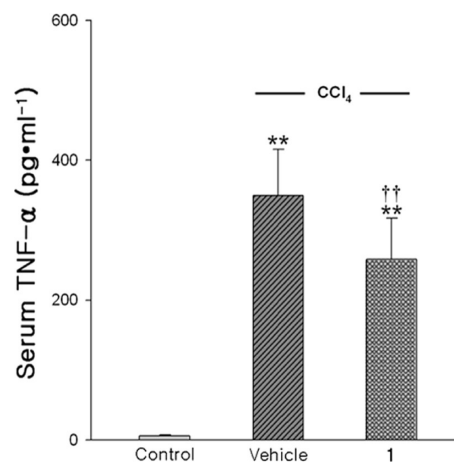
**Nrf2 Protein Expression.** The Nrf2-Kelch-like ECH-associated protein 1 pathway has been characterized as an important endogenous mechanism for combating oxidative stress. Nrf2 is a transcription factor that induces expression of various cytoprotective



**Figure 1.** Effect of hyperoside (**1**) on the histological changes in the liver of the  $\text{CCl}_4$ -treated group (original magnification  $\times 100$ ). (A) Control group: normal lobular architecture and cell structure; (B) vehicle-treated  $\text{CCl}_4$  group: extensive hepatocellular damage with the presence of portal inflammation, centrilobular necrosis, and Kupffer cell hyperplasia; (C) **1** ( $100 \text{ mg} \cdot \text{kg}^{-1}$ )-treated  $\text{CCl}_4$  group: mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia; (D) silymarin ( $800 \text{ mg} \cdot \text{kg}^{-1}$ )-treated  $\text{CCl}_4$  group: mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia. The arrows indicate necrotic areas.

enzymes possessing ARE in the promoter region.<sup>14</sup> Nrf2-target genes include GPx, GST, glutamate cysteine ligase, NAD(P)H: quinone oxidoreductase, and HO-1. Recent studies have demonstrated that hepatotoxins such as bromobenzene, furosemide, and  $\text{CCl}_4$  are capable of inducing a significant increase in Nrf2 accumulation in hepatic nuclei.<sup>20</sup> In this study, nuclear accumulation of Nrf2 was increased by  $\text{CCl}_4$  administration. Of particular interest, nuclear levels of Nrf2 protein expression further increased in the **1**-treated  $\text{CCl}_4$  group (Figure 3), which implies a molecular basis by which this compound stimulates the activity of antioxidants and phase 2 detoxifying enzymes.

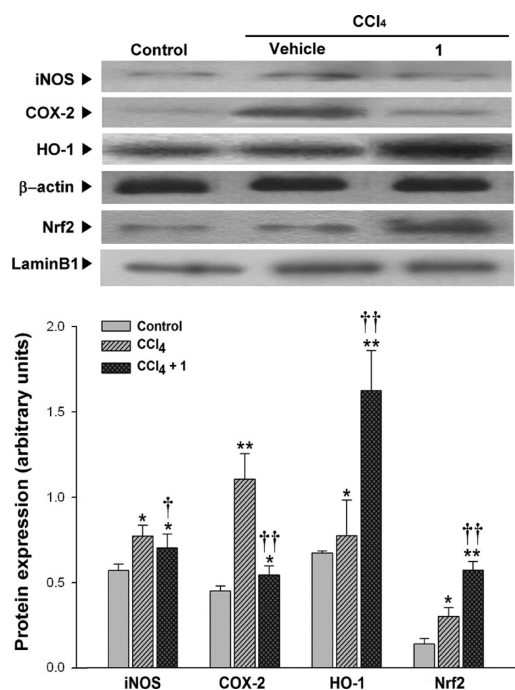
**Serum TNF- $\alpha$  Level and Inducible Nitric Oxide Synthase (iNOS) Protein and mRNA Expression.** In response to oxidative stress, pro-inflammatory cytokines are often overproduced and, in turn, can also cause oxidative stress in target cells. This vicious cycle of oxidative stress and overproduction of pro-inflammatory cytokines can be inhibited by activation of the Nrf2/ARE system.<sup>21</sup> In a dextran sulfate sodium-mediated mouse colitis model, Nrf2 knockout mice exhibited markedly increased production of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ .<sup>22</sup>  $\text{CCl}_4$  rapidly induces pro-inflammatory cytokines such as TNF- $\alpha$  by Kupffer cells and recruits stromal cells of the liver to participate in this inflammatory response via paracrine production of cytokines. TNF- $\alpha$  stimulates the release of cytokines from other macrophages and induces a phagocyte oxidative metabolism and nitric oxide (NO) production. NO can exacerbate oxidative stress by reacting with ROS, particularly with the superoxide anion, and formation of peroxynitrite. Moreover, overproduction of NO by iNOS may



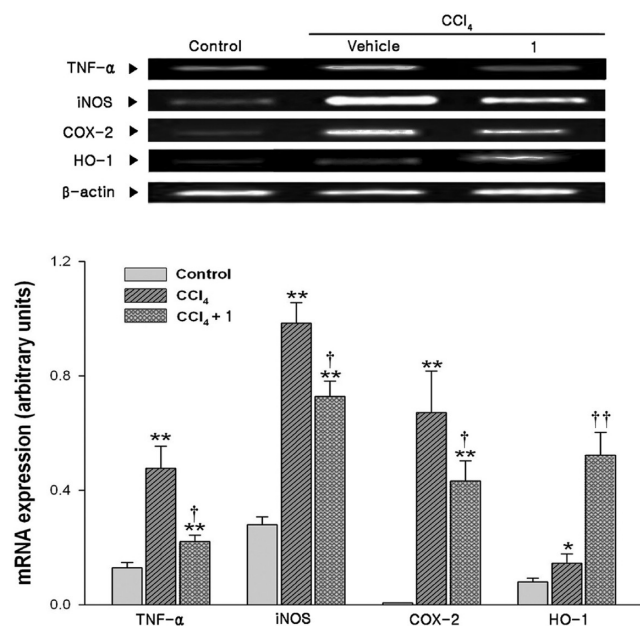
**Figure 2.** Effect of hyperoside (**1**) ( $100 \text{ mg} \cdot \text{kg}^{-1}$ ) on the serum TNF- $\alpha$  level after carbon tetrachloride ( $\text{CCl}_4$ ) administration. Values are presented as the means  $\pm$  SEM of 10 mice per group. \*\*Significantly different ( $p < 0.01$ ) from the control group. ††Significantly different ( $p < 0.01$ ) from the vehicle-treated  $\text{CCl}_4$  group.

mediate  $\text{CCl}_4$ -induced acute hepatotoxicity through up-regulation of inflammatory responses.<sup>23</sup> In the present study, the levels of serum TNF- $\alpha$  and iNOS protein and their mRNA expression increased after  $\text{CCl}_4$  injection, and **1** attenuated these increases (Figures 2–4). Therefore, **1** may regulate TNF- $\alpha$  and iNOS production at the transcriptional level.





**Figure 3.** Effect of hyperoside (**1**) ( $100 \text{ mg} \cdot \text{kg}^{-1}$ ) on iNOS, COX-2, HO-1, and Nrf2 protein expression after carbon tetrachloride ( $\text{CCl}_4$ ) administration. Values are presented as the means  $\pm$  SEM of 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  $\text{CCl}_4$  group.



**Figure 4.** Effect of hyperoside (**1**) ( $100 \text{ mg} \cdot \text{kg}^{-1}$ ) on TNF- $\alpha$ , iNOS, COX-2, and HO-1 mRNA expression after carbon tetrachloride ( $\text{CCl}_4$ ) administration. Values are presented as the means  $\pm$  SEM of 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  $\text{CCl}_4$  group.

**Cyclooxygenase (COX)-2 Protein and mRNA Expression.** COX-2 is one isoform of the COX enzyme family and is the

rate-limiting enzyme for prostaglandin synthesis involved in the inflammatory response.  $\text{CCl}_4$  has been shown to induce prostaglandin formation through the COX pathway. Previously, activation of Nrf2 was suggested to interfere directly with c-Jun N-terminal kinase/c-Jun signaling activity and subsequently reduces COX-2 expression in human chondrocytes.<sup>24</sup> Furthermore, our recent study has shown that pretreatment with the HO-1 inducer, hemin, regulates the protein and mRNA expression levels of iNOS and COX-2 during hepatic ischemia/reperfusion.<sup>25</sup> Increased levels of COX-2 protein and mRNA expression after  $\text{CCl}_4$  injection were attenuated by **1** (Figures 3 and 4).

In summary, the present findings suggest that hyperoside (**1**) is protective against  $\text{CCl}_4$ -induced liver injury, and it enhances the antioxidative defense system and suppresses the inflammatory response. This study is supportive of **1** being further investigated for its possible use in the treatment of hepatic injury.

## EXPERIMENTAL SECTION

**General Experimental Procedures.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were determined using a JEOL JNM ECP-400 spectrometer at 400 and 100 MHz in the denaturated solvent dimethylsulfoxide ( $\text{DMSO}$ )- $d_6$ . EIMS were recorded on a Hewlett-Packard 5989B spectrometer (Agilent Technologies, Santa Clara, CA) and a JEOL JMS-700 spectrometer (Tokyo, Japan). Reversed-phase HPLC was performed to check the compound purity on a JASCO HPLC system (Tokyo, Japan), consisting of a PU-1580 Intelligent HPLC pump, a LG-1580-04 quaternary gradient unit, a UV-1575 Intelligent UV/vis detector, a PG-1580-54 4-line degasser, and a CO-1560 Intelligent column thermostat. A Borwin chromatographic system (Le Fontanil, France) was used for HPLC data analysis. Column chromatography was conducted using silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany) and Diaion HP20 (250–850  $\mu\text{m}$ ; Sigma, St. Louis, MO). All TLC was conducted on precoated Merck Kieselgel 60 F254 plates (20  $\times$  20 cm, 0.25 mm; Merck), and 50%  $\text{H}_2\text{SO}_4$  was used as a visualization reagent.

**Plant Material.** The whole plants of *A. capillaris* were collected from Kyeongju, Korea, in August 2009 and authenticated by Prof. Je-Hyun Lee, College of Oriental Medicine, Dongguk University, Kyeongju, Korea. A voucher specimen (20093008) was deposited in the laboratory of one of the authors (J. S. Choi) for future reference.

**Extraction and Isolation of Hyperoside (1).** Whole plants of *A. capillaris* were dried and ground to a powder. The dried powder (19.0 kg) was extracted with hot MeOH (50.0 L  $\times$  3 times) for 3 h. After filtration, the total filtrate was concentrated to dryness in vacuo at 40  $^\circ\text{C}$  to afford a MeOH extract (1.81 kg). This was suspended in distilled water–MeOH (9:1) and partitioned successively with  $\text{CH}_2\text{Cl}_2$ , EtOAc, and *n*-BuOH to yield dried  $\text{CH}_2\text{Cl}_2$  (707 g), EtOAc (282 g), and *n*-BuOH (387 g) extracts, respectively, as well as a  $\text{H}_2\text{O}$  residue. The *n*-BuOH extract was chromatographed over HP-20 Diaion using  $\text{H}_2\text{O}$ –MeOH (1:0  $\rightarrow$  6:4  $\rightarrow$  4:6  $\rightarrow$  0:1) gradient solvent systems to afford four subfractions [H<sub>2</sub>O (264.17 g), 40% MeOH (60.82 g), 60% MeOH (40.95 g), and MeOH (12.76 g), respectively]. The 40% MeOH subfraction was chromatographed over a silica gel column using  $\text{CH}_2\text{Cl}_2$ –MeOH (10:1), with gradual increases in the amount of MeOH, to obtain 15 subfractions (4F-1 to 4F-15). After filtration of 4F-6 to 4F-8, the precipitate was triturated using MeOH to afford **1** (3.52 g) as a yellow amorphous powder. This compound was identified by comparison of its spectroscopic data ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, MS) with literature values.<sup>26</sup> The percent purity of the isolated **1** was found to be 99%, as determined by HPLC.

**Animals and Experimental Treatments.** All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the U.S.

National Institutes of Health. Male ICR mice (25–30 g) were obtained from Daehan Biolink, Co. (Eumseong, Korea). The animals were fasted for 16 h before CCl<sub>4</sub> treatment and given access to water and food throughout the experiments ad libitum. The mice were divided randomly into six groups of 10 animals each. Mice in group 1 (control) received only olive oil (10 mL·kg<sup>-1</sup>, ip). In groups 2 to 6, CCl<sub>4</sub> dissolved in olive oil (1:499, v/v) was administered intraperitoneally with a final concentration of 20 μL·kg<sup>-1</sup>. Groups 1 and 2 (vehicle) were treated with saline (10 mL·kg<sup>-1</sup>, ip). Groups 3 to 5 were treated with **1** (50, 100, and 200 mg·kg<sup>-1</sup>·10 mL<sup>-1</sup>, ip), and group 6 was treated with silymarin (800 mg·kg<sup>-1</sup>, ip; Sigma), 30 min before and 2 h after administration of CCl<sub>4</sub>. The dose and timing of the treatment with **1** were selected based on previous reports.<sup>10,27</sup> Twenty-four hours after injection of CCl<sub>4</sub>, blood was taken from the abdominal aorta and the liver was isolated and stored at -75 °C for analysis, except for the left lobe, which was used for histological studies.

**Serum Aminotransferase Activities.** ALT and AST activities were determined using ChemiLab ALT and AST assay kits (IVDLab Co., Ltd., Uiwang, Korea), respectively.

**Histological Analysis.** The liver was fixed by immersion in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5 μm sections, and stained with hematoxylin-eosin, followed by blind histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated.<sup>28</sup> Histological changes were evaluated in nonconsecutive, randomly chosen 100× histological fields.

**Hepatic Lipid Peroxidation Levels and GSH Content.** The steady-state level of MDA, which is the end product of lipid peroxidation, was measured in liver homogenates by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at 535 nm, as described by Buege and Aust.<sup>29</sup> The level of total GSH was determined in liver homogenates after precipitation with 1% picric acid using yeast-glutathione reductase, 5,5'-dithiobis(2-nitrobenzoic acid), and NADPH, at 412 nm.<sup>30</sup>

**Preparation of Protein Extracts.** Freshly isolated liver tissues were homogenized in PRO-PREP (iNtRON Biotechnology Co., Ltd., Seongnam, Korea) for preparation of whole protein extracts. NE-PER (Pierce Biotechnology, Rockford, IL) was used for extraction of nuclear proteins, according to the manufacturer's instructions. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology).

**Western Blot Immunoassay.** A 15 μg sample of protein from liver homogenates was loaded per lane on 7.5–10.0% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and 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. Blots were then incubated overnight at 4 °C with primary antibodies. The next day, the blots were incubated in appropriate secondary antibodies and detected with an ECL detection system (iNtRON Biotechnology Co., Ltd.), according to the manufacturer's instructions. Visualized immune-reactive bands were evaluated densitometrically with ImageQuant TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ). The following primary antibodies were used: iNOS (BD Biosciences, Los Angeles, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), HO-1 (Stressgen Bioreagents Corp., Ann Arbor, MI), Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin and lamin B1 (Abcam, Cambridge, UK). The signals were normalized to that of β-actin or lamin B1.

**Measurement of Serum TNF-α Level.** The serum TNF-α level was quantified using a commercial TNF-α ELISA assay kit (eBiosciences Co., San Diego, CA) according to the manufacturer's instructions.

**Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated using the method described by Chomczynski and Sacchi.<sup>31</sup> Reverse transcription

of the total RNA was carried out for synthesis of first-strand cDNA using an oligo(dT)<sub>12–18</sub> primer and SuperScript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR reaction was performed with a diluted cDNA sample and amplified in each 2 μL reaction volume. Final reaction concentrations were as follows: primers, 10 μM; dNTP mix, 250 mM; 10× PCR buffer; Ex Taq DNA polymerase, 0.5 U/reaction. All PCR reactions had an initial denaturation step at 94 °C for 5 min and a final extension at 72 °C for 7 min using a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA). PCR amplification cycling conditions were as follows: 28 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (30 s) for TNF-α; 35 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s) for iNOS; and 35, 30, and 25 cycles of 94 °C (30 s), 56 °C (30 s), and 72 °C (30 s) for COX-2, HO-1, and β-actin. After RT-PCR, 10 μL samples of the amplified products were resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The intensity of each PCR product was evaluated semiquantitatively using a digital camera (DC120; Eastman Kodak, Stamford, CT) and a densitometric scanning analysis program (1D Main; Advanced American Biotechnology, Fullerton, CA).

**Statistics.** All the results are presented as the means ± 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.

## AUTHOR INFORMATION

### Corresponding Author

\*Tel: +82 31 290 7712. Fax: +82 31 292 8800. E-mail: sunmee@skku.edu.

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