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Protective Activities of Stilbene Glycosides from *Acer mono* Leaves against H_2O_2-Induced Oxidative Damage in Primary Cultured Rat Hepatocytes

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In our previous study, we isolated two new hepatoprotective stilbene glycosides, 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-glucopyranoside (MRA) and 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (MRAG), from the methanolic extract of *Acer mono* leaves. Thereby, we have attempted to elucidate the hepatoprotective mechanism of these compounds, focusing on antioxidative effects, using hydrogen peroxide (H_2O_2)-injured primary cultures of rat hepatocytes. Both MRA and MRAG showed potent hepatoprotective activities in pretreatment but showed little effects in posttreatment. In addition, they increased the glutathione (GSH) level in the normal control cultures and significantly prevented the depletion of GSH in H_2O_2-injured primary cultured rat hepatocytes. Moreover, these compounds significantly restored the level of GSH depleted by buthionine sulfoximine or diethylmaleate in the presence or absence of H_2O_2. Furthermore, these compounds preserved the activities of antioxidant enzymes such as superoxide dismutase, glutathione reductase, and glutathione peroxidase reduced by H_2O_2 insults. Meanwhile, MRA and MRAG showed moderate scavenging effects with IC_{50} values of 103.6 and 80.5 μM, respectively, as determined by 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging activity. Taken together, these results suggest that MRG and MRAG exert significant hepatoprotective activities against H_2O_2-induced hepatotoxicity by maintaining the antioxidative defense system rather than scavenging free radicals.

KEYWORDS: ***Acer mono*; antioxidants; hydrogen peroxide; hepatoprotective; primary cultures of rat hepatocytes; 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-glucopyranoside (MRA); 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (MRAG)**

INTRODUCTION

Oxidative stress caused by increased accumulation of reactive oxygen species (ROS) has been implicated in the pathogenesis of various diseases (*1*−*3*). Mitochondria and cytochrome P450 enzymes are the main sources of ROS in hepatocytes acutely and/or chronically exposed to a "toxic" injury (environmental drugs, alcohol, therapeutical drugs, viruses, etc.) (*4*−*6*). ROSs are also derived from Kupffer and inflammatory cells, in particular neutrophils (*7*). Therefore, oxidative stress in the liver has been linked to chemical-derived hepatitis, cirrhosis, and other pathological conditions (*8*−*10*).

As a defense system against oxidative stress caused by ROS, cells possess many endogenous nonenzymatic antioxidant molecules such as vitamins A and E and glutathione (GSH) as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase (GR), and glutathione peroxidase (GSH-px) (*11*, *12*). Thus, a large number of studies have focused on the pathogenetic significance of oxidative stress in liver injury as well as on therapeutic intervention with antioxidant scavengers.

We have been searching for hepatoprotective compounds from natural products using primary cultures of rat hepatocytes injured by hydrogen peroxide (H_2O_2), a direct mediator of oxidative injury (*4*), as a screening system. In the course of screening extracts of natural products, we found that the ethyl acetate (EtOAc) fraction of *Acer mono* Maximowicz (Aceraceae) leaves showed significant hepatoprotective activity (*13*). *A. mono* is widely distributed in Korea, the People's Republic of China, and Japan. The leaves of *A. mono* have been used in Korean folk medicine for hemostasis, and the roots have been used for the treatment of arthralgia and cataclasis. The sap of *A. mono* has been employed for the treatment of difficulty in urination, constipation, other gastroenteric disorders, gout, and neuralgia (*14*). Bioactivity-guided fractionation of the EtOAc fraction of *A. mono* leaves yielded two new hepatoprotective stilbene glycosides, 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-glucopyranoside (MRG) and 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (MRAG) (**Figure 1**) (*13*). Therefore, we have attempted to further elucidate the hepatoprotective

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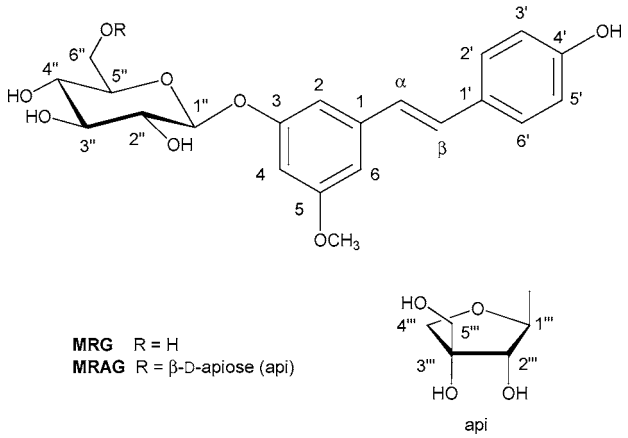

Figure 1. Structures of MRG and MRAG.

mechanism of MRG and MARG using H_2O_2-injured primary cultured rat hepatocytes. In addition, to evaluate their direct scavenging effects on free radicals, we determined the 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, scavenging activity.

MATERIALS AND METHODS

Chemicals. MRG and MRAG were isolated from the EtOAc fraction of *A. mono* leaves by successive column chromatography and reverse-phase high pressure liquid chromatography, as we reported previously (*13*). Supplements for cell culture and other reagents used in the study were obtained from Sigma (St. Louis, MO). The kit for assessing glutamic pyruvic transaminase (GPT) activity was purchased from Youngdong Pharmaceutical (Seoul, Korea). All other chemicals were of the highest purity available.

Animals. Male Wistar rats (150−200 g body weight) were provided by the Laboratory Animal Center, Seoul National University. Rats were kept on standard chow with free access to tap water in temperature- and humidity-controlled animal quarters under a 12 h light−dark cycle. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Seoul National University with special care taken to avoid any undue animal pain or suffering.

Isolation and Culture of Rat Hepatocytes. Isolated hepatocytes were prepared from Wistar rat by the collagenase perfusion method as we described previously (*15*). Cells were purified by several centrifugations and inoculated onto collagen-precoated culture plates at a density of 5×10^5 cell/mL. Then, cells were incubated at 37 °C in a humidified incubator gassed with 5% CO_2:95% air.

Test compounds were dissolved in dimethyl sulfoxide (final culture concentration, 0.1%). One day after isolated rat hepatocytes were plated, cultures were treated with test compounds for 1 h. Cultures were then exposed to 12 mM H_2O_2 and incubated further for 1.5 h (pretreatment). In some experiments, the cultures were exposed to 12 mM H_2O_2 for 30 min and then test compounds were treated for 1 h (posttreatment). Hepatotoxicity was assessed by measuring the GPT activity released into the culture medium. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (*16*). For the measurements of antioxidant enzyme activity and GSH level, the cells were harvested and the supernatant was prepared as described previously (*17*).

To reduce the cellular GSH level, cultures were pretreated with 1 mM buthionine sulfoximine (BSO) or diethylmaleate (DEM) for 2 h before administration of the test compounds (*17*, *18*). After the treatment with test compounds for 1 h, the cultures were followed by H_2O_2 challenge for 1.5 h.

Determination of GSH Level. The level of GSH was determined spectrophotometrically using the enzymatic cycling method of Tietze (*19*) with minor modification.

Determination of Antioxidant Enzyme Activity. The SOD activity was determined according to the method of McCord and Fridovich (*20*) using xanthine−xanthine oxidase reaction. The catalase activity was determined according to the method of Beers and Sizer (*21*) based on H_2O_2 decomposition. GR activity, based on the reduction of GSSG by GR and β-nicotinamide adenine dinucleotide phosphate (NADPH), was measured according to the method of Carlberg and Mannervik (*22*). The GSH-px activity was determined according to the method of Flohe and Gunzler (*23*) by qualifying the rate of oxidation of GSH to glutathione disulfide (GSSG) by cumene hydroperoxide, a reaction catalyzed by GSH-px.

Protein Assay. The protein content was measured using bicinchoninic acid with bovine serum albumin as a standard.

Determination of the DPPH Radical Scavenging Activity. The scavenging activity of DPPH radical was measured using a modified method of Soares et al. (*24*). The aqueous solution of each compound was added to a solution of DPPH (120 μM) in EtOH to give final compound concentrations of 0.1, 1, 10, 25, 50, and 100 μM. The control used distilled water in place of test compound solution. The reaction mixture was shaken vigorously and allowed to react at room temperature. After 30 min, the remaining DPPH was determined colorimetrically at 517 nm. L-Ascorbic acid was used as a positive control. The absorbance of each compound alone was subtracted as the blank from that of the reaction mixture. The radical scavenging activity was calculated as $100 \times (OD_{control} - OD_{test\ compound})/OD_{control}$. The IC_{50} value was defined as the concentration (μM) of the test compounds required to inhibit the formation of DPPH radical by 50%.

Statistical Analysis. One-way analysis of variance procedures were used to assess significant differences among treatment groups. The criterion for statistical significance was set at $p < 0.05$, $p < 0.01$, or $p < 0.001$.

RESULTS

Hepatoprotective Activity against H_2O_2-Induced Toxicity. We previously reported that MRG and MRAG from *A. mono* leaves showed significant hepatoprotective activities against H_2O_2-induced toxicity in pretreatment at the concentrations ranging from 10 to 100 μM in a dose-dependent manner (*13*). To clarify the mode of protection, the hepatoprotective activity by a timed exposure to compounds after H_2O_2 challenge was evaluated first. As shown in **Figure 2**, these stilbene glycosides significantly suppressed the toxicity induced by H_2O_2 at the concentrations of 50 μM in pretreatment paradigm (viability, 100.6 ± 2.8 and 93.8 ± 3.8%; GPT activity, 84.0 ± 5.3 and 81.6 ± 3.4%, respectively). In contrast, the posttreatments of these stilbene glycosides at the concentration of 50 μM after the H_2O_2 insults on the primary cultured hepatocytes did not affect the cellular viabilities and hepatoprotective activities against H_2O_2-induced toxicity.

Effects on Antioxidative Defense System. GSH is a major endogenous antioxidant and plays an important role in protection from oxidative stress (*17*, *25*). As such, we evaluated the effects of MRG and MRAG on the GSH level in H_2O_2-injured rat hepatocytes. As shown in **Table 1**, the GSH level in H_2O_2-injured cells was decreased to 39.3 ± 4.1 nmol/mg protein as compared to a normal value of 54.5 ± 0.7 nmol/mg protein. MRG and MRAG increased the GSH level even in the normal control cultures (65.1 ± 3.4 and 56.1 ± 3.7 nmol/mg protein, respectively) and almost completely prevented the depletion of GSH by H_2O_2 (55.0 ± 3.3 and 53.6 ± 3.9 nmol/mg protein, respectively).

The effect of these stilbene glycosides on GSH level was further investigated using such GSH depletors as BSO and DEM. BSO is known to deplete GSH by inhibition of γ-glutamylcysteine synthetase, a key enzyme in GSH synthesis and DEM to deplete GSH via a reaction catalyzed by GSH-*S*-transferase (*17*). Treatment with BSO or DEM caused the

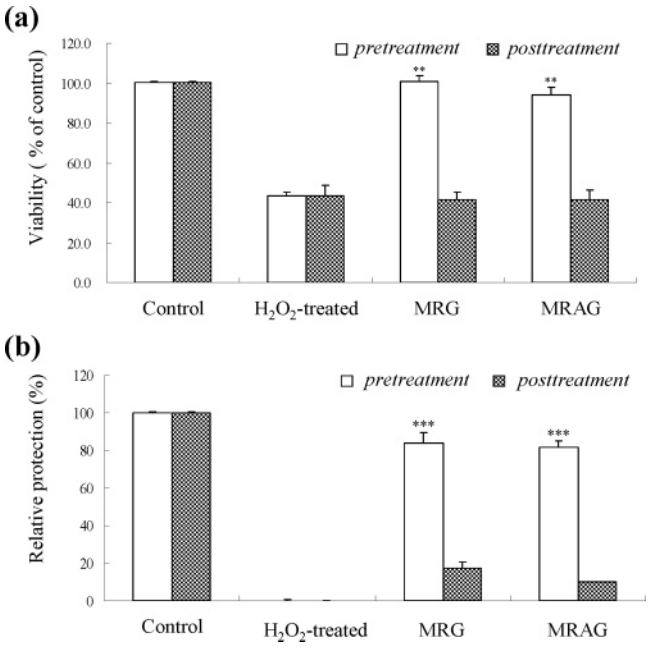

Figure 2. Hepatoprotective activities of MRG and MRAG in H_2O_2-injured primary cultures of rat hepatocytes. The control is the value of hepatocytes not challenged with H_2O_2. Each value represents the mean ± SD (n = 3). Significantly different from H_2O_2-treated values: *p < 0.05, **p < 0.01, and ***p < 0.001. (**a**) Cell viability was measured by the MTT assay. Optical densities (ODs) of the control and H_2O_2-treated were 3.95 ± 0.1 and 1.72 ± 0.2, respectively. (**b**) The control and H_2O_2-treated values for GPT were 26.3 ± 3.0 and 65.0 ± 2.2 IU/L, respectively. The % value of protection was calculated as 100 × (value of H_2O_2-treated − value of test compound)/(value of H_2O_2-treated − value of control).

Table 1. Effect of MRG and MRAG on GSH Levels in BSO-, DEM-, and/or H_2O_2-Injured Primary Cultures of Rat Hepatocytes[a]

	GSH (nmol/mg protein)		
	none[d]	MRG	MRAG
control	54.5 ± 0.7	65.1 ± 3.4	56.1 ± 3.7
BSO-treated[b]	21.1 ± 5.1	24.8 ± 5.2	26.1 ± 0.6
DEM-treated[c]	16.9 ± 1.0	25.4 ± 4.2	21.4 ± 6.0
H_2O_2-treated	39.3 ± 4.1	55.0 ± 3.3**	53.6 ± 3.9*
BSO + H_2O_2-treated	18.8 ± 2.4	31.2 ± 3.8**	30.4 ± 1.8**
DEM + H_2O_2-treated	13.0 ± 3.1	18.3 ± 2.4	17.8 ± 7.0

[a] Each value represents the mean ± SD (n = 3). BSO-, DEM-, and/or H_2O_2-treated values differ significantly from the control at a level of p < 0.001. Significantly different from each value in the absence of samples: *p < 0.05, **p < 0.01, and ***p < 0.001. [b,c] Primary cultures of rat hepatocytes were preincubated with 1 mM BSO or 1 mM DEM for 2 h before treatment with samples (50 μM). [d] None is the value of hepatocytes, which were not challenged with samples.

depletion of GSH in cells regardless of H_2O_2 insult (**Table 1**). MRG and MRAG did not prevent the depletion of GSH level in the BSO- or DEM-treated hepatocytes. However, they significantly restored the GSH level reduced by the treatment of BSO followed by H_2O_2 challenge.

Antioxidant enzymes are also involved in the defense against oxidative stress (*12*, *13*). Therefore, we investigated the effects of MRG and MRAG on antioxidant enzymes such as SOD, GR, GSH-px, and catalase. Exposure of hepatocytes to H_2O_2 decreased the activities of antioxidant enzymes. MRG and MRAG preserved the activities of SOD, GR, and GSH-px in H_2O_2-injured primary cultures of rat hepatocytes. However, these compounds showed little effects on catalase activity (**Table 2**).

Table 2. Effect of MRG and MRAG on Activities of Antioxidant Enzymes (SOD, GR, and GSH-px) in H_2O_2-Injured Primary Cultures of Rat Hepatocytes[a]

	SOD (Unit/ mg protein)	GR (mUnit/ mg protein)	GSH-px (mUnit/ protein)
control[b]	25.5 ± 1.4	78.0 ± 1.0	34.8 ± 3.1
H_2O_2-treated[c]	15.7 ± 2.5	53.8 ± 3.8	18.8 ± 2.4
MRG	22.1 ± 1.1*	75.4 ± 2.7**	31.7 ± 2.9*
MRAG	18.3 ± 5.5	71.8 ± 8.7*	33.0 ± 4.1

[a] Each value represents the mean ± SD (n = 3). Significantly different from H_2O_2 value: *p < 0.05 and **p < 0.01. Samples were treated at the concentration of 50 μM. [b] Control is the value of hepatocytes, which were not challenged with H_2O_2. [c] H_2O_2 is the values of hepatocytes, which were challenged with H_2O_2.

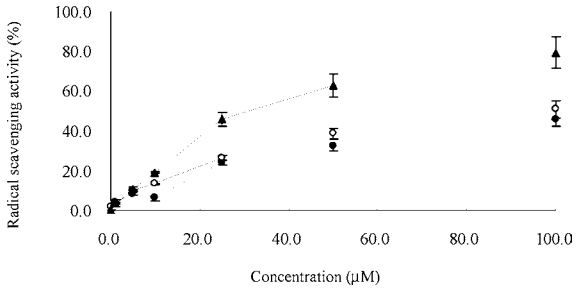

Figure 3. Radical scavenging activity of MRG and MRAG. Each value represents the mean ± SD (n = 3). The radical scavenging activity was calculated as 100 × ($OD_{control}$ − $OD_{test\ compound}$)/$OD_{control}$. ●, MRG; ○, MRAG; and ▲, L-ascorbic acid.

DPPH Radical Scavenging Activity. To evaluate the direct scavenging effects of these stilbene glycosides on free radicals, we determined the DPPH, a stable free radical, scavenging activity. As shown in **Figure 3**, MRG and MRAG reduced the level of DPPH radical at the concentrations up to 100 μM and showed the free radical scavenging effects with IC_{50} values of 103.6 and 80.5 μM, respectively. L-Ascorbic acid, a well-known antioxidant (*25*), showed the free radical scavenging effect with an IC_{50} value of 27.3 μM.

DISCUSSION

In our present study, we further investigated the action mechanism of two new hepatoprotective stilbene glycosides, MRG and MARG, from *A. mono* leaves (*13*). H_2O_2 is a direct mediator of oxidative injury. H_2O_2 itself is an active oxygen species and can also react with superoxide radicals to form more reactive hydroxyl radicals in the presence of Fe(II) or Cu(I) (*4*, *26*). The hydroxyl radicals initiate self-propagating reactions leading to cascade of biological oxidations resulting in the rapid modification of cytoplasmic constituents, the depletion of intracellular GSH and ATP, a decrease in NAD^+ level, an increase in free cytosolic Ca(II), and lipid peroxidation (*27*). These stilbene glycosides significantly suppressed hepatotoxicity induced by H_2O_2 in pretreatment whereas the posttreatments of these stilbenes exerted weak hepatoprotective activities (**Figure 2**), indicating that these compounds mainly act in the early stage in H_2O_2-induced toxicity rather than restore the H_2O_2-injured hepatocyte functions.

GSH is a key component of the cell defense system, and GSH depletion causes serious impairments of cellular defense against some toxicants such as H_2O_2 or other oxidants (*28*). Antioxidant enzymes such as SOD, catalase, GR, and GSH-px are also modulated in various diseases by free radical attacks and

maintain the balance between the rate of radical generation and the rate of radical scavenging in an essential part of biological homeostasis (*11*, *12*). MRG and MRAG increased the GSH level in the normal control cultures and prevented the depletion of GSH in H_2O_2-induced toxicity. Further investigation using GSH depletors, BSO and DEM, showed that treatment with BSO or DEM caused the depletion of GSH in cells regardless of H_2O_2 insult and the increase in the sensitivity to H_2O_2-induced toxicity (**Table 1**). These stilbene glycosides restored the level of GSH decreased by BSO in the presence of H_2O_2 but had no significant effects on the GSH depletion caused by DEM. Therefore, these results suggest that MRG and MRAG prevent the depletion of GSH induced by H_2O_2 but do not influence the synthesis of GSH. Moreover, these stilbene glycosides reduced the decrease in the activity of GR, suggesting that they might be involved in restoring and recycling GSH from GSSG in H_2O_2-injured hepatocytes. These compounds also prevented the decrease in the activities of antioxidant enzymes, SOD, and GSH-px (**Table 2**) but had no significant effect on catalase activity (no data shown). Therefore, MRG and MRAG increased the GSH levels and preserved the activities of antioxidant enzymes in H_2O_2-injured primary cultures of rat hepatocytes.

Despite their effects on the regulation of antioxidantive defense system, MRG and MRAG hold moderate DPPH radical scavenging activities (IC_{50} values of 103.6 and 80.5 μM, respectively).

In conclusion, we evaluated the effects of MRG and MRAG on the antioxidative defense system against H_2O_2-induced oxidative damage in primary cultured rat hepatocytes. We also measured the DPPH free radical scavenging activities. From the results, we could demonstrate that MRG and MRAG exert significant hepatoprotective effects on cultured rat hepatocytes mainly by maintaining the antioxidative defense system rather than free radical scavenging activities. Further studies are necessary to fully understand the cellular and molecular mechanisms underlying the action of these stilbene glycosides.

ABBREVIATIONS USED

ROS, reactive oxygen species; GSH, glutathione; SOD, superoxide dismutase; GSH-px, glutathione peroxidase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; EtOAc, ethyl acetate; BSO, buthionine sulfoximine; DEM, diethylmaleate; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; GPT, glutamic pyruvic transaminase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; GSSG, glutathione disulfide; NADPH, β-nicotinamide adenine dinucleotide phosphate.

LITERATURE CITED

(1) Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of aging. *Nature* **2000**, *408*, 239−247.

(2) Kaplowitz, N. Cell death at the millennium. Implications for liver diseases. *Clin. Liver Dis.* **2000**, *4*, 1−23.

(3) Czaja, M. J.; Liu, H.; Wang, Y. Oxidant-induced hepatocyte injury from menadione is regulated by ERK and AP-1 signaling. *Hepatology* **2003**, *37*, 1405−1413.

(4) Mitsuyoshi, H.; Nakashima, T.; Sumida, Y.; Yoh, T.; Nakajima, Y.; Ishikawa, H.; Inaba, K.; Sakamoto, Y.; Okanoue, T.; Kashima, K. Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 537−542.

(5) Bailey, S. M.; Cunningham, C. C. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radical Biol. Med.* **2002**, *32*, 11−16.

(6) Bailey, S. M.; Cunningham, C. C. Acute and chronic ethanol increases reactive oxygen species generation and decreases viability in fresh, isolated rat hepatocytes. *Hepatology* **1998**, *28*, 1318−1326.

(7) Loguercio, C.; Federico, A. Oxidative stress in viral and alcoholic hepatitis. *Free Radical Biol. Med.* **2003**, *34*, 1−10.

(8) Mehendale, H. M.; Roth, R. A.; Gandolfi, A. J.; Klaunig, J. E.; Lemasters, J. J.; Curtis, L. R. Novel mechanisms in chemically induced hepatotoxicity. *FASEB J.* **1994**, *8*, 1285−1295.

(9) Stohs, S. J. The role of free radicals in toxicity and disease. *J. Basic Clin. Physiol. Pharmacol.* **1995**, *6*, 205−228.

(10) Black, D.; Bird, M. A.; Samson, C. M.; Lyman, S.; Lange, P. A.; Schrum, L. W.; Qian, T.; Lemasters, J. J.; Brenner, D. A.; Rippe, R. A.; Behrns, K. E. Primary cirrhotic hepatocytes resist TGFβ-induced apoptosis through a ROS-dependent mechanism. *J. Hepatol.* **2004**, *40*, 942−951.

(11) Somani S. M.; Husain, K.; Schlorff, E. C. Response of antioxidant system to physical and chemical stress. In *Oxidants, Antioxidants, and Free Radicals*; Baskin, S. I., Salem, H., Eds.; Taylor and Francis: Washington, DC, 1997; pp 125−141.

(12) Lee, S. E.; Hwang, H. J.; Ha, S. J.; Jeong, H. S.; Kim, S. H. Screening of medicinal plant extracts for antioxidant activity. *Life Sci.* **2003**, *73*, 167−179.

(13) Yang, H.; Sung, S. H.; Kim, Y. C. Two new hepatoprotective stilbene glycosides from *Acer mono* leaves. *J. Nat. Prod.* **2005**, *68*, 101−103.

(14) Bae, K. H. *Medicinal Plants of Korea*; Kyo-Hak Publishing Co., Ltd.: Seoul, 2002; p 302.

(15) Lee, E. J.; Kim, S. R.; Kim, J.; Kim, Y. C. Hepatoprotective phenylpropanoids from *Scrophularia buergeriana* roots against CCl_4-induced toxicity: Action mechanism and structure−activity relationship. *Planta Med.* **2002**, *68*, 407−411.

(16) Hansen, M. B.; Nielsen, S. E.; Berg, K. Reexamination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* **1989**, *119*, 203−210.

(17) Kim, S. R.; Park, M. J.; Lee, M. K.; Sung, S. H.; Park, E. J.; Kim, J.; Kim, S. Y.; Oh, T. H.; Markelonis, G. J.; Kim, Y. C. Flavonoids of *Inula britannica* protect cultured cortical cells from necrotic cell death induced by glutamate. *Free Radical Biol. Med.* **2002**, *32*, 596−604.

(18) Gebhardt, R. Antioxidative and protective properties of extracts from leaves of the Artichoke (*Cynara scolymus* L.) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* **1997**, *144*, 279−286.

(19) Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **1969**, *27*, 502−522.

(20) McCord, J. M.; Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969**, *244*, 6049−6055.

(21) Beers, R. F.; Sizer, I. W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **1952**, *195*, 133−140.

(22) Carlberg, I.; Mannervik, B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* **1975**, *250*, 5475−5480.

(23) Flohe, F.; Gunzler, W. A. Assays of glutathione peroxidase. *Methods Enzymol.* **1984**, *105*, 114−121.

(24) Soares, D. G.; Andreazza, A. C.; Salvador, M. Sequestering ability of butylated hydroxytoluene, propyl gallate, resveratrol, and vitamins C and E against ABTS, DPPH, and hydroxyl free radicals in chemical and biological systems. *J. Agric. Food Chem.* **2003**, *51*, 1077−1080.

(25) Sciuto, A. M. Antioxidant properties of glutathione and its role in tissue protection. In *Oxidants, Antioxidants, and Free Radicals*; Baskin, S. I., Salem, H., Eds.; Taylor and Francis: Washington, DC, 1997; pp 171−191.

(26) Acworth, I. N.; McCabe, D. R.; Maher, T. J. The analysis of free radicals, their reaction products, and antioxidants. In *Oxidants, Antioxidants, and Free Radicals*; Baskin, S. I., Salem, H., Eds.; Taylor and Francis: Washington, DC, 1997; pp 23−77.

(27) Kanno, S.; Ishikawa, M.; Takayanagi, M.; Takayanagi, Y.; Sasaki, K. Characterization of hydrogen peroxide-induced apoptosis in mouse primary cultured hepatocytes. *Biol. Pharm. Bull*. **2000**, *23*, 37−42.

(28) Haidara, K.; Moffatt, P.; Denizeau, F. Metallothionein induction attenuates the effects of glutathione depletors in rat hepatocytes. *Toxicol. Sci.* **1999**, *49*, 297−305.

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