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Hepatoprotective Effect of *Diospyros lotus* Leaf Extract against Acetaminophen-induced Acute Liver Injury in Mice

Byoung Ok Cho[1,2], Hong Hua Yin[1], Chong Zhou Fang[2], Sang Jun Kim[3], Seung Il Jeong[3], and Seon Il Jang[1,2,*]

[1]*Ato Q&A Corporation, Jeonju, Jeonbuk 55069, Korea*
[2]*Department of Health & Science, Jeonju University, Jeonju, Jeonbuk 55069, Korea*
[3]*Jeonju Biomaterials Institute, Jeonju, Jeonbuk 54810, Korea*

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*Corresponding Author
Tel: +82-63-220-3124
Fax: +82-63-220-2054
E-mail: sonjjang@jj.ac.kr

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Abstract The aim of this study was to investigate the protective effect of *Diospyros lotus* leaf extract (DLE) against acetaminophen (APAP)-induced acute liver injury in mice. Administration of DLE significantly attenuated the levels of serum aspartate aminotransferase, alanine aminotransferase, and liver lipid peroxidation in APAP-treated mice. Histopathological examination showed that DLE treatment decreased the incidence of liver lesions in APAP-treated mice. DLE treatment markedly increased superoxide dismutase, catalase, glutathione peroxidase activity, and glutathione levels in APAP-treated mice. Furthermore, DLE treatment significantly suppressed the production of pro-inflammatory factors such as the nitric oxide, IL-6, TNF-α, and iNOS in APAP-treated mice. These results suggest that DLE protects the liver from APAP-induced hepatic injury via antioxidant and anti-inflammatory effects.

Keywords: *Diospyros lotus*, acetaminophen, liver injury, antioxidants, anti-inflammation

Introduction

The liver is a critical organ regulating synthesis, metabolism, storage and redistribution of carbohydrates, proteins and lipids (1). In addition, the liver plays an important role in detoxifying toxic substances such as xenobiotics and toxins. During the removal of xenobiotics and toxins, various liver diseases including alcoholic liver injury can be caused by these toxic substances (2). Acetaminophen (APAP) is widely used as analgesic and antipyretic agent. APAP is usually safe at therapeutic doses, but overuse causes severe and fatal hepatotoxicity (3). In the United States and Europe, overdose usage of APAP commonly occurs because of accessibility to the general public drug and causes liver injury, accounting for the most frequent form of acute liver failure (4,5). APAP is metabolized by the liver cytochrome P450 into reactive metabolites, such as N-acetyl-*p*-benzoquinoneimine (NAPQI) which leads to the depletion of glutathione (GSH) (6). NAPQI binds to cellular proteins and causes the formation of reactive oxygen species (ROS) and peroxynitrite (7). GSH depletion caused by NAPQI induces oxidative stress and finally leads to cell death (7).

Diospyros lotus L. is a deciduous tree that belongs to the Ebenaceae family and is native to China and Asia. Its fruits have been used traditionally as a sedative, astringent, nutritive, anti-septic, anti-diabetic, anti-tumor, laxative, and nutritive as a febrifuge. It is also used to treat diarrhea, dry cough and hypertension. Chemical investigation have elucidated that the fruits of *D. lotus* contain some fatty acids, sugars phenolic compounds and non-volatile acids (8,9). Several studies have shown that roots of *D. lotus* exhibit anti-nociceptive, anti-inflammatory, antipyretic and sedative activities in mice (9,10). Recently, extract of *D. lotus* seeds have been reported to indicate antioxidant, anti-hemolytic, and nephroprotective activities (11). In previous studies, we have shown extracts and fractions from leaves of *D. lotus* indicate antioxidant, anti-allergy, and anti-pruritic effects (12-14). A previous study has demonstrated that extract of *D. lotus* leaves exhibits antioxidant, anti-inflammatory, analgesic and hepatoprotective activities (15). However, the detailed mechanism of the hepatoprotective effects of *D. lotus* leaf extract (DLE) has not yet been fully clarified. The objective of the current study was to explore DLE protection of murine liver from APAP-induced injury by attenuating oxidative stress and suppressing the inflammatory response.

Materials and Methods

Reagents Gallic acid, caffeic acid, chlorogenic acid, myricitrin, kaempferol, quercetin, ferulic acid, formic acid, APAP, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kaempferitin was purchased from ChemFaces (Wuhan, China). Antibodies for iNOS and β-actin were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). The goat anti-rabbit IgG HRP-conjugated antibody was purchased from Zymed (San Francisco, CA, USA). The analytical column was selected from Phenomenex (Torrance, CA, USA). The analytical organic solvents of HPLC grade were purchased from J.T. Baker (Phillipsburg, NJ, USA). All chemicals used were of reagent grade and were purchased from Sigma-Aldrich unless otherwise stated.

Plant material and extract preparation *D. lotus* leaves were collected on June 2014 from Bugui-myen, Jinan-gun (Jeonbuk, Korea). The plant was identified and authenticated by Professor Hong-Jun Kim at the College of Oriental Medicine, Woosuk University (Wanju, Korea). A voucher specimen (#2014-07-08) was deposited in the author's laboratory (Professor Seon Il Jang). The extract preparation was performed according to as described previously (12). Briefly, the collected leaves were washed in distilled water, steamed for 5 min, and dried using a dry oven at 40°C for 12 h. The dried leaves (100 g) were chopped, crushed, and extracted in 2 L methanol for 7 days and filtered through a membrane filter with a 0.45 μm pore. The filtrate was evaporated and freeze-dried to powdered form and the resulting powder was approximately 10.3 g and finally stored at −20°C until further analysis. The desired dose was then prepared in distilled water.

HPLC analysis HPLC was performed using an Agilent 1200 series (Agilent, Santa Clara, CA, USA) with a degasser (G1379A), a binary pump (G1312A), an autoinjector (G1329A), a column oven (G1316A) and a diode array detector (DAD) (G1315B). The separation of polyphenolic compounds was performed on Phenomenex Gemini-NX C18 column (4.6×250 mm ID, 5.0 micrometer particles) through the gradient elution with 0.5% aqueous formic acid (A) and acetonitrile (B): 0 min, 5% B; 3 min, 5% B; 8 min, 10% B; 12 min, 10% B; 15 min, 15% B; 20 min, 15% B; 27 min, 40% B; 32 min, 40% B; 39 min, 65% B; 43 min, 65% B; 48 min, 30% B; 50 min, 10% B. The mobile phase was retained at a flow rate of 0.8 mL/min and the column oven was set at a temperature of 30°C. Fifteen microliters of the extracts have been injected. The profiled wavelength of DAD was set at the 190-420 nm range, and was quantified at 254, 280, and 370 nm, respectively. The integration of each phenolic component on the chromatograms was processed using software Agilent Chemstation.

Animals Male Balb/c mice (7 weeks old) were obtained from Central Lab Inc. (Seoul, Korea), the Korean branch of Charles River Japan (Kanagawa, Japan). They were housed under standard environmental conditions and fed with a commercial standard laboratory diet and water *ad libitum*. The mice were maintained under constant conditions (22±2°C, 55±5% humidity and 12/12 h light-dark cycle) in an air-conditioned room. All experimental procedures were performed according to the Jeonju University Institutional Animal Care and Use Committee guidelines.

Experimental design The mice were randomly divided into six groups with six mice per group as follows: group 1, control; group 2, APAP 300 mg/kg; group 3, APAP plus DLE 50 mg/kg; group 4, APAP plus DLE 100 mg/kg; group 5, APAP plus DLE 200 mg/kg; group 6 (a positive control), APAP plus silymarin 50 mg/kg. DLE and silymarin were prepared in saline. APAP was prepared in vehicle (1% Et-OH and Tween-20). Groups 1 and 2 were administered the saline, and groups 3-6 were administered DLE and silymarin orally every day for 7 days. One hour after the final administration, groups 1 were treated intraperitoneally with the vehicle. Groups 2-6 were treated intraperitoneally with APAP at a dose of 300 mg/kg of body weight and fasted for 16 h. All groups were subsequently euthanized. Blood was obtained by the cardiac puncture after the mice had been anesthetized with ether. The blood samples were allowed to clot for 30 min at room temperature. The blood serum was separated by centrifugation at 2,000×*g* for 15 min at 4°C. Liver samples were also collected and rinsed with ice cold saline. The liver samples were quickly frozen with liquid nitrogen and stored at −80°C until used for further studies. A portion of each liver sample was fixed in 10% formalin for histopathological analysis.

AST and ALT assay Hepatic enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using an AST and ALT assay kit (ASAN Pharmaceutical., Seoul, Korea) according to the manufacturer's protocol.

Histopathology The liver tissue from different groups were collected and fixed in 10% formalin, dehydrated in graduated ethanol 50 to 100%, cleared in xylene, and embedded in paraffin. Sections 5 μm thick were prepared, stained with hematoxylin and eosin (H&E, St. Louis, MO, USA) dye, and examined for histopathological changes under a microscope (Leica, Wetzlar, Germany).

Lipid peroxidation Lipid peroxidation was measured using a malondialdehyde enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol.

Liver SOD, catalase, and GPx assay Liver superoxide dismutase (SOD), catalase, and glutathione peroxidase (GD) activities were measured using commercially available assay kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol.

Liver GSH assay Liver GSH was measured using a GSH assay kits (United States Biological, Salem, MA, USA) according to the manufacturer's protocol.

Serum NO assay The serum nitrite/nitrate concentration was measured using a QuantiChrom™ Nitric Oxide assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

The absorbance was measured at 540 nm with a microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) that was directly proportional to the serum nitrite/nitrate concentration.

Serum TNF-α and IL-6 assay The quantities of TNF-α and IL-6 in serum were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Western blot The liver tissues were homogenized in 10 mL of ice cold lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% nonidet-P40, 0.02% NaN_3, and 1 mM Phenyl-methylsulfonyl fluoride (PMSF)) containing a protease inhibitor cocktail per gram and centrifuged at 12,000×*g* for 15 min at 4°C. An equal amount of proteins was separated on 10% sodium dedecyl sulfate (SDS) polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% skim-milk in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h and subsequently incubated with primary antibodies (iNOS and β-actin) at 4°C overnight. The membranes were washed three times with TBS-T for 10 min and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Membranes were washed again, and the blotted proteins were detected using an enhanced chemiluminescence detection system (GE Heathcare, Bucks, UK).

Statistical analysis All data are presented as the mean±SD. The significance of differences between the means of the treated and untreated groups was determined by a Student's *t*-test. A $p<0.05$ value was considered to be significant.

Results and Discussion

Identified phenolic compounds from DLE A comparative analysis of the levels of each compound within the native plant was performed using HPLC analysis. The nature of each peak in the HPLC trace was doubly verified by comparison with retention time of the compound. Phenolic compounds for the analysis of DLE were identified the standard commercial compounds. Gallic acid (1), caffeic acid (2), myricitrin (3), chlorogenic acid (4), kaempferol (5) and quercetin (6) were provided by Sigma-Aldrich. Figure 1 showed the comparative chromatogram for each component. In addition,

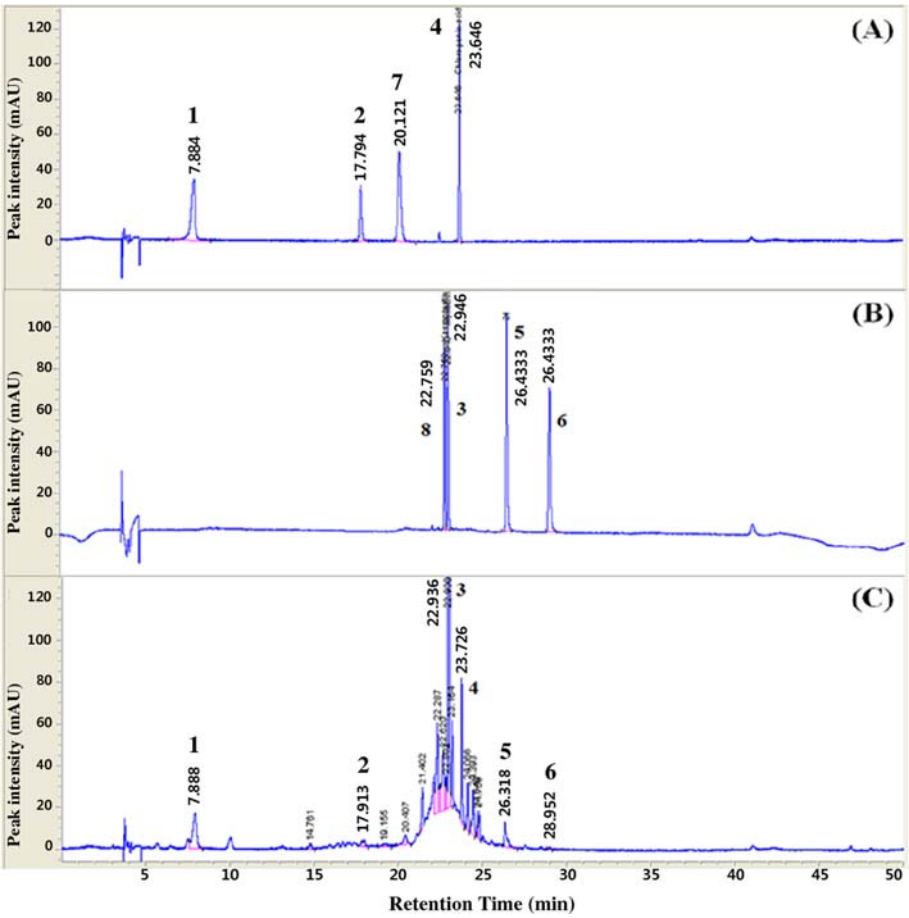

Fig. 1. Representative HPLC chromatogram of standard compounds (A and B) and methanol extract of *Diospyros lotus* leaf (C). Each sample was eluted by Phenomenex Gemini NX C18 column (4.6×250 mm, 5 μm) and scanned from UV 254 to 370 nm. Peaks: 1, gallic acid; 2, caffeic acid; 3, myricitrin; 4, chlorogenic acid; 5, kaempferol; 6, quercetin; 7, ferulic acid; 8, kaempferitin

Table 1. Content (μg/g) of phenolic components in methanol extracts of *D. lotus* leaves[1)]

Sample ID	Acquired results (μg/g)					
	1[2)]	2	3	4	5	6
D. lotus leaves	418.83	67.03	10599.81	474.02	95.62	30.66

[1)]The concentration was expressed as free form.
[2)]1: Gallic acid; 2: caffeic acid; 3: myricitrin; 4: chlorogenic acid; 5: kaempferol, 6: quercetin

compound 7 and 8 as ferulic acid and kaempferitin were under studying in DLE. HPLC analysis of DLE was performed and peaks were identified as gallic acid, caffeic acid, myricitrin, chlorogenic acid, kaempferol, quercetin, respectively (Fig. 1). The content of those compounds was 418.83, 67.03, 10599.81, 474.02, 95.62, 30.66 μg/g, respectively (Table 1). In the previous studies, eight compounds were identified as gallic acid, methylgallate, ellagic acid, kaempferol, quercetin, myricetin, myricetin 3-*O*-β-glucuronide, and myricetin 3-*O*-α-rhamnoside (also known as myricitrin) from the fruits and leaves of *D. lotus* (8,15). Among them, methylgallate was reported to possess antioxidant and anti-inflammatory activities (16). Reports have shown that gallic acid, methylgallate, ellagic acid, kaempferol, quercetin, myricetin, and myricitrin have strong antioxidant activities as well as hepatoprotective effects (17-22). In the HPLC analysis, we found DLE to contain 6 flavonoids: gallic acid, caffeic acid, myricitrin, chlorogenic acid, kaempferol, and quercetin. A previous study has described chlorogenic acid to exhibit protective activities against APAP-induced oxidative liver injury in mice (4). Many studies demonstrated that caffeic acid can prevent ischemic brain injury and oxidative stress in mice (23,24). Our *in vitro* results showed that DLE exhibits strong scavenging activities of DPPH and ABTS radical (12,14). DLE also significantly indicated the anti-inflammatory effect in phorbol 12-myristate 13-acetate plus calcium ionophore A23187-stimulated rat peritoneal mast cells and carrageenan-injected rat (13,15). Therefore, we postulated that DLE can have antioxidant effect and may protect against APAP-induced mouse liver injury based on our data and above mentioned previous reports.

Effects of DLE on serum AST and ALT levels in APAP-treated mice APAP has been used to investigate liver toxicity associated to oxidative stress (25). Liver toxicity induced by APAP causes necrosis of hepatocytes and as a result releases AST and ALT into the circulating blood (25). Serum AST and ALT levels are diagnostic biomarkers of liver injury and measurement of these enzymes levels are widely used to evaluate the levels of liver injury (25-27). The levels of AST and ALT in the serum were investigated to assess liver injury. The levels of serum AST and ALT were significantly increased in APAP-treated mice, compared with the control group (Fig. 2). However, mice pretreated with DLE showed dose-dependently decreases in the levels of AST and ALT enzymes, compared with APAP alone. Silymarin treatment (a positive control) also decreased APAP-induced increase of serum AST and ALT levels. These results showed that DLE exhibited a hepatoprotective capability against APAP-induced liver injury. These results correspond with the previous report (15), suggesting hepatoprotective activity.

Effects of DLE on histopathological changes in APAP-treated mouse liver APAP overdose could cause a variety of hepatic histopathological changes such as hepatocellular necrosis, leucocyte infiltration, and hepatic architecture damage (28). To further investigate the effect of

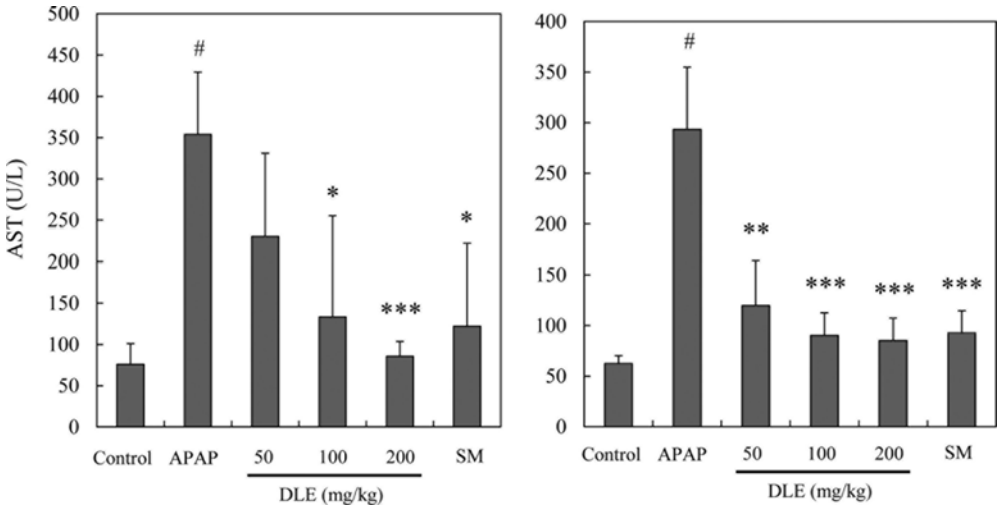

Fig. 2. Effect of DLE on the levels of serum AST and ALT in APAP-treated mice. Mice were treated with 300 mg/kg APAP, APAP plus 50 mg/kg DLE, APAP plus 100 mg/kg DLE, APAP plus 200 mg/kg DLE, or 50 mg/kg silymarin, as described in the Materials and Methods. Silymarin was used as a positive control. Blood samples were prepared from mice and tested for AST and ALT levels. Data are presented as the mean±SD of six mice. #p<0.001 vs. control, *p<0.05 vs. APAP, **p<0.01 vs. APAP, ***p<0.001 vs. APAP.

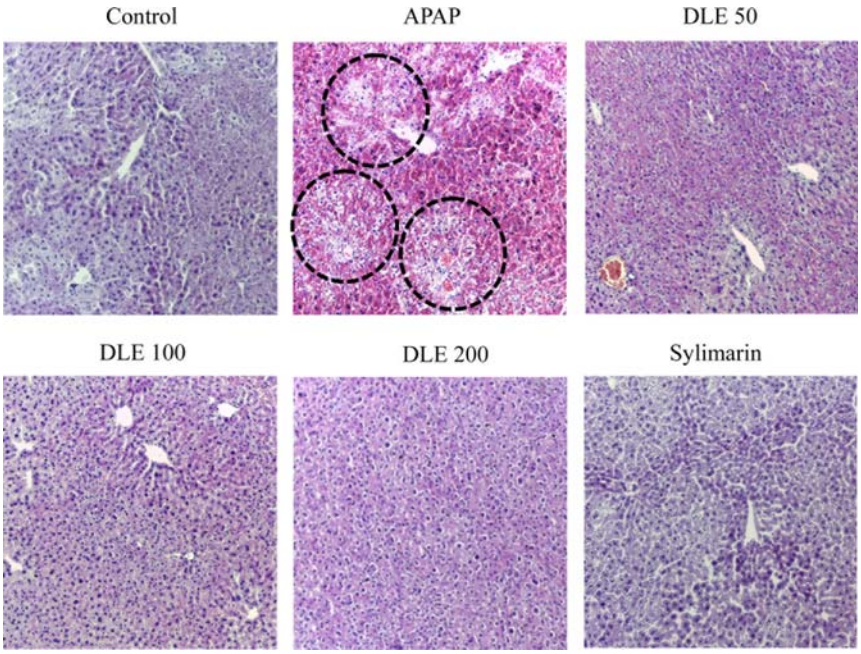

Fig. 3. Effect of DLE on the histological changes in APAP-treated mice. Liver sections were stained with hematoxylin and eosin. All sections were photographed with a microscope (100×magnification). The dotted circle regions showed the portions of parenchyma collapse, extensive hepatocyte necrosis, and leukocyte infiltration.

DLE on liver injury in APAP-treated mice, we determined the histopathological changes using H&E staining. As shown in Fig. 3, a liver section of APAP-treated mice exhibited histological changes such as the collapse of parenchyma, extensive hepatocyte necrosis and leukocyte infiltration, compared with the control group. However, APAP-induced liver injury was ameliorated in mice pretreated with DLE. Silymarin also recovered the histopathological changes similar to the effect of DLE in the APAP-induced liver injury model. The present studies suggest that DLE significantly suppresses APAP-induced liver injury through the recovery of liver function and architecture of liver tissue.

Effects of DLE on lipid peroxidation and antioxidant system in APAP-treated mouse liver APAP hepatotoxicity is initiated by NAPQI, which binds to mitochondrial proteins and leads to initial mitochondrial oxidative stress by producing ROS including superoxide anion radical and finally results in hepatocellular necrosis (25). APAP-induced ROS could cause lipid peroxidation and protein oxidation, and leads to depletion of an antioxidant system including activities of antioxidant enzymes (SOD, catalase, GPx, and glutathione reductase) and nonenzymatic antioxidants (GSH, vitamin C, and vitamin E) (29). Malondialdehyde (MDA) has been used as a major oxidative stress marker in APAP-induced liver injury (28). Previous studies have demonstrated that APAP administration in mice showed a significant increase in hepatic MDA levels and decreased hepatic GSH levels and the activities of antioxidant enzymes (6,30). These statuses were reversed by extract or chemical from plants (6,30). As shown in Fig. 4, a significant increase in the hepatic level of MDA was observed in

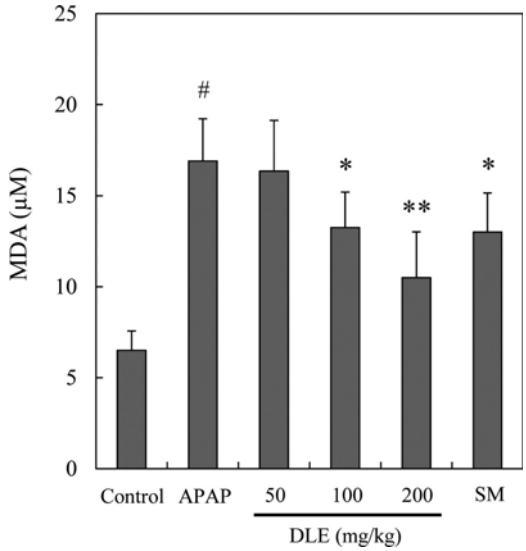

Fig. 4. Effect of DLE on the levels of lipid peroxidation in APAP-treated mice. Tissue homogenates were prepared from mouse livers and tested for levels of lipid peroxidation. Data are presented as the mean±SD of six mice. $^{\#}p<0.001$ vs. control, $^{*}p<0.05$ vs. APAP, $^{**}p<0.01$ vs. APAP.

mice treated with APAP alone, compared with the control group. However, APAP-induced elevation of MDA concentration was dose-dependently decreased in mice pretreated with DLE. Silymarin treatment also decreased APAP-induced increase of hepatic level of MDA. The present study also analyzed the effects of DLE on SOD, catalase (CAT), and GPx activities and GSH levels in APAP-treated mouse liver. As shown in Fig. 5, the activities of the antioxidant

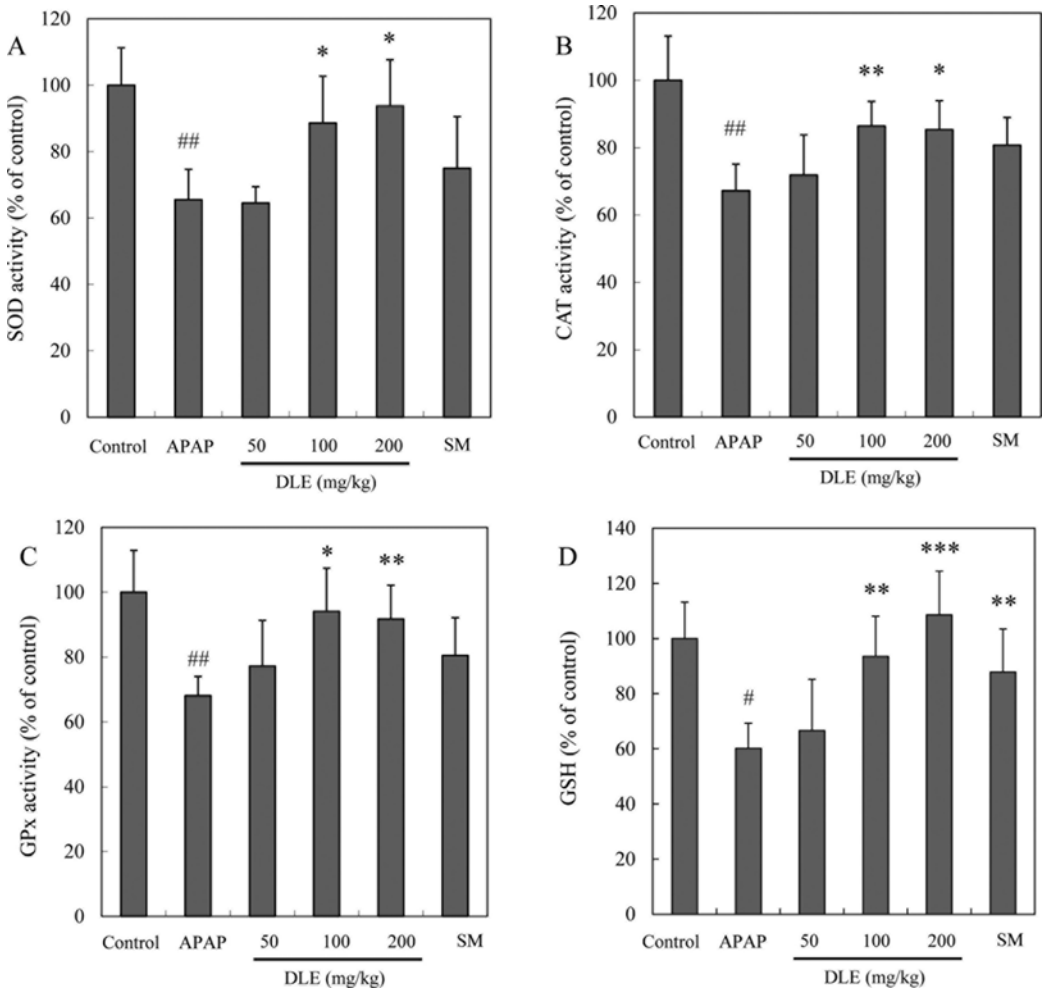

Fig. 5. Effect of DLE on the SOD, CAT, and GPx activity and GSH levels in APAP-treated mice. Tissue homogenates were prepared from mouse liver and tested for SOD activity (A), CAT activity (B), GPx activity (C), and GSH levels (D). Data are presented as the mean±SD of six mice. $^{\#}p<0.001$ vs. control, $^{\#\#}p<0.01$ vs. control, $^{*}p<0.05$ vs. APAP, $^{**}p<0.01$ vs. APAP, $^{***}p<0.001$ vs. APAP.

enzymes SOD, CAT, and GPx were significantly decreased in APAP-treated mice, compared with the control group. However, mice pretreated with DLE at 100 and 200 mg/kg showed significant increases in SOD, CAT, and GPx activities, compared with the APAP alone. Silymarin treatment also slightly increased the enzymatic activities of those, but there were no significant differences, compared with APAP alone. The levels of liver GSH amount were significantly decreased in APAP-treated mice, while DLE treatment reversed APAP-decreased liver GSH amount in a dose dependent manner. Silymarin treatment also increased the APAP-decreased liver GSH amount. These data suggest that DLE could attenuate oxidative stress by direct and indirect scavenging of APAP-induced ROS through the inhibition of lipid peroxidation and the activation of antioxidant system.

Effects of DLE on inflammatory response in APAP-treated mice

To further elucidate the mechanism of the protective effect of DLE against liver injury, inflammatory mediators such as TNF-α, IL-6, NO, and iNOS were investigated. As shown in Fig. 6, the increases in levels of TNF-α, IL-6, and NO were dose-dependently decreased in mice pretreated with DLE. In addition, the up-regulation of iNOS expression was markedly reduced in mice pretreated with DLE in a dose-dependent manner. Silymarin treatment also inhibited the elevation of these inflammatory mediators similar to the effect of DLE in the APAP-induced liver injury model. Many studies have indicated that inflammatory mediators including TNF-α, IL-6, IFN-γ, iNOS, and NO are decisively involved in the development of acute liver injury by APAP (3,5). In addition to hepatocellular necrosis by oxidative stress, APAP hepatotoxicity can also activate Kupffer cells, the phagocytic macrophages of the liver and cause liver inflammation. Activated Kupffer cells release pro-inflammatory cytokines including IL-12, IFN-γ, and TNF-α (3,5). Cytokines such as IL-6, IL-1β, and IL-10 significantly are elevated in APAP-induced hepatotoxic mice via the production of ROS (31). Inflammatory cytokines including TNF-α and IL-6 were reported to be involved in the iNOS-mediated DNA damage (32,33). The NO increased by iNOS expression is a highly reactive free radical.

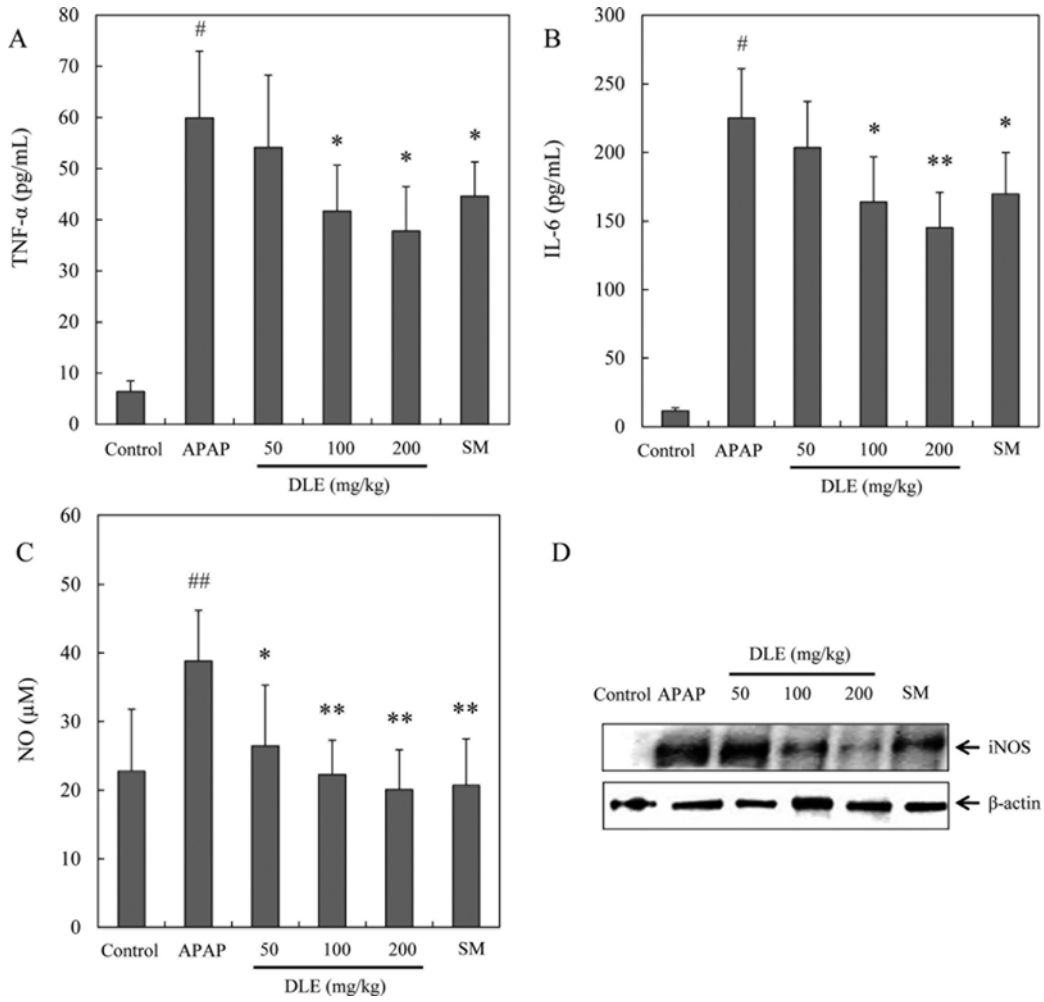

Fig. 6. Effect of DLE on the serum TNF-α (A), IL-6 (B), and NO (C) levels in APAP-treated mice. Data are presented as the mean±SD of six mice. $^{\#}p<0.001$ vs. control, $^{\#\#}p<0.05$ vs. control, $^{*}p<0.05$ vs. APAP, $^{**}p<0.01$ vs. APAP. (D) Effect of DLE on the expression of iNOS in APAP-treated mouse liver. Tissue homogenates were prepared from mouse liver, and 50 μg of protein was electrophoresed on a 10% polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and detected by western blot analysis. The protein of β-actin served as a control.

The production of excessive NO could cause hepatocellular damage by oxidizing and nitrating cellular macromolecules via a reaction with a superoxide anion (3,34,35). In addition, excessive NO has been reported to be involved in the depletion of intracellular GSH, increasing the susceptibility to oxidative stress (3). The present study suggests that DLE could ameliorate APAP-induced liver injury through suppressing the inflammatory response as well as elevating antioxidant capacity. Moreover, major compound of DLE was myricitrin, as shown in Table 1. A previous study has demonstrated that myricitrin exhibits a significant hepatoprotective activity (17). Therefore, we speculate that myricitrin is the most active compound for the hepatoprotective effect.

In conclusion, DLE suppressed increases in AST and ALT levels, and lipid peroxidation through increased activities of antioxidant enzymes and the attenuation of inflammatory cytokines, NO production and iNOS expression in APAP-treated mice. The present study suggests that DLE protects the liver from APAP-induced hepatic injury, potentially due to antioxidant capabilities and inhibition of the inflammatory response. Therefore, DLE may be a potential therapeutic materials to prevent hepatic injury and further study requires for confirm the potential of DLE.

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Disclosure The authors declare no conflict of interest.

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