



Contents lists available at ScienceDirect

## Food and Chemical Toxicology

journal homepage: [www.elsevier.com/locate/foodchemtox](http://www.elsevier.com/locate/foodchemtox)

## Palmitine attenuates D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in mice

Woo-Cheol Lee<sup>a</sup>, Joon-Ki Kim<sup>a</sup>, Jung-Woo Kang<sup>a</sup>, Woo-Yong Oh<sup>a</sup>, Joo-Yeon Jung<sup>a</sup>, Yeong Shik Kim<sup>b</sup>, Hyun Ah Jung<sup>c</sup>, Jae Sue Choi<sup>c</sup>, Sun-Mee Lee<sup>a,\*</sup>

<sup>a</sup> College of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, Republic of Korea

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

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

## ARTICLE INFO

## Article history:

Received 8 August 2009

Accepted 1 October 2009

Available online xxxxx

## Keywords:

Apoptosis

Fulminant hepatic failure

D(+)-Galactosamine

Inflammation

Lipopolysaccharide

Palmitine

## ABSTRACT

Palmitine is an isoquinoline alkaloid from *Coptis chinensis*, an herbal medicine used to treat various inflammatory diseases such as gastritis, edema and dermatitis. The present study examined the cytoprotective properties of palmitine on D(+)-galactosamine (GalN)/lipopolysaccharide (LPS)-induced fulminant hepatic failure. Mice were intraperitoneally given GalN (700 mg/kg)/LPS (10 µg/kg). Palmitine (25, 50, 100, and 200 mg/kg) was administered 1 h before GalN/LPS. GalN/LPS increased the mortality and serum aminotransferase activities. These increases were attenuated by palmitine. GalN/LPS increased hepatic lipid peroxidation and decreased the contents of reduced glutathione. Palmitine did not affect the lipid peroxidation and glutathione content. GalN/LPS increased the circulating levels of tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and IL-10. Palmitine prevented the increase of serum TNF-α and augmented that of serum IL-10. GalN/LPS treatment also increased the levels of TNF-α, IL-6 and IL-10 mRNA expression in liver tissue. Palmitine decreased the TNF-α mRNA expression and increased the IL-10 mRNA expression. Palmitine attenuated the apoptosis of hepatocytes, as evidenced by the TUNEL method and caspase-3 analysis. Our data suggest that palmitine alleviates GalN/LPS-induced liver injury by modulating the cytokine response and inhibiting apoptosis.

© 2009 Elsevier Ltd. All rights reserved.

### 1. Introduction

Fulminant hepatic failure is a life-threatening clinical syndrome that results from severe impairment of liver function. Galactosamine (GalN) and lipopolysaccharide (LPS)-induced liver failure is a widely used model that resembles human liver failure (Nakama et al., 2001). Lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria, is an endotoxin that is thought to contribute significantly to hepatic failure (Bohlinger et al., 1996). Galactosamine is a specific hepatotoxic agent that depletes the uridine triphosphate pool and thereby inhibits macromolecule synthesis (Decker and Keppler, 1974). As a transcriptional inhibitor, GalN potentiates the toxic effects of LPS in liver (Xiong et al., 1999).

**Abbreviations:** ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; ELISA, enzyme-linked immunosorbent assay; GalN, galactosamine; GSH, glutathione; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MDA, malondialdehyde; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nicked end labeling.

\* Corresponding author. Address: College of Pharmacy, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon, Gyeonggi-do 440-746, Republic of Korea. Tel.: +82 31 290 7712; fax: +82 31 292 8800.

E-mail address: [sunmee@skku.edu](mailto:sunmee@skku.edu) (S.-M. Lee).

The toxic effects of endotoxin are exerted through the generation of endogenous cytokines. High levels of circulating tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-10, IL-12 and interferon-γ (INF-γ) were reported during endotoxemia (van Deuren et al., 1992). Most recently, it was reported that TNF-α-induced apoptosis was associated with Fas and FasLigand up-regulation. Within this context, neutralization of TNF-α partially prevented GalN/LPS-induced hepatocellular apoptosis (Kuhla et al., 2008). IL-10 is an anti-inflammatory cytokine known to decrease circulating TNF-α and to offer protection against endotoxic shock (Howard et al., 1993).

*Coptis chinensis* (Ranunculaceae), a traditional herbal medicine, is used as a remedy for abdominal pain, enteritis and gastritis. In experimental animal models, such as ear edema, acetic acid-induced capillary permeability test, and cotton pellet-induced inflammation, *C. chinensis* has protective effects on acute and chronic inflammation (Park et al., 2007). Total alkaloids from *C. chinensis* have protective effects against gastric ulcers induced by water-immersion stress, intragastric ethanol, acetic acid erosion and pylorus ligation (Li et al., 2005). Palmitine is an isoquinoline alkaloid that can be extracted and purified from *C. chinensis*. Palmitine inhibits serotonin-induced hind paw edema and acetic acid-induced transition of vascular permeability (Kupeli et al., 2002). Its

inhibitory effect on outward potassium currents and  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  current could be one of the mechanisms by which palmatine exerts a protective effect on isolated rat hepatocytes (Wang et al., 2003).

The aim of this study was to investigate the hepatoprotective effects of palmatine in fulminant hepatic failure, particularly on the extent of inflammation and apoptosis.

## 2. Materials and methods

### 2.1. Extraction and isolation of palmatine

The powdered rhizome of *C. chinensis* (10 kg) was refluxed with methanol (MeOH) for 3 h ( $3 \times 10$  L). The total filtrate was then concentrated to dryness *in vacuo* at 40 °C, in order to render the MeOH extract (2.2 kg). This extract was suspended in distilled water ( $\text{H}_2\text{O}$ ) and then successively partitioned with methylene chloride ( $\text{CH}_2\text{Cl}_2$ ), *n*-butanol (BuOH), to yield  $\text{CH}_2\text{Cl}_2$  (230 g), *n*-BuOH (1.1 kg) fractions, respectively, as well as  $\text{H}_2\text{O}$  residue (840 g). A portion of *n*-BuOH fraction (316 g) was initially chromatographed over a silica gel column using a mixed solvent of EtOAc, MeOH, and  $\text{H}_2\text{O}$  (EtOAc:MeOH: $\text{H}_2\text{O}$  21:4:3 → EtOAc:MeOH:2% HCl 21:10:3, gradient conditions) to yield 14 subfractions (BF1–BF18). BF02 (9.3 g) was repeatedly chromatographed over a silica gel column using a mixed solvent of EtOAc, MeOH, and  $\text{H}_2\text{O}$  (EtOAc:MeOH: $\text{H}_2\text{O}$  21:4:3) to yield palmatine (3 g). The chemical structure of palmatine was elucidated on the basis of spectroscopic evidences and by comparison with published data (Grycová et al., 2007; Lee and Kim, 1997). The NMR data of palmatine were as follows.

Palmatine  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  9.78 (1H, s, H-8), 8.88 (1H, s, H-13), 8.10 (1H, d,  $J = 8.0$  Hz, H-11), 8.00 (1H, d,  $J = 8.0$  Hz, H-12), 7.63 (1H, s, H-1), 7.03 (1H, s, H-4), 4.87 (2H, m, H-6), 4.19 (3H, s, 9-OCH<sub>3</sub>), 4.08 (3H, s, 10-OCH<sub>3</sub>), 3.97 (3H, s, 2-OCH<sub>3</sub>), 3.92 (3H, s, 3-OCH<sub>3</sub>), 3.52 (2H, m, H-5);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  152.6 (C-3), 150.7 (C-10), 149.7 (C-2), 145.2(C-8), 144.5 (C-9), 138.6 (C-13a), 134.0 (C-12a), 128.9 (C-4a), 126.8 (C-12), 123.3(C-11), 122.1 (C-13b), 120.1 (C-13), 119.3 (C-8a), 111.0 (C-4), 108.7 (C-1), 61.3 (9-OCH<sub>3</sub>), 56.4 (10-OCH<sub>3</sub>), 56.1 (2-OCH<sub>3</sub>), 55.8 (3-OCH<sub>3</sub>), 55.4 (C-6), 26.6 (C-5); LC–ESI–MS/MS  $m/z$  352 [M]<sup>+</sup>, 337, 322, 308.

### 2.2. Treatment of animals

Male ICR mice (20–22 g) were fasted overnight but given access to water *ad libitum*. All animals were treated humanely under the Sungkyunkwan University Animal Care Committee guidelines. To induce fulminant hepatic failure with GalN/LPS, the mice (except for the control) were injected intraperitoneally with GalN (700 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) and LPS (10  $\mu\text{g}/\text{kg}$  Escherichia coli 026:B6, Sigma) dissolved in phosphate-buffered saline. Palmatine was suspended in 10% Tween 80–saline (vehicle) and administered intraperitoneally at a dose of 25, 50, 100, or 200 mg/kg 1 h before the GalN/LPS treatment. The doses of palmatine were selected based on previous reports (Kim et al., 2009; Xu and Malavé, 2001). Eight treatment groups were examined: The mice in group I (control) received only vehicle. The mice in group II (palmatine) were treated with palmatine (100 mg/kg, i.p.) dissolved in vehicle. In groups III to VIII, the mice were treated with GalN/LPS. The mice in groups IV to VII were pretreated with palmatine (25, 50, 100 and 200 mg/kg, i.p.), and the mice in group VIII were pretreated with silym-

arin (positive control, 200 mg/kg, i.p.). The mice were sacrificed by decapitation at 1, 2, 4 and 8 h after GalN/LPS injection. Blood was collected from the abdominal aorta at each time point. The liver was isolated and used immediately to prepare mRNA; both the mRNA and liver tissues were stored at  $-75$  °C for later analysis.

### 2.3. Mortality and histopathological analysis

The survival rate of the mice was monitored for 24 h after GalN/LPS injection. Liver specimens for histopathological analysis were obtained 8 h after GalN/LPS administration. The liver sample was fixed in 10% neutral-buffered formalin, then embedded in paraffin, sliced into 5- $\mu\text{m}$  sections, and stained with hematoxylin–eosin for blind histological assessment. The morphologic criteria used to determine the degree of necrosis included portal inflammation, hepatocellular necrosis, inflammatory cell infiltration, and loss of cell architecture. The histological changes were evaluated in nonconsecutive, randomly chosen X200 histological fields. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-labeling (TUNEL) method using an *in situ* apoptosis detection kit (TaKaRa Co., Shiga, Japan). Under microscopy, the number of TUNEL-positive cells in X200 histological fields was counted.

### 2.4. Serum aminotransferase activities

Plasma samples were taken from the mice at 8 h after GalN/LPS injection. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined with a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan).

### 2.5. Hepatic lipid peroxidation and glutathione contents

The hepatic lipid peroxidation levels and glutathione content were measured 8 h after the GalN/LPS injection. The steady-state level of malondialdehyde, a lipid peroxidation end-product, was analyzed by measuring the level of thiobarbituric acid reactive substances spectrophotometrically at a wavelength of 535 nm according to the method reported by Buege and Aust (1978) using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, USA) as the standard. The total glutathione in the liver homogenate was determined spectrophotometrically at a wavelength of 412 nm, with yeast glutathione reductase, 5,5'-dithio-bis (2-nitrobenzoic acid) and NADPH, according to the method reported by Tietze (1969). The GSSG level was measured by the same method in the presence of 2-vinylpyridine (Griffith, 1980), and the GSH level was determined as the difference between the total glutathione and the GSSG levels.

### 2.6. Serum cytokine levels

Circulating levels of the cytokines TNF- $\alpha$ , IL-6 and IL-10 were quantified at 1, 2, 4 and 8 h after GalN/LPS injection using commercial mouse ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

### 2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted as reported by Chomczynski and Sacchi (1987). First strand cDNA was synthesized by reverse transcription of the total RNA using the oligo(dT) primer and SuperScript™ II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen

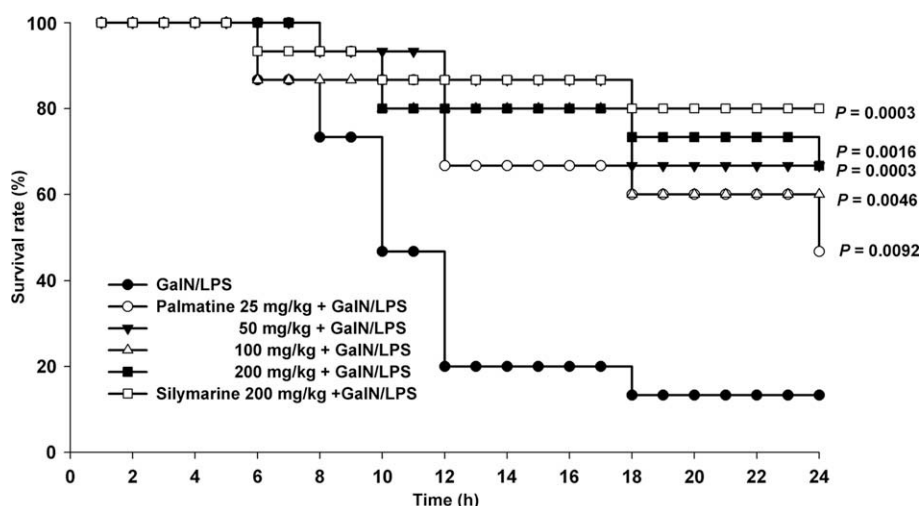


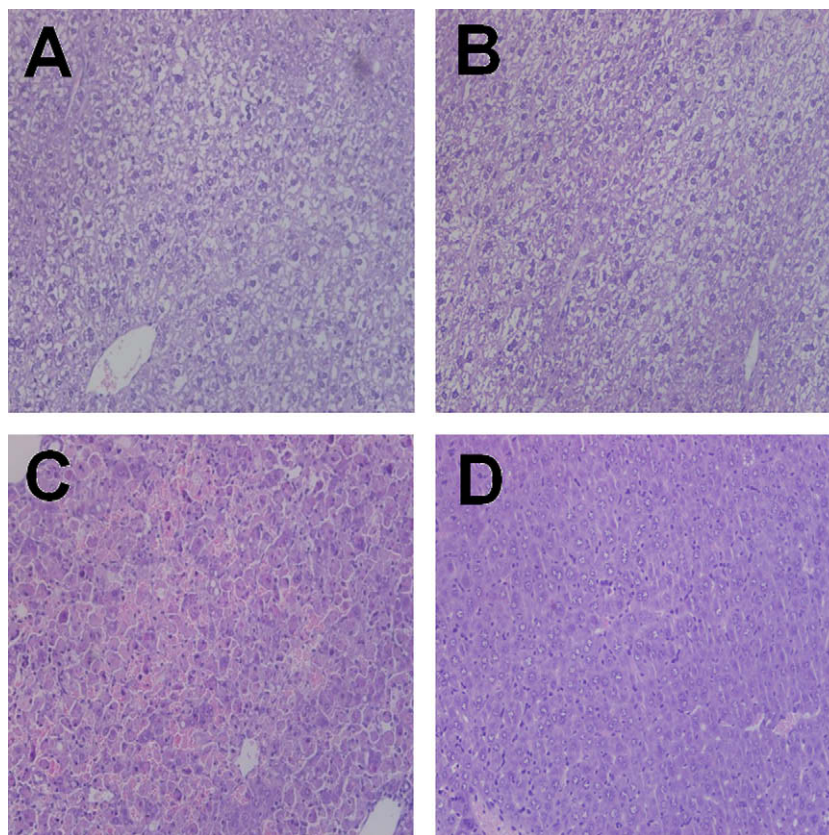
Fig. 1. Effect of palmatine on lethality induced by GalN/LPS. GalN, galactosamine; LPS, lipopolysaccharide.

Tech-Line™, Carlsbad, CA, USA). The PCR reaction was carried out with a diluted cDNA sample in a 20- $\mu$ l reaction volume. The final reaction concentrations were as follows: primers, 10 pmol; dNTP mix, 250  $\mu$ M; 10 $\times$  PCR buffer; and Ex Taq DNA polymerase, 0.5 U per reaction. RT-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 amplification cycling conditions were as follows: for *TNF- $\alpha$* , 28 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; for *IL-6*, 40 cycles at 94 °C for 30 s, 57.6 °C for 30 s, and 72 °C for 45 s; for *IL-10*, 40 cycles at 94 °C for 30 s, 62.9 °C for 30 s, and 72 °C for 45 s; for  *$\beta$ -actin*, 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After RT-PCR, 10- $\mu$ l 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) and analysis software.

### 2.8. Caspase-3 activity

The caspase-3 activity was measured using an *in vitro* fluorogenic peptide substrate, N-acetyl-Asp-Glu-Val-Asp-7-amino-trifluoromethyl-cumarine (DEVD-AFC; BioMol, Plymouth Meeting, PA, USA), according to the procedure reported by Morin et al. (2004). Eight hours after the GalN/LPS treatment, a sample of liver tissue (1 g) was homogenized in 6 ml of buffer containing 25 mM Tris, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 50  $\mu$ l of protease inhibitor cocktail (Sigma). The homogenate was centrifuged for 15 min at 40,000g and the resulting supernatant was collected for determining the



**Fig. 2.** H&E staining of livers after GalN/LPS administration. Typical images were chosen from each experimental group (original magnification 200 $\times$ ). (A) Control group: normal lobular architecture and cell structure; (B) palmatine (100 mg/kg)-treated group; (C) GalN/LPS group; multiple and extensive areas of portal inflammation and hepatocellular necrosis, and a moderate increase in inflammatory cell infiltration; (D) palmatine (100 mg/kg) and GalN/LPS-treated group: minimal hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation. GalN, galactosamine; H&E, hematoxylin and eosin; LPS, lipopolysaccharide.

**Table 1**

Effect of palmatine on aminotransferase activities, lipid peroxidation and reduced glutathione levels in mice after GalN/LPS-treatment.

Group	Dose (mg/kg i.p.)	ALT (U/L)	AST (U/L)	Malondialdehyde (nmol/mg protein)	GSH ( $\mu$ mol/g liver)
Control	–	41.3 $\pm$ 4.2	52.9 $\pm$ 1.4	0.33 $\pm$ 0.01	6.67 $\pm$ 0.34
Palmatine	100	49.1 $\pm$ 5.5	53.7 $\pm$ 3.3	0.35 $\pm$ 0.02	6.23 $\pm$ 0.39
GalN/LPS					
Vehicle	–	4606.4 $\pm$ 630.3**	5342.7 $\pm$ 1304.3**	0.85 $\pm$ 0.03**	1.94 $\pm$ 0.44**
Palmatine	25	4004.3 $\pm$ 1193.6**	4255.3 $\pm$ 964.4**	0.81 $\pm$ 0.11*	2.30 $\pm$ 0.38*
	50	896.4 $\pm$ 229.3***	1304.4 $\pm$ 373.5***	0.78 $\pm$ 0.10**	2.71 $\pm$ 0.24**
	100	276.2 $\pm$ 78.5***	407.4 $\pm$ 86.4***	0.79 $\pm$ 0.09**	2.92 $\pm$ 0.11**
	200	532.7 $\pm$ 365.6***	583.8 $\pm$ 89.9***	0.77 $\pm$ 0.06**	2.36 $\pm$ 0.17**
Silymarin	200	410.1 $\pm$ 159.0**	697.2 $\pm$ 179.1***	0.45 $\pm$ 0.03***	4.79 $\pm$ 0.51**

Each value is the mean  $\pm$  SEM of eight animals per group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GalN, galactosamine; GSH, glutathione; LPS, lipopolysaccharide.

\* Significant differences from the control group ( $p < 0.05$ ).

\*\* Significant differences from the control group ( $p < 0.01$ ).

\* Significant differences from the vehicle-treated GalN/LPS group ( $p < 0.05$ ).

\*\* Significant differences from the vehicle-treated GalN/LPS group ( $p < 0.01$ ).

caspase-3 activity. Dithiothreitol (10 mM) was added to the samples immediately before freezing. The caspase-3 activity was assayed in a total volume of 100  $\mu$ l. A 30- $\mu$ g sample of cytosolic protein was incubated at room temperature for 15 min in buffer containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100, and 10 mM dithiothreitol. The reaction was initiated by adding 200  $\mu$ M DEVD-AFC and the samples were then incubated at 37  $^{\circ}$ C. The change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min.

### 2.9. Statistical analysis

Survival data were analyzed by the Kaplan–Meier curve and log-rank test. All other data were analyzed by one-way analysis of variance (ANOVA), and the Bonferroni test was used for *post hoc* comparisons. The differences between the groups were considered statistically significant at a *p* value < 0.05. The results are presented as mean  $\pm$  S.E.M.

## 3. Results

### 3.1. Mortality and histological change

In the GalN/LPS group, the mice began to die 6 h after GalN/LPS injection. The mortality was 40% at 10 h and stabilized at 87% 24 h after GalN/LPS injection. However, pretreatment with palmatine (25, 50, 100 and 200 mg/kg) before GalN/LPS markedly reduced the mortality. In silymarin-treated mice (positive control), the mortality was 20% 24 h after GalN/LPS (Fig. 1). The histological analysis of the liver in the control group revealed normal liver lobular architecture and cell structure. However, livers exposed to GalN/LPS showed multiple and extensive areas of portal inflammation and cellular necrosis and a moderate increase in inflammatory cell infiltration. These changes were attenuated by palmatine (100 mg/kg) treatment (Fig. 2).

### 3.2. Serum aminotransferase activities, lipid peroxidation and reduced glutathione levels

In the control group, the serum levels of ALT and AST, markers of hepatocyte necrosis, were quite low. In the GalN/LPS-treated group, the serum levels of ALT and AST increased by approximately 112-fold and 100-fold over the control, respectively, at 8 h after GalN/LPS injection, which indicated severe liver injury. These increases were attenuated by administration of 50, 100 and 200 mg/kg palmatine and 200 mg/kg silymarin. The hepatic MDA level was  $0.33 \pm 0.01$  nmol/mg protein in the control. However, GalN/LPS increased the hepatic MDA level by approximately 2.6-fold over that of the control animals. The GSH level was  $6.7 \pm 0.3$   $\mu$ mol/g in control and significantly decreased to  $1.9 \pm 0.4$   $\mu$ mol/g 8 h after GalN/LPS treatment. Palmatine did not affect the changes in MDA and GSH levels after GalN/LPS. In contrast, silymarin significantly decreased the MDA level and increased the GSH level after GalN/LPS injection (Table 1).

### 3.3. Serum levels of cytokines

The regulatory effects of palmatine (100 mg/kg) on systemic secretion of circulating cytokines, such as TNF- $\alpha$ , IL-6 and IL-10, were examined by ELISA. As shown in Fig. 3, the serum level of TNF- $\alpha$  significantly increased ( $845 \pm 106$  pg/ml) 1 h after GalN/LPS, and this elevation was attenuated by palmatine treatment. The serum levels of IL-6 and IL-10 began to increase 1 h after GalN/LPS and were maximal ( $3379 \pm 767$  and  $605 \pm 48$  pg/ml, respectively) after 2 h. Palmatine augmented the IL-10 level but did not affect the IL-6 level 2 h after GalN/LPS.

### 3.4. TNF- $\alpha$ , IL-6, and IL-10 mRNA expression

As shown in Fig. 4, the hepatic level of TNF- $\alpha$  mRNA expression in GalN/LPS-treated mice was approximately 5.5-fold higher than

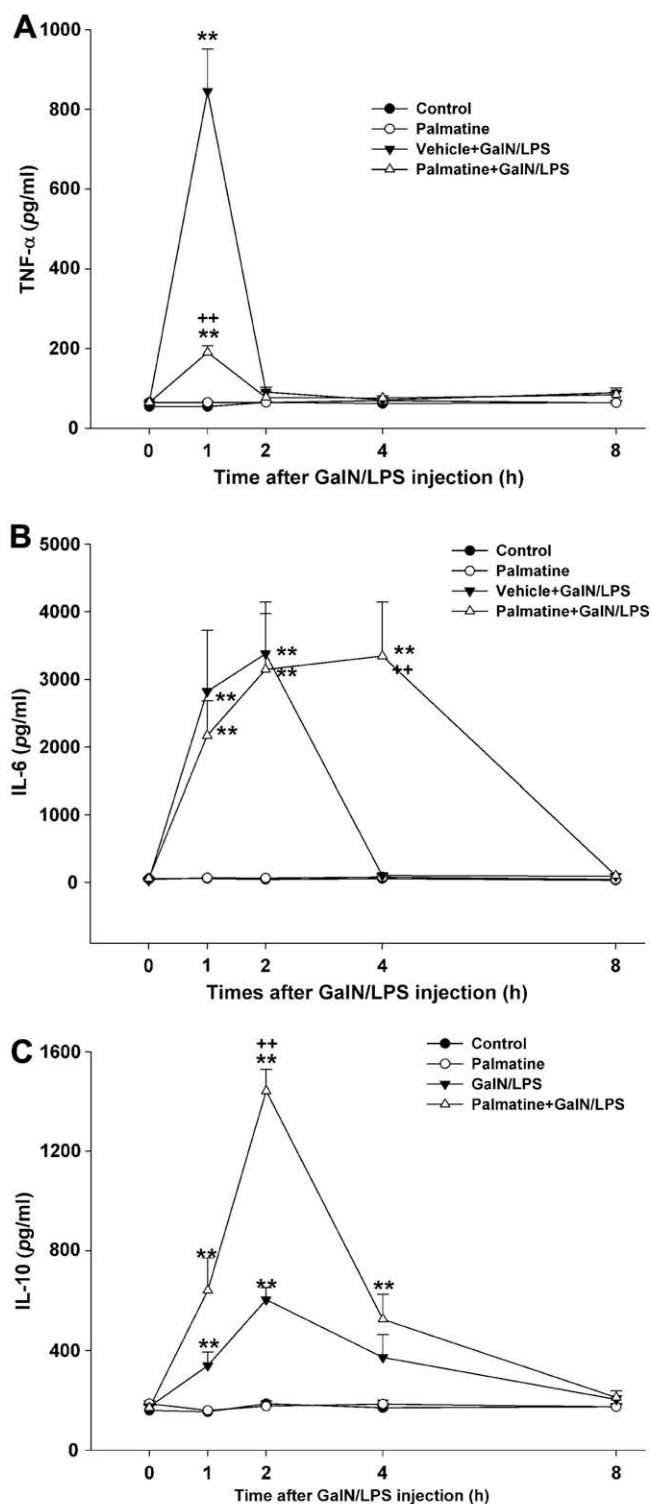
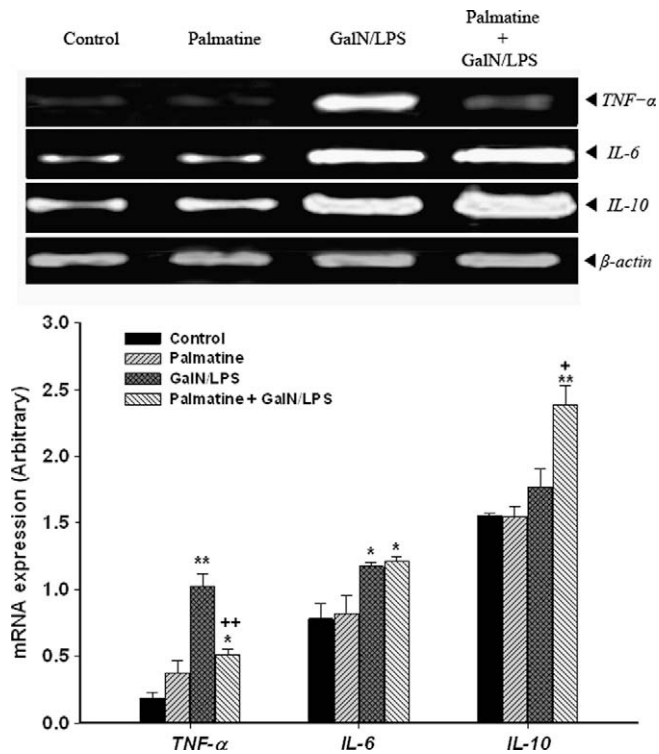


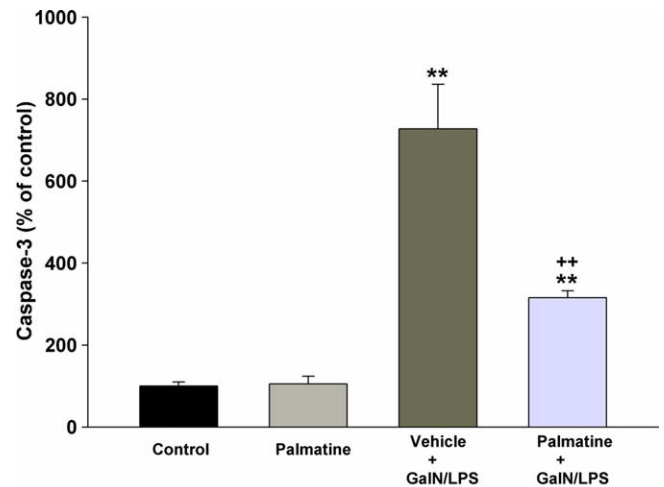
Fig. 3. Effect of palmatine (100 mg/kg) on systemic secretion of TNF- $\alpha$ , IL-6 and IL-10 after GalN/LPS administration. (A) TNF- $\alpha$ , (B) IL-6 (C) IL-10. The results are presented as the mean  $\pm$  S.E.M. of 8 animals per group. \* Denotes significant differences from the control group (*p* < 0.01); \*\* denotes significant differences from the GalN/LPS group (*p* < 0.01). GalN, galactosamine; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

that in the control mice. This increase was attenuated by palmatine. GalN/LPS also increased the hepatic level of IL-6 mRNA expression by approximately 1.5-fold over the control levels. Palmatine did not affect the increase in IL-6 mRNA expression.



**Fig. 4.** Effect of palmatine (100 mg/kg) on *TNF-α*, *IL-6*, and *IL-10* mRNA expression after GalN/LPS administration. The results are presented as the mean  $\pm$  S.E.M. of eight animals per group. \*, \*\* Denotes significant differences from the control group ( $p < 0.05$  and  $p < 0.01$ ); +, ++ denotes significant differences from the GalN/LPS group ( $p < 0.05$  and  $p < 0.01$ ). GalN, galactosamine; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

The hepatic level of *IL-10* in the GalN/LPS group increased approximately 1.13-fold compared with the control level. *IL-10* mRNA expression was augmented by palmatine treatment. Palmatine alone did not affect the levels of *TNF-α*, *IL-6* and *IL-10* mRNA expression.



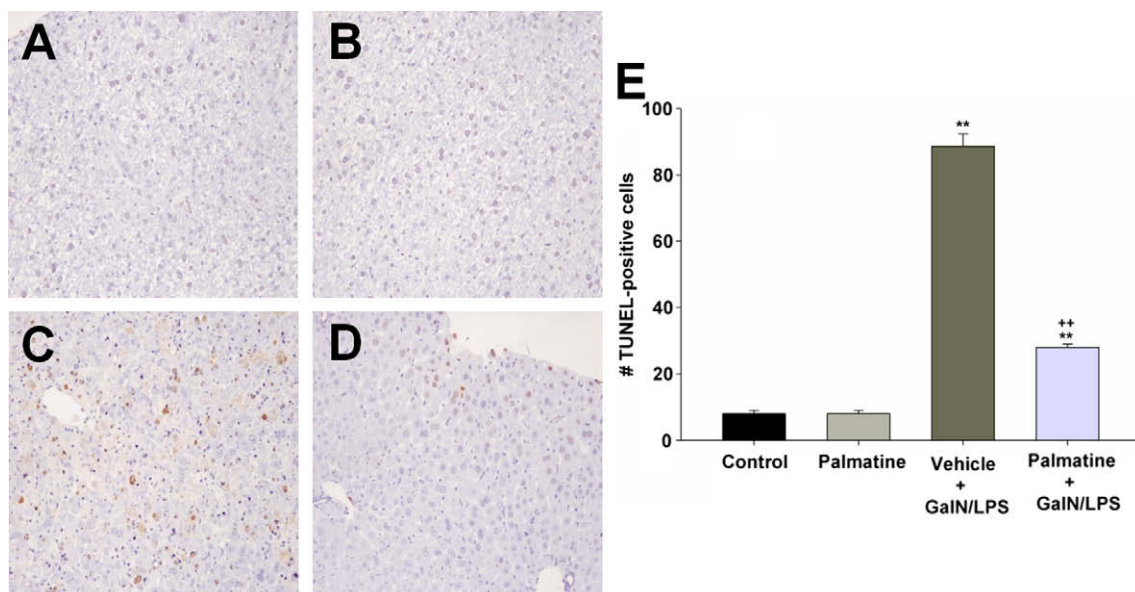
**Fig. 5.** Effect of palmatine (100 mg/kg) on caspase-3 activity after GalN/LPS administration. The results are presented as the mean  $\pm$  S.E.M. of eight animals per group. \*, \*\* Denotes significant differences from the control group ( $p < 0.01$ ); +, ++ denotes significant differences from the GalN/LPS group ( $p < 0.01$ ). GalN, galactosamine; LPS, lipopolysaccharide.

### 3.5. Caspase-3 activity

Caspase-3 plays a central role in the execution phase of cell apoptosis. We examined the caspase-3 activity in the cytosol fraction of liver isolated 8 h after GalN/LPS treatment. As shown in Fig. 5, GalN/LPS significantly increased caspase-3 activity by approximately 4-fold over the control level. However, the increase of caspase-3 activity was attenuated by palmatine treatment.

### 3.6. Apoptotic cells in liver

Apoptotic hepatocytes were detected by TUNEL. The liver was isolated 8 h after GalN/LPS treatment. In the GalN/LPS-treated group, a large number of TUNEL-positive hepatocytes were



**Fig. 6.** Detection of apoptotic hepatocytes in the liver of mice after GalN/LPS injection (original magnification 200 $\times$ ). (A) Control group; (B) palmatine (100 mg/kg)-treated group; (C) GalN/LPS group; (D) palmatine (100 mg/kg) and GalN/LPS-treated group; (E) TUNEL-positive hepatocyte. Numbers are apoptotic cells in randomly chosen 400  $\times$  histological fields. \*, \*\* Denotes significant differences from the control group ( $p < 0.01$ ); +, ++ denotes significant differences from the GalN/LPS group ( $p < 0.01$ ). GalN, galactosamine; LPS, lipopolysaccharide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

observed. However, few TUNEL-positive hepatocytes were observed in livers from palmatine-treated mice (Fig. 6).

#### 4. Discussion

Palmatine, a member of the protoberberine class of isoquinoline alkaloids, is a bioactive herbal ingredient isolated from *C. chinensis*. It has been reported that palmatine might contribute to the protective effects of *Coptidis rhizoma* against oxidative stress, and that it inhibits cellular peroxynitrite generation in renal tubular epithelial cells (Yokozawa et al., 2005). Also, palmatine exhibits hypoglycemic and hypolipidemic effects (Yuan et al., 2006). However, the protective effect of palmatine against acute liver injury has not been studied. This study demonstrated that palmatine treatment confers protection against GalN/LPS-induced fulminant hepatic failure in mice.

Administration of GalN with LPS induces liver damage that closely resembles human viral hepatitis in its morphological and functional features; this method is therefore widely used as experimental liver injury model for elucidating the mechanisms of clinical liver dysfunction and for evaluating the efficacy of hepatoprotective agents (Nakama et al., 2001). In this study, GalN/LPS-treated mice began to die at 6 h and most mice died within 24 h after GalN/LPS injection. However, pretreatment with palmatine before GalN/LPS markedly reduced the mortality. Furthermore, at 8 h after GalN/LPS injection, liver damage, including increases of serum ALT and AST levels, was detected. These increases were attenuated by treatment with palmatine. In histological analysis, palmatine administration significantly attenuated the structural degeneration observed at 8 h after GalN/LPS. These results suggest that palmatine may have potential clinical application in the treatment of liver disease.

ROS play a major role in the onset of hepatic damage in the pathogenesis of GalN/LPS-induced hepatic failure (Xiong et al., 1999). An ROS attack on biological membranes can lead to the oxidative destruction of the polyunsaturated fatty acids in the membrane through lipid peroxidation. Lipid hydroperoxides and their breakdown products are involved in the deactivation and degradation of mitochondrial enzymes and transport proteins (Chen and Yu, 1994). GSH serves as an antioxidant system to counteract the deleterious effects of ROS. Hence, we analyzed the effect of palmatine on GSH and lipid peroxidation in fulminant hepatic failure. In GalN/LPS-treated mice, GSH levels significantly decreased and lipid peroxidation increased compared with control. However, palmatine did not affect these changes. This suggests that anti-oxidative properties may be not essential for hepatoprotection by palmatine in GalN/LPS-induced fulminant hepatic failure.

In GalN/LPS-induced fulminant hepatic failure, TNF- $\alpha$  plays an important role in the inflammatory response (Enomoto et al., 2003). TNF- $\alpha$  can trigger an inflammatory cascade involving the induction of other pro-inflammatory cytokines including IFN- $\gamma$ , IL-1 $\beta$  and IL-6, which are essential for inflammation and consequent liver damage (Tiegs et al., 1989). TNF- $\alpha$  release is closely followed by enhanced secretion of IL-10 as a counter-regulatory cytokine. Studies have demonstrated that IL-10 can protect mice from lethal endotoxemia concomitantly or following LPS challenge (Bean et al., 1993; Howard et al., 1993). In the present study, the serum levels of TNF- $\alpha$  and IL-6 increased in the GalN/LPS-treated group and the increase in TNF- $\alpha$  was attenuated by palmatine. The serum level of IL-10, an anti-inflammatory cytokine, increased in GalN/LPS-treated mice and this increase was augmented by palmatine. Similar to serum levels of TNF- $\alpha$ , IL-6 and IL-10, the levels of TNF- $\alpha$ , IL-6 and IL-10 mRNA expression increased compared with those in control. Palmatine attenuated the increase in TNF- $\alpha$  mRNA and augmented the increase in IL-10 mRNA expression.

These results suggest that palmatine largely regulates the GalN/LPS-induced production of TNF- $\alpha$  and IL-10 at the transcriptional level.

TNF- $\alpha$  induces apoptosis in a variety of cell types. In the liver, TNF- $\alpha$ -induced apoptosis is thought to contribute to viral and alcoholic hepatitis, fulminant hepatitis, and injury from hepatotoxins (Yamada et al., 2008). TNF- $\alpha$  is a key cytokine that induces massive apoptosis of hepatocytes in mice with GalN/LPS-induced fulminant hepatic failure (Arvelo et al., 2002; Nakama et al., 2001). TNF- $\alpha$ -induced apoptosis is mediated by TNF receptors (TNFR-1) (Luedde and Trautwein, 2006). TNFR-1 recruits the adaptor protein Fas-associated protein with death domain (FADD) via homotypic interactions of the death domain. FADD contains a death effector domain that recruits and cleaves procaspase-8, resulting in homodimerization and activation (Malhi et al., 2006). Caspase-8 then triggers the activation of caspase-3, a downstream cysteine proteinase, through multiple apoptosis signal pathways (Hishinuma et al., 1990). In this study, the activity of caspase-3 and the number of apoptotic hepatocytes increased after GalN/LPS injection and these increases were attenuated by palmatine. These results suggest that anti-apoptotic properties may be one mechanism by which palmatine protects against GalN/LPS-induced liver damage.

In conclusion, palmatine attenuates mortality and liver injury in fulminant hepatic failure by inhibiting the inflammatory response and apoptosis. Thus, we propose that palmatine might be useful as a potential therapeutic medication for attenuating the fulminant hepatic failure.

#### Conflict of interest

None declared.

#### Acknowledgements

This work was supported by a grant from the Korea Food and Drug Administration (Studies on the Identification of Efficacy of Biologically Active Components from Oriental Herbal Medicines).

#### References

- Arvelo, M.B., Cooper, J.T., Longo, C., Daniel, S., Grey, S.T., Mahiou, J., Czismadia, E., Abu-Jawdeh, G., Ferran, C., 2002. A20 protects mice from D-galactosamine/lipopolysaccharide acute toxic lethal hepatitis. *Hepatology* 35, 535–543.
- Bean, A.G., Frejberg, R.A., Andrade, S., Menon, S., Zlotnik, A., 1993. Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock. *Infect. Immun.* 61, 4937–4939.
- Bohlinger, I., Leist, M., Gantner, F., Angermuller, S., Tiegs, G., Wendel, A., 1996. DNA fragmentation in mouse organs during endotoxic shock. *Am. J. Pathol.* 149, 1381–1393.
- Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. *Methods Enzymol.* 52, 302–310.
- Chen, J.J., Yu, B.P., 1994. Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic. Biol. Med.* 17, 411–418.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Decker, K., Keppler, D., 1974. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev. Physiol. Biochem. Pharmacol.* 77–106.
- Enomoto, N., Takei, Y., Hirose, M., Kitamura, T., Ikejima, K., Sato, N., 2003. Protective effect of thalidomide on endotoxin-induced liver injury. *Alcohol Clin. Exp. Res.* 27, 25–6S.
- Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207–212.
- Grycová, L., Dostál, J., Marek, R., 2007. Quaternary protoberberine alkaloids. *Phytochemistry* 68, 150–175.
- Hishinuma, I., Nagakawa, J., Hirota, K., Miyamoto, K., Tsukidate, K., Yamanaka, T., Katayama, K., Yamatsu, I., 1990. Involvement of tumor necrosis factor-alpha in development of hepatic injury in galactosamine-sensitized mice. *Hepatology* 12, 1187–1191.
- Howard, M., Muchamuel, T., Andrade, S., Menon, S., 1993. Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med.* 177, 1205–1208.

- Kim, Y.M., Ha, Y.M., Jin, Y.C., Shi, L.Y., Lee, Y.S., Kim, H.J., Seo, H.G., Choi, J.S., Kim, Y.S., Kang, S.S., Lee, J.H., Chang, K.C., 2009. Palmatine from *Coptidis rhizoma* reduces ischemia-reperfusion-mediated acute myocardial injury in the rat. *Food Chem. Toxicol.* 47, 2097–2102.
- Kuhla, A., Eipel, C., Siebert, N., Abshagen, K., Menger, M.D., Vollmar, B., 2008. Hepatocellular apoptosis is mediated by TNF alpha-dependent Fas/FasLigand cytotoxicity in a murine model of acute liver failure. *Apoptosis* 13, 1427–1438.
- Kupeli, E., Kosar, M., Yesilada, E., Husnu, K., Baser, C., 2002. A comparative study on the antiinflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish Berberis species. *Life Sci.* 72, 645–657.
- Lee, H.Y., Kim, C.W., 1997. Studies on the constituents of *Berberis amurensis* Ruprecht. *Kor. J. Pharmacogn.* 28, 257–263.
- Li, B., Shang, J.C., Zhou, Q.X., 2005. Study of total alkaloids from Rhizoma *Coptis chinensis* on experimental gastric ulcers. *Chin. J. Integr. Med.* 11, 217–221.
- Luedde, T., Trautwein, C., 2006. Intracellular survival pathways in the liver. *Liver Int.* 26, 1163–1174.
- Malhi, H., Gores, G.J., Lemasters, J.J., 2006. Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 43, S31–S44.
- Morin, D., Pires, F., Plin, C., Tillement, J.P., 2004. Role of the permeability transition pore in cytochrome C release from mitochondria during ischemia-reperfusion in rat liver. *Biochem. Pharmacol.* 68, 2065–2073.
- Nakama, T., Hirono, S., Moriuchi, A., Hasuiki, S., Nagata, K., Hori, T., Ido, A., Hayashi, K., Tsubouchi, H., 2001. Etoposide prevents apoptosis in mouse liver with D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure resulting in reduction of lethality. *Hepatology* 33, 1441–1450.
- Park, E.K., Rhee, H.I., Jung, H.S., Ju, S.M., Lee, Y.A., Lee, S.H., Hong, S.J., Yang, H.I., Yoo, M.C., Kim, K.S., 2007. Antiinflammatory effects of a combined herbal preparation (RAH13) of *Phellodendron amurense* and *Coptis chinensis* in animal models of inflammation. *Phytother. Res.* 21, 746–750.
- Tiegs, G., Wolter, M., Wendel, A., 1989. Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. *Biochem. Pharmacol.* 38, 627–631.
- Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27, 502–522.
- van Deuren, M., Dofferhoff, A.S., van der Meer, J.W., 1992. Cytokines and the response to infection. *J. Pathol.* 168, 349–356.
- Wang, F., Zhou, H.Y., Cheng, L., Zhao, G., Zhou, J., Fu, L.Y., Yao, W.X., 2003. Effects of palmatine on potassium and calcium currents in isolated rat hepatocytes. *World J. Gastroenterol.* 9, 329–333.
- Xiong, Q., Hase, K., Tezuka, Y., Namba, T., Kadota, S., 1999. Acteoside inhibits apoptosis in D-galactosamine and lipopolysaccharide-induced liver injury. *Life Sci.* 65, 421–430.
- Xu, X., Malavé, A., 2001. Protective effect of berberine on cyclophosphamide-induced haemorrhagic cystitis in rats. *Pharmacol. Toxicol.* 88, 232–237.
- Yamada, I., Goto, T., Takeuchi, S., Ohshima, S., Yoneyama, K., Shibuya, T., Kataoka, E., Segawa, D., Sato, W., Dohmen, T., Anezaki, Y., Ishii, H., Ohnishi, H., 2008. Mao (Ephedra sinica Stapf) protects against D-galactosamine and lipopolysaccharide-induced hepatic failure. *Cytokine* 41, 293–301.
- Yokozawa, T., Satoh, A., Cho, E.J., Kashiwada, Y., Ikeshiro, Y., 2005. Protective role of *Coptidis rhizoma* alkaloids against peroxynitrite-induced damage to renal tubular epithelial cells. *J. Pharm. Pharmacol.* 57, 367–374.
- Yuan, L., Tu, D., Ye, X., Wu, J., 2006. Hypoglycemic and hypocholesterolemic effects of *Coptis chinensis* franch inflorescence. *Plant Foods Hum. Nutr.* 61, 139–144.