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Protective Effects of Methanol Extract and Alisol B 23-acetate of *Alisma orientale* on Acetaminophen-Induced Hepatotoxicity in Rats

Ki Ho Yang[1,2], Seong Hee Choi[3] and Jong Cheol Park[1,4,*]

[1]*Research Institute of Korean Oriental Medicine, Sunchon National University, Suncheon, Jeonnam 540-742, Korea*
[2]*Susung Oriental Medicine Hospital, 4256 Seongnam-dong, Jungwon-gu, Seongnam, Kyunggi-do 462-829, Korea*
[3]*Department of Food Science, Sunmoon University, Asan, Chungnam 336-708, Korea*
[4]*Department of Oriental Medicine Resources, Sunchon National University, Suncheon, Jeonnam 540-742, Korea*

Abstract – Hepatoprotective effects of methanol extract and alisol B 23-acetate of *Alisma orientale* were studied in acetaminophen (APAP)-treated rats. APAP increased hepatic content of lipid peroxide, which was suppressed by methanol extract and alisol B 23-acetate. The liver of rats treated with APAP had higher P-450, aminopyrine N-demethylase and aniline hydroxylase activities than those of normal control rats. The increases in hepatic drug metabolizing enzymes by the *i.p.* injection of APAP were significantly alleviated by the administration of methanol extract or alisol B 23-acetate. The injection of APAP also resulted in a substantial reduction of hepatic glutathione content and glutathione S-transferase activity, and the decreases were partially, but significantly, restrained by the oral administration of methanol extract prior to the *i.p.* injection of APAP. Hepatic activities of glutathione reductase (GR) and γ-glutamylcystein synthetase (γ-GCS) were also decreased significantly in APAP-treated rats. The decreases in hepatic GR and γ-GCS activities by APAP injection were improved partially, but significantly, with administration of methanol extract of *A. orientale*. Treatment with alisol B 23-acetate also improved the hepatic γ-GCS activity significantly, but not GR.
Keywords – Hepatoprotective effect, Alisol B 23-acetate, *Alisma orientale,* Acetaminophen, Lipid peroxide, Hepatic drug metabolizing enzymes

Introduction

Alisma orientale is a perennial marshy plant used as an oriental medicine in Korea. In *Dongeui Bogam* written by Huh, Jun, it is described to be cold in nature, sweet and salty in taste without toxicity, and it is effective in facilitating urination and bringing down heat in bladder (Hur, 1994). In traditional oriental medicine it has been used for removing damp spirit of body, smoothing urination, ceasing diarrhea and easing edema. It is also used for curing unconscious emission in men. It has also been used as a folklore remedy for diabetes and as a diuretic (Park *et al*., 2005).

Several phytochemicals including triterpenoids and sequiterpenoids and hemagglutinating lectin have been isolated from the plant (Matsuda *et al*., 1999; Nakajima *et al*., 1994; Oshima *et al*., 1983; Yoshikawa *et al*., 1993; Yoshikawa *et al*., 1997; Park *et al*., 1995). Alisol compounds isolated from the plant have been found to have hepatoprotective activities against CCl_4 intoxication and hypochoesterolemic effects in mice and rats (Chang *et al*., 1982; Imai *et al*., 1970). Some terpene components of the plant have also been reported to have anti-complementary and anti-allergic activities (Lee *et al*., 2003; Kubo *et al*., 1997).

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP), a widely used analgesic and antipyretic drug, is safe at therapeutic doses, but when given as large overdoses severe liver injury and acute liver failure may occur in experimental animals and human (Thomas, 1993; Cover *et al*., 2006). The metabolic events of toxicity have been studied and are believed to be due to the metabolic conversion of APAP to a highly reactive electrophilic intermediate, *N*-acetyl *p*-benzoquinonimine (NAPQI), which is generated by cytochrome P-450 mediated oxidases (Jaeschke *et al*., 2003; Knight *et al*., 2003). This toxic metabolite is eliminated from hepatocyte by reacting with reduced glutathione (GSH) (Chasseaud, 1979). The relevant decrease in liver GSH is harmful because it is a basic cytosolic antioxidant.

Hundreds of natural resources and plants have been

*Author for correspondence
Tel: +82-61-750-3662; E-mail: jcpark@sunchon.ac.kr

used as medicinal stuffs for curing diseases in oriental medicine or as foodstuffs. Recently much attention has been focused on the medicinal plants as targets for research to develop noble medicines. In the present study, the effects of the methanol extract of *A. orientale* and its major compound, alisol B 23-acetate, on lipid peroxidation and the activities of enzymes involved in drug metabolism and glutathione homeostasis were examined in the liver of APAP-treated rats.

Experimental

Plant material – The aerial part of *A. orientale* was collected from a field in Suncheon, Korea in April, 2004. A voucher specimen (specimen No. NM1104) has been deposited at the Herbarium of the Department of Oriental Medicine Resources at Sunchon National University.

Extraction and isolation of alisol B 23-acetate – The air-dried rhizome of *A. orientale* (15 kg) was extracted 3 times with methanol by refluxing for 4 hrs each time and concentrated *in vacuo*. An aliquot of the methanol extract was partitioned with organic solvents of different polarity to obtain dichloromethane, ethyl acetate, and aqueous fractions. The dichloromethane fraction was subjected to silica gel chromatography (SiO_2: 500 g, column: 6.2 cm × 42.5 cm), using a solvent gradient of CH_2Cl_2-MeOH (20 : 1 → 5 : 1). The effluent of silica gel chromatography was further chromatographed on a Sephadex LH-20 to obtain alisol B 23-acetate (Fig. 1): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.90, 0.94, 0.96, 0.97, 1.07, 1.24, 1.26 (3H each, s), 0.99 (3H, *d*, *J* = 6.36 Hz), 1.64 (1H, *d*, *J* = 10.70 Hz, H-9), 2.00 (3H, s, OAc), 2.49 (1H, *dd*, *J* = 5.69, 13.20 Hz, Ha-12), 2.66 (1H, *d*, *J* = 8.52 Hz, H-24), 3.74 (1H, *ddd*, *J* = 5.73, 10.72, 10.73 Hz, H-11), 4.54 (1H, *ddd*, *J* = 2.71, 8.53, 10.64 Hz, H-23). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 18.38 (C-26), 19.02 (C-29), 19.06 (C-6), 19.09 (C-21), 20.17 ($OCO\underline{C}H_3$), 22.15 (C-18), 22.83 (C-30), 23.68 (C-27), 24.64 (C-19), 26.83 (C-20), 28.15 (C-16), 28.54 (C-28), 29.65 (C-15), 29.94 (C-1), 32.72 (C-22), 33.18 (C-7), 33.50 (C-12), 35.76 (C-2), 35.93 (C-10), 39.72 (C-8), 45.94 (C-4), 47.48 (C-5), 48.98 (C-9), 56.03 (C-14), 57.45 (C-25), 64.08 (C-24), 69.21 (C-11), 70.52 (C-23), 133.17 (C-17), 137.12 (C-13), 169.02 ($O\underline{C}OCH_3$) 219.13 (C-3) (Park *et al*., 2005).

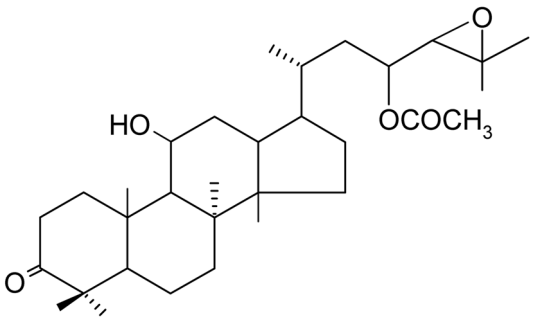

Fig. 1. Structure of alisol B 23-acetate isolated from the rhizome of *Alisma orientale.*

Animals – Male Sprague-Dawley rats (Daehan BioLink, Eumsung, Korea), weighing 200 ± 10 g, were fed *ad libitum* with a commercial standard rat diet based on AIN-93G, and maintained at 20 ±1 °C with a 12 hr light/dark cycle. The animals were cared for under the guidelines for the care and use of laboratory animals established by the Institute of Laboratory Animal Resources, U.S.A.

Animals were orally administered daily for 2 weeks with 250 or 500 mg of the methanol extract of the plant/kg of body weight or with 5, 10 or 20 mg/kg body weight of alisol B 23-acetate that had been isolated from the methanol extract. On the last day of oral treatment of the plant materials, rats were injected *i.p.* with APAP (800 mg/kg) (Choi and Park, 1996). At 24 hr after the injection of APAP, the animals were sacrificed under anesthesia with CO_2. Animals were starved for 24 hr before sacrifice.

Preparation of enzyme sources – The liver, which had been exhaustively perfused with ice-cold 0.9% NaCl, was homogenized with 4 volumes of an ice-cold 0.1 M potassium phosphate buffer, pH 7.5. An aliquot of the homogenate was used for the determination of lipid peroxide and glutathione contents. The remaining homogenate was centrifuged at 600 × g for 10 min, and the resulting supernatant was recentrifuged at 10,000 × g for 20 min. The supernatant was further centrifuged at 105,000 × g for 60 min to obtain the upper fraction as cytoplasm. The pellet was resuspended in the same volume of the 0.1 M potassium phosphate buffer and centrifuged at 105,000 × g for 60 min to obtain the microsomal fraction. The cytoplasmic fraction was used as the enzyme source of glutathione S-transferase, glutathione reductase and γ-glutamylcysteine synthetase, and the microsomal fraction was used to measure the activities of cytochrome P-450, aniline N-demethylase and aniline hydroxylase.

Assay of serum ALT, AST and SDH – As a marker of acute liver damage in animals, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by using a commercial enzyme assay kit AM 101-K (Asan Phamaceutical Co Ltd., Seoul, Korea) based on the method of Reitman and Frankel (1957). Serum sorbitol dehydrogenase (SDH)

was assessed spectrophotometrically by the method of Wiesner *et al.* (1965) using a kit purchased from Sigma chemical Co. (St. Louis, MO, USA).

Measurement of lipid peroxide level – As a marker for lipid peroxidation in the liver, the content of thiobarbituric acid reactive substances (TBARS) was measured by the method of Ohkawa *et al*. (1979). Briefly, a mixture of 0.4 ml of 10% liver homogenate, 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution was heated at 95 ºC for 1 hr. After cooling, 5.0 ml of *n*-butanol-pyridine (15 : 1) was added, and the absorbance of the *n*-butanol-pyridine layer was measured at 532 nm.

GSH content – Reduced glutathione (GSH) content of liver was measured by a colorimetric method (Boyne and Ellman, 1972). A mixture of 0.5 ml of liver homogenate and 0.5 ml of 4% sulfosalicylic acid was centrifuged at 2500 rpm for 10 min. To 0.3 ml of the resulting supernatant 2.7 ml of disulfide reagent was added and absorbance at 412 nm was measured after standing at room temperature for 20 min. GSH levels were calculated using an extinction coefficient of 1.36×10^5 M^{-1} cm^{-1}. Results are expressed in μmol GSH/g tissue.

γ-Glutamylcysteine synthetase activity – Reaction mixture for the determination of γ-glutamylcysteine synthetase consisting of 0.1 M tris HCl (pH 8.0), 8.9 mM L-glutamic acid, 0.94 mM EDTA, 3.2 mM $MgCl_2$, 1.35 mM ATP, 1.0 mM L-α-aminobutyric acid and enzyme (100 - 200 g protein) in a total volume of 1.0 ml was incubated at 37 ºC for 10 min. The reaction was stopped by adding 10% TCA. P*i* in supernatant was measured by absorbance at 600 nm after addition of molybdic acid and aminonaphthol sulfonic acid. Enzyme activity was expressed as nmols of P*i* formed/mg protein/min (Richman and Meister, 1975).

Glutathione reductase activity – Glutathione reductase activity was determined by oxidation of NADPH by measuring the decrease in absorbance at 340 nm (Mize and Langdon, 1962). Reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.5), 0.94 mM EDTA, 4.6 mM oxidized glutathione, 0.16 mM NADPH and enzyme solution containing 400 - 600 mg of protein in a total volume of 3.0 ml. Enzyme activity was expressed as nmols of glutathione reduced/mg protein/min.

Cytochrome P-450 activity – The concentration of cytochrome P-450 was estimated from CO difference spectrum of liver microsome reduced by the addition of dithionite using molar extinction coefficient of 91 mM^{-1} cm^{-1}, based on the method of Omura and Sato (1964). The microsomal suspension (containing 1 mg protein/ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 20% glycerol, 0.5% sodium cholate and 0.4% Triton N-101 was mixed with sodium dithionite after bubbling with CO for 1 min. Absorbance of the mixture was measured at 450 and 490 nm against a blank prepared without CO bubbling. The cytochrome P-450 activity was calculated from the difference between A_{450} and A_{490} using 91 $mM^{-1}cm^{-1}$ of the extinction coefficient.

Aminopyrine N-demethylase activity – The aminopyrine N-demethylase activity was assayed by measuring the production of formaldehyde formed by the demethylation of aminopyrine (Nash, 1953). The reaction mixture consisted of 300 - 400 mg of microsomal protein, a 0.1 M potassiumphosphate buffer (pH 7.5) and 2.0 mM aminopyrine in a total volume of 2.0 ml. The mixture was preincubated for 3 min at 37 ºC, and 0.5 mM NADPH was added to initiate the reaction. The reaction was stopped after 30 min by adding 0.5 ml of 15% $ZnSO_4$ and saturated $Ba(OH)_2$, and the resulting mixture was cooled to room temperature. After centrifuging at 1,000 × g for 10 min, 1 ml of the supernatant was mixed with 5 ml of the Nash reagent. The tubes were then capped and heated at 60 ºC for 30 min. After cooling in tap water, the absorbance was read at 415 nm against a water blank. The activity was expressed as nmol of formaldehyde/mg of protein/min.

Aniline hydroxylase activity – The aniline hydroxylase activity was measured by determining the *p*-aminophenol formation from aniline (Bidlack and Lowery, 1982). The reaction mixture consisted of 300 - 400 mg of microsomal protein, a 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 150 mM KCl, 1.0 mM aniline-HCl and 0.5 mM NADPH in a total volume of 2.0 ml. After incubation for 20 min at 37 ºC, the reaction was terminated by adding 0.5 ml of 20% trichloroacetic acid. The mixture was centrifuged at 1,000 × g for 10 min, and 1 ml of the supernatant was added to 1 ml of 0.2 N NaOH containing 2% phenol. After mixing, 1 ml of 10% Na_2CO_3 was then added. The mixture was kept for 20 min at room temperature, before the absorbance was read at 640 nm against a water blank. The activity was expressed as nmol of *p*-aminophenol/mg protein/min.

Glutathione S-transferase activity – The glutathione S-transferase activity was assayed by measuring the conjugated glutathione 2,4-dinitrobenzene formation from 1-chloro-2,4-dinitrobenzene (Habig *et al*., 1974). The reaction mixture consisted of 100 μl of the cytosol fraction, a 0.1 M potassium phosphate buffer (pH 6.5), 1.0 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione in a total volume of 3.5 ml. The mixture was incubated at

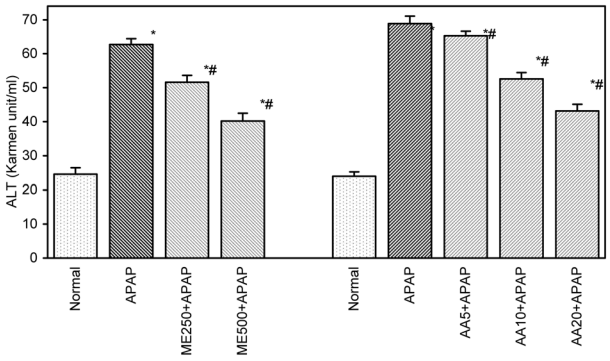

Fig. 2. Changes in serum alanine aminotransferase (ALT) level of the acetaminophen-overdosed rats pretreated with methanol extract or alisol B 23-acetate of *Alisma orientale*. Rats were administered orally with the methanol extract (ME) at a dose of 250 or 500 mg/kg of body weight or alisol B 23-acetate (AA) at a dose of 5, 10 or 20 mg/kg of body weight daily for 2 weeks, and acetaminophen (APAP), at a dose of 800 mg/kg body weight, was *i.p.* injected on the last day of oral treatment of plant materials. Each bar represents the mean ± S.D. of 8 animals. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

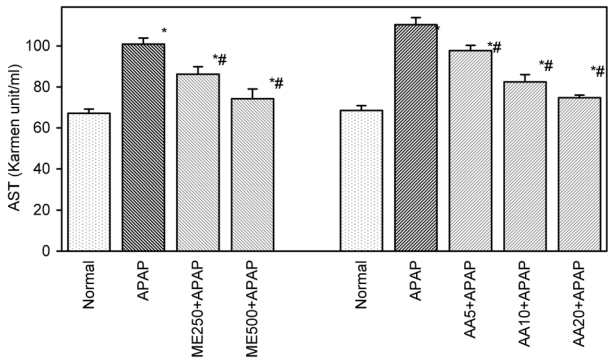

Fig. 3. Changes in serum aspartate aminotransferase (AST) level of the acetaminophen-overdosed rats pretreated with methanol extract or alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

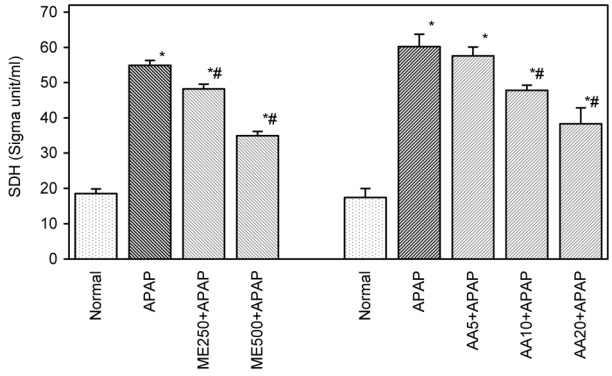

Fig. 4. Changes in serum sorbitol dehydrogenase (SDH) level of the acetaminophen-overdosed rats pretreated with methanol extract or alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

25 °C for 2 min. The spectrometric change per unit time at the maximal absorbance wavelength (340 nm) was calculated with a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. The activity was expressed as nmol of 1,2-dinitro-4-nitrobenzene/mg of protein/min

Protein assay – Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical analysis – Statistical analyses were performed with the statistical package SAS (SAS Institute, Inc., Cary, NC). The statistical differences among the experimental groups were evaluated by analysis of variance, and the differences between groups were determined by Student's *t*-test. Differences were considered significant at $P \leq 0.05$. Data are expressed as mean ± SD of 8 animals.

Results

Treatment of male SD rats with 800 mg/kg APAP induced liver injury as indicated by the rise in plasma ALT, AST and SDH activities (Fig. 2,3,4). The rise in hepatic enzymes in plasma was attenuated significantly by oral administration of methanol extract of the aerial part of *A. orientale* or alisol B 23-acetate isolated from the plant for 2 weeks prior to the *i.p.* injection of APAP. The hepatoprotective effects of the methanol extract and alisol B 23-acetate were dose dependent at the doses from 250 to 500 and from 5 to 20 mg/kg, respectively.

The effects of the methanol extract of *A. orientale* or alisol B 23-acetate isolated from the plant on the lipid peroxide content were measured in the liver of the rats treated with APAP (Fig. 5). The *i.p.* injection of APAP resulted in increase of the hepatic lipid peroxide content to 43.3 and 48.9 nmol of TBARS/g from the normal values of 18.4 and 18.7 nmol, respectively. However, the increase in TBARS content was suppressed to 30.5 and 28.5 nmol/g by treating the animal with the methanol extract (500 mg/kg) and alisol B 23-acetate (20 mg/kg), respectively, prior to the *i.p.* injection of APAP.

Changes in the activities of hepatic cytochrome P-450, aminopyrine N-demethylase and aniline hydroxylase are shown in Fig. 6-8. The liver of rats treated with APAP had higher P-450, aminopyrine N-demethylase and aniline hydroxylase activities than those of normal control rats. The increases in hepatic drug metabolizing enzymes by *i.p.* injection of APAP were significantly alleviated by

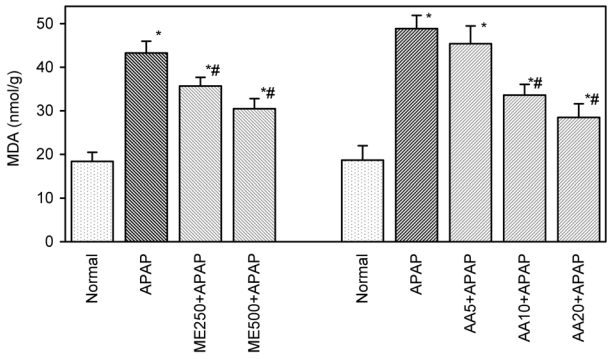

Fig. 5. Effects of the methanol extract and alisol B 23-acetate of *Alisma orientale* on the hepatic lipid peroxide contents in acetaminophen-treated rats. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

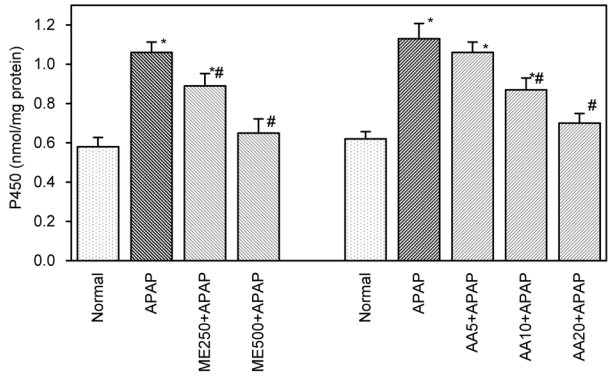

Fig. 6. Effects of the methanol extract or alisol B 23-acetate of *Alisma orientale* on the hepatic cytochrome P450 activity in acetaminophen-treated rats. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

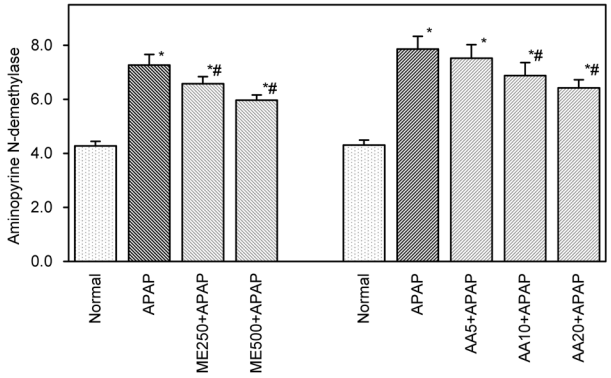

Fig. 7. Aminopyrine N-demethylase activity in the liver of acetaminophen-intoxicated rats treated with the methanol extract or alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. Unit: nmol formaldehyde/mg of protein/min. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

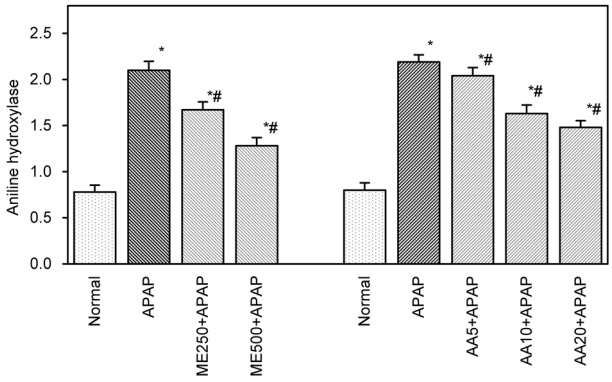

Fig. 8. Aniline hydroxylase activity in the liver of acetaminophen-intoxicated rats treated with the methanol extract or alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. Unit: nmols of *p*-aminophenol/mg protein/min. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

Table 1. Hepatic glutathione S-transferase activity in the liver of acetaminophen-overdosed rats pretreated with the methanol extract or alisol B 23-acetate of *Alisma orientale*

Treatment†	Glutathione S-transferase (nmol/mg protein/min)
Normal	201.9 ± 30.2
APAP	93.5 ± 21.3*
APAP + ME250	160.3 ± 18.5*#
APAP + ME500	178.6 ± 15.8#
Normal	203.6 ± 30.5
APAP	98.8 ± 12.7*
APAP + AA5	110.3 ± 13.8*
APAP + AA10	118.6 ± 18.9*
APAP + AA20	121.2 ± 21.3*

†Treatments and abbreviations are as in Fig. 2. Data are mean ± S.D. (n = 8). Unit: nmols of conjugated 1,2-dinitro-4-nitrobenzen/mg protein/min. *Significantly different from normal group ($p < 0.05$). #Significantly different from APAP group ($p < 0.05$).

the administration of methanol extract of *A. orientale* or alisol B 23-acetate isolated from the plant.

The injection of APAP resulted in a substantial reduction of glutathione S-transferase activity to 93.5 and 98.8 from the normal value of 201.9 and 203.6 nmol/mg of protein/min, respectively (Table 1). The decrease in hepatic glutathione S-transferase activity by APAP was partially, but significantly, restrained by the oral administration of methanol extract of *A. orientale* prior to the *i.p.* injection of APAP. However, the treatment with alisol B 23-acetate which had been isolated from the plant did not show any effect on the enzyme activity.

Table 2 shows the changes in hepatic glutathione content when APAP was injected to the rats treated with the methanol extract or alisol B 23-acetate isolated from

Table 2. Hepatic glutathione contents of acetaminophen-overdosed rats pretreated with the methanol extract or alisol B 23-acetate of *Alisma orientale*

Treatment[†]	Glutathione (μmol/g tissue)
Normal	5.27 ± 0.59
APAP	2.17 ± 0.21[*]
APAP + ME250	2.98 ± 0.35[*#]
APAP + ME500	3.89 ± 0.33[#]
Normal	5.43 ± 0.48
APAP	2.10 ± 0.19[*]
APAP + AA5	2.27 ± 0.33[*]
APAP + AA10	2.36 ± 0.40[*]
APAP + AA20	2.43 ± 0.27[*]

[†]Treatments and abbreviations are as in Fig. 2. Data are mean ± S.D. (n = 8). [*]Significantly different from normal group ($p < 0.05$). [#]Significantly different from APAP group ($p < 0.05$).

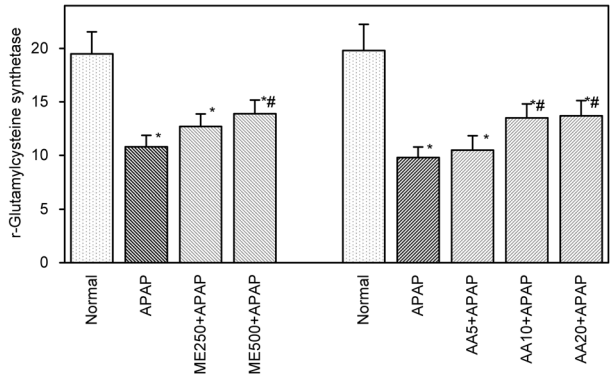

Fig. 9. γ-Glutamylcysteine synthetase activity in the liver of acetaminophen intoxicated rats treated with the methanol extract and alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. Unit: nmols of P*i* formed /mg protein/min. [*]Significantly different from normal group ($p < 0.05$). [#]Significantly different from APAP group ($p < 0.05$).

A. orientale. The glutathione content was lowered by more than half in liver of rats treated with APAP in comparison with the normal control rats. The reduction in hepatic glutathione content by APAP injection was prevented partially, but significantly, by administration of methanol extract of *A. orientale*. The protective effect of the methanol extract was in a dose dependent manner at the dose range from 100 to 250 mg/kg body weight. However, the treatment with alisol B 23-acetate which had been isolated from the methanol extract did not show any effect on the hepatic level of glutathione.

Hepatic activities of γ-glutamylcysteine synthetase (γ-GCS) (Fig. 9) and glutathione reductase (GR) (Fig. 10) were significantly decreased in APAP-treated rats. The decreases in hepatic γ-GCS and GR activities by APAP injection were improved partially, but significantly, by

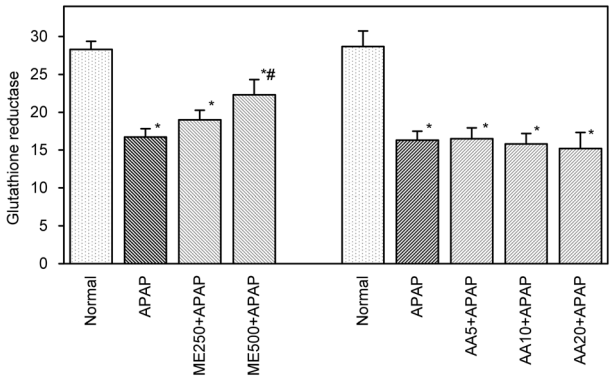

Fig. 10. Glutathione reductase activity in the liver of acetaminophen intoxicated rats treated with the methanol extract and alisol B 23-acetate of *Alisma orientale*. Treatments and abbreviations are as in Fig. 2. Each bar represents the mean ± S.D. of 8 animals. Unit: nmols of glutathione reduced/mg protein/min. [*]Significantly different from normal group ($p < 0.05$). [#]Significantly different from APAP group ($p < 0.05$).

administration of methanol extract of *A. orientale*. The improvements of hepatic γ-GCS and GR activities by the methanol extract were in dose dependent at the dose range from 100 to 250 mg/kg body weight. Treatment with alisol B 23-acetate which had been isolated from the methanol extract also improved the hepatic γ-GCS activity significantly, but not GR. The decrease in GR activity by APAP treatment was not affected by the administration of alisol B 23-acetate.

Discussion

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is a widely used analgesic, but when it is ingested in high doses it causes fatal liver injury and acute liver failure in experimental animals and human (Thomas, 1993; Cover *et al*., 2006). In the present study, increased serum levels of ALT, AST and SDH in rats treated with APAP implicate a deterioration of the hepatic functions due to the toxic effects of the drug. The increases in tissue TBARS contents accompanied by a significant reduction in glutathione level also indicate APAP-induced oxidative liver injury.

The metabolic events of toxicity induced by APAP overdose have been studied and are believed to be associated with the metabolic conversion of APAP to a highly reactive electrophilic intermediate, *N*-acetyl *p*-benzoquinonimine (NAPQI), which is generated by microsomal P-450 mediated reactions (Jaeschke *et al*., 2003; Knight *et al*., 2003). The bioconversion to reactive intermediates capable of reacting to critical cellular macromolecules and destroying cellular function is a

common toxicological pathway shared by a wide variety of xenobiotics (Cohen e*t al*., 1997; Pumford and Halmes, 1997).

Detoxification of xenobiotics occurs in 2 phases, phase I and phase II. Among the phase I enzymes, cytochrome P-450 has been studied most, being divided into type I and type II based on the drug binding type and site existence (Imai *et al*., 1996). The phase I enzymes include those catalyzing aminopyrine and aniline as a substrate, which are associated with the production of free radicals in the microsomal system. The activities of microsomal aminopyrine N-demethylase and aniline hydroxylase in the liver were significantly increased in the present study by APAP injection. The increase in the activities of phase I enzymes by the APAP injection was prevented significantly by pretreatment of the animals with the methanol extract or alisol B 23-acetate of *A. orientale*.

At therapeutic doses, the reactive electrophilic intermediate NAPQI is normally eliminated from hepatocyte by conjugation with hepatic glutathione (GSH) (Chasseaud, 1979). The enzyme responsible for the conjugation reaction is a phase II enzyme, glutathione S-transferase. An overdose of APAP resulted in a significant suppression of the enzyme. The suppression, however, was not occurred in rats administered with methanol extract of *A. orientale*.

When the generation of reactive metabolite exceeds the availability of hepatic GSH for conjugation, the metabolite binds covalently to cellular macromolecules being associated with liver injury induced by this drug (Mitchell *et al*., 1973a; Mitchell *et al*., 1973b; Moore *et al*., 1985).

GSH, a tripeptide containing a sulfhydryl group, is present in relatively high concentrations as reduced form in most mammalian tissues. It is the main component of endogenous non-protein sulfhydryl pool, and is known to be a major low molecular weight scavenger of free radicals in the cytoplasm (Ross, 1988; Shaw *et al*., 1990). This endogenous tripeptide plays a major protective role against toxicity of xenobiotics and normal oxidative products of cellular metabolism. It acts as a nucleophilic scavenger for numerous toxic chemicals or their metabolites, as in the case of NAPQI, the reactive cytochrome P450-reactive metabolite formed by APAP, which becomes toxic when GSH is depleted by an overdose of APAP. Glutathione in this capacity binds to NAPQI as a suicide substrate and in the process detoxifies it. It takes the place of cellular protein thiol groups which would otherwise be covalently modified. When all GSH has been spent, NAPQI begins to react with the cellular proteins, killing the cells in the process.

The GSH also acts as a substrate in the reduction of hydroperoxides catalyzed by glutathione peroxidase. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. The selenium-containing enzyme protects tissues from oxidative damage by removing peroxides through oxidation of glutathione, which is a part of the body's antioxidant protection. Thus a significant depletion of GSH from normal level may depreciate the defense process of an organism leading to severe cell injury and death (Reed *et al*., 1986). Following toxic doses of APAP, the cell depletes of GSH, a cellular protectant against reactive oxygen species (ROS), thus leading to oxidative stress. Since GSH inhibits lipid peroxidation in liver mitochondria and microsomes, the decrease in GSH content in the liver could well give rise to an increase in malondialdehyde formation, thus explaining the enhanced peroxidation in liver of APAP overdosed rats.

In many cell types, depletion of GSH results in oxidative stress and increase cytotoxicity associated with prooxidant drug and chemical exposures. The role of GSH as a protectant against drug-induced oxidative damage has been studied (Reiter *et al*., 2002; Sener *et al*., 2003). Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind a variety of electrophilic radicals and metabolites that may damage the cells (Szabo *et al*., 1992). NAPQI reacts rapidly with GSH, leading to a substantial depletion of the sulfhydryl antioxidant (Nelson and Bruschi, 2003). At sufficiently high doses of APAP, GSH becomes depleted, leaving NAPQI free to bind to essential hepatocellular proteins as APAP-adducts and causes hepatic necrosis. The toxicity of APAP is, therefore, a function of the amount of NAPQI formed and the availability of hepatic GSH for detoxification of this toxic metabolite (Banerjee *et al*., 1998; Dimova *et al*., 2000).

Hepatic lipid peroxide as measured by TBARS in the present study was elevated significantly by an over dose of APAP (Fig. 5), and the increase in TBARS content was accompanied with a decrease in GSH content (Table 2). GSH plays an important role in defending against lipid peroxidation. Since GSH inhibits lipid peroxidation in liver mitochondria and microsomes, the decrease in GSH content in the liver could well give rise to an increase in malondialdehyde formation, thus explaining the enhanced peroxidation in liver of APAP overdosed rats.

The factors attributable to decrease in tissue GSH level involve limited GSH synthesis, enhanced GSH utilization, and limited intracellular reduction of oxidized glutathione

(GSSG) into GSH. Intracellular synthesis of GSH occurs in two-step process including γ-glutamylcysteine synthetase and glutathione synthetase. γ-Glutamylcysteine synthetase, the rate-limiting enzyme, catalyzes the formation of a peptide linkage between the g-carboxyl group of glutamic acid and the amino group of cysteine, and subsequently the addition of glycine is catalyzed by glutathione synthetase. GSH is also formed by reduction of GSSG, which is catalyzed by glutathione reductase widely distributed in mammalian tissues, especially the liver, kidney, heart and erythrocytes. Thus, the depletion of hepatic GSH in the present study would be due, at least in part, to decrease in the activities of γ-glutamylcysteine synthetase (Fig. 9) and glutathione reductase (Fig. 10).

On the other hand, the administration of methanol extract of *A. orientale* to rats before treating APAP resulted in a significant prevention of hepatic lipid peroxidation (Fig. 5) along with a high level of GSH content (Table 2). Since GSH protects liver from lipid peroxidation, the high level of GSH content could give rise to a decrease in TBARS formation in liver methanol extract-treated rats. The methanol extract also increased the activities of γ-glutamylcysteine synthetase (Fig. 9) and glutathione reductase (Fig. 10). Thus the relatively high content of hepatic GSH in rats treated with methanol extract of *A. orientale* was supposed to be resulted from both enhanced synthesis of GSH and increased reduction of GSSG.

The hepatic lipid peroxidation by APAP was also prevented significantly by treating the animals with alisol B 23-acetate of *A. orientale* prior to the intoxication with APAP (Fig. 5). However, the treatment of alisol B 23-acetate did not show any effect on the hepatic level of glutathione. The protective effect of alisol B 23-acetate against APAP-induced lipid peroxidation would be through some mechanisms other than glutathione.

In conclusion, the findings of the present study elucidate that the methanol extract and alisol B 23-acetate of *A. orientale*, as antioxidant agents, protect liver against APAP-induced oxidative injury, and thus deserve consideration as potential agents in controlling the drug-induced hepatotoxicity.

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