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Protective Effect of Water Extract of *Fraxinus Rhynchophylla* Leaves on Acetaminophen-induced Nephrotoxicity in Mice and Its Phenolic Compounds

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Abstract The protective effect of the water extract of *Fraxinus rhynchophylla* leaves (FLE) was determined using an animal model of acetaminophen (AAP)-induced nephrotoxicity. The BALB/c male mice used in this study were divided into 3 groups; the normal, AAP-administered, and FLE-pretreated AAP groups. A single dose of AAP induced necrosis of renal tubules and congestion along with edema to a remarkable degree as observed by hematoxylin and eosin stain, and also increased the numbers of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive renal tubular epithelial cells. Blood urea nitrogen and plasma creatinine levels were determined to be significantly higher in the AAP group than in the normal group. However, FLE pretreatment resulted in an attenuation of renal tubule necrosis. Regeneration and dilatation of renal tubules were noted, and the numbers of TUNEL-positive cells were reduced in the FLE-pretreated groups. In an effort to detect the bioactive compounds exerting protective effects in FLE, the analysis of phenolic compounds via gas chromatography/mass spectrometry (GC/MS) were performed, and identified esculetin and esculin. The present study indicates that these compounds may exert a protective effect against AAP-induced nephrotoxicity.

Keywords: *Fraxinus rhynchophylla* leaf, acetaminophen, renal pathology, phenolic compound, gas chromatography/mass spectrometry (GC/MS)

Introduction

Plants are valuable and readily accessible sources of natural products. Many studies indicate that among most plants, the leaves have become received much interest as a tea material because of its desirable taste and refreshing effects (1). In addition, plant leaves contain rich chlorophyll, carotene, ascorbic acid, and phenolic compounds, and their water extract have been used for many medicinal purposes (2). Some of these extracts have been shown to exert anti-obesity, anti-carcinogenic effects, and defenses against aging-associated degenerative diseases (3, 4).

Fraxinus rhynchophylla hance is widely cultivated throughout Asia, Europe, and North America, and these trees have often been planted in urban areas. The bark, a Chinese herbal medicine, has been proven to be effective in the treatment of diarrhea and dysentery due to intense heat, and is especially effective for dysentery accompanied by blood stool and for lung disease with fever, cough, and dyspnea (5). In Korea, the bark has also been traditionally used as an agent for the treatment of gout and neuralgia, and as an antipyretic agent (6). The bark has recently attracted a considerable attention due to anti-diarrheal properties in ion transport in rat intestinal epithelia (7).

Acetaminophen (AAP), *n*-acetyl-*p*-aminophenol, is an analgesic and antipyretic drug in common usage. AAP-induced toxicity in rodents is an animal model that is extensively used to assess the hepatic and renal protective activities of novel compounds (8-10). It has been proven to be very safe at therapeutic dosages. AAP overdoses in humans are fairly common, and induce fulminating renal necrosis, both in experimental animals and human beings (8-10). Tubular cell necrosis is a characteristic feature both of acute renal failure and chronic renal disease (10). The precise mechanism by which AAP induces renal injury is believed to involve the metabolic conversion of AAP to its reactive intermediate, *n*-acetyl *p*-benzoquinone imine. This metabolite causes rapid depletion to intracellular glutathione (GSH) (10, 11), resulting in cell necrosis. The breakdown of the GSH-dependent antioxidant defensive system augments the intracellular flux of oxygen-free radicals, and initiates apoptosis (10, 12). Tubular cell loss is observed in cases in which cell death predominates over mitosis. Apoptosis is an active form of cell death, which provides an opportunity for therapeutic intervention (8, 10-12).

The bark of *F. rhynchophylla* is known to contain coumarin and its ester components, which has been identified as esculin, esculetin, fraxetin, fraxin, and stylosin by a variety of analysis techniques (6, 13-15). However, regardless of the widespread use of bark in traditional medical practices, no reports have yet been made available regarding the bioactive components and biological activity of those components in with regard to the possible renal protective effects evidenced by these leaves. We have found that the tree leaves protect against paracetamol-induced damages by blocking oxidative stress, as well as CYP2E1-mediated paracetamol bio-activation (8, 9).

In an attempt to develop new natural materials from *F.*

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rhynchophylla leaves, the renal protective effects of a water extract of *F. rhynchophylla* leaves were investigated, using an AAP-induced animal model. To determine bioactive components existed in the water extract of *F. rhynchophylla* leaves possessing renal protective activity, the identification of phenolic compounds was performed using gas chromatography/mass spectrometry (GC/MS).

Materials and Methods

Chemicals Three standard analyzes (esculetin and esculin) and acetaminophen were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Analysis for blood urine nitrogen and creatinine performed with commercial kits (Asan Pharm Co., Seoul, Korea) and apoptag apoptosis detection kit (Intergen Co., Purchase, NY, USA) were used in this experiment. Pyridine, 1,1,1,3,3,3-hexamethyl-disilazane and trifluoroacetic acid for derivatization were obtained from Sigma Chemical Co. All other chemicals were of GC-MS analytical reagent grade.

Preparation of the *F. rhynchophylla* leaves extract The *F. rhynchophylla* leaves were collected from the Department of Forestry of Daegu-si, Korea, in June 2005. A voucher specimen was deposited in the Herbarium of the College of Medicine, Yeungnam University (FR leaf 201). The leaves were extracted using 50 times of hot water for 20 min at 90°C. The extract was then filtered and adjusted to pH 2.0 with 1.0 N HCl, then centrifuged, resulting in the separation of a cloudy precipitate. The supernatant successively partitioned with ethyl acetate (EtOAc) at a solvent-to-water phase ratio of 1:1 (EtOAc fr.). Total phenolic compound of the ethyl acetate fraction were quantitatively determined using Prussian blue reagent (16), and the content was 28.52±0.63%. The aquous layer was then partitioned further with *n*-butanol (BtOH), in a procedure similar to that utilized in the formation of EtOAc fr., as shown Fig. 1 (BtOH fr.). The 2 fractions were combined, dehydrated with anhydrous sodium sulfate, filtered, and then evaporated to dryness under nitrogen. The dried fraction extract (FLE) was stored at 4°C until use.

Animal care and induction of renal toxicity by AAP Specific pathogen-free BALB/c mice (male, 6-8 weeks of age) were purchased from the Hyochang Science Co. (Daejeon, Korea), and were housed in a temperature-controlled room under a constant 12-hr/12-hr light/dark photocycle. The mice were subjected to a 1-week adaptation period and were randomly divided into 3 groups of mice for the experiment: the normal, AAP-administered (AAP), and FLE-pretreated AAP (FLE+AAP) groups. Experimental kidney damage was induced via injection of AAP at a dosage of 800 mg/kg BW weight, a dosage which was sufficient to cause the development of evident kidney injury, but was not sufficiently severe to cause death.

The FLE and AAP were dissolved in warm PBS (+Tween 20). The FLE was administered orally at a dose of 200 mg/kg once daily for 7 consecutive days. On the 7th day, 3 hr after FLE treatment, mice were injected intraperitoneally with AAP solution. Animals in the normal group were administered only vehicle. All animals were fed on standard mouse chow (Sam Yang Food Co., Wonju, Ganwon, Korea), and were given free access to water. The care of the animals was consistent with the guidelines of the National Institutes of Health regarding the care and use of laboratory animals. Nephrotoxicity was evaluated 24 hr after AAP-induction, and the mice were subjected to a fast 15 hr prior to sacrifice. The animals were anesthetized using sodium pentothal (40 mg/kg).

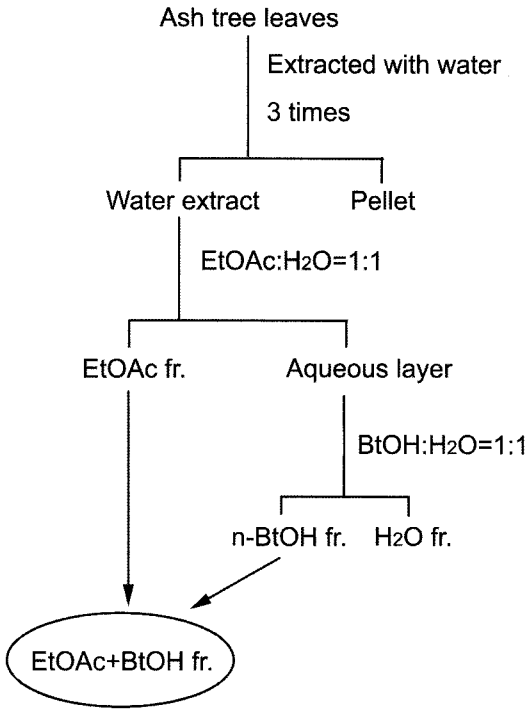

Fig. 1. Schematic diagram of extraction for the identification of phenolic compounds in the water extracts of *Fraxinus rhynchophylla* leaves. EtOAc, ethyl acetate; BtOH, butanol.

Biochemical assay Blood was collected from the abdominal aorta of each mouse, using heparinized syringes. The blood was then centrifuged for 5 min at 5,000×g at 4°C in order to separate the plasma. Then, in order to diagnose impaired kidney function, blood urine nitrogen (BUN) and plasma creatinine levels were analyzed using commercial kits (Asan Pharm Co., Seoul, Korea).

Histological examination Three sections of the kidney of each animal were used for the histological examination. Fresh kidney tissue was fixed for 24 hr in 4% paraformaldehyde prior to routine histological processing. The kidney was subsequently dehydrated using a series of ethanol solutions from 75 to 100% prior to embedding in paraffin wax. Five μm thick cross-sections were then cut and stained with hematoxylin and eosin (H&E) stain. The assessment of the extent of the injury of the kidney was performed under the light microscope by the observer that was unaware of the treatment of protocols.

TUNEL assay Detection of apoptotic cell death was

performed with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL). An Apoptag apoptosis detection kit (Intergen Co., Purchase, NY, USA) was utilized in accordance with the manufacturer's introductions. Total number of TUNEL-positive cells within kidney cortex and medulla was counted on three kidney sections of each animal and the average number of TUNEL-positive cells/mm^2 was calculated. For each treatment group the results were expressed as the average number of TUNEL-positive cells/mm^2 ± standard errors (SE).

Trimethylsilated (TMS) derivatization of phenolic compounds TMS derivatization was conducted in accordance with the method described by Kim and Park (17). Briefly, to perform the TMS derivatives of phenolic compounds, the dried EtOAc + BtOH fr. was added to 0.3 mL pyridine and 0.4 mL of hexamethyldisilazane plus 0.1 mL of trifluoroacetic acid. The mixture was then vigorously shaken, maintained for 5 min at 70°C, and dried at 40°C *in vacuo* using a rotary evaporator.

GC/MS The TMS derivatives of the phenolic compounds were separated on a GC-MS (GCT-TOF, Micromass Co., Manchester, UK) equipped with a flame ionization detector. An HP-5 capillary column (0.2 mm × 0.25 μm × 25 m, nonpolar, Hewlett Packard, Ramsey, MN, USA) was employed in this procedure. The head pressure of the carrier gas (helium) was 10 psi. The injector and detector temperatures were 230 and 250°C, respectively. The oven temperature was programmed to operate from 150 to 300°C, maintaining the temperature at 150°C for 5 min, and then increasing it from 150 to 300°C at 7°C/min, and finally maintained for 10 min at 300°C. The split ratio was 1:50 and the volume of each of the injected samples was 1.0 μL. Mass spectrum acquisition was conducted in a mass range from 40 to 800 *m/z*. ionization using 70 eV electrons. Mass spectrometry and comparison of gas chromatographic retention times were utilized for the identification of the individual phenolic compounds isolated using the GC. Isolated compounds were identified via comparisons of the retention times with those of the authentic compounds, coupled with the spectral data acquired from the Willey and National Institute Standards and Technology (NIST) libraries. Each determination was conducted in duplicate.

Table 1. Effect of extract of *Fraxinus rhynchophylla* leaves on blood urea nitrogen and creatinine of AAP-induced renal injury in mice

Group[1)]	BUN	Creatinine
	Unit/L	
Normal	37.41±0.88[2)]	26.81±0.50
AAP	116.46±2.30*	97.72±1.64*
FLE+AAP	76.94±2.44**	58.12±1.20**

[1)]Normal, Normal+PBS; AAP, AAP+PBS; FLE+AAP, FLE-pretreated group once daily for 7 days prior to AAP administration. Renal toxicity was determined 24 hr after AAP-induction.
[2)]Means±SE of 10 mice, *$p<0.05$ vs. Normal, **$p<0.05$ vs. AAP

Statistical analysis Results are expressed as the means±SE. For multiple comparisons, one-way analysis of variance (ANOVA) was employed. When ANOVA revealed significant differences, post-hoc analyses were conducted using the Newman-Keuls multiple range test, with the SPSS software package.

Results and Discussion

BUN and plasma creatinine levels BUN and plasma creatinine levels were used for the assessment of impaired renal function in the AAP-induced mice. Mice induced with AAP alone developed significant renal injury, as was evident from a significant ($p<0.05$) increase in the plasma levels of BUN and creatinine when compared to the normal group (Table 1). Pretreatment of the mice with FLE at doses of 200 mg/kg effected a marked reduction of the elevated BUN and plasma creatinine levels (Table 1).

Histological findings On light microscopic examination, the normal group showed a morphologic characteristic of renal parenchyma with well-designated glomeruli and tubules (Fig. 2A). According to the histopathologic

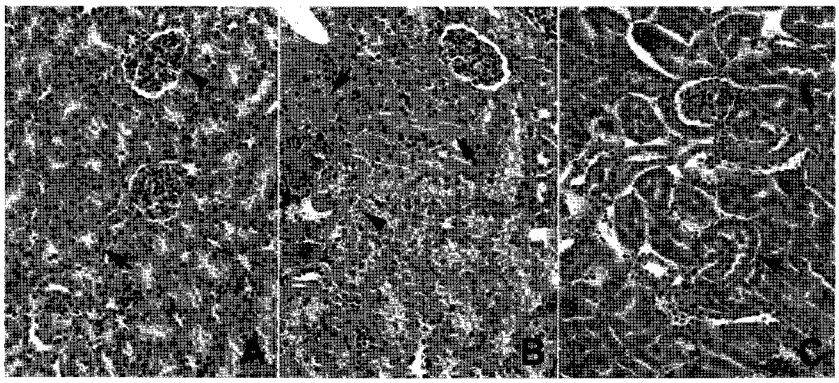

Fig. 2. Representative H&E stained kidney sections. (A) Normal group. The renal tubules (arrow) and glomeruli (arrowhead) are normal (H&E stain, 200×). (B) AAP group. The necrosis (arrows) of renal tubules and congestion (arrowhead) are present (H&E stain, 200×). (C) FLE+AAP group. The regeneration and dilatation of renal tubules (arrows) are present. Renal tubule necrosis is significantly attenuated (H&E stain, 200×).

findings of the AAP group, necrosis and degeneration of renal tubules was observed (Fig. 2B). Congestion, interstitial edema, and inflammation were also detected in the AAP group. The partial decrease of the degenerative changes observed in the FLE pretreatment group. Regeneration and mild dilatation of renal tubules were observed in the groups pretreated with FLE prior to the injection of AAP. The extent to which tubular necrosis, congestion, and inflammatory cell infiltration in the FLE-pretreated group was significantly less than those were observed in the AAP alone group (Fig. 2C).

TUNEL assay On TUNEL staining, only few TUNEL-positive cells were observed in the normal group (Fig. 3A). Large areas of TUNEL-positive renal cells were detected at the brush border of proximal convoluted tubules in the AAP group (Fig. 3I-B), but the pretreatment of FLE strongly decreased the numbers of TUNEL-positive tubular epithelial cells A shown in Fig. 3II, the numbers of TUNEL-positive cells/mm^2 in normal, AAP, and FLE+AAP group were 0.8±0.13, 22.5±5.62, and 6.5± 2.58, respectively. The TUNEL positive cells of the kidney treated by AAP shows a significantly increased number of TUNEL positive cells (p<0.05).

The identification of phenolic compounds in *F. rhynchophylla* leaves via GC-MS analysis A total ion chromatogram of the phenolic compounds from ash leaf water extracts is presented in Fig. 4. As the results indicate, the respective peaks were separated quite well. Two phenolic compounds were simultaneously isolated as TMS derivatives after purification, and were identified via the present method, using the Wiley and NIST libraries. The 2 principal phenolic compounds existed in the extract were identified as esculetin (t_R; 24.61 min) and esculin (t_R; 40.39 min), respectively. On the other hand, most of peaks, with the exception of those reflective of the 2 phenolic compounds, were identified as glucose, fructose, and sucrose or inositol, as confirmed by the mass spectra and gas chromatographs of authentic standards. At this time, the contents of esculetin and esculin in FLE were 229.4±3.49 and 137.1±10.21 mg/100 g, respectively. Figure 5 shows the mass spectra and structure information for the TMS derivatives of the 2 coumarins; esculetin (6, 7-dihydroxycoumarin) evidenced base peaks at the [M-15]$^+$ ion, a peak typical of the TMS derivatives of phenolic compounds, and esculin (6,7-dihydroxy 6-glucoside coumarin) evidenced a base peak at the *m/z* 217 ion, respectively.

The *F. rhynchophylla*, a Chinese medicinal plant, has been commonly used for the treatment of diarrhea and dysentery due to fever (5). The observed pharmacological activities showed that the bark is able to inhibit the growth of dysentery bacillus and *Staphylococcus*, and also showed its antitussive and expectorant functions (15). Thus far, plant bark has been widely studied with regard to its pharmacological actions and possible uses, but little remains known regarding the qualities of the plant leaves. We have recently conducted an investigation into the antioxidant activities of *F. rhynchophylla* leaves in order to determine their relevant properties (8, 9).

In our results, histological findings indicated that the administration of AAP caused renal tubular necrosis,

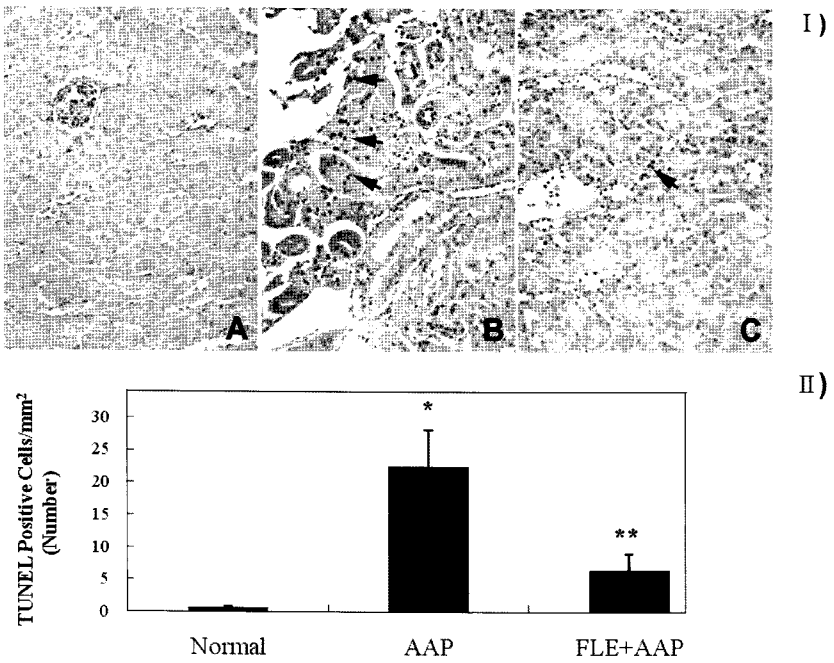

Fig. 3. Histological appearance of kidney sections stained via TUNEL assay (I). (A) Normal group. No TUNEL-positive cells are present (TUNEL staining, 200×). (B) AAP group. Increased numbers of TUNEL-positive tubular epithelial cells (arrows) are present (TUNEL staining, 200×). (C) FLE+AAP group. The number of TUNEL-positive tubular epithelial cells (arrow) is reduced (TUNEL staining, 200×). TUNEL-positive tubular epithelial cells of all groups (II). AAP group showed a significantly increased number of TUNEL-positive cells. Means±SE of 10 mice, $^*p$<0.05 vs. normal, $^{**}p$<0.05 vs. AAP.

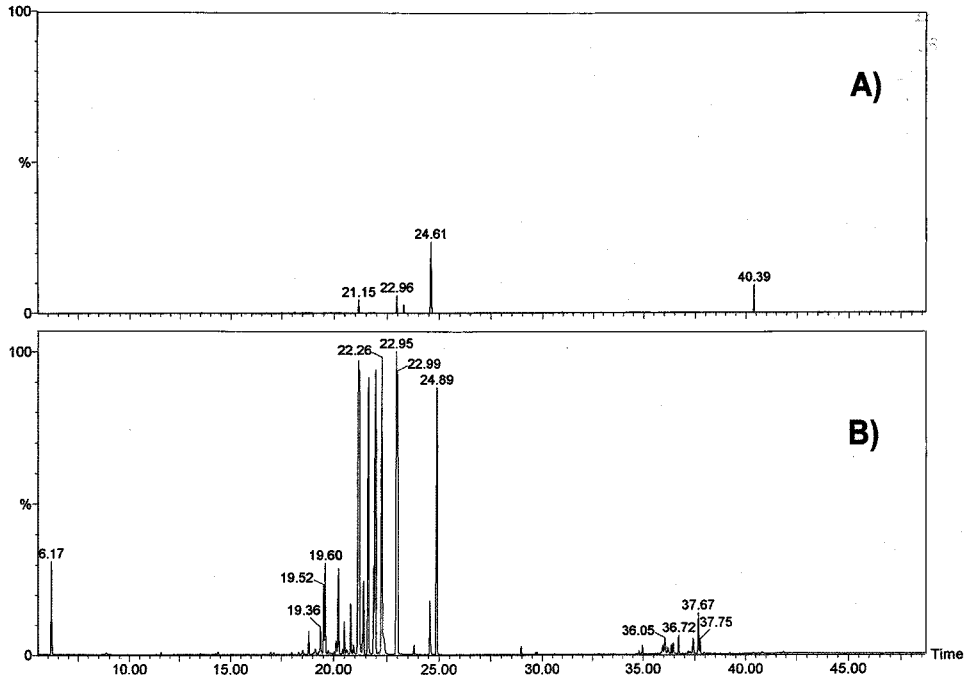

Fig. 4. Total ion chromatograms of the TMS derivatives obtained from EtOAc+BtOH fr. (A) and water extract (B) obtained from ash leaves. A shows total ion chromatogram for determination of esculetin and esculin in EtOAc+BtOH fractioned from *Fraxinus rhynchophylla* leaf (water) extract. The esculetin and esculin were detected at around 24.6 and 40.4 min in this system and confirmed by GC/MS system, respectively.

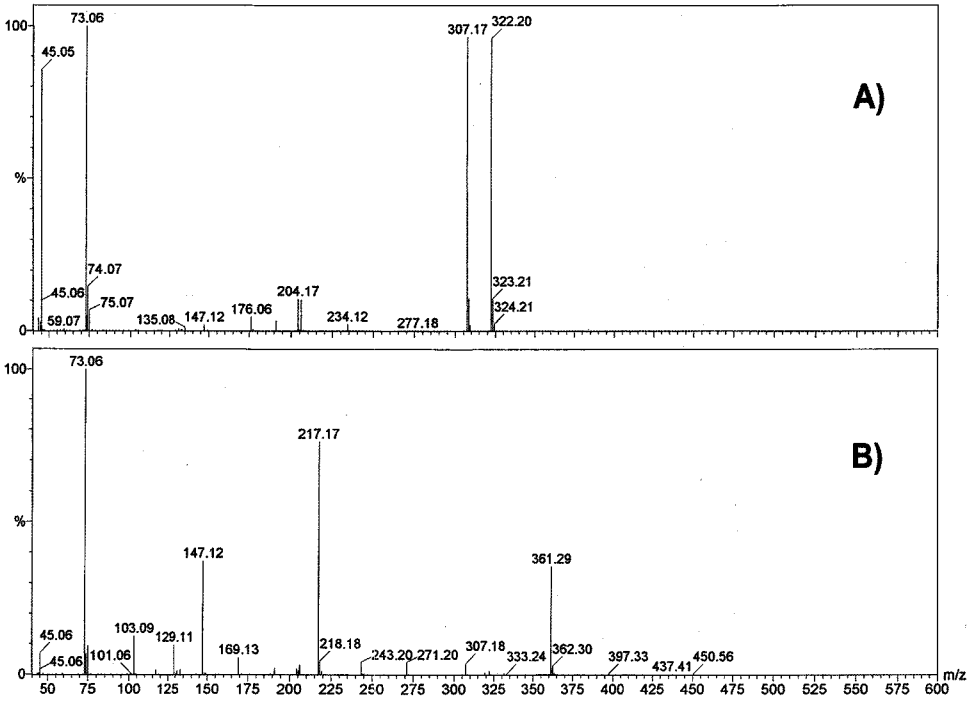

Fig. 5. Mass spectra and structure information for the TMS derivatives of the compounds separated from EtOAc+BtOH fr. of *Fraxinus rhynchophylla* leaf water extract. A) esculetin, B) esculin.

degeneration, congestion, and inflammation. However, the observed renal damage was alleviated significantly via pretreatment with FLE prior to AAP administration, and AAP-intoxificated changes such as tubular necrosis were not observed in FLE-pretreated mice. The partial decrease of the degenerative changes observed in the FLE pretreatment group prior to drug treatment indicates that FLE prevents damage to the kidneys of AAP-induced mice. Histological findings of TUNEL staining showed that FLE pretreatment significantly attenuated the number of

TUNEL-positive renal cells when compared to that of the AAP group. The results supports that FLE provides a protective effect against acute renal damage such as the necrosis and apoptosis induced by AAP. The result of biochemical analysis, which revealed a significant alleviation of the BUN and plasma creatinine levels elevated by FLE pretreatment, is generally consistent with the histological findings.

In order to analysis the active components of FLE showing a protective effect against AAP-induced nephrotoxicity, a rapid, accurate, and simultaneous analytical method for the identification of phenolic compounds using GC/MS was applied in this study (17). According to the result of our preliminary experiments, the ethyl acetate soluble fraction showed the strongest electron-donating, and free radical-scavenging activities among its several polar solvent fractions obtained from the water extract of *F. rhynchophylla* leaves (data not shown).

In our results, the 2 steps of fractionation by EtOAc and BtOH led to the isolation of 2 phenolic compounds: esculetin and esculin. In general, these phenolic compounds are as coumarin derivatives, which have been reported multiple biological activities, including the inhibition of xanthine oxidase activity, antioxidant activity, antitumor activity, and an inhibitory effect on the growth of human breast cancer cells (13, 18, 19). Pharmacological experiment shows that esculin can prolong the hypnotic effects of hexobarbital in mice, and that esculetin can exert antihistaminic effects and relax the smooth muscles of guinea pig'trachea *in vitro* (15). Also, the analgesic effect of esculetin is stronger than that of aspirin (15). Esculetin has been shown to inhibit the oxidative damage induced by *t*-butyl hydroxyl peroxide, as well as 5'-lipoxygenase and leukotriene biosynthesis (20, 21).

Therefore, the presence of these compounds (esculetin and esculin) in *F. rhynchophylla* leaves may explain their protective effects against the AAP-induced renal toxicity observed in this study, and supports the traditional use of these plant leaves against kidney damage. Further investigations are necessary to establish the protective mechanism of the relevant phenolic compounds isolated from the plant leaves in a model of AAP-mediated renal injury.

Acknowledgments

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