


Protective effects of *Gastrodia elata Blume* on acetaminophen-induced liver and kidney toxicity in rats

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Abstract An overdose of acetaminophen (AAP) causes hepatic and renal toxicity. This study examined the protective effects of *Gastrodia elata Blume* (GEB) on hepatic and renal injury induced by AAP. Rats were orally administered distilled water or GEB for 14 days and injected with AAP 1 h after the oral last administration; control rats were administered water without AAP injection. All rats were sacrificed 24 h after AAP injection. The GEB pretreatment group showed decreased necrosis and the expression of pro-inflammatory cytokines in the liver and kidney. TUNEL-positive cells and oxidative stress marker, such as malondialdehyde, were decreased. However, antioxidant enzymes, such as glutathione and superoxide dismutase, were increased. The expression of CYP2E1 and *N*-acetyl-beta-D-glucosaminidase was decreased in the GEB pretreatment group. This study shows that GEB prevents AAP-induced liver and kidney injury.

Keywords Acetaminophen · *Gastrodia elata Blume* · Liver · Kidney · Rats

Introduction

Acetaminophen (AAP) is one of the most widely used non-steroidal anti-inflammatory drugs (Abdel-Hafez et al., 2017). An overdose of AAP causes both hepatic and renal toxicity. Although renal toxicity is rarely observed compared to hepatotoxicity, it may lead to isolated organ damage or fatal multisystem organ failure (Mazer and Perrone, 2008). Acute kidney injury induced by AAP overdose is caused by circulatory failure and is associated with hepatic failure (Inoue et al., 2017). Acute liver and kidney injuries are caused by the overexpression of cytochrome P450s. Cytochrome P450 2E1 (CYP2E1) metabolizes AAP to *N*-acetyl p-benzoquinoneimine (NAPQI) in the liver (Xu et al., 2017).

Gastrodia elata Blume (GEB, tian ma) is used as a traditional herb in many Asian countries for the treatment of many diseases, such as depression, epilepsy, obesity, asthma, and inflammation (Chen et al., 2016; Jang et al., 2010). In previous studies, GEB decreased lipid peroxide levels and was found to have free radical-scavenging ability in rats (Yu et al., 2005). GEB showed improved cognitive and learning abilities in mice (Mishra et al., 2011). The GEB have phenolic compounds (Kim et al., 2007) and there are currently more than 81 compounds of GEB. p-Hydroxybenzyl alcohol (HBA) is considered to be the main active component of GEB (Wang et al., 2016), and HBA is a typical pleiotropic agent and is known to have a great influence on cellular mechanisms. It is used widely as an anti-convulsant, analgesic, and sedative for vertigo, paralysis, epilepsy, and tetanus (Xu and Guo, 2000). In addition, many investigators have used in vitro and in vivo experiments to demonstrate the potential neuroprotective properties of HBAs for nerve injury. The HBA protected the hippocampus from gerbils after transient

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cerebral ischemic brain ischemia and HBA blocks excitotoxicity by increasing GABA transaminase (Kim et al., 2007). HBAs enhance learning and facilitate memory consolidation and discovery. Also, HBA has anti-inflammatory action, which is thought to be due to inhibition of nitric oxide production (Lim et al., 2007).

In this study, we investigated whether pretreatment with GEB extract protects against AAP-induced hepatic and kidney injury in rats and examined the underlying mechanisms.

Materials and methods

Preparation of the GEB extract

An aqueous extract of GEB was kindly provided by MJ Health Foods Co. (Muju, Jeollabukdo, Korea). Briefly, roots of GEB were subjected to hot air-drying. Dried roots of GEB were cut and extracted with 10 volumes of distilled water at 110°C for more than 20 h. After removing the insoluble portion by filtration twice (with 25- and 5- μ m diameter cartridges), the filtrate was concentrated under vacuum, lyophilized, and powdered. The dried GEB was sterilized by adding distilled water and concentrated under reduced pressure.

Animals

Six-week-old male Sprague–Dawley rats (Orientbio Co, Gyunggi-do, Korea), weighing 180–220 g, were housed under standard conditions (12-h light/day cycle with $22 \pm 2^\circ\text{C}$ temperature and $50 \pm 10\%$ humidity) and were provided free access to commercial food (Purina Inc., Gyunggi-do, Korea) and water. Water and food intake was measured twice a week for 14 days. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Eulji University.

Experimental design

After 7 days of adaptation, rats were randomized into the following 3 groups (9 rats per group): (1) a group of rats administered distilled water (10 mL/kg) for 14 days (control group), (2) a group of rats administered distilled water (10 mL/kg) for 14 days and injected with AAP (AAP group), and (3) a group of rats administered GEB (10 mL/kg) for 14 days and injected with AAP (GEB group). All rats were orally administered distilled water or GEB daily. AAP (1 g/kg) was intraperitoneally injected 1 h after the last distilled water or GEB administration. AAP was injected at a volume of 20 mL/kg body weight. All animals were autopsied 24 h after AAP injection. Body weights of

rats were measured during the experiment, and organ weights were measured at the time of autopsy. The left lobe of the liver and the left kidney were fixed for the observation of histological changes, immunohistochemistry (IHC), and TUNEL assay. The remaining portions of the liver and the right kidney were frozen for the measurement of oxidative stress markers and western blot analysis.

Serum biochemistry

During sacrifice, blood samples were collected from rats under isoflurane anesthesia (Hana Pharm. Co., Hwasung, Korea). All blood samples were centrifuged at $1000 \times g$ and 4°C for 15 min using clotting-activated serum tubes. Aspartate aminotransferase (AST), alanine aminotransaminase (ALT), blood urea nitrogen (BUN), and creatinine levels were measured in the serum.

Observation of histological changes

The liver and kidney were routinely processed, embedded in paraffin, and sectioned at 4 μ m thickness. These sections were deparaffinized, rehydrated, and stained with HE or PAS for microscopic examination. All sections were evaluated by microscopic examination. The liver and kidney sections were scored according to scoring method (Aycaan et al., 2015; Onyekwere et al., 2015).

IHC

For IHC, Sects. (4 μ m) were mounted on silane-coated glass slides (Matsunami, Japan). After deparaffinization, the slides were incubated with 0.1% trypsin and blocked by H_2O_2 in methanol for 30 min at room temperature. Then, the slides were incubated first with normal goat serum, and then overnight at 4°C with primary antibodies, TNF- α (diluted 1:200, Abcam Inc., Cambridge, UK), IL-1 β (diluted 1:200, Abcam Inc.), and nitrotyrosine (diluted 1:200, Abcam Inc.). Further, the slides were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and avidin–biotin complex (Vector Laboratories). After washing, the slides were visualized using 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA).

TUNEL assay

The TUNEL assay was used to assess DNA damage. Apoptosis was detected by the TUNEL assay using an in situ cell death detection kit (Roche, Indianapolis, IN, USA). TUNEL-positive cells are expressed as a percentage of the total number of cells.

Measurement of oxidative stress markers

Liver and kidney tissues (100 mg) from all rats were homogenized and centrifuged, and the supernatants were obtained according to the manufacturer's instructions. Malondialdehyde (MDA) content, total glutathione (GSH) content, and total superoxide dismutase (SOD) activity level in each sample were measured in duplicate with commercial kits (Cell Biolabs, San Diego, CA, USA; STA-312,330 and Cayman, Michigan, USA, 706002).

Western blot analysis

For western blot analysis, equal amounts of protein (50 µg) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with 5% skim milk solution for 1 h and then washed in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). They were probed with the following primary antibodies: anti-CYP2E1 (diluted 1:2500, Abcam Inc.), anti-*N*-acetyl-beta-D-glucosaminidase (NAG) (diluted 1:1000, Abcam Inc.) and β-actin (diluted 1:10,000, Abcam Inc.) at 4°C for 16 h. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Pierce, Rockford, IL, USA). Immunoreactivity was detected using film.

Statistical analyses

The significance of the differences in mean values among the experimental groups was determined using one-way ANOVA, followed by least significant difference multiple comparison test. The level of statistical significance was set at $p < 0.05$ and $p < 0.01$. SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA) was used to calculate probability values. All the results in this study are expressed as mean ± standard error of mean (SEM).

Results and discussion

General observation

In all AAP treated groups, relative organ weights were higher than that in the control group. AAP overdose induced cell damage, such as swelling and AAP accumulation in organ, that increased organ weights. There was no significant difference between organ weights in the AAP and GEB groups. During administration, body weights steadily increased, and there was no significant difference among the groups (data not shown).

Serum biochemistry of GEB

AST, ALT, BUN, and creatinine levels were higher in the AAP group than in the GEB group (Fig. 1). High levels of AST and ALT in the serum indicate cellular leakage, cell membrane damage, and the loss of integrity in the liver (Drotman and Lawhorn, 1978). In this study, elevated serum ALT and AST levels in the AAP group verify liver damage due to AAP. The accumulation of BUN and creatinine in the serum indicate defective renal functioning (Reshi et al., 2017). These levels were lower in the GEB group than in the AAP group. Therefore, the administration of GEB reduces cell damage and the extent of cellular leaks.

Histological observation

HE-stained liver tissues in the control group showed normally structured hepatocytes and sinusoids, which branched out of the central vein. However, hepatocytes in the AAP group showed necrosis around the central vein, congestion, the infiltration of inflammatory cells, and the swelling of hepatocytes. Tissues in the GEB group exhibited decreased cell damage, such as cell swelling, congestion, and necrosis near the central vein. Kidney tissues in the control group showed normally structured glomerulus and proximal tubules. In the AAP group, proximal tubular epithelial detachment, necrosis, degeneration, the infiltration of inflammatory cells, and the dilation of tubules were observed; however, these effects were decreased in the GEB group.

The liver and kidney tissues were scored following specific scoring methods (Aycan et al., 2015; Onyekwere et al., 2015). By these methods, we scored the severity of necrosis, inflammation, and hepatocyte ballooning in liver and the loss of brush border of tubular cells in the kidney. These scores were the highest in the AAP group. However, they were significantly lower in the GEB group than in the AAP group.

PAS-stained liver tissues in the control group showed brush borders of normal proximal tubules. In the AAP group, the basement membrane was thinner in the glomerulus and proximal tubules than in the control group. However, the basement membrane in the glomerulus around the site of necrosis in the GEB group was similar to that in the control group (Fig. 2). Moreover, the necrosis and swelling of the glomerulus and proximal tubules was reduced in the GEB group.

To investigate inflammation markers, such as IL-1β and TNF-α, which are pro-inflammatory cytokines, IHC was performed. IL-1β- and TNF-α-positive cells focally appeared in the central venous region of the liver tissues and in the proximal tubules of the kidney tissues in the AAP group. These cells were higher in the AAP group than

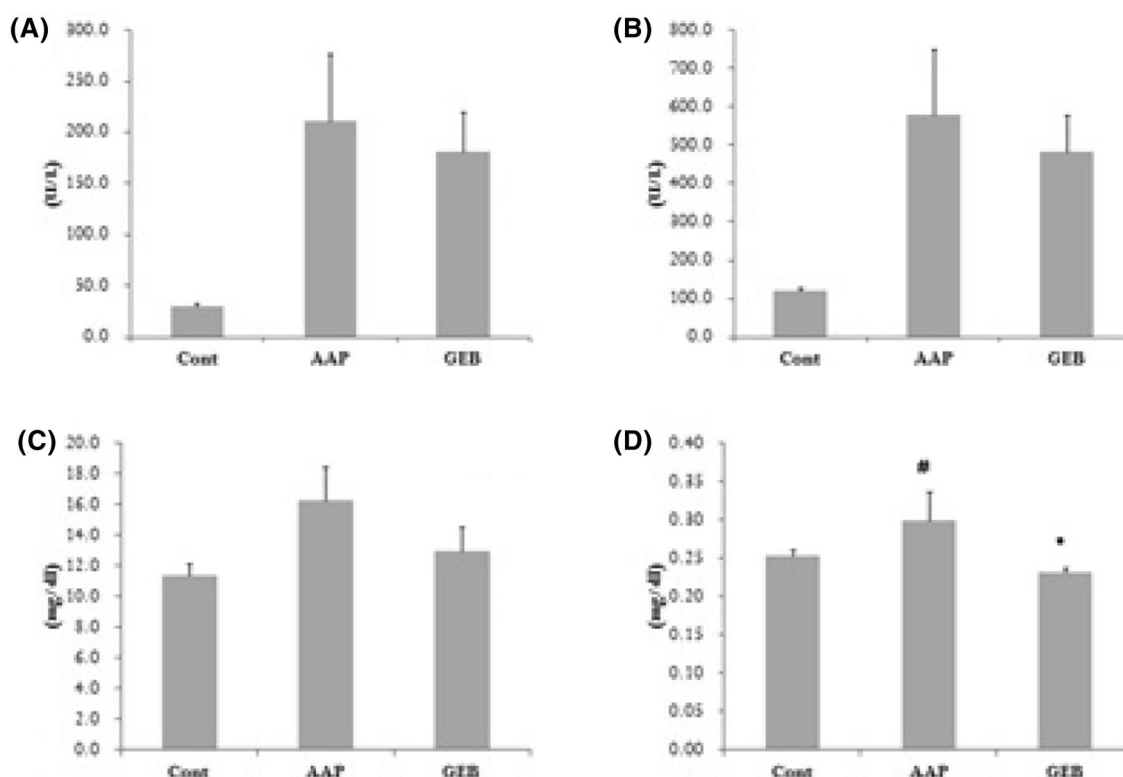


Fig. 1 Acetaminophen (AAP)-induced liver and kidney injury significantly increases serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels. Reduced ALT, AST, BUN, and creatinine levels were

observed in the *Gastrodia elata Blume* (GEB) group compared with those in the AAP group. (A) ALT, (B) AST, (C) BUN, (D) creatinine. The data are presented as mean \pm standard error of mean (SEM). # Versus control group ($p < 0.05$), * versus AAP group ($p < 0.05$)

in the control group; however, the expression of these pro-inflammatory cytokines was significantly lower in the GEB group than in the AAP group. Nitrotyrosine, a metabolite of peroxynitrite, was also quantified by IHC to confirm the production of peroxynitrite. Nitrotyrosine in the AAP group was expressed in the central venous region and proximal tubules, and it significantly decreased in the GEB group (Fig. 3).

The number of TUNEL-positive cells was higher in the AAP group than in the control group. However, it was significantly lower in the GEB group than in the AAP group in the liver and kidney (Fig. 4).

Hepatotoxicity and nephrotoxicity occur due to AAP overdose by a complex sequence of events (Hinson et al., 2010). In most cases, nephrotoxicity is followed by hepatotoxicity (Canayakin et al., 2016). The liver is the major organ with metabolic functions and can be intoxicated by drug exposure (Parameshappa et al., 2012). In the histopathological analysis, centrilobular necrosis, vacuolization, and degeneration in liver tissues were found. The centrilobular zone contains abundant CYP2E1, an enzyme involved in the generation of free radicals that cause hepatocyte damage by covalent bonding (Li et al., 2017). A similar pattern was found for the expression of IL-1 β , TNF- α , and nitrotyrosine in this study. After AAP

injection, the expression of nitrotyrosine and pro-inflammatory cytokines, such as IL-1 β and TNF- α , increased in the vicinity of the central vein. The expression of nitrotyrosine near the central vein has been confirmed in other studies (Cho et al., 2016). Other studies have demonstrated an increase in pro-inflammatory cytokine levels following AAP injection (Ghosh et al., 2010). Pretreatment with GEB reduced this inflammation.

The kidney is one of the organs related to the detoxification process. Several drugs, chemicals, and heavy metals exert toxic effects on the kidney by changing its structure and function (Parameshappa et al., 2012; Priyamvada et al., 2010). AAP overdose causes acute renal failure, whereas chronic exposure to AAP can cause chronic kidney failure (Fored et al., 2001). These toxic effects might be mediated by AAP metabolism by CYP2E1 that produces a high concentration of NAPQI (Posadas et al., 2010). In this study, the control group showed normally structured glomerulus and proximal tubules. In the AAP group, cell damage, such as proximal tubular epithelial detachment, necrosis, degeneration, inter-tubular cell infiltration, the interstitial infiltration of inflammatory cells, the atrophy and dilation of tubules, resulted in the damage of the basement membranes of the glomerulus and proximal tubules. Pretreatment with GEB attenuated cellular damage and the basement membranes in

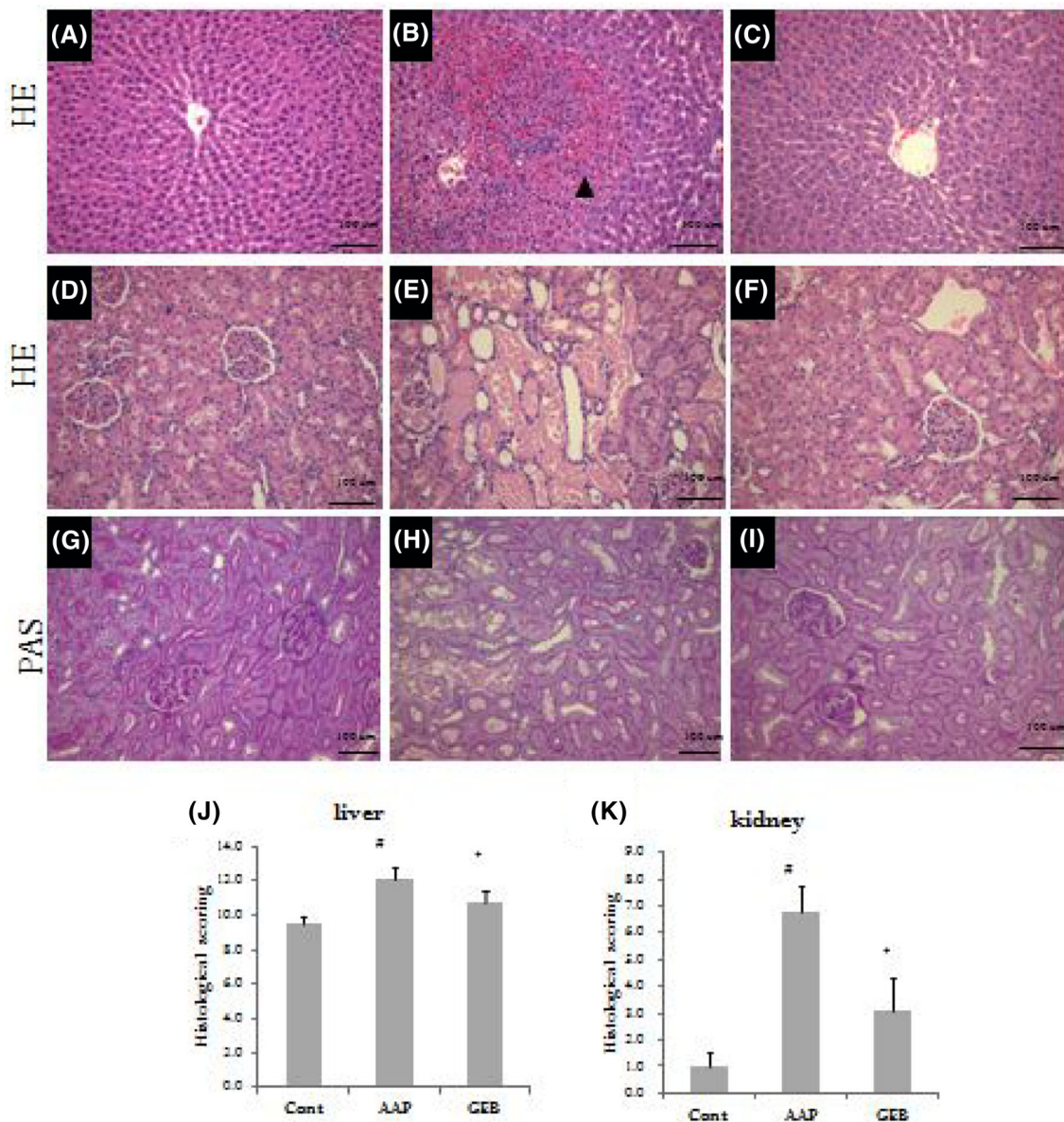


Fig. 2 Histopathological changes in liver and kidney tissues in different groups after HE and PAS staining. (A) Hepatocytes in the control group were normal. (B) However, those in the acetaminophen (AAP) group were abnormal and exhibited congestion and necrosis (arrow head). (C) *Gastrodia elata Blume* (GEB) treatment reduced histological damage and areas of necrosis. In the kidney, (D) brush borders of normal proximal tubules were observed in the control group. (E) Tubular necrosis and extremely damaged brush borders

were observed in the AAP group. (F) Less damaged brush borders were observed in the GEB group. After PAS staining, (G) brush borders of normal proximal tubules were observed in the control group. (H) Damaged basement membrane was observed in the AAP group. (I) The basement membrane was maintained in GEB group, and it was similar to that in the control group. (J) Liver and (K) kidney tissues were scored based on histopathology. # Versus control group ($p < 0.05$), * versus AAP group ($p < 0.05$)

these tissues were normal. These results have also been reported in other studies (Das et al., 2010; Ghosh et al., 2010). Renal damage is common in the proximal tubule, which seems to be related to the absorption of one of the major functions of the kidney. Kidney damage seems to be related to the expression of CYP2E1 in the liver. In the GEB group, the expression of pro-inflammatory cytokines, such as IL-1 β and TNF- α , and nitrotyrosine decreased. After AAP

injection, necrosis, inflammation, and apoptosis increased in the liver and kidney, indicating cellular damage. Cell damage was also confirmed by elevated serum ALT, AST, BUN, and creatinine levels. However, these levels were significantly lower in the GEB group than in the AAP group.

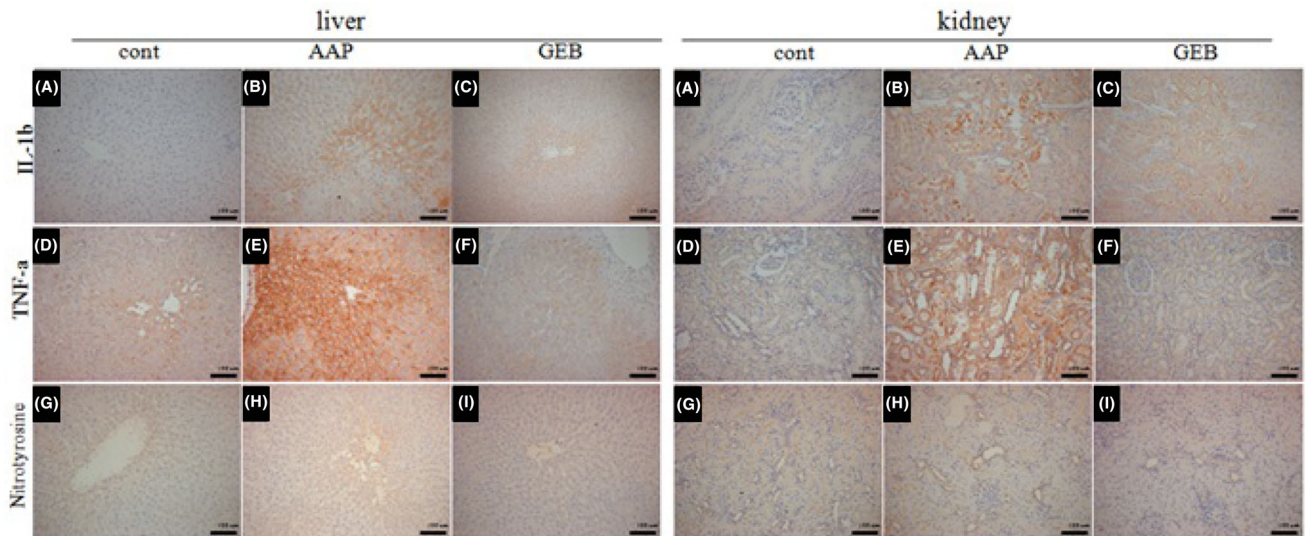
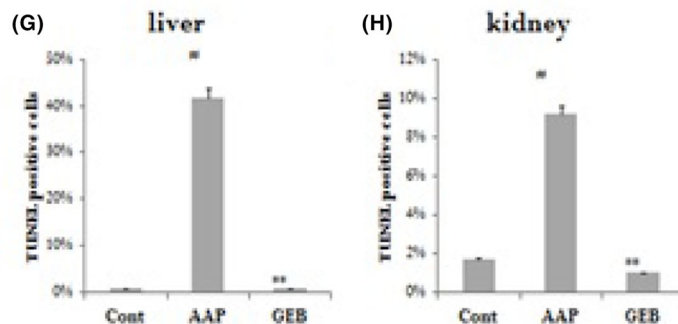
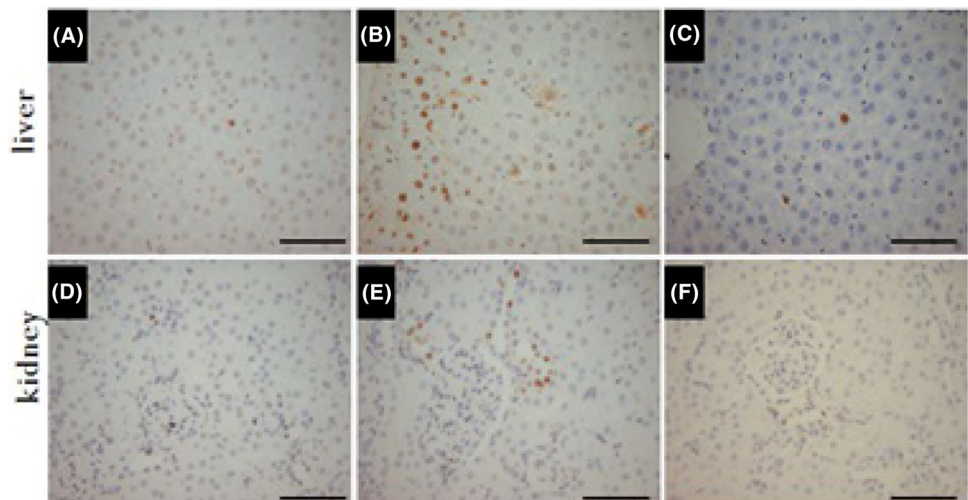


Fig. 3 Reduced expression of IL-1 β , TNF- α , and nitrotyrosine in liver and kidney injury in rats. Increased IL-1 β , TNF- α , and nitrotyrosine expression was observed in the acetaminophen (AAP) group in the centrilobular zone and proximal tubule (B, E,

H) compared to the control group (A, D, G). The expression of IL-1 β , TNF- α , and nitrotyrosine was decreased in the *Gastrodia elata Blume* (GEB) group (C, F, I)

Fig. 4 Effects of *Gastrodia elata Blume* (GEB) on apoptosis as measured by the TUNEL assay in liver and kidney injury induced by acetaminophen (AAP) in rats. Few TUNEL-positive cells were observed around the central vein and proximal tubule in the control group (A, D). The number of TUNEL-positive cells was significantly increased in the AAP group (B, E), whereas it was decreased in the GEB group (C, F) compared to the AAP group. Liver and kidney scores increased in the AAP group compared to the control group; however, they were lower in the GEB group than in the AAP group (G, H). # Versus control group ($p < 0.05$), ** versus AAP group ($p < 0.01$)

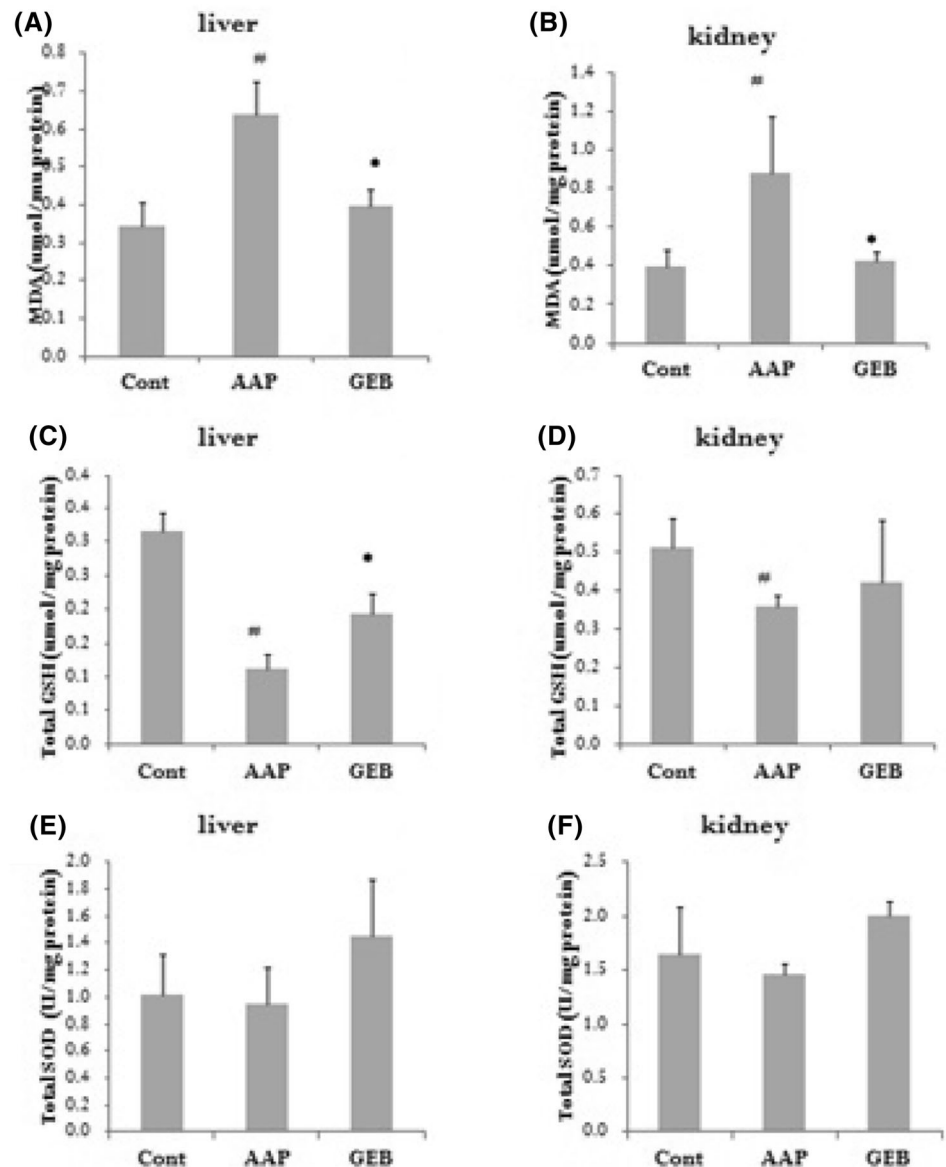


Oxidative stress markers

MDA was measured in liver and kidney tissues as an indicator of oxidative stress. Increased oxidative stress

leads to cell damage and increases MDA. MDA level in the AAP group was higher than that in the control group. However, it was significantly lower in the GEB group than in the AAP group in the liver and kidney [Fig. 5(A, B)].

Fig. 5 Effects of pretreatment with *Gastrodia elata* Blume (GEB) on the production of malondialdehyde (MDA), total glutathione (GSH), and total superoxide dismutase (SOD) in liver and kidney injury induced by acetaminophen (AAP) in rats. MDA, a lipid peroxidation marker, significantly increased in the AAP group compared to the control group (A, B). However, it significantly decreased in the GEB group. Total GSH significantly increased in the AAP group compared to the control group (C, D). However, it significantly decreased in the GEB group. Total SOD activity significantly decreased in the GEB group compared to that in the AAP group (E, F). # Versus control group ($p < 0.05$), * versus AAP group ($p < 0.05$), ** versus AAP group ($p < 0.01$)



GSH is an important antioxidant protein that eliminates AAP metabolites produced by CYP2E1. GSH levels were measured in liver and kidney tissues. GSH in both liver and kidney tissues were significantly decreased in the AAP group but significantly increased in the GEB group [Fig. 5(C, D)].

The activity of SOD, one of the antioxidants, was also measured in liver and kidney tissues. SOD hydrolyzes superoxide into hydrogen peroxide when superoxide is produced. SOD activity in the AAP group was lower than that in the control group, and it was higher in the GEB group than in the AAP group. However, no significant changes were observed [Fig. 5(E, F)].

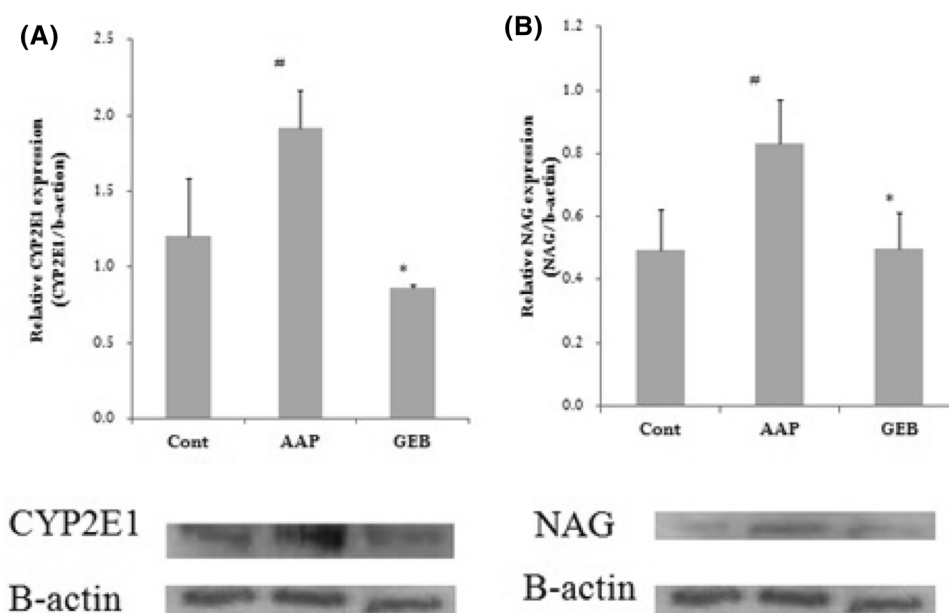
AAP-induced oxidative stress is cleared by antioxidant enzymes, such as GSH and SOD. SOD decomposes superoxide anion into hydrogen peroxide, and this

hydrogen peroxide is removed by antioxidant components, such as catalase and GSH. As expected, AAP administration increased MDA level and decreased GSH and SOD levels. However, pretreatment with GEB prevented the decrease in GSH and SOD levels and the increase in MDA level. MDA is one of the principal causes of AAP-induced toxicity, which is mediated by the production of NAPQI, an AAP metabolite (Şener et al., 2005). Pretreatment with GEB inhibited cell damage and promoted cellular antioxidant defense mechanism.

Effects of pretreatment with GEB on the expression of CYP2E1 and NAG

CYP2E1 is typically expressed in the liver and oxidizes AAP. In the AAP group, the expression of CYP2E1 was

Fig. 6 Western blot analysis of the expression of CYP2E1 and NAG in rats treated with acetaminophen (AAP) and *Gastrodia elata Blume* (GEB). β -actin was used as a loading control. The bar graphs show relative levels of CYP2E1 and NAG expression. The data are presented as mean \pm standard error of mean (SEM). # Versus control group ($p < 0.05$), * versus AAP group ($p < 0.05$), ** versus AAP group ($p < 0.01$)



significantly increased, and it was decreased in the GEB group [Fig. 6(A)]. NAG is an enzyme that increases when inflammation, necrosis, and fibrosis occur. NAG was also increased in the AAP group and significantly decreased in the GEB group [Fig. 6(B)].

CYP2E1 is expressed abundantly in the liver. The CYP2E1 inactivates most drugs either directly or by stimulating their excretion from the body (Shimada et al., 1994). After AAP injection, CYP2E1 was increased in the liver. These results have also been reported in other studies (Murad et al., 2016). Pretreatment with the GEB extract decreased the expression of CYP2E1 and ameliorated AAP-induced liver and kidney injury as indicated by histological changes, the expression of pro-inflammatory cytokines, TUNEL assay, and MDA level. 4-Methylpyrazole, an inhibitor of CYP2E1, significantly reduces AAP metabolism (Hazai et al., 2002). The suppression of CYP2E1 reduces the levels of reactive metabolites, thereby decreasing tissue injury (Ko et al., 2017). In this study, the decreased expression of CYP2E1 in the GEB group decreased cell damage, such as necrosis, inflammation, apoptosis, and oxidative stress.

NAG level is used as an index of liver damage in addition to AST and ALT. It is known to be elevated by inflammation, necrosis, and fibrosis (Wu et al., 2003). No fibrosis was observed in the AAP group when staining was conducted to investigate fibrosis in the liver because AAP was used for acute disease induction. Further studies are required to examine the effects of GEB in chronic disease models induced by AAP. In this experiment, the increase in NAG in the acute disease model after single AAP administration was due to inflammation and cell necrosis. However, NAG level decreased in the GEB group. These results

were also confirmed by decreased cell necrosis observed in HE-stained tissues and decreased pro-inflammatory cytokines observed by IHC. Therefore, GEB protects against liver injury induced by AAP.

Liver and kidney damage after AAP administration can proceed through several pathways. AAP produces NAPQI after being metabolized by CYP2E1 in the liver. GSH in the liver binds and neutralizes this NAPQI. If GSH is depleted or is overwhelmed by the elevated oxidative stress, NAPQI causes cell damage. At the same time, levels of MDA and pro-inflammatory cytokines are increased and SOD activity is decreased. It affects mitochondrial ATP levels and causes cell and mitochondrial dysfunction, leading to mitochondrial swelling. Mitochondrial swelling results in mitochondrial permeability transition, which is one of the mechanisms of hepatic injury induced by AAP. These effects can cause cell death (Bovedan et al., 2017). Antioxidant components, such as SOD and GSH, form the first-line defense system that scavenges free radicals (Adil et al., 2016). The balance between antioxidant components (SOD and GSH) and reactive oxygen species is important for controlling cellular oxidative stress (Adil et al., 2016; Kandhare et al., 2015). AAP is known to decrease SOD and GSH and increase MDA level (Naguib et al., 2014). The GEB extract could maintain the antioxidant enzyme level and scavenge reactive oxygen species. It had protective effects against hepatotoxicity and nephrotoxicity induced by AAP in rats. This protective effect of GEB is thought to be due to the increase in antioxidant enzymes with a reduction in oxidative stress and inflammation through the inhibition of CYP2E1 expression.

Results of this study indicate that pretreatment with GEB extract may protect against AAP-induced hepatic and

renal toxicity by inhibiting CYP2E1 expression, apoptosis, and oxidative stress.

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