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ORIGINAL ARTICLE

Yijin-tang, an Oriental Herbal Formula Reduces Ethanol-induced Acute Gastric Injury in Rats

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Abstract Protective effects of Yijin-tang extract (YTE) were investigated on ethanol-induced acute gastric injury. Simultaneous determination of six components, homogentisic aicd, liquiritin, hesperidin, neohesperidin, poncirin, and glycyrrhizin was performed in YTE by high performance liquid chromatography-photodiode. Acute gastric lesion was induced by oral administration of absolute ethanol (5 mL/kg). The positive control (omeprazole, 50 mg/kg) and YTE groups (200 and 400 mg/kg) were administered by oral gavage 2 h prior to ethanol treatment. The animals were sacrificed 1 h after receiving ethanol treatment. Acute toxicity study was performed to evaluate the safety of YTE. YTE protected gastric mucosa against ethanol-induced acute gastric injury including hemorrhage and hyperemia. YTE reduced the elevated lipid peroxidation of stomach and increased the activities of antioxidant enzyme. In the acute toxicity study, YTE did not cause any toxic effect at the dose level of 2000 mg/kg in rats. These results showed that YTE protects gastric mucosa from ethanol-induced acute gastric injury via increasing the antioxidant status. We suggest that YTE has a potential as a therapeutic agent for acute gastric injury.

Keywords antioxidant · cute gastric injury · ethanol · oriental herbal formula · Yijin-tang

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Introduction

The gastric mucous membrane is persistently exposed to various factors such as ethanol, acetic acid, bacterial products, and nonsteroidal anti-inflammatory drugs (Suresh et al., 1999; Sugimoto et al., 2000; Ozdil et al., 2004). These agents increase the production of reactive oxygen species (ROS) and decrease the activity of antioxidant enzymes, leading to acute gastric damage including hemorrhage, congestion, edema, erosion, and ulcers (Medeiros et al., 2008; Silva et al., 2009). Although the mechanism of ethanol-induced gastric lesion is unclear, ethanol-induced acute gastric lesions occur mainly by the production of ROS, modulation of nitric oxide system, reduction of mucosal blood flow, and depletion of sulfhydryl groups (Rao et al., 2004). In particular, ROS were considered to have an important role in the pathogenesis of ethanol-induced acute gastric injury (Ozdil et al., 2004; Kanter et al., 2005). Based on previous studies, several researchers considered the possibility that antioxidants or ROS scavengers have protective activity against ethanol-induced acute gastric injury. Actually, antioxidants such as quercetin, curcumin, and Vitamin C exhibited protective effects against ethanol-induced acute gastric injury (Suzuki et al., 1998; Koyuturk et al., 2004; Tuorkey and Karolin, 2009).

Yijin-tang (Nichin-to in Japanese and Er-chen-tang in Chinese), an oriental herbal formula, is composed of five different herbs; *Citrus unshiu, Glycyrrhiza uralensis, Pinellia ternate, Poria cocos*, and *Zingiber officinale*. It is traditionally used for treatment of gastrointestinal disorders such as irritable bowel syndrome, gastroesophageal reflux disease, gastritis, and gastric ulcer (Lee et al., 2010). Previous studies showed that YTE has protective effects against ethanol-induced gastric damage in certain animal models (Ok et al., 2003; Choi et al., 2007). Recently, researchers have demonstrated that YTE possesses antioxidant activity based on the results of *in vitro* and *in vivo* experiments (Lee et al., 2003; Park et al., 2010). Additionally, Yokozawa et al. (2005) reported

that crude herbs in Yijin-tang formulation also possess antioxidant activity. However, until recently the protective effects of YTE on ethanol-induced acute gastric injury may be related to the antioxidant activity was not known. Considering the properties of YTE, we predicted that YTE would reduce ethanol-induced acute gastric injury, possibly through antioxidant effects.

Acute toxicity is the toxicity produced by a pharmaceutical when administered in one or more doses during a single period that does not exceeding 24 h. To evaluate the acute toxicity of a pharmaceutical, many researchers performed acute toxicity test in compliance with regulatory guidelines. Data from acute toxicity tests can be used to screen for toxicity of a pharmaceutical or to determine if a compound is toxic. Thus, acute toxicity studies in animals are necessary for single-dose administered pharmaceuticals that are intended for human use.

In the present study, the protective effects of YTE on ethanol-induced acute gastric injury in rats, were evaluated. In addition, to confirm the safety of YTE, an acute toxicity study was conducted according to Organization for Economic Cooperation and Development (OECD) Testing Guideline TG 423.

Materials and Methods

Reagents and materials. Homogentisic acid was purchased from Sigma-Aldrich (St Louis, MO). Liquiritin, and glycyrrhizin were purchased from Wako (Osaka, Japan). Hesperidin, Neohesperidin and poncirin were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China), CromaDex (Irvine, CA), and Roth (Karlsruhe, Germany), respectively. The purity of all reference standards were more than 98.0%. High performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ). Glacial acetic acid was of analytical reagent grade, procured from Junsei (Tokyo, Japan). The materials forming YTE were purchased from Omniherb (Yeongcheon, Korea) and HMAX (Jecheon, Korea). A voucher specimen (2008-KE08-1–KE08-5) has been deposited at the Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine.

Preparation of standard solutions and calibration curves. Standard stock solutions of homogentisic acid (2,000 μg/mL), liquiritin (500 μg/mL), hesperidin (2,000 μg/mL), neohesperidin (2,000 μg/mL), poncirin (200 μg/mL), and glycyrrhizin (1,000 μg/mL) were prepared in methanol and stored below 4°C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. All calibration curves were obtained by assessment of peak areas from standard solutions in the concentration ranges: homogentisic acid, 0.10–100.00 μg/mL; liquiritin and glycyrrhizin, 0.25–250.00 μg/mL; hesperidin, 0.25–500.00 μg/mL; neohesperidin and poncirin, 0.05–50.00 μg/mL.

Preparation of YTE. YTE was prepared with a mixture of chopped crude herbs. *C. unshiu, P. ternate and Z. officinale were* purchased from Omniherb and (Yeongcheon, Korea) and *G. uralensis* and *P. cocos* were obtained from HMAX (Jecheon, Korea). Identity of each crude herbs was confirmed by professor Je-Hyun Lee of Dongguk, Gyeongju, Korea. YTE was prepared with *C. unshiu* (629.29 g), *G. uralensis* (314.64 g), *P. ternate* (1258.58 g), *P. cocos* (629.29 g) and *Z. officinale* (629.29 g), and its extract was obtained by boiling the herbs in distilled water (35 L) at 100°C for 2 h. The solution was evaporated and freeze-dried (640.3 g, yield: 18.5%). Lyophilized YTE extract (500 mg) was dissolved in distilled water (25 mL) and mixed. The solution was filtered through a SmartPor GHP syringe filter (0.2 μm pore size; Woongki Science, Seoul, Korea).

HPLC analysis. Simultaneous analyses were performed using a Shimadzu LC-6A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit (LC-6AD), an on-line degasser (DGU-14A), an auto sample injector (SIL-10AF), and a PDA detector (SPD-M10A). The data processor employed LCsolution software (Version 1.24, Shimadzu Co., Kyoto, Japan). The analytical column used was a Luna C18 column (250 mm × 4.6 mm; particle size 5 μm, Phenomenex, Torrance, CA). The mobile phases consisted of 1.0% (v/v) aqueous acetic acid (A) and 1.0% (v/v) acetic acid in acetonitrile (B). The gradient flow was as follows: (A)/(B)=90/10 (0 min) → (A)/(B)=35/65 (35 min) (A)/(B)=0/100 (45 min; hold for 5 min) (A)/(B)=90/10 (52 min). The analysis was carried out at 1.0 mL/min with PDA detection at 254 nm (for glycyrrhizin) and 280 nm (for homogentisic acid, liquiritin, hesperidin, neohesperidin, and poncirin). The injection volume was 10 μL.

Ethanol-induced gastric injury. Specific pathogen-free male Sprague-Dawley rats, weighing 200–250 g (aged 6 weeks) were purchased from the Orient Co. (Seoul, Korea) and used after 1 week of quarantine and acclimatization. The animals were kept in a room at 23±3°C with a relative humidity of 50% under a controlled 12 h/12 h light/dark cycle. The rats were given a standard rodent chow and sterilized tap water *ad libitum*. All experimental procedures were performed in compliance with the NIH Guidelines for the care and use of laboratory animals and the National Animal Welfare Law of Korea.

Acute gastric lesions were induced by intragastric administration of absolute ethanol according to a previously described method (Robert et al., 1979). A total of 45 rats were divided into 5 groups and fasted for 18 h before the experiment. Rats in the control group were given PBS orally as the vehicle, and the absolute-ethanol group (EtOH group) received absolute ethanol (5 mL/kg body weight) by oral gavage. Rats in the positive control group were given oral omeprazole (50 mg/kg body weight) 2 h prior to the administration of absolute ethanol. Omeprazole was used as the positive control drug, because it possesses anti-inflammatory and antioxidant activity, and it has been widely used for the treatment of gastritis (Lapenna et al., 1996; Sener-Muratoglu et al., 2001). The fourth and fifth groups received YTE (200 and 400 mg/kg body weight, respectively) 2 h prior to absolute ethanol intake. The application volumes (5 mL/kg body weight) of PBS, omeprazole, and YTE were calculated in advance based on the

recored body weight of the individual animal before performing the present study.

Animals were sacrificed with an overdose of 50 mg/kg pentobarbital 1 h after receiving the absolute ethanol treatment. The stomach was removed from each animals, and opened along the greater curvature. The tissue was gently rinsed in PBS. The stomach was stretched on a piece of cork with the mucosal surface facing upward, and was then examined in a standard position for a gross examination of the gastric mucosal lesions. Photographs of hemorrhagic erosions in the stomach were taken with a Photometric Quantix digital camera (Nikon, Japan). After the gastric lesions were photographed, the stomach tissues were stored at –70°C for biochemical analysis. Quantitative analysis of gastric mucosal injury was performed using an image analyzer (Molecular Devices, Inc., Sunnyvale, CA).

Biochemical analysis. The stomach tissue was cut into small pieces and homogenized (1/10 w/v) with tissue lysis/extraction reagent along with a protease inhibitor (Sigma, Saint Louis, MI). The homogenates were centrifuged at 12,000 rpm for 10 min at 4°C to discard all cell debris, and the supernatant was used to measure malondialdehyde (MDA), reduced glutathione (GSH), catalase, glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD). Total proteins were determined using a protein assay reagent (Bio-Rad, Hercules, CA).

Lipid peroxidation was estimated by determination of MDA using a thiobarbituric acid reactive substances (TBARS) assay kit (BioAssay Systems, Hayward, CA). In brief, 100 μL of homogenates was mixed with 100 μL of 10% trichloroacetic acid and incubated for 15 min on ice. The mixture was centrifuged at 12,000 rpm for 5 min at 4°C. Subsequently, 200 μL of supernatant was mixed with 200 μL of thiobarbituric acid and incubated at 100°C for 60 min. After the mixture was cooled, the absorbance at 535 nm was measured. The results were expressed as nmol of MDA/mg protein.

The levels of GSH were measured using a GSH assay kit (Cayman, Ann Arbor, MI), and the results were expressed as ìmol/mg protein. The activity of antioxidative enzymes including catalase, GST, GPx, GR, and SOD were quantified using a commercial kit (Cayman) according to the manufacturer's protocols. The results were expressed as U/mg protein.

Acute toxicity study. Five-week-old male and female Sprague-Dawley rats were purchased from a specific pathogen-free facility at the Orient Bio Co. (Seoul, Korea) and were used after one week of quarantine and acclimatization. All animals were housed in a room maintained at 23±3°C with a relative humidity of 50%, artificial lighting from 08:00 to 20:00 and with 10 to 20 air changes per hour. The animals were fed a commercial pellet diet (PMI Nutrition International, Richmond, VA) and sterilized tap water *ad libitum* following UV irradiation and filtration. The acute toxicity study was performed in compliance with the test guidelines of the Korea Food and Drug Administration (KFDA) under the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies. Protocol of the study was approved by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology (earned by AALAC International, 1998).

In the preliminary study, a single oral administration of YTE did not induce any toxic effects at dose levels up to 2,000 mg/kg. Based on the results, 2,000 mg/kg was selected as a limited dose recommended by OECD test guidelines. Ten rats of each sex were randomly assigned to two groups, with five rats in each group, and the animals received a single 2,000 mg/kg dose through gavage. The vehicle control rats received an equivalent volume of distilled water. After oral administration, all abnormal clinical signs were recorded before and after dosing at least twice a day, and body weight was measured immediately on the day of dosing (Day 1), before treatment as well as on days 2, 4, 8, and 15. At scheduled termination (Day 15), all surviving animals were anesthetized by carbon dioxide and sacrificed by exsanguination from the aorta. Complete gross postmortem examinations were performed on all animals.

Statistical analysis. Data are expressed as means ± SD. Statistical significance was determined using analysis of variance (ANOVA). If the tests showed a significant difference among the groups, the data were analyzed by a multiple comparison procedure using Dunnett's test (1964). Statistical analysis was performed by using the Path/Tox System (Ver. 4.2.2, Xybion Medical System, Cedar Knolls, NJ). The level of significance was defined as $p < 0.05$ or 0.01.

Results

HPLC analysis of YTE. Three-dimensional chromatogram was obtained using HPLC-PDA detector. Under optimized chromatography conditions, six components were eluted before 35 min in sample analysis using mobile phases consisting of solvent A (1.0%, v/v, aqueous acetic acid) and solvent B (1.0%, v/v, acetic acid in acetonitrile). Simultaneous determination of six components, homogentisic aicd in *P. ternate*, liquiritin and glycyrrhizin in *G uralensis*, and hesperidin, neohesperidin, and poncirin in *C. unshiu* by HPLC-PDA. Three-dimensional HPLC chromatogram of YTE is shown in Fig. 1. The retention times of the components were 7.6 (homogentisic acid), 17.5 (liquiritin), 19.0 (hesperidin), 19.6 (neohesperidin), 23.6 (poncirin), and 29.7 min (glycyrrhizin). The linearity of the peak area (y) versus concentration (x, μg/mL) curve for each component was used to calculate the contents of the main components in YTE. The correlation coefficient (r2) of calibration curves on six constituents was greater than 0.9995. The contents of the six components in YTE range form 0.07–13.10 mg/g and are summarized in Table 2.

Acute toxicity of YTE. Acute toxicity of YTE was evaluated to confirm the safety of oral YTE administration. No significant differences in body weight changes were observed between the control and YTE-treatment groups except for those on Days 4 and 8 in the 2,000 mg/kg female group (Fig. 2). Throughout the study

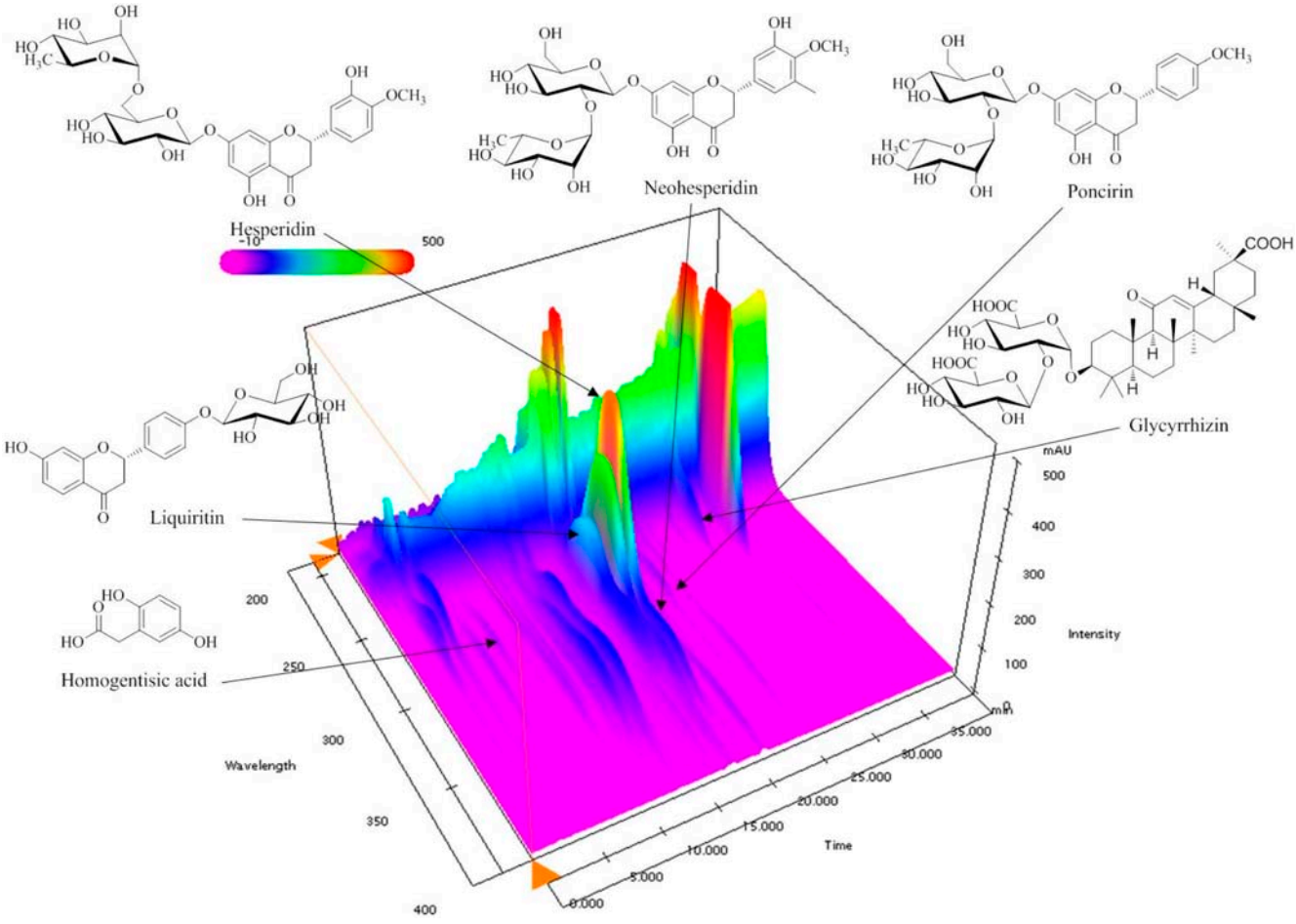

Fig. 1 Three-dimensional chromatogram of YTE by HPLC-PDA.

Table 1 Contents of six Components in YTE

Content (mg/g)								
Homogentisic acid			Liquiritin			Hesperidin		
Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
1.85	1.15×10^{-2}	0.62	5.93	5.97×10^{-2}	1.01	13.10	9.45×10^{-2}	0.72
Content (mg/g)								
Neohesperidin			Poncirin			Glycyrrhizin		
Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
0.63	1.35×10^{-3}	0.21	0.07	1.50×10^{-4}	0.22	7.14	9.55×10^{-2}	1.34

period, no treatment-related deaths were observed. Furthermore, YTE treatment related clinical signs and gross pathological findings were not observed up to dose levels of 2,000 mg/kg body weight.

Effect of YTE on ethanol-induced acute gastritis. In the gross examination of gastric mucosa, the EtOH group had gastric mucosal injuries including hemorrhage and hyperemia (Fig. 3A), whereas omeprazole-treated group attenuated gastric mucosal injuries. YTE treatment also attenuated the gastric mucosal injuries compared to the EtOH group and the Omeprazole-treated group. In addition, Quantative analysis of gastric mucosal injury showed that the EtOH group statistically increased the gastric mucosal injury index compared with the control group. However, omeprazole-treated group and YTE-treated groups showed significant reduction than the EtOH group (Fig. 3B)

Effects of YTE on lipid peroxidation and GSH contents in ethanol-induced acute gastritis. The level of MDA, an end product of lipid peroxidation, in the EtOH group was increased

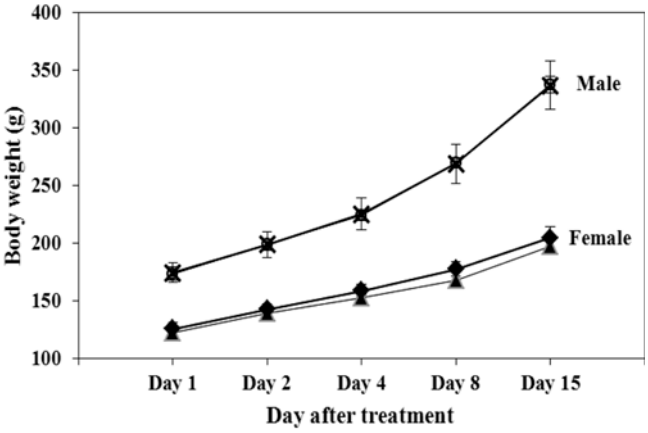

Fig. 2 Body weight changes of animals treated with YTE at dose levels of 0 (×) and 2,000 mg/kg (○) in males and 0 (▲) and 2,000 (◆) mg/kg in females. There were no significant differences in body weight between the YTE-treated and control groups. However, significant increases in body weight of females were observed on days 4 and day 8 (p <0.05).

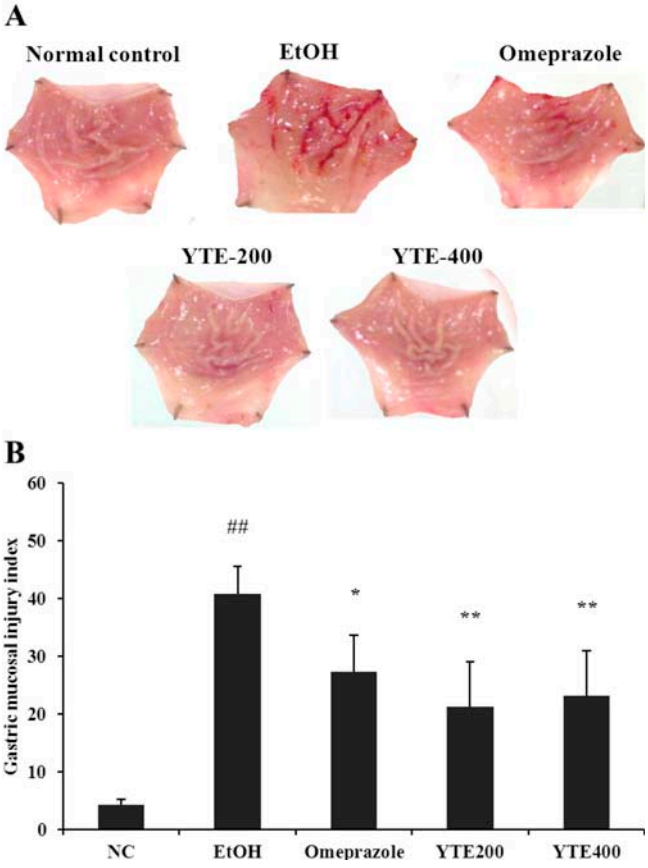

Fig. 3 Representative photographs of gastric mucosa (A) and gastic mucosal injury index (B) with absolute ethanol-induced gastric injuries. Absolute ethanol induced hemorrhagic and hyperemia in gastric mucosa. In contrast, YTE attenuated the gastric mucosal injury induced by absolute ethanol. Gastric mucosal injury index showed that YTE-treated groups significant decreased compared with the EtOH group. ##Significant difference at p <0.01 compared to the control group. *Significant difference at p <0.05 and **at p <0.01 compared to the EtOH group.

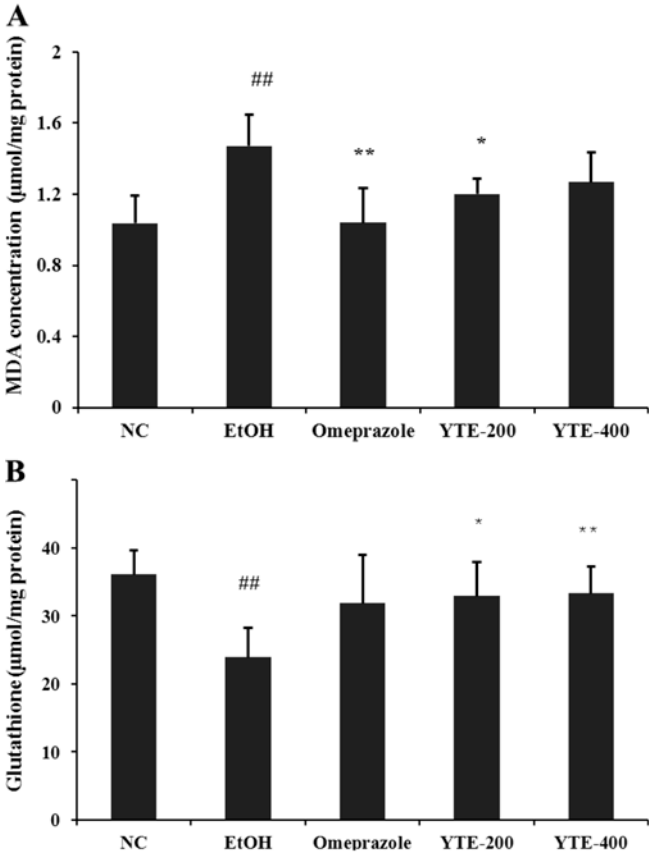

Fig. 4 Effects of YTE on the gastric MDA concentration (A) and GSH contents (B) on absolute ethanol-induced gastric injury in rats. Each bar represents the mean ± SD of six rats. #Significant difference at p <0.05 and ##Significant difference at p <0.01 compared to the control group. *Significant difference at p <0.05 and **at p <0.01 compared to the EtOH group.

more than the concentration in the control group (Fig. 4A). In contrast, in the omeprazole-treated group, the MDA level decreased compared with the EtOH group. Similar to omeprazole-treated group, MDA level of the YTE-treated groups decreased compared with the EtOH group. A significant difference was detected in 200 mg/kg group of YTE, but not in 400 mg/kg group.

Conversely, the content of GSH in the stomachs of the EtOH group was significantly decreased compared to the control group (Fig. 4B). Althought no significant significant difference was observed, omeprazole-treated group showed increased GSH contents compared with the EtOH group. On the other hand, YTE-treated groups showed significant increases in gastric GSH content compared to that of the EtOH group.

Effects of YTE on antioxidant enzymes in ethanol-induced acute gastritis. Catalase activity in the EtOH group decreased compared to the control group (Fig. 5A). In contrast, omeprazole-treated group significantly increased catalase activity compared with the EtOH group. YTE-treated groups showed increased catalase activities compared with the EtOH- and omeprazole-

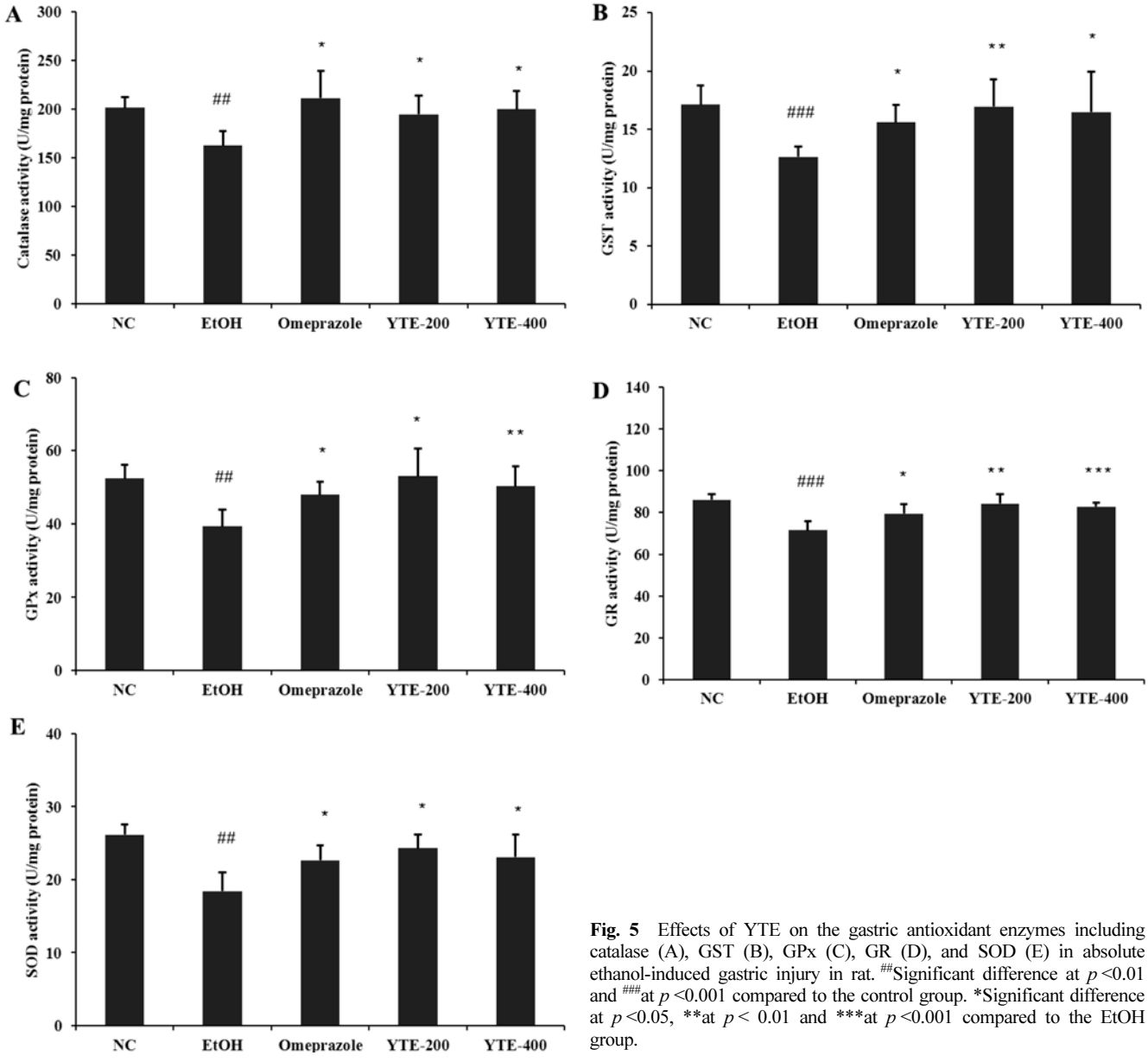

Fig. 5 Effects of YTE on the gastric antioxidant enzymes including catalase (A), GST (B), GPx (C), GR (D), and SOD (E) in absolute ethanol-induced gastric injury in rat. $^{\#\#}$Significant difference at $p<0.01$ and $^{\#\#\#}$at $p<0.001$ compared to the control group. *Significant difference at $p<0.05$, **at $p<0.01$ and ***at $p<0.001$ compared to the EtOH group.

treated groups. Similar to the results of catalase activity, GST, GPx, GR, and SOD activities in the EtOH group were markedly reduced compared with the control group and omeprazole-treated group was increased the GST, GPx, GR, and SOD activities compared with the EtOH group. Furthermore, YTE-treated groups showed increased GST, GPx, GR, and SOD acitivities significantly increased compared with the EtOH group (Fig. 5).

Discussion

Ethanol-induced gastric lesions in rats are considered to be a reliable tool for studying the pathogenesis of acute gastric injury (Robert et al., 1979). Ethanol-induced acute gastric lesions are characterized by pathological changes such as hemorrhage, edema, inflammatory infiltration, and loss of epithelial cells (Medeiros et al., 2008; Silva et al., 2009). These characteristics were consistent with the lesions observed in the present study. Gastric lesions of rats treated orally with YTE had reduced gastric injuries compared to rats treated only with absolute ethanol. Considering these observations, oral administration of YTE appears to attenuate ethanol-induced acute gastric injury.

In normal metabolism, there is a balance between the generation of ROS and the antioxidant defense system. The antioxidant defense system includes GSH, catalase, GST, GPx, GR, and SOD, which are known to be major constituents of the

intracellular protective mechanism against oxidative injury (Sener-Muratoglu et al., 2001). Pathological conditions induced by various chemicals and stress could lead to an increased production of ROS above normal conditions, thereby causing increase in lipid peroxidation and decrease in the activity of antioxidant enzymes including GSH, catalase, GST, GPx, GR, and SOD (Johansen et al., 2005; Valko et al., 2007). Ethanol-induced acute gastric lesions are consistent with these pathological changes. According to several researchers, ethanol induces increase in lipid peroxidation and reduction in activity of antioxidant enzymes, which are one of the important factors in the pathogenesis of ethanol-induced gastric injury (Ozdil et al., 2004; Kanter et al., 2005). Thus, several studies focusing on reductions in oxidative stress have been conducted. In the present study, ethanol-induced gastric lesions increased the level of lipid peroxidation and reduced the levels of GSH, catalase, GST, GPx, GR, and SOD. In contrast, administration of YTE decreased the level of lipid peroxidation in ethanol-induced acute gastric lesions. YTE also increased gastric GSH contents as well as the activity levels of GST, catalase and SOD compared to ethanol-induced acute gastric lesions. These results were similar to those of previous studies that evaluated the protective effects of antioxidants such as quercetin and curcumin using the ethanol-induced gastric injury model (Suzuki et al., 1998; Koyuturk et al., 2004; Tuorkey and Koralin, 2009). Crude herbs in YTE were used for treatment of various diseases due to their anti-inflammatory and antioxidant effects. *G. uralensis*, *Z. officinale*, *P. cocos*, and *Citrus unshiu* (Sugiura et al., 2006; Park et al., 2009; Oboh et al., 2010; Wu et al., 2011) produce anti-inflammatory and antioxidant effects in *in vivo* and *in vitro* experiments. Moreover, previouse studies on *G. uralensis* and *Z. officinale* indicated anti-ulcer effects in experimentally induced gastric injury (Dehpour et al., 1994; Khushtar et al., 2009). The antioxidant effects of YTE were also reported (Lee et al., 2003; Park et al., 2010). These properties of YTE were consistent with the results of the present study. Therefore, protective effects of YTE against acute gastric injury was considered to be closely related to their antioxidant effects.

Additionally, acute toxicity study was conducted in accordance with the OECD guidelines. YTE did not cause deaths during the experimental period. Body weight changes were significantly increased on Days 4 and 8 in the YTE-treated group compared with animlas in the control group. Because these changes occurred temporarily and were not accompanied by clinical signs or gross lesions, they were not considered to be YTE-treatment-related effects. As for clinical signs and gross findings, there were no observed treatment-related adverse effects in any of the groups. Based on these results, YTE could be regarded as a safe material when a single dose of YTE is administered by oral gavage to rats at a dose level of 2,000 mg/kg.

In conclusion, upon investigation of the protective effects of YTE on ethanol-induced acute gastric injury in rats, YTE was found to decrease gastric injuries and lipid peroxidation in ethanol-induced acute gastric lesions as well as increase the levels of antioxidants. These results indicate that the protective effect of YTE against ethanol-induced acute gastric injury was caused in part by the antioxidant activities of YTE. In addition, acute toxicity study revealed that YTE is a safe drug. Thus, results of the present study suggest that YTE can be developed as a useful drug for the treatment of acute gastric injury.

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