

<http://dx.doi.org/10.4314/ajtcam.v10i2.18>

## GASTROPROTECTIVE EFFECTS OF LEEJUNG-TANG, AN ORIENTAL TRADITIONAL HERBAL FORMULA, ON ETHANOL-INDUCED ACUTE GASTRIC INJURY IN RATS

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### Abstract

Leejung-tang (LJT, Rechu-to in Japanese and Lizhong-tang in Chinese) is an oriental traditional traditional herbal formula. LJT has been used for treatment of gastrointestinal disorders in Korea, Japan, and China for a long time. In present study, we investigated the protective effects of LJT against absolute ethanol induced gastric injuries. Rats in the control group were given PBS orally (5 mL/kg body weight) 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 omeprazole orally (50 mg/kg body weight) 2 h prior to the administration of absolute ethanol. The treatment groups received LJT (400 mg/kg body weight) 2 h prior to absolute ethanol administration. All rats were sacrificed 1 h after receiving the ethanol treatment. The stomach was excised for macroscopic examination and biochemical analysis. The administration of LJT protected gastric mucosa against ethanol-induced acute gastric injury, including hemorrhage and hyperemia. LJT reduced the increase in lipid peroxidation in ethanol-induced acute gastric lesions. LJT increased GSH content and activities of the antioxidant enzymes, catalase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase, and superoxide dismutase. These results indicate that LJT protects gastric mucosa against ethanol-induced acute gastric injury by increasing their antioxidant content. We suggest that LJT can be developed as an effective drug for the treatment of acute gastric injury.

**Key words:** Herbal formula, Leejung-tang, Absolute ethanol, Acute gastric injury, Antioxidant

### Introduction

Acute gastric injury is induced by agents such as ethanol, stress, and nonsteroidal anti-inflammatory drugs (NSAIDs) when the gastric mucosal membrane is continuously exposed to them (Suresh et al., 1999; Sugimoto et al., 2000; Kanter et al., 2005). The mechanism underlying ethanol-induced gastric injury is unclear but several factors have been implicated. Of these factors, reactive oxygen species (ROS) in particular seem to play an important role in gastric injury, including inflammation, hemorrhage, erosion, and ulceration of the gastric mucosa, as they may increase oxidative stress and deplete antioxidants (Ozdil et al., 2004; Kanter et al., 2005). Previous studies showed that induction of ROS overproduction using various agents increased oxidative stress and markedly increased gastric mucosal injury (El-Missiry et al., 2001; Ozdil et al., 2004; Rios et al., 2010). Therefore, antioxidants and ROS scavengers should decrease ethanol-induced gastric injury (La Casa et al., 2000). Indeed, many studies have shown that various antioxidants have protective effects against ethanol-induced acute gastric injury (Suzuki et al., 1998; Koyuturk et al., 2004; Tuorkey and Karolin, 2009).

Leejung-tang (Lizhong-tang in Chinese and Rechu-to in Japanese) is a Korean traditional herbal formula composed of four different crude herbs: *Radix ginseng*, *Rhizoma atractylodis*, *Radix glycyrrhizae*, and *Rhizoma zingiberis*. Leejung-tang water extract (LJT) has been used for treatment of gastrointestinal disorders such as vomiting, stomach pain, chronic gastritis, and gastrointestinal ulceration in Korea, Japan, and China for many years. LJT has proven therapeutic effects such as immunomodulation, anticancer activity, antitoxic effect and modulates gastrointestinal motility and secretion of gastric acid (Kwak et al., 1987; Ha and Lee, 1998; Kim et al., 2008; Seo et al., 2009). In addition, many *in vivo* and *in vitro* experiments have demonstrated that the crude herbs of which LJT is composed possess antioxidant activity (Yokozawa et al., 2005; Rhyu et al., 2007; Du et al., 2010). However, no studies have been conducted to evaluate the gastroprotective effects of LJT on ethanol-induced acute gastric injury. We evaluated the protective effects of LJT against absolute ethanol-induced gastric injury and assessed antioxidant activity in damaged gastric tissue.

### Materials and Methods

#### Preparation of LJT

LJT was prepared in our laboratory from a mixture of chopped herbs purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). The identity of each herb was confirmed by a pharmacist and professors at a college of oriental medicine. LJT was prepared as described in Table 1 and an extract was created by boiling the herbs in distilled water at 100 °C for 2 h. The solution was evaporated and freeze-dried (yield: 18.5%).

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## Animals

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 a week of quarantine and acclimatization. The animals were kept in a room maintained at  $23 \pm 3$  °C with a relative humidity of  $50 \pm 10\%$  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.

## Absolute ethanol-induced gastric injury

Acute gastric lesions were induced by intragastric administration of absolute ethanol in according to a previously described method with some modification (Robert et al., 1979). In preliminary study, animals were administered by oral gavage at doses of 400 and 800 mg/kg of LJT. The protocols of preliminary study were the same as those of present study. At the scheduled termination, we measured the gross pathological examination on gastric mucosa. The animals treated with 400 and 800 mg/kg decreased the gross pathological findings compared with the EtOH group. However, 400 mg/kg treated animals is more effective than 800 mg/kg treated animals. Based these results, we determined 400 mg/kg as effective dose. Twenty-eight rats were divided into four groups and fasted for 18 h before the experiment. Rats in the control group were given PBS orally (5 mL/kg body weight) 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 omeprazole (50 mg/kg body weight) orally 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 is widely used for the treatment of gastritis (Lapenna et al., 1996; Sener-Muratoglu et al., 2001). The treatment group received LJT (400 mg/kg body weight) 2 h prior to absolute ethanol intake.

## Gross pathological findings in gastric mucosa

Animals were sacrificed using an overdose of pentobarbital (50 mg/kg) administered 1 h after they received the absolute ethanol treatment. The stomach was removed, opened along the greater curvature, and gently rinsed in PBS. The stomach was then laid into corkboard with the mucosal surface facing up, and examined from a standard position for the gastric mucosal lesions. Photographs of hemorrhagic erosions were taken with a Photometric Quantix digital camera (Quantix 1400; Roper Scientific, Tucson, AZ). After the gastric lesions were photographed, the stomach tissue was stored at -70 °C until biochemical analysis. Quantitative analysis of gastric mucosal injury was performed using an image analyzer (Molecular Devices Inc., CA, USA).

## Preparation of gastric tissue homogenate

The stomach tissue was cut into small pieces and homogenized (1/10 w/v) with tissue lysis/extraction reagent and protease inhibitor cocktail (Sigma, MI, USA) using a homogenizer. The homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C to precipitate cell debris, and the protein concentrations of the supernatant fractions were determined using Bradford reagent (Bio-Rad, Hercules, CA).

## Lipid peroxidation and GSH level

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

The levels of reduced glutathione (GSH) were measured using a GSH assay kit (Cayman, Ann Arbor, MI), which involves an optimized enzymatic recycling method and glutathione reductase. The sulfhydryl group of GSH reacts with 5,5-dithio-*bis*-2-nitrobenzoic acid (DTNB) and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). A mixed disulfide, GSTNB (between GSH and TNB), is produced concomitantly and is reduced by glutathione reductase to recycle the GSH, producing more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. The absorbance of TNB at 410 nm was used to estimate the amount of GSH in the sample. The GSH level was expressed as  $\mu$ mol/mg protein.

## Antioxidative enzyme activity

Superoxide dismutase (SOD) activity was measured using a SOD assay kit (Cayman), in which a tetrazolium salt is used for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Glutathione-S-transferase (GST) activity was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione using GST assay kit (Cayman). Conjugation is accompanied by an increase

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in absorbance at 340 nm and rate of increase is directly proportional to the GST activity in the sample.

Glutathione peroxidase (GPx) activity was measured indirectly via a coupled reaction with glutathione reductase using a GPx assay kit (Cayman). Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to NADP<sup>+</sup> is accompanied by a decrease in absorbance at 340 nm.

Glutathione reductase (GR) activity was determined by measuring the rate of NADPH oxidation using a GR assay kit (Cayman). Oxidation of NADPH to NADP<sup>+</sup> is accompanied by a decrease in absorbance at 340 nm.

Catalase activity was measured according to the peroxidatic function of catalase using a catalase assay kit (Cayman). This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced is measured colorimetrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Activities of SOD, catalase, GST, GPx and GR, were expressed as U/mg protein.

### Statistical analysis

Data are expressed as the means  $\pm$  standard deviation (SD). Statistical significance was determined using analysis of variance (ANOVA). If the test showed a significant difference between groups, the data were analyzed by a multiple comparison procedure using Dunnett's test (Dunnett, 1964). The level of significance was defined as  $P < 0.05$  or  $P < 0.01$ .

## Results

### LJT decreased ethanol-induced gastric injury

The EtOH group developed gastric mucosal injuries, including hemorrhage and hyperemia (Figure 1A). The omeprazole treated group and LJT groups had less severe gastric mucosal injuries than the EtOH group. In quantitative analysis, EtOH group showed the significant increase in gastric mucosal injury index compared with the control group (Fig. 1B). By contrast, LJT group significantly increased the gastric mucosal injury index compared with the EtOH group.

**Table 1:** Crude components of LJT

Latin name	Scientific name	Amount (g)	Company of purchase	Source
Ginseng Radix	<i>Panax ginseng</i>	7.50	Omnierb	Geumsan, Korea
Atractylodes Rhizoma	<i>Atractylodes macrocephala</i>	7.50	Omnierb	China
Glycyrrhizae Radix	<i>Glycyrrhiza uralensis</i>	3.75	HMAX	China
Zingiberis Rhizoma Crudus	<i>Zingiber officinale</i>	7.50	Omnierb	Yeongcheon, Korea
Total amount		26.25		

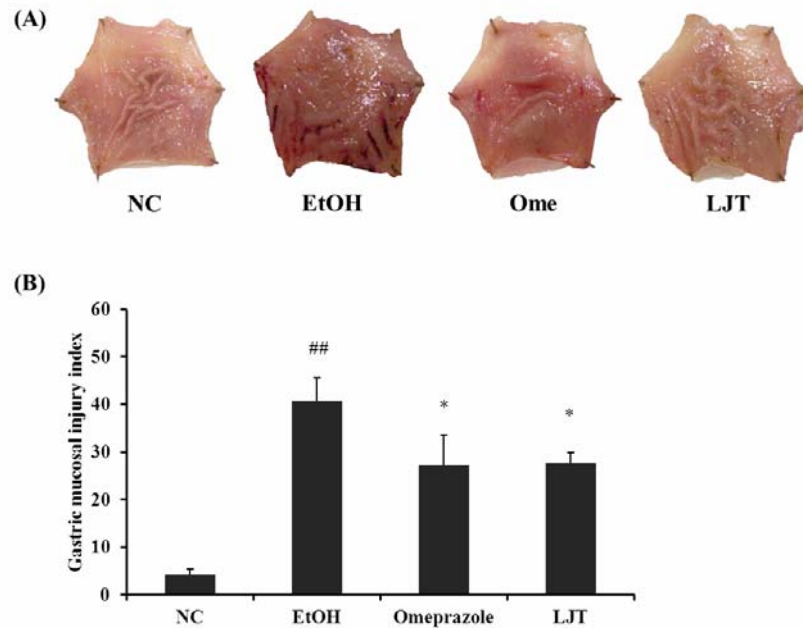
### LJT reduced the lipid peroxidation and increased GSH

The concentration of MDA, an end product of lipid peroxidation, in the EtOH group ( $13.32 \pm 1.32$  nmol/mg protein,  $P < 0.05$ ) was markedly increased compared with the control group ( $10.09 \pm 0.66$  nmol/mg protein). The omeprazole group had a significantly lower MDA concentration ( $8.73 \pm 1.08$  nmol/mg protein,  $P < 0.01$ ) than the EtOH group. Also, LJT-treated group was observed the significant reduction ( $11.62 \pm 0.67$ ,  $P < 0.05$ ) compared with the EtOH group (Figure 2A). In contrast to the MDA responses, the GSH contents of gastric tissue was significantly less in the EtOH group ( $32.30 \pm 1.09$   $\mu$ mol/mg protein,  $P < 0.01$ ) than in the control group ( $45.99 \pm 2.05$   $\mu$ mol/mg protein). On the other hand, the GSH content of gastric tissue was significantly greater in the LJT group ( $46.44 \pm 10.82$   $\mu$ mol/mg protein,  $P < 0.05$ ) than in the EtOH group (Figure 2B).

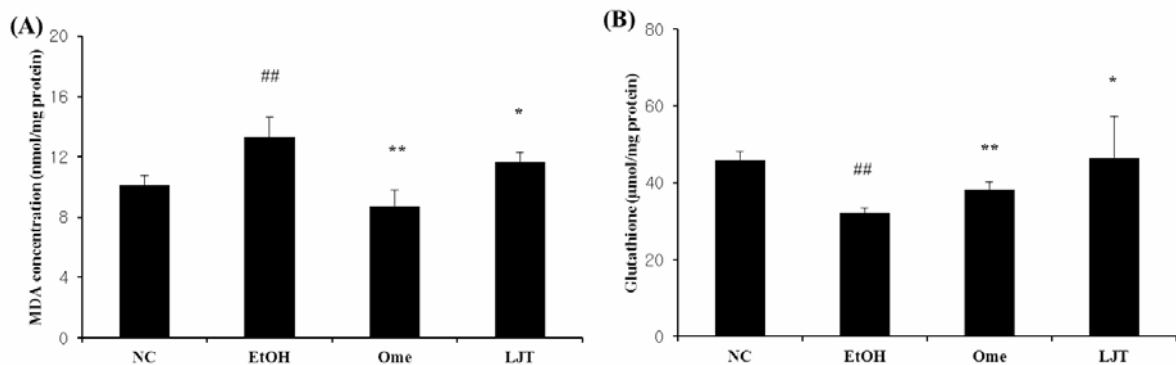
### LJT increased the activities of antioxidant enzymes

As presented in Figure 3A, SOD activity in the EtOH group ( $2.39 \pm 0.35$  U/mg protein,  $P < 0.01$ ) was significantly less compared with the control group. SOD activity was significantly greater in the LJT group ( $3.10 \pm 0.41$  U/mg protein,  $p < 0.01$ ) than in the EtOH group. The responses of the other antioxidant enzymes were consistent with the responses in SOD

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**Figure 1:** Representative photographs (A) and quantitative analysis (B) of gastric mucosa with absolute ethanol-induced gastric injuries. Absolute ethanol induced hemorrhages and hyperemia in gastric mucosa. In contrast, LJT attenuated ethanol-induced gastric mucosal injury. Each bar represent the mean ± SD for six rats. ##Significant difference at  $P < 0.01$  compared to the control group. \*Significant difference at  $P < 0.05$  compared to the EtOH group.



**Figure 2:** Effects of LJT on gastric MDA concentration (A) and GSH contents (B) of gastric tissues exposed to absolute ethanol. Each bar represents the mean ± SD for six rats. ##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.

activity (Figure 3B, C, and D). The activities of GST, GPx and GR in the EtOH group were  $8.63 \pm 1.34$  U/mg protein ( $P < 0.05$ ),  $55.12 \pm 4.83$  U/mg protein ( $P < 0.01$ ) and  $57.34 \pm 6.06$  U/mg protein, respectively, and were markedly lower than those of the control group. However, in the LJT group, the activities of GST ( $11.00 \pm 1.27$  U/mg protein,  $P < 0.01$ ), GPx ( $70.36 \pm 6.82$ ,  $P < 0.01$ ) and GR ( $71.25 \pm 5.93$ ,  $P < 0.05$ ) were greater than those of the EtOH group.

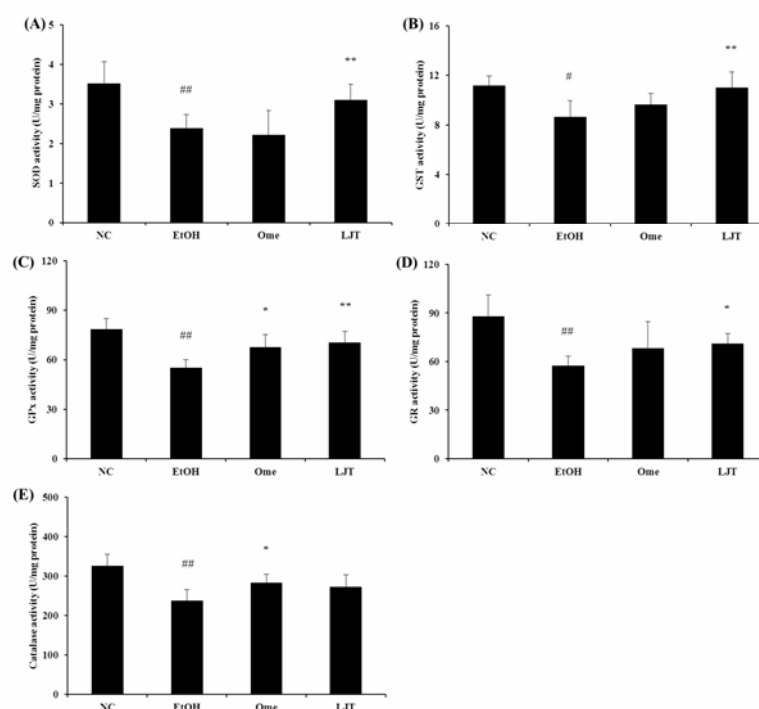
Catalase activity in the EtOH group ( $238.13 \pm 28.07$  U/mg protein,  $P < 0.01$ ) was less than that of the control group ( $326.23 \pm 29.08$  U/mg protein). However, catalase activity in the LJT group ( $272.47 \pm 31.44$  U/mg protein) did not differ from that in the EtOH group (Fig. 3E).

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## Discussion

We evaluated the protective effects of LJT against absolute ethanol-induced gastric injury in rats. Absolute ethanol induced gastric lesions and caused hemorrhage, hyperemia, and increased lipid peroxidation in gastric tissue. It also decreased the antioxidant enzymes of gastric tissue, including SOD, GST, GR, GPx, catalase, and GSH. However, LJT decreased the severity of ethanol-induced gastric lesions. It also reduced lipid peroxidation and increased levels of antioxidant enzymes in gastric tissue that was exposed to ethanol.

Lipid peroxidation refers to the oxidative degradation of lipids. It is induced by free radicals, which attack polyunsaturated fatty acids in cell membranes, producing MDA, the end product of lipid peroxidation (Moore and Roberts, 1998). According to Theophile et al. (2006) modulation of lipid peroxidation is a good indication that a plant possesses antioxidant properties. In present study, absolute ethanol induced an increase in MDA level in gastric tissue compared with the controls. However, LJT attenuated the ethanol-induced increase in MDA level. These results are in agreement with previous reports that demonstrated a significant reduction in lipid peroxidation after administration of absolute ethanol (Ozdil et al., 2004; El-Moselhy et al., 2009).



**Figure 3:** Effects of LJT on SOD (A), GST (B), GPx (C), GR (D), and catalase (E) activities of gastric tissue exposed to absolute ethanol. #Significant difference at  $P < 0.05$  and ##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.

Glutathione is an antioxidant and prevents ROS from damaging important cellular components. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. GSH donates a reducing equivalent to ROS, forming GSSG. Thus, the level of GSH is considered indicative of oxidative stress (Suresh et al., 1999; Yang et al., 2008). As in previous studies, administration of absolute ethanol decreased the level of GSH in gastric tissue, but LJT attenuated the ethanol-induced reduction in GSH. This indicates that LJT reduces ethanol-induced oxidative stress.

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes including SOD, catalase, GST, GPx, and GR (Sies, 1997). These antioxidant enzymes induce direct or indirect enzymatic processes that decrease oxidative stress caused by ROS in cells. SOD catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide and catalase is catalyzes the conversion of hydrogen peroxide to water and oxygen (Bannister et al., 1987; Chelikani et al., 2004). GST catalyzes the conjugation of GSH with many xenobiotics and their reactive metabolites and GR reduces glutathione disulfide (GSSG) to the sulfhydryl form, GSH, which is an important cellular antioxidant (Meister, 1998; Sheehan et al., 2001). GPx reduces lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water (Muller et al., 2007). In this study, oral administration of absolute ethanol notably decreased the activities of SOD, GST, GPx, and GR, but administration of LJT significantly elevated the activities of these enzymes in gastric tissue. Although LJT did not significantly increase catalase activity; however, the lack of an increase in catalase activity may reflect a lack of substrate

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for this enzyme (Bilici et al., 2002). Our results for antioxidant enzymes are in good agreement with those of recent studies on the gastroprotective effects of antioxidants against absolute ethanol-induced gastric injury (Ozdil et al., 2004; El-Moselhy et al., 2009). Thus, our data may be interpreted as signifying that LJT possesses the ability to induce the antioxidant defense system.

Ethanol-induced acute gastric lesions are characterized by pathological alterations such as hemorrhage, edema, and ulceration (Medeiros et al., 2008; Silva et al., 2009). In our study, absolute ethanol induced pathological changes such as hemorrhage and hyperemia in the gastric tissue. On the other hand, LJT administration reduced these ethanol-induced pathological alterations in the gastric mucosa according to macroscopic examinations.

In conclusion, this study shows that the protective effect of LJT against ethanol-induced gastric injury is caused in part by the antioxidant activity of LJT. Our results strongly suggest that LJT may be a useful agent for treatment of gastric injuries.

## Acknowledgements

This study was part of a project (Evidence-Based Medicine for Herbal Formulas) funded by the Herbal Medicine EBM Research Center of the Korea Institute of Oriental Medicine. In-Sik Shin<sup>#</sup>, Mee-Young Lee contributed equally to the work.

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