Regular Article

Protective Effects of Manassantin A against Ethanol-Induced Gastric Injury in Rats

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Manassantin A, a neolignan isolated from *Saururus chinensis*, is a major phytochemical compound that has various biological activities, including anti-inflammatory, neuroleptic, and human acyl-CoA : cholesterol acyltransferase (ACAT) inhibitory activities. In this study, we investigated the protective effects of manassantin A against ethanol-induced acute gastric injury in rats. Gastric injury was induced by intragastric administration of 5 mL/kg body weight of absolute ethanol to each rat. The positive control group and the manassantin A group were given oral doses of omeprazole (20 mg/kg) or manassantin A (15 mg/kg), respectively, 1 h prior to the administration of absolute ethanol. Our examinations revealed that manassantin A pretreatment reduced ethanol-induced hemorrhage, hyperemia, and epithelial cell loss in the gastric mucosa. Manassantin A pretreatment also attenuated the increased lipid peroxidation associated with ethanol-induced acute gastric lesions, increased the mucosal glutathione (GSH) content, and enhanced the activities of antioxidant enzymes. The levels of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β were clearly decreased in the manassantin A-pretreated group. In addition, manassantin A pretreatment enhanced the levels of cyclooxygenase (COX)-1, COX-2, and prostaglandin E_2 (PGE_2) and reduced the inducible nitric oxide synthase (iNOS) overproduction and nuclear factor kappa B (NF-κB) phosphorylation. Collectively, these results indicate that manassantin A protects the gastric mucosa from ethanol-induced acute gastric injury, and suggest that these protective effects might be associated with COX/PGE_2 stimulation, inhibition of iNOS production and NF-κB activation, and improvements in the antioxidant and anti-inflammatory status.

Key words manassantin A; ethanol; gastric injury; antioxidant; anti-inflammatory

Gastric ulcer is a major gastrointestinal disorder, affecting approximately 8–10% of the world's population.[1] The disease, which has a multifactorial etiology, is caused by an imbalance between the harmful and gastro-protective factors of the gastric mucosa.[2,3] Other factors, such as excessive drinking, a poor diet, a stressful lifestyle, smoking, *Helicobacter pylori*, and excessive ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) can contribute to the pathogenesis of gastric ulcers.[4]

The basic components of gastric mucosal defense include an intact mucus barrier, adequate mucus secretion and mucosal blood flow, prostaglandins, and the activities of antioxidant and anti-inflammatory compound.[5] The drug treatments used against gastric ulcer typically seek to either counteract aggressive factors or stimulate mucosal defenses. Numerous drugs have been used to treat gastric ulcers, including antacids, histamine receptor antagonists, muscarinic antagonists, and proton pump inhibitors.[6] However, many of these drugs not only produce undesirable adverse effects, including arrhythmia, hematopoietic disorders, hypersensitivity, impotence, and gynecomastia[7] they are also expensive.[8,9] Therefore, researchers are continuing to seek new agents for the management of gastrointestinal diseases that may present fewer side effects while also being safer, more efficient and less expensive.

Saururus chinensis (Saururaceae) is a perennial herb that is distributed throughout China and southern Korea. Its aerial portion has been used in Korean folk medicine for the treatment of edema, gonorrhea, jaundice, and several inflammatory diseases.[10] Studies have shown that *S. chinensis* has various activities, including anti-inflammatory, anti-oxidant,[11,12] anti-angiogenic,[13] anti-asthmatic,[14] and anti-hypertensive[15] activities, as well as therapeutic activity against atopic dermatitis.[16] Among the bioactive compounds isolated from *S. chinensis*, manassantin A is a major phytochemical composition that has various biological activities, including anti-inflammatory, neuroleptic, and human acyl-CoA : cholesterol acyltransferase (ACAT) inhibitory activities.[17] Multiple reports have also described manassantin A as having various beneficial effects, including the abilities to reduce hyperpigmentation disorders, prevent atherosclerosis, and lower cholesterol.[17–19] However, the potential anti-ulcer activity of manassantin A has not previously been reported. In the present study, we evaluated the protective effect of manassantin A on the gastric mucosa of rats subjected to ethanol-induced gastric ulceration.

MATERIALS AND METHODS

General Procedures Melting points were measured using an Electrothermal 9100 capillary melting point apparatus (Essex, U.K.). Optical rotations were measured using

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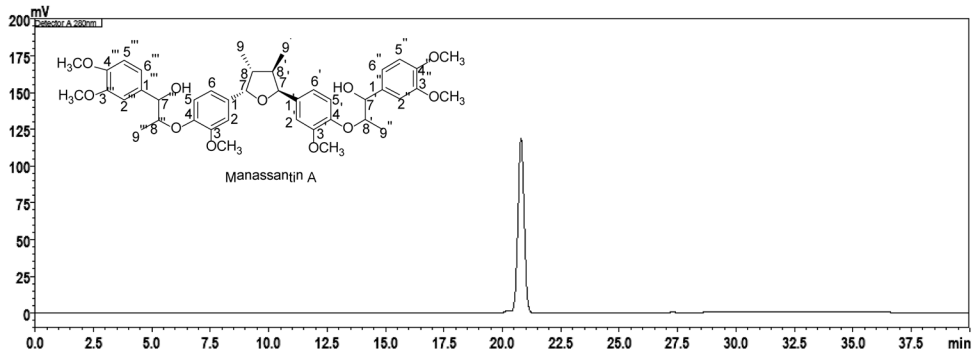

Fig. 1. Structure of Manassantin A

a JASCO DIP-1000 automatic digital polarimeter (Tokyo, Japan). The NMR spectra were recorded on a Bruker 250 MHz spectrometer (DMX 250, Karlsruhe, Germany) using the manufacturer's standard pulse program. Samples were dissolved in deuterated chloroform ($CDCl_3$-d_1), and chemical shifts were reported in ppm downfield from tetramethyl silane (TMS). FAB-MS was performed using a JMS700 spectrometer (JEOL, Japan). Column chromatography was performed using Silica gel 60 (70–230 and 230–400 mesh) and TLC plates (Silica-gel 60 F254, 0.25 mm) purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation and by spraying with 10% H_2SO_4 followed by heating.

Plant Material The roots of *S. chinensis* were purchased in February 2003 from a folk medicine market called Yakryong-si (Daegu, Republic of Korea). This material was taxonomically confirmed by Professor Ki-Hwan Bae at Chungnam National University (Daejeon, Republic of Korea). A voucher specimen (YNSC2004) has been deposited at the College of Pharmacy at Yeungnam University (Gyongsan, Republic of Korea).

Isolation of Manassantin A Manassantin A was isolated from the ethyl acetate fraction of *S. chinensis* by open silica gel chromatography. Briefly, the dried roots of *S. chinensis* (9.7 kg) were extracted with 70% methanol by reflux for 24 h. The methanol extract (1.0 kg) was suspended in distilled water (1.4 L), and the resulting water layer was successively partitioned with *n*-hexane, ethyl acetate, and butanol (each 1.4 L×3). The ethyl acetate fraction (130 g) was subjected to silica gel column separation (60×12 cm, Silica-gel 70–230 mesh; Merck, Darmstadt, Germany), and the desired sample was eluted with *n*-hexane–ethyl acetate (stepwise gradient from 100% *n*-hexane to 100% ethyl acetate followed by ethyl acetate-methanol (stepwise gradient from 100% ethyl acetate to 100% methanol). The eluates (500 mL in each flask) were combined on the basis of TLC. Consequently, 39 fractions (SCE1-39) were obtained. Manassantin A was obtained as a single compound in SCE34 [8.2 g, amorphous powder, *n*-hexane : ethyl acetate=2 : 8, *Rf*: 0.25, mp 80–82°C, $[\alpha]_D^{25}$: −107.6° (c=0.64, $CHCl_3$)]. The chemical structure of manassantin A (Fig. 1) was determined by comparison to the spectroscopic data, including NMR and MS, reported in the literature.[20]

Animals Specific-pathogen-free (SPF) male Sprague-Dawley rats (6–7 weeks old; 200–250 g) were purchased from Orient Bio (Republic of Korea) and used after one week of quarantine and acclimatization. The animals were housed under environmentally controlled conditions (22±2°C; relative humidity, 50±5%) with a 12 h light/dark cycle. The rats were provided with a standard rodent chow and sterilized tap water *ad libitum*. The experiment procedures were conducted in compliance with the National Institutes of Health Guide for care and use of the laboratory animals, and were approved by the Animal Experimental Ethics Committee of Chungnam National University.

Ethanol-Induced Gastric Injury Acute gastric lesions were induced *via* intragastric administration of absolute ethanol according to the previously described method.[21] The animals were randomly divided into four groups (n=6–7 per group), fasted for 24 h with free access to drinking water, and then orally pretreated with phosphate buffered saline (PBS; 5 mL/kg body weight, normal control and ethanol-treated group), omeprazole (20 mg/kg body weight; Sigma-Aldrich, U.S.A.), or manassantin A (15 mg/kg body weight). One hour later, the rats (with the exception of those in the normal control group) were orally treated with absolute ethanol (100% ethanol, 5 mL/kg body weight). The utilized dosage of manassantin A was selected based on the results of a preliminary dosing experiment performed on rats subjected to ethanol-induced ulceration. All animals were sacrificed *via* cervical dislocation 1 h after the absolute ethanol treatment.

Macroscopic Evaluation of the Gastric Ulcer Area Stomachs were rapidly removed, opened along the greater curvature, and rinsed with cold saline to remove the gastric contents and blood clots. The flattened stomach samples were photographed, and the ulcer area (UA, mm^2) was measured using image analysis software (ImageJ 46a; NIH, U.S.A.). The percentage of the UA relative to the total stomach area was determined. The inhibition percentage was calculated using the following formula: [(UA control−UA treated)/UA control)]×100%.

Histopathological Examination For histological evaluation, specimens of the gastric walls were fixed with 10% buffered formalin, processed, sectioned (4-μm) and stained with hematoxylin and eosin (H&E).

Measurement of Glutathione (GSH), Superoxide Dismutase (SOD), Malonaldehyde (MDA), and Catalase (CAT) Frozen stomach tissue samples were cut into small pieces, homogenized with the appropriate tissue lysis buffer for each assay, and then centrifuged at 12000×g and 4°C for 10 min. The concentration of protein in each supernatant was determined by the Bradford method, with bovine serum albumin (BSA) used as the standard. The supernatants were then assayed for CAT activity, SOD activity, and the levels of GSH and MDA, using commercial assay kits according to the

manufacturer's instructions (Cell Biolabs, U.S.A.).

RNA Isolation and Real-Time Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Total RNA was extracted using an RNeasy mini kit (Qiagen, U.S.A.). The concentration of RNA was determined based on the absorbance at 260 nm, and the purity was evaluated by measuring the A_{260}/A_{280} ratio. cDNA was generated from 1 μg total RNA using a reverse transcription kit (Qiagen) according to the manufacturer's instructions. PCR was performed in an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, U.S.A.) using the SYBR Green PCR Master Mix (Life Technologies) as recommended by the manufacturer. The utilized PCR primers were as follows: interleukin (IL)-1β, 5′-AGG ACC CAA GCA CCT TCT TT-3′ (forward) and 5′-AGA CAG CAC GAG GCA TTT T-3′ (reverse); tumor necrosis factor-α (TNF-α), 5′-GTC TGT GCC TCA GCC TCT TC-3′ (forward) and 5′-CCC ATT TGG GAA CTT CrC CT-3′ (reverse); IL-6, 5′-TAG TCC TTC CTA CCC CAA CT-3′ (forward) and 5′-TTG GTC CTT AGC CAC TCC TT-3′ (reverse); cyclooxygenase (COX)-1, 5′-TGA CTA TCT GGC GGG TGA CT-3′ (forward) and 5′-CTT GCT GGA CAT TGG GTT CT-3′ (reverse); COX-2, 5′-CGG ACT TGC TCA CTT TGT TG-3′ (forward) and 5′-CTC TCT GCT CTG GTC AAT GG-3′ (reverse); and glyceraldehyde-3-phosphatedehydrogenase (GAPDH), 5′-ACA GCA ACA GGG TGG TGG AC-3′ (forward) and 5′-TTT GAG GGT GCA GCG AAC TT-3′ (reverse). The PCR data were analyzed using the Applied Biosystems 7500 Real-Time PCR System software, and the fold change in the cDNA expression of the target gene relative to the endogenous control (β-actin) was calculated using the $2^{-\Delta\Delta Ct}$ method.

Determination of Prostaglandin E_2 (PGE_2) PGE_2 levels were determined in stomach tissues obtained from rats subjected to ethanol-induced gastric ulceration. The stomach tissue samples were homogenized and centrifuged, and the supernatant was subjected to determination of PGE_2 using a commercially available kit (Cayman, U.S.A.). The optical density was measured at 450 nm, and the results were expressed as ng/mg protein.

Nuclear and Cytosolic Protein Extraction Stomach was homogenized in lysis buffer containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg Aprotinin, 10 μg Leupeptin and 10 mM DTT). Samples were then allowed to incubate for 30 min on ice and were then centrifuged at 1200×***g*** for 5 min at 4°C. The supernatants (cytosolic fraction) were removed and frozen at −70°C. The remaining pellet was resuspended in 100 μL of ice-cold resuspension buffer (10 mM HEPES, pH 7.9; 10 mM $MgCl_2$; 0.3 mM EDTA and 10% glycerol) and protease inhibitors as described above. The pellets were completely resuspended by pipetting and were incubated for 20 min on ice with gentle resuspension of the pellets every ten minutes. After incubation, the pellets were centrifuged at 25000×***g*** for 30 min at 4°C. The supernatant was collected (nuclear fraction) and frozen at −70°C. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.).

Western Blotting Equal amounts of total gastric proteins (35 μg) were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes at 30 V for 2 h. The membranes were blocked for 1 h with Tris-buffered saline containing 0.05% Tween 20 (TBST) plus 5% skim milk and were incubated with anti-inducible nitric oxide synthase (iNOS; Cell Signaling Technology, U.S.A.; 1 : 1000), nuclear factor kappa B p65 (NF-κB p65; Cell Signaling Technology; 1 : 1000), or anti-β-actin (Sigma Chemical Co.; 1 : 1500) antibodies overnight at 4°C. The membranes were washed three times with TBST, and then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were again washed three times with TBST, and then developed using an enhanced chemiluminescence kit (Thermo Scientific, U.S.A.).

Statistical Analysis The data are expressed as the mean±standard error of the mean (S.E.M.). The statistical significance of each between-group difference was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. $p<0.05$ was considered significant.

RESULTS

Effects of Manassantin A on Ethanol-Induced Acute Gastric Injury As shown in Fig. 2A, no macroscopic lesion was observed in the normal control group. Intragastric administration of ethanol induced severe hemorrhagic ulcers with elongated-band erosions in the glandular portion of the stomach (Fig. 2B), whereas pretreatment with omeprazole or manassantin A attenuated these ethanol-induced gastric mucosal injuries (Figs. 2C, D). The UA of the ethanol-treated group was 116.7±29.7 mm^2, while the UA of rats pretreated with omeprazole or manassantin A were 49.8±23.0 mm^2 (57% inhibition) and 51.8±32.2 (56% inhibition), respectively (Table 1).

Histopathological examination revealed that the ethanol-treated group exhibited hemorrhage and the loss of gastric mucosa from the stomach tissue (Fig. 3). However, the omeprazole- or manassantin A-pretreated groups both showed reductions in the acute gastric damage induced by absolute ethanol (Fig. 3).

Effects of Manassantin A on MDA, GSH, CAT, and

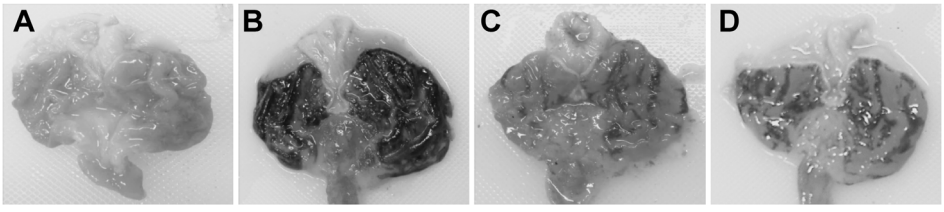

Fig. 2. Effects of Manassantin A on the Macroscopic Appearance of the Gastric Mucosa in Rats Subjected to Ethanol-Induced Gastric Mucosal Lesions

(A) Normal control group. (B) Ethanol-treated group. (C) Omeprazole (20 mg/kg)+ethanol-treated group. (D) Manassantin A (15 mg/kg)+ethanol-treated group.

SOD The MDA concentration was higher in the ethanol-treated group (138.8±20.2 μmol/mg protein) than in the normal control group (65.0±12.4 μmol/mg protein, Fig. 4A). However, the MDA concentration was significantly lower in the omeprazole (82.2±9.4 μmol/mg protein)- or manassantin A (68.9±24.4 μmol/mg protein)-pretreated groups compared with the ethanol-treated group (Fig. 4A).

In contrast to the MDA results, the GSH content in the stomach tissues of ethanol-treated rats (0.1±0.0 μmol/mg protein) was significantly lower than that in the normal control group (0.3±0.0 μmol/mg protein), while those of omeprazole (0.3±0.1 μmol/mg protein)- or manassantin A (0.3±0.1 μmol/mg protein)-pretreated rats were higher than that of the ethanol-treated group (Fig. 4B). The CAT activity in the ethanol-treated group (284.5±65.0 U/mg protein) was lower than that in the normal control group (623.1±223.9 U/mg protein). However, the omeprazole (668.9±202.7 U/mg protein)- or manassantin A (564.3±124.5 U/mg protein)-pretreated rats showed significant increases in CAT activity compared with the ethanol-treated group (Fig. 4C). Similarly, the SOD activity increased to a greater extent in the omeprazole (3.2±0.6 U/mg protein)- or manassantin A (5.3±1.0 U/mg protein)-pretreated groups compared with the ethanol-treated group (1.6±0.2 U/mg protein) (Fig. 4D).

Effects of Manassantin A on the mRNA Expression Levels of TNF-*α*, IL-6, and Il-1*β* RT-qPCR revealed that

Table 1. Effects of Manassantin A on Gastric Ulcer Area (UA, mm^2) and Percentage Inhibition

Group	Treatment	UA (mm^2)	Inhibition (%)
NC	PBS/PBS	0.0±0.0	—
EtOH	PBS/Ethanol	116.7±29.7##	—
OME+EtOH	Omeprazole/Ethanol	49.8±23.0**	57
Man A+EtOH	Manassantin A/Ethanol	51.8±32.2**	56

The results were expressed as the mean±S.E.M. ##$p<0.01$ compared to the NC group. **$p<0.01$ compared to the EtOH group.

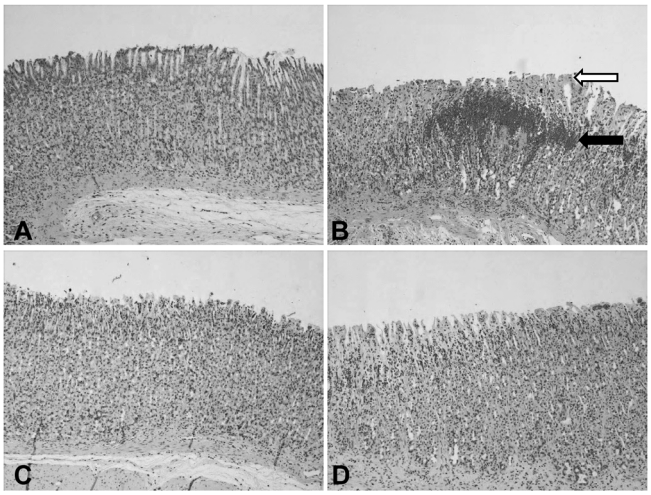

Fig. 3. Effects of Manassantin A on the Microscopic Appearance of the Gastric Mucosa in Rats Subjected to Ethanol-Induced Gastric Mucosal Lesions

(A) Normal control group. (B) Ethanol-treated group. (C) Omeprazole (20 mg/kg)+ethanol-treated group. (D) Manassantin A (15 mg/kg)+ethanol-treated group. No disturbance to the gastric mucosa is observed in the normal control group, whereas the ethanol-treated group shows severe destruction of the surface epithelium and necrotic lesions. The black arrow indicates severe hemorrhage and disruption of the deep mucosal layer. The white arrow indicates disruption of the surface epithelium (magnification ×10).

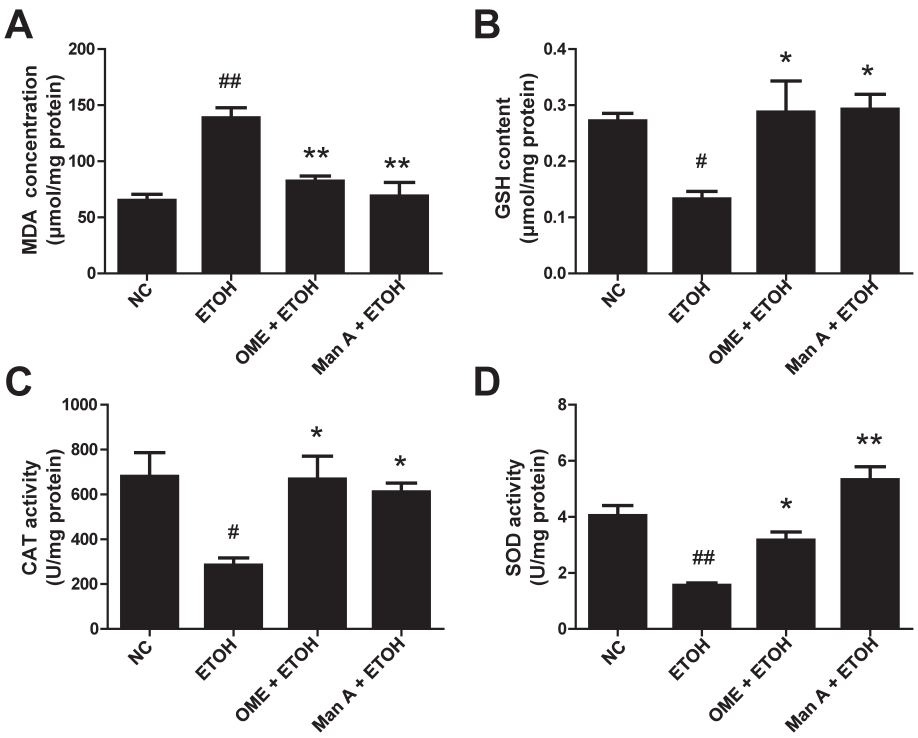

Fig. 4. Effects of Manassantin A on Gastric MDA, GSH, CAT, and SOD in Rats Subjected to Ethanol-Induced Gastric Injury

(A) MDA concentration. (B) GSH content. (C) CAT activity. (D) SOD activity. Abbreviations: NC, normal control group; EtOH, ethanol-treated group; OME+EtOH, omeprazole (20 mg/kg)+ethanol-treated group; and Man A+EtOH, manassantin A (15 mg/kg)+ethanol-treated group. Data are expressed as the mean±S.E.M. #$p<0.05$ and ##$p<0.01$, respectively, compared to the NC group; *$p<0.05$ and **$p<0.01$, respectively, compared to the EtOH group.

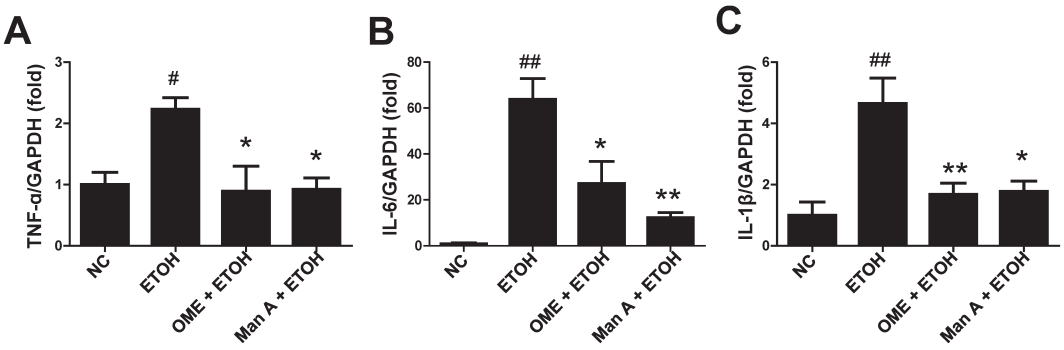

Fig. 5. Effects of Manassantin A on the Levels of Pro-inflammatory Cytokines in Gastric Tissues

(A) The expression of TNF-α in gastric tissues. (B) The expression of IL-6 in gastric tissues. (C) The expression of IL-1β in gastric tissues. Abbreviations: NC, normal control group; EtOH, ethanol-treated group; OME+ETOH, omeprazole (20 mg/kg)+ethanol-treated group; and Man A+EtOH, manassantin A (15 mg/kg)+ethanol-treated group. Data are expressed as the mean±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$, respectively, compared to the NC group; $^{*}p<0.05$ and $^{**}p<0.01$, respectively, compared to the EtOH group.

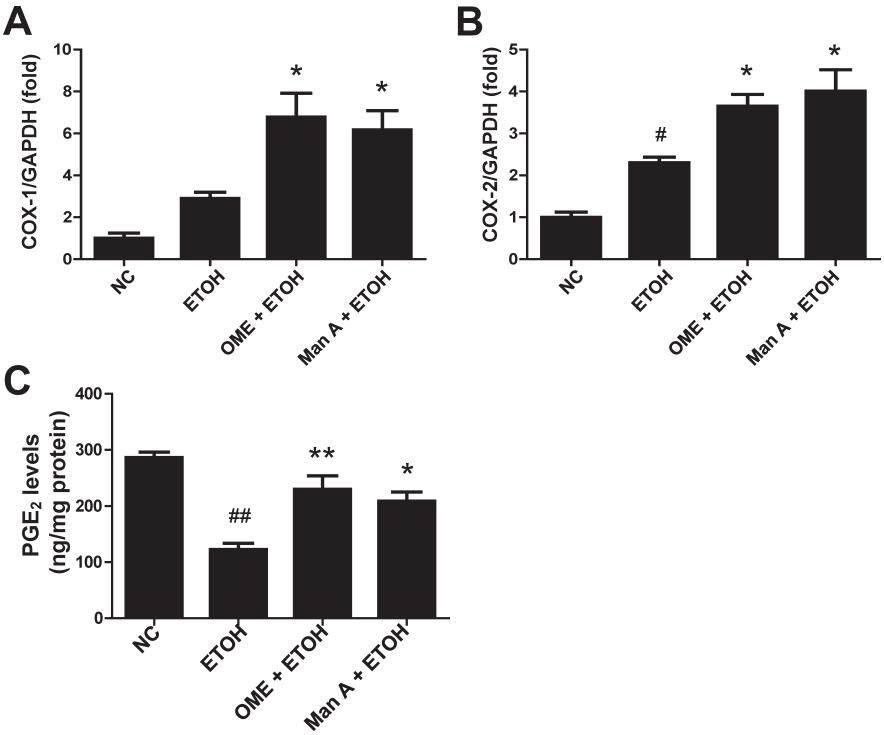

Fig. 6. Effects of Manassantin A on the Levels of COX-1 and COX-2 and the Production of PGE_2 in Rats Subjected to Ethanol-Induced Gastric Injury

(A) The expression of COX-1 in gastric tissues. (B) The expression of COX-2 in gastric tissues. (C) The expression of PGE_2 in gastric tissues. Abbreviations: NC, normal control group; EtOH, ethanol-treated group; OME+EtOH, omeprazole (20 mg/kg)+ethanol-treated group; and Man A+EtOH, manassantin A (15 mg/kg)+ethanol-treated group. Data are expressed as the mean±S.E.M. $^{\#}p<0.05$ and $^{\#\#}p<0.01$, respectively, compared to the NC group; $^{*}p<0.05$ and $^{**}p<0.01$, respectively, compared to the EtOH group.

ethanol treatment increased TNF-α expression in the gastric mucosa by 2.2-fold compared with that observed in the normal control group (Fig. 5A). The omeprazole (0.9-fold)- or manassantin A (0.9-fold)-pretreated rats showed significant reductions in this elevation of TNF-α, compared to the ethanol-treated group (Fig. 5A). The mRNA expression levels of IL-6 and IL-1β were also elevated after ethanol administration (63.8-fold and 4.7-fold, respectively), and these increases were significantly reduced by pretreatment with omeprazole (to 27.1-fold and 1.7-fold, respectively) or manassantin A (to 12.3-fold and 1.8-fold, respectively) (Figs. 5B, C).

Effects of Manassantin A on the Production of COX-1, COX-2, and PGE_2 The mRNA expression levels of COX-1 and COX-2 were elevated after ethanol administration (2.9-fold and 2.3-fold, respectively), and these increases were significantly enhanced by pretreatment with omeprazole (to 6.8-fold and 3.7-fold, respectively) or manassantin A (to 6.2-fold and 4.0-fold, respectively) (Figs. 6A, B). The levels of PGE_2 were clearly lower in rats subjected to intragastric administration of ethanol (122.7±22.7 ng/mg protein) compared with the normal control group (286.5±16.6 ng/mg protein), but this level was significantly increased by pretreatment with omeprazole (229.7±41.5 ng/mg protein) or manassantin A (209.0±28.2 ng/mg protein) (Fig. 6C).

Effects of Manassantin A on the Expression of iNOS Western blot results showed that the protein expression of iNOS was significantly increased after intragastric administration of ethanol. However, pretreatment with omeprazole or manassan-

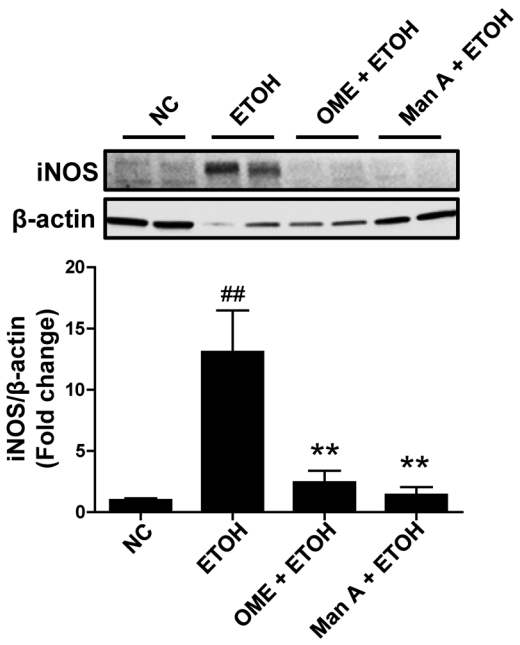

Fig. 7. Effects of Manassantin A on the Expression of iNOS in Rats Subjected to Ethanol-Induced Gastric Injury

Abbreviations: NC, normal control group; EtOH, ethanol-treated group; OME+EtOH, omeprazole (20 mg/kg)+ethanol-treated group; and Man A+EtOH, manassantin A (15 mg/kg)+ethanol-treated group. Data are expressed as the mean±S.E.M. $^{\#\#}p<0.01$ compared to the NC group; $^{**}p<0.01$ compared to the EtOH group.

tin A markedly reduced iNOS protein expression (Fig. 7).

Effects of Manassantin A on the Expression of NF-κB The nuclear translocation of NF-κB was increased after intragastric administration of ethanol, but it was decreased by pretreatment with omeprazole or manassantin A (Fig. 8).

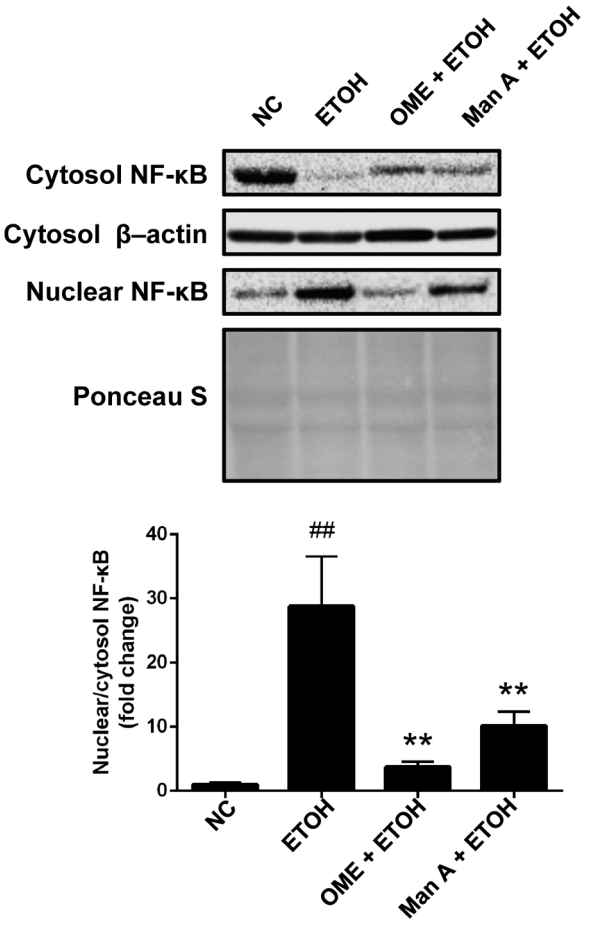

Fig. 8. Effects of Manassantin A on the Expression of NF-κB in Rats Subjected to Ethanol-Induced Gastric Injury

Abbreviations: NC, normal control group; EtOH, ethanol-treated group; OME+EtOH, omeprazole (20 mg/kg)+ethanol-treated group; and Man A+EtOH, manassantin A (15 mg/kg)+ethanol-treated group. Data are expressed as the mean±S.E.M. $^{\#\#}p<0.01$ compared to the NC group; $^{**}p<0.01$ compared to the EtOH group.

DISCUSSION

The present study investigated the gastroprotective effects of manassantin A. We found that oral administration of manassantin A effectively protected against absolute-ethanol-induced acute gastric mucosal injury in rats. Manassantin A-pretreated animals showed a lower gastric injury index, less lipid peroxidation, and increased anti-oxidant activities compared with rats that received ethanol alone. Moreover, pretreatment with manassantin A markedly reduced pro-inflammatory cytokine expression and promoted gastric PGE_2.

Ethanol-induced acute gastric lesions are characterized by pathological changes, such as hemorrhage, edema, inflammatory infiltration, and epithelial cell loss.[22,23] In the present study, a single oral administration of ethanol was found to cause acute gastric bleeding and hemorrhagic lesions in the rat stomach, confirming the detrimental effect of ethanol on the gastric mucosa. Manassantin A pretreatment markedly attenuated this gastric injury, however, suggesting that manassantin A protects against ethanol-induced gastric mucosal injury.

Alcohol causes severe oxidative stress in gastric tissue, which is manifested as an enhancement in lipid peroxidation that occurs *via* an increase in the MDA content and a decrease in the gastric GSH content.[24] Lipid peroxidation is a major outcome of free-radical-mediated injury, which causes immediate damage to the cell membrane and is related to DNA damage.[25] MDA is a final product of lipid peroxidation, and its level is commonly measured as an indication of lipid peroxidation levels in tissues.[26] GSH, the most abundant antioxidant in cells, plays a major role in the defense against oxidative stress-induced cellular injury and is essential for the maintenance of the intracellular redox balance.[27] Consistent with previous results, the administration of ethanol significantly increased the concentration of MDA and decreased that of GSH in the gastric tissues. Furthermore, the levels of antioxidant enzymes (CAT and SOD) were markedly reduced in the ethanol-treated group compared to the normal control group. CAT is a classical oxidative biomarker that exists mainly in the peroxisomes of all aerobic cells, and serves to protect the cells against damage from hydrogen peroxide.[28] SOD is a metalloenzyme that can convert the oxygen produced during oxidative stress to hydrogen peroxide.[29] Here, we observed that pretreatment with manassantin A prior to ethanol-induced acute gastric lesioning inhibited the increase in lipid peroxidation, increased the mucosal GSH content and enhanced the activities of the antioxidant enzymes, CAT and SOD, in a rat model. These results suggest that manassantin A can act as an antioxidant to further reduce ethanol-induced gastric injury.

In addition to generating ROS, ethanol administration

provokes an inflammatory response that releases numerous inflammatory cytokines, such as TNF-α, IL-6, and IL-1β.[30] TNF-α is a major inflammatory cytokine that is secreted by macrophages during inflammation and plays a key role in the induction of gastric mucosal damage.[31] TNF-α reduces the gastric microcirculation around an ulcer and delays gastric ulcer healing by potentiating the inflammatory response.[32] Elevated levels of IL-6 activate neutrophils, lymphocytes and monocytes/macrophages at the inflammatory site, which in turn initiates oxidative bursts, toxic metabolites, and the lysosomal enzymes responsible for local tissue damage in peptic ulcer disease.[33] IL-1β plays a crucial role in inducing the systemic acute-phase response and the release of other members of the pro-inflammatory cytokine cascade, such as TNF-α, platelet activating factor (PAF), prostaglandins and pro-inflammatory interleukins.[34] Our observation that pretreatment with manassantin A reverses the ethanol-induced increases in the mucosal mRNA expression levels of TNF-α, IL-6, and Il-1β indicates that the therapeutic effect of manassantin A on ethanol-induced gastric ulcer involves the inhibition of local inflammatory processes. This finding is consistent with the previous observation that manassantin A attenuates the lipopolysaccharide (LPS)-induced expressions of TNF-α, IL-6, and IL-1β in dendritic cells (DCs) and inhibits the activations of nuclear factor kappa B (NF-κB), extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinases (JNK).[35] Moreover, previous studies have shown that administration of manassantin A inhibits the TNF-α-induced expression of cell adhesion molecules by human umbilical vein endothelial cells[17] and the IL-6-induced activation of signal transducer and activator of transcription 3 (STAT3) in Hep3B cells.[36]

Gastric mucus, which is the first line of defense against acid, adheres with epithelium-secreted bicarbonate to serve as a barrier against self-digestion.[37] Among the humoral factors in the mucosa, PGE_2 is known to play an important protective role by stimulating the secretion of mucus and bicarbonate, maintaining the local blood flow, and increasing the resistance of epithelial cells against potential damage by cytotoxins.[38] Prostaglandins (PGs) were synthesized mainly through COX isoforms, COX-1 and COX-2.[39] COX-1 appears to be responsible for the production of PGs that is physiologically important for homeostatic functions, such as maintenance of the mucosal integrity and mucosal blood flow. Under physiological conditions, prostanoid synthesis depends upon the availability of arachidonic acid and the COX-1 activity.[40] While COX-2 is not constitutively expressed in most of the tissues but is dramatically up-regulated during inflammation and injury.[41] In our study, exposure to ethanol produced a significant fall in PGE_2 production in the gastric mucosa despite overexpression of COX-1 and COX-2. This result might be explained by other reports described that in the presents of oxidative damage, the PGs could be converted into products of oxidation such as 8-iso-PGF_2alpha.[42] In addition, oxidative stress could inhibit COX activity, thus reducing PG levels.[43] We further found that this fall in PGE_2 level was counteracted by manassantin A, indicating that PGE_2 were responsible for the putative beneficial effects of manassantin A in ethanol-induced mucosal injury. Based on the previous studies demonstrating PGE_2 was related to the up-regulation of COX-2, manassantin A-enhanced synthesis of PGE_2 might be related to the over-expression of COX-2. Our results were in agreement with recent report showing that ethanol-induced gastric lesions were characterized by increasing gastric COX-2 expression and decreasing PGE_2 synthesis, but the compound 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a classic inhibitor of anion transport, was beneficial in rat model of gastric injury through enhancing the expression of COX-2 and reversing the decrease of PGE_2.[44]

Nitric oxide (NO) is involved in the modulation of the gastric mucosal integrity and the role of endogenous NO in physiological processes has been established in many tissues, including the gastrointestinal tract. NO dilates blood vessels, increases blood flow and stimulates gastric angiogenesis in the healing process of ulcers.[45] NO also stimulates cell proliferation of the gastric mucosa and granulation tissue formation at the base of an ulcer.[45] Previous research reported that the enhanced ulcerogenic response is mediated by endogenous NO, mainly produced by iNOS.[46] In the present study, treatment with ethanol significantly increased expression of iNOS in gastric tissue when compared normal group. Pretreatment with manassantin A prominently decreased iNOS expression, suggesting that a reduction iNOS expression is possible involved in the gastroprotective action of manassantin A.

It is well known that NF-κB is a vital transcription factor which regulates many immune and acute phase inflammatory processes. Previous study has indicated that activated NF-κB triggers transcription up-regulation of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β.[47] Besides cytokine regulation, NF-κB has also been related to the expression of iNOS and the release of NO.[48] Therefore, to further investigate the effect of manassantin A against ethanol-induced gastric lesions, we first targeted the transcription factor NF-κB. Ethanol administration remarkably triggered the nuclear translocation of NF-κB, which was significantly suppressed by pretreatment of manassantin A. These results are consistent with previous studies showing that manassantin A is potent inhibitors of NF-κB activation by the suppression of transcriptional activity of RelA/p65 subunit of NF-κB[20] and manassantin A inhibits nitric oxide production by macrophages through the inhibition of NF-κB activation.[35] Therefore, it suggests that manassantin A has anti-oxidant and anti-inflammatory effects on ethanol-induced gastric lesions by regulation of NF-κB/iNOS or NF-κB/TNF-α pathway, respectively.

In conclusion, the present study provides the first evidence that manassantin A has potent gastroprotective effects against ethanol-induced gastric injury, as demonstrated by significant reductions in the ulcerated area. The remarkable gastroprotective effects of manassantin A appear to be at least partially mediated *via* its ability to reduce pro-inflammatory cytokines, stimulate PGE_2 secretion, inhibit iNOS and NF-κB, and attenuate oxidative stress.

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Conflict of Interest The authors declare no conflict of interest.

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