INTRODUCTION

The pathogenesis of gastric ulcer appears to be multifactorial, involving an imbalance between ‘aggressive’ (e.g. gastric acid and pepsin) and ‘defensive’ (e.g. prostaglandin production, mucus/mucosal barrier, bicarbonate production, blood flow and cell regeneration) factors. Historically, the management of peptic ulcer disease has focused on controlling gastric acid secretion (Shay et al., 1945). One of the greatest concerns is to ascertain whether H. pylori-induced gastritis may lead to gastric cancer. According to several epidemiological studies concerning the association between gastric cancer and H. pylori, the antibodies for H. pylori were increased and higher in patients with gastric cancer than in the control group (Kusters et al., 2006). Additionally, gastrointestinal lesions, such as gastric ulcers, and gastric cancers are strongly associated with H. pylori infection. One way in which it may be possible to prevent carcinogenesis would be to reduce ROS damage to cellular constituents, especially DNA. It has been demonstrated that eradication of H. pylori leads to a reduction in ROS activity in the gastric mucosa (Drake et al., 1998).

The immature fruits of Poncirus trifoliata (L.) (Rutaceae), Ponciri Fructus (PF), are widely used in Oriental medicine for uterine contraction, relaxation and cardiovascular diseases (Kim et al., 1997). Pharmacological studies of PF extracts show several biological activities such as antiplatelet (Teng et al., 1992) and antiinflammatory (Shin et al., 2006). In addition, this crude drug has a beneficial effect on type I hypersensitivity, IgE production and IgE-mediated local anaphylaxis (Kim et al., 1999). In addition, PF inhibits both tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) secretion from rat peritoneal mast cells stimulated by stem cell factors. These findings provide evidence that PF inhibits the chemotactic response and inflammatory cytokine secretion to stem cell factor in mast cells (Na et al., 2003).

Numerous compounds, including flavonoids, coumarin and alkaloids, were isolated from PF (Park et al., 2005), and it was found that the several coumarin derivatives are potent antiplatelet constituents (Chen et al., 1996). Methoxyhispidol A, which is isolated from PF, was suggested as an antitumor agent against human hepatocarcinoma cells by arresting the cell cycle and inducing apoptosis (Hong et al., 2008).

The aim of this study was to investigate the potential antiulcer and antiinflammatory activities of PF ethanol extract and several constituents isolated from PF.

MATERIALS AND METHODS

Materials. The dried fruits of P. trifoliata (Ponciri Fructus, PF) were purchased from Tokyo, Japan in March 2005. The ethanol extract of PF and isolated compounds were provided from Professor S. H. Lee, College of Pharmacy, Yeungnam University, Korea. A voucher specimen (YU-594) was deposited at the herbarium of the College of Pharmacy, Yeungnam University, Korea.

Keywords: Poncirus trifoliata; neohesperidin; poncirin; gastritis; antioxidant activity.
Fetal bovine serum (FBS), RPMI medium 1640 and Hank’s balanced salt solution were obtained from Gibco Co. (Grand Island, NY). Dantrolene sodium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), trypan blue, probenecid, dimethyl sulfoxide (DMSO), sodium bicarbonate, penicillin-streptomycin, trypsin-EDTA, cimetidine and ampicillin were obtained from Sigma Chemical Co. (St Louis, MO). HCl, ethanol and other solvents were purchased from Duksan Pure Chemical Co. Ltd (Kyunggi-do, Korea). Silica gel 60, Kieselgel 60, Kieselgel 77 and TLC plates were purchased from Merck Ltd (Darmstadt, Germany). All other reagents and solvents for extraction were of pharmaceutical or analytical grade.

Animals. Male Sprague-Dawley rats, weighing 180–200 g, were purchased from Samyook Animal Laboratories, Kyunggi-do, Korea, and were acclimatized to standard laboratory conditions (24 ± 2 °C, 55 ± 5% humidity and 12 h light/dark cycle) for 14 days in the animal facility in Dukuns Women’s University. The experimental procedures for rats were conducted in accordance with the Guidelines of the Care and Use of Laboratory Animals, Dukuns Women’s University. The animals were allowed free access to food (standard pellet diet) and water ad libitum. This study was carried out in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 1999-61) issued by the Korea Food and Drug Administration, the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies (Notification No. 2000-63) issued by the Korea Food and Drug Administration, and the Principles of Good Laboratory Practice issued by the Organization for Economic Cooperation and Development.

Preparing extract and isolation constituents from PF. The dried PF (10 kg) was extracted three times with 70% ethanol at room temperature. The PF ethanol extract was concentrated under reduced pressure to give a residue (500 g) and then was further fractionated between H2O and chloroform. The chloroform extract (160 g) was loaded on a silica gel column (80 ¥ 12 cm) and eluted with n-hexane/EtOAc (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, each 4 L) in a gradient mode to give 10 fractions (PF1–PF10). Fraction PF4 (9.4 g) was separated on a silica gel column (70 ¥ 6 cm) and eluted with n-hexane/acetone (9:1, 8:2, each 4 L) to give three fractions (PF41–PF43). Fraction PF42 was further separated over a reverse phase column (50 ¥ 4 cm) eluting with MeOH/H2O (6:4, 7:3, 8:2, 9:1, each 4 L) to give 3 fractions (PF421–PF423), and fraction PF422 was recrystallized in MeOH to afford aurapten (3.2 g, Fig. 1a). The H2O extract (80 g) was separated on a MCI column (60 ¥ 5 cm) and eluted with a gradient of MeOH/H2O (2:8, 3:7, 4:6, 5:5, 9:1, each 4 L) to give five fractions (PFW1–PFW5). Fractions PFW2 (8.5 g) and PFW4 (18.5 g) were recrystallized in MeOH/CHCl3 (1:1) to afford poncirin (4.5 g, Fig. 1b) and naringin (14.2 g, Fig. 1c), respectively. Fraction PFW5 (8.5 g) was chromatographed on a silica gel column (60 ¥ 5 cm) and eluted with a gradient of MeOH/CH2Cl2 (1.9, 1.5:8.5, 2.8, 3:7, 5:5, 7:3, each 4 L) to give 16 fractions (PFW11–PFW16). Fraction PFW14 was purified by HPLC to afford hesperidin (60 mg, Fig. 1d). Fraction PFW19 was chromatographed on a reverse phase column (50 ¥ 4 cm) eluting with MeOH/H2O (1:9, 3:7, 5:5, 9:1, each 4 L) to afford neohesperidin (80 mg, Fig. 1e). The compounds were identified by NMR and LC/MS and confirmed by comparison with its spectral data with those reported in the literature data (Vasconcelos et al., 1998; Okamura et al., 1994).

Drug administration in vitro and in vivo. For the in vitro assay, the samples were prepared as 50 mg/mL stock in DMSO, and diluted with adequate solvent for each assay. For in vivo administration, the samples dissolved in saline were administered at a volume of 0.5 mL per 100 g (body weight). Saline was given to the control group. The routes and timings of administration are mentioned in each experimental description.

Acid-neutralizing capacity. One gram of extracts/compounds was added to 100 mL of 0.1 N HCl and then incubated for 1 h at 37 °C with shaking. The acid-neutralizing capacity was determined by titrating with

Figure 1. Chemical structure of aurapten (a), poncirin (b), naringin (c), hesperidin (d) and neohesperidin (e).
0.1 n NaOH using methyl orange as an indicator. Hydro-
talcite and cimetidine were used as a positive control.

**Antioxidant activity.** One milliliter of DPPH (150 µM) in methanol was added to 4 mL of extracts/compounds (2.5–120 µg/mL), and then the mixture was stirred. After 30 min incubation at room temperature, the absorb-
ance of the mixture was read against a blank at 520 nm. Scavenging DPPH free radical as a percentage (I%) was calculated as follows:

$$I\% = \frac{(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}} \times 100$$

where $A_{\text{blank}}$ is the absorbance of the mixture without samples and $A_{\text{sample}}$ is the absorbance of the mixture with samples. The concentration providing 50% inhibi-
tion (IC50) was determined from the graph plotting the inhibition percentage against concentration. L-Ascorbic acid was used as a positive control.

**Anti-*H. pylori* activity.** The *H. pylori* strain (HP 43504) was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The inhibitory effect of PF ethanol extract and its constituents on the growth of *H. pylori* was investigated. Six hundred microliters of samples was mixed to 5.4 mL of brucella agar medium containing 7% horse serum in the petri dish. *H. pylori* (5 × 10⁶ CFU) was seeded in this media and then incu-
bated for 3 days at 37 °C in an incubator (AnaeroPak Campylo: 85% N₂, 10% CO₂, 5% O₂). Ampicillin was used as a positive control.

**Cell culture.** AGS and SNU638 human gastric cancer cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Gastric cancer cells were grown at 37 °C in a 5% CO₂ humidified incubator in MEM containing 10% FBS, 200 000 IU/L penicillin, 200 mg/L of streptomycin and 1 mx sodium pyruvate. After reaching confluence, the cells were subcultured by trypsinization. SNU and AGS cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum (which can inhibit trypsin) and were subdivided using 0.05% trypsin with 0.53 mM EDTA.

**Cytotoxicity assay.** Cell viability was assessed by the MTT method. The cells were seeded in 24-well plates at a density of 5 × 10⁵ cells/well. After 24 h incubation with sample, 100 µL of MTT (5 µg/L in H₂O) was added to every well and the plates were incubated for 4 h. Two hundred microliters of DMSO was added to every well and mixed by pipetting to dissolve MTT formazan completely. The relative cell viability was obtained by measuring the absorbance using an ELISA reader (Molecular Devices, Menlo Park, CA) at 540 nm.

**HCl/ethanol-induced gastric lesion.** The rats, which were fasted for 24 h with free access to water prior to the experiment, were administered orally with compounds (Mizui and Dodeuchi, 1983). After 30 min, 0.5 mL/100 g of a HCl/ethanol (150 mx HCl in 60% ethanol) solution was given orally for the induction of gastric lesions. One hour later, the animals were anesthetized with ether, and their stomachs were removed and fixed with 2% formalin for 10 min. The amount of hemorrhage on the glandular portion was measured by summing the total length (mm) of each lesion and expressed as a lesion index.

**Gastric secretion.** The rats were immediately administered with neohesperidin and poncirein or cimetidine intra-
duodenally (Shay et al., 1945). At 4 h after the pyloric ligation, the animals were killed, and the contents of the stomach were collected and centrifuged at 1050 × g for 10 min. The total volume of gastric juice and pH were measured, and an acid output (mEq/mL) was deter-
mined by titrating the gastric juice with 0.05 n NaOH using phenolphthalein as an indicator.

**Mucus secretion.** After the rats were fasted for 24 h before the experiment with free access to water, oral adminis-
tration of compounds was performed. Thirty minutes later, the absolute ethanol (1 mL/100 g) was given orally for induction of gastric lesions in rats. One hour later, the animals were killed and the secreted mucus was determined (Kitagawa et al., 1986). The glandular portion separated from the excised stomach was opened along the lesser curvature and everted. The stomach was soaked in 0.1% alcian blue 8GX dissolved in 0.16 m sucrose buffer with 0.05 m CH₃COONa (adjusted to pH 5.8 with HCl) for 2 h. The mucus combined with the alcian blue was extracted with 20 mL of 70% ethanol containing 30% dioctyl sodium sulfosuccinate and centrifuged for 10 min at 500 × g. The optical density of the supernatant was measured at 620 nm in an UV-spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA).

**Statistical analysis.** All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM), and were analysed using one-way analysis of variance (ANOVA) and Student-Newman-
Keul’s test for individual comparisons. Values of *p* < 0.05 are considered statistically significant.

**RESULTS**

Quantitative determination of DPPH-radical scavenging activities was performed for measuring the antioxidant activity of the PF extract and its constituents (Table 1). Neohesperidin had a significant free radical scavenging activity (IC50 = 22.31 µg/mL). The IC50 of L-ascorbic acid, a positive control, was <5 µg/mL. The PF ethanol extract and its other constituents showed low antioxidant activities with >160 µg/mL of IC50.

Hesperidin had slightly inhibitory effects on the growth of *H. pylori* as shown in Table 2. The PF ethanol extract and its other constituents did not have suppressive activity for colorization of *H. pylori*, which causes several gastric diseases.

<table>
<thead>
<tr>
<th>Table 1. Scavenging activities of 1,1-diphenyl-2-picrylhydrazyl radical by Ponciri Fructus extract and its constituents</th>
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<tr>
<td>Material</td>
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<tr>
<td>Ponciri Fructus 70% ethanol extract</td>
</tr>
<tr>
<td>Aurapten</td>
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<tr>
<td>Hesperidin</td>
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<td>Naringin</td>
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<td>Neohesperidin</td>
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<td>Poncirin</td>
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<td>L-Ascorbic acid</td>
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Cytotoxicity of the PF extract and its constituents was assessed by measuring the cell viability of AGS and SNU638 human gastric cancer cell lines. The PF 70% ethanol extract showed significant cytotoxicity against AGS gastric cancer cell (IC$_{50}$ = 85.39 μM) (Table 3). The PF 70% ethanol extract and its several compounds did not have a cytotoxic effect against the SNU638 gastric cancer cell line.

The acid-neutralizing capacity of the PF extract and its constituents was investigated by measuring the volume of NaOH consumption. The PF 70% ethanol extract inhibited NaOH consumption by approximately 11.38% compared with the control, which is similar to the inhibitory activity (12.94%) of cimetidine, as a positive control (Table 4). In addition, aurapten had a partial inhibitory activity (5.34%), whereas other constituents showed no acid-neutralizing capacity.

The effects of the PF constituents on the HCl/ethanol-induced lesion were investigated (Table 5). The lesion index of the control group was 66.7 ± 6.77 mm. Ponciri (50 mg/kg) slightly decreased the total acid output, but increased pH (Table 6). In addition, neohesperidin (100 mg/kg) significantly decreased the gastric secretion and acid output, and increased the pH, compared with the control. Ponciri (50 mg/kg) slightly decreased the total acid output, but increased pH (Table 6). In addition, neohesperidin (100 mg/kg) significantly decreased the mucus secretion model, even though ethanol reduced the amount of mucus secretion in the rats, neohesperidin (100 mg/kg) increased the mucus secretion.

DISCUSSION

Antioxidant action plays an important role for the inhibition of oxidation processes, which are involved in the mechanisms of several gastric disorders including ulceration (La Casa et al., 2000). The involvement of reactive oxygen species is well established in the pathogenesis of ischaemic injury of the gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol and the infection with H. pylori (Drake et al., 1998). Naringin and hesperidin, which are natural flavonoids, suppressed the generation of superoxide anion from the opsonized (50 mg/kg) significantly decreased the gastric secretion and acid output, and increased the pH, compared with the control. Ponciri (50 mg/kg) slightly decreased the total acid output, but increased pH (Table 6). In addition, neohesperidin (100 mg/kg) significantly decreased the mucus content to 148.3 ± 6.27 μg, which was almost the same as that of sucralfate, a positive control (Table 7). In the mucus secretion model, even though ethanol reduced the amount of mucus secretion in the rats, neohesperidin enhanced the mucus secretion.
zimysan-stimulated human neutrophils in inflammation (Zielińska-Przyjemska and Ignatowicz, 2008). As shown in Table 1, neohesperidin, which is a derivative of hesperidin, with free radical scavenging activity is expected to protect against gastric mucosal injury through the antioxidative mechanism.

*H. pylori* is the major cause of bacterial gastrointestinal infections. Also, it is believed to be a major cause of peptic ulcer (Gerrits et al., 2006) and gastric cancer (Matysiak-Budnik and Megraud, 2006). Antibiotic therapy, which uses the combination of two or three drugs, has been used widely to eradicate these infections. However, drug resistant bacteria necessitate new sources of drugs. Plants seem to be ideal sources of new antibacterial compounds. Indeed, the medicinal plants and their chemical components have been reported to have a potential benefit in eradicating such problems (Nostro et al., 2005). Phenolic phytochemicals such as cinnamic acids, cinnamaldehydes, coumarins, capsaicin, flavanoids and tannins, which are purified from peppers, wine and many other natural products, showed high anti-*H. pylori* activity (Bae et al., 1999). In patients with mucosal *H. pylori* infection, the eradication of this microorganism seems to cure both infection and ulcer disease (Marshall et al., 1988). Also, *H. pylori* infection increases free radical production, and a delicate balance between the generation of free radicals and endogenous as well as exogenous antioxidants such as superoxide dismutase (SOD) and ascorbic acid is critically important for physiological functions of cells. Hesperidin is cytotoxic for *H. pylori* so might decrease the risk of pathogen-derived gastritis through the inhibition of *H. pylori* survival (Table 2).

Gastric cancer is a leading cause of cancer-related deaths, being second only to lung cancer as a cause of death. For the prevention of gastric cancer disease, it is very important to develop a natural herbal medicine to act on the cancer cells directly. Dietary components including flavonoids and carotenoids have potentially beneficial effects on gastric cancers. Recently, the development of new anticancer drugs is a key issue for cancer chemotherapy because of the reality that cancer cells, which are resistant to chemotherapy, will eventually cause mortality. Herbal medicines as substitutes for cancer remedies have attracted a great deal of interest, because of their low toxicity and costs. As shown in Table 3, the PF ethanol extract is expected to have a suppressive effect on the AGS human gastric cancer cell line, in addition to other antibacterial biological functions.

| Table 7. Effects of neohesperidin and poncirin on mucus secretion from ethanol-induced gastric lesion |
| Material | Dose (mg/kg) | Mucus content (µg as alcian blue) |
| Control |  | 122.8 ± 7.91 |
| Neohesperidin | 50 | 118.9 ± 5.78 |
| | 100 | 148.33 ± 6.27a |
| Poncirin | 50 | 100.71 ± 6.18 |
| | 100 | 122.5 ± 5.63 |
| Sucrafate | 375 | 155.6 ± 8.23a |

The values are mean ± SEM of six animals. *p < 0.01, compared with the control group.

Table 6. Effect of neohesperidin and poncirin on gastric secretion in pylorus-ligated rats

| Material | Dose (mg/kg) | Volume (mL) | pH | Total acid output (mEq/4 h) |
| Control | – | 5.4 ± 2.53 | 0.7 ± 0.04 | 0.47 ± 0.02 |
| Neohesperidin | 50 | 3.1 ± 1.20a | 1.4 ± 0.02 | 0.19 ± 0.01b |
| | 100 | 7.2 ± 0.66 | 1.1 ± 0.03 | 0.66 ± 0.02 |
| Poncirin | 50 | 5.5 ± 1.23 | 1.4 ± 0.04 | 0.39 ± 0.03 |
| | 100 | 6.6 ± 1.97 | 1.2 ± 0.03 | 0.59 ± 0.02 |
| Cimetidine | 100 | 2.8 ± 1.35b | 2.7 ± 0.02 | 0.09 ± 0.01c |

Total gastric juice volume and pH were measured 4 h after the pyloric ligation.

The values are mean ± SEM of six animals.

*p < 0.05, b p < 0.01, a p < 0.001 compared with the control group.

The values are mean ± SEM of six animals. *p < 0.01, compared with the control group.
of gastric mucosal lesions by necrotizing agents such as HCl and ethanol is known to be associated with the depression of gastric defensive mechanisms (Kinosita et al., 1995). Therefore, regulators such as proton pump inhibitors for acid production accelerate the healing of gastric ulcers and gastritis by potent and long-lasting antisecretory actions (Welage, 2003), and are widely used in the clinic (Pisegna, 2002). Additionally, the anti-inflammatory activity of bioflavonoid hesperidin elucidated that the majority of antiinflammatory agents have deleterious actions on stomach mucosa through inhibition of nitric oxide (NO) and inflammatory mediator production, such as tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) (Rao et al., 2008). Plant-derived naringin was effective in the ethanol- and pylorus ligated ulcer models in rats (Borreli and Izzo, 2000). Tables 6 and 7 suggest that the mechanism of neohesperidin, showing antiagastic ulcer and antiagastritis effects, may be due to the suppression of aggressive factors such as the inhibition of gastric acid secretion and, in turn, the augmentation of defensive factors such as proton pump inhibitors. Therefore, the gastroprotective activity of neohesperidin seems to originate from the stimulation of mucus secretion.

In this study, the PF ethanol extracts showed acid-neutralizing capacities and cytotoxicity against the human AGS gastric cancer line. Neohesperidin and poncirin isolated from PF had a potent protective effect against HCl/ethanol-induced gastric mucosal lesions, and this effect partly depended upon the suppression of aggressive factors (i.e. inhibition of gastric acid secretion) and in turn, the augmentation of defensive factors (i.e. stimulation of mucus secretion) and antioxidative activity. Therefore, PF ethanol extract, neohesperidin and poncirin are expected to have a protective effect against gastritis.

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References