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ORIGINAL ARTICLE

Ameliorating Effects of *Geranium koreanum* Kom on Esophageal Damage in Reflux Esophagitis via Nuclear Factor-κB Signaling-Mediated Anti-Inflammatory Activities

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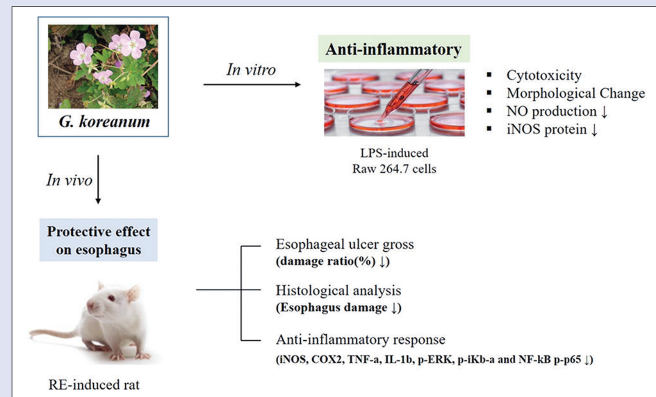
ABSTRACT

Background: *Geranium koreanum*, an herbaceous perennial plant, is widely used as a Chinese herbal medicine to treat several diseases. **Objective:** In the present study, we wanted to demonstrate the anti-inflammatory effects of *G. koreanum* extracts on esophagus damage in acute reflux esophagitis (RE)-induced rats. **Materials and Methods:** The anti-inflammatory effects of *G. koreanum* measured in nitric oxide (NO) production and inducible NO synthase (iNOS) protein expression on lipopolysaccharide (LPS)-induced Raw 264.7 macrophages. To evaluate the improvement in RE, rats were per orally treated with *G. koreanum* 100 and 200 mg/kg 90 min before RE induction surgery. After 5 h, the rats were sacrificed to confirm the degree of esophageal injury. The esophageal mucosal ulcer ratio and histological changes were examined using Image J program and hematoxylin and eosin (H and E) staining of the rat esophagus, respectively. In addition, expression levels of pro-inflammatory proteins on esophagus in RE rat were measured by western blotting. **Results:** *G. koreanum* exhibited anti-inflammatory effects against LPS-stimulated cells by significantly inhibiting NO production and iNOS protein expression. In addition, the LPS-induced morphological transformation of cells was recovered following pretreatment with *G. koreanum*. *In vivo*, the result show that *G. koreanum* 200 mg/kg extract-treated group was ameliorated esophageal damage on histological analysis. The inflammatory mechanism (nuclear factor-κB and mitogen-activated protein kinases signaling pathways) involved in the esophageal damage was investigated by using western blotting with esophageal tissue, and *G. koreanum* clearly inhibited the inflammatory response in esophagitis-induced rats. **Conclusions:** This study showed that *G. koreanum* has anti-inflammatory and ameliorating effects in acute RE.

Key words: Acute reflux esophagitis, anti-inflammatory, gastroesophageal reflux disease, *Geranium koreanum*, *in vivo*, nuclear factor-κB

SUMMARY

- *Geranium koreanum* showed the presence of Geraniin
- *G. koreanum* has shown significant anti-inflammatory potential in the macrophage
- *G. koreanum* has shown protective activity on esophagus mucosa damage in reflux esophagitis rats via nuclear factor-κB signaling-mediated anti-inflammatory activities.



Abbreviations used: RE: Reflux esophagitis; G. koreanum: Geranium koreanum; NO: Nitric oxide; iNOS: inducible nitric oxide synthase; H and E: Hematoxyline and eosin

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INTRODUCTION

Reflux esophagitis (RE), a gastroesophageal reflux disease (GERD), is characterized by esophagus damage and inflammation arising from reflux of the gastric contents into the esophagus.[1] RE is usually caused by gastric acid reflux resulting from dysfunction of the lower esophageal sphincter. In addition, this disease is related to stress, crapulence, irregular living habits, and westernization of lifestyle and diet. Inflammation such as mucosal damage caused by regurgitation of gastric acid induces symptoms such as heartburn, burning pain in the chest, nausea, pharyngeal pain, chronic cough, and asthma.[2,3] Although H2-receptor antagonists and proton-pump inhibitors (PPIs) have been widely used to treat GERD, they cause many complications, and their long-term use leads to resistance.[4]

Inflammatory responses are well-known to be a part of the physiological innate defense system that maintains immune homeostasis and prevents multiple diseases, including cancer, diabetes, atherosclerosis, autoimmune disorders, and arthritis.[5,6] During the immune response, the inflammatory process is regulated by nuclear factor (NF)-κB transcription factors, mediated by subunits such as p65 and the inhibitor of NF-κB-alpha (IκB-α) complex.[7,8] The activation of NF-κB leads to the transcription of inflammatory cytokines and proteins, including tumor

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necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1 β), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2. Recent studies have shown that both the production of inflammatory cytokines such as TNF-α and IL-1 β and the expression of inflammatory proteins such as iNOS and COX-2 induce esophageal tissue damage.[2,3,9,10] Besides, the inflammatory cytokines and stress lead to increased phosphorylation and activation of MAPKs, such as extracellular-signal-regulated kinase (ERK), and also activate NF-κB.[11,12]

Geranium koreanum Kom, an herbaceous perennial plant that belongs to the Geraniaceae family, mainly grows in Korean and northeast China. Conventionally, *G. koreanum* is widely used as a Chinese herbal medicine to treat several diseases and symptoms such as itching, bruising, shigellosis, enteritis, chronic diarrhea, and liver disorder and it contains tannins, quercetin, succinic acid, and garllic acid. A previous study reported that *G. koreanum* extracts have antibacterial and hepatoprotective effects.[13,14] Geraniin, the main polyphenolic compound isolated from various Geranium species, is also an important Chinese herbal medicine.[15] Previous studies have shown that geraniin has a wide range of pharmacological effects, including radioprotective, antioxidant, and antitumor activities, as well as inhibitory effects on NF-κB activity.[16-19]

The aim of the present study was to demonstrate the improvement in esophageal tissue damage by *G. koreanum*, potentially mediated by anti-inflammatory activities through the NF-κB and ERK/mitogen-activated protein kinases (MAPK) signaling pathways. To this end, we used esophageal tissue from RE-induced rats to investigate the effects of a *G. koreanum* extract on cell viability and inflammatory mediator production in Raw 264.7 macrophages as well as its protective effect on RE-induced inflammatory damage *in vivo*.

MATERIALS AND METHODS

Chemicals

Geraniin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). lipopolysaccharidea (LPS), dimethyl sulfoxide (DMSO), and sulfanilamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Wel Gene (St. Namchen, Gyeongsan, Korea). N-(1-naphthyl) ethylene-diamine dihydrochloride (NED), potassium chloride, 4-(2-Hydroxyethyl) piperazine-1-ehanesulfonic acid (HEPES), hematoxylin, eosin, and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for lamin B and phospho-ERK were obtained from cell signaling (Danvers, MA, USA). Further, iNOS, COX-2, β-actin, TNF-α, IL-1 β, phospho-IκB-α, and phospho-NF-κB were purchased from Santa Cruz (Dallas, TX, USA). The secondary antibodies goat anti-rabbit and goat anti-mouse were purchased from Santa Cruz (Dallas, TX, USA), and western blotting luminal reagent was purchased from Santa Cruz (Dallas, TX, USA). Isoflurane was obtained from Hana Pharm (Kyonggi-do, Korea). The BSA protein kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Chromatographic analyses of *Geranium koreanum* using high-performance liquid chromatography

All chromatographic analyses were performed using a Waters Sunfire C_{18} column (250 mm × 4.6 mm, i. d. 5 µm). The mobile phase was composed of 0.1% formic acid in distilled water and 90% acetonitrile. The gradient program was set as follows; 0–2 min, 5% B; 2–28 min, 5%–60% B, and 28–35 min 60%-5% B. The chromatogram was detected at a wavelength of 220 nm using PDA, and the injection volumes of sample solutions were fixed at 10 µL. We first established the standard working curves of Geraniin and *G. koreanum* extracts, and analyzed the samples by using high-performance liquid chromatography (HPLC).

Plant materials and extraction

The whole plants of *G. koreanum* were collected from Gangwon-do, Korea; the material was authenticated by the Korean Institute of Oriental Medicine. The collected *G. koreanum* samples were cleaned and dried, and then a 100 g sample was extracted three times with 70% ethanol for 3 h (100 mL each time) (yield: 18.01%). The extract was filtered and lyophilized (IlShin, Korea). A fraction in DMSO as the stock was stored at −20°C, and subsequently used as a sample for measurement.

Cell culture

The Raw 264.7 macrophage cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Raw 264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C in a 5% CO_2 incubator (SANTO, Sakata, Japan). The cells were pretreated with different concentrations (0, 100 and 200 µg/mL) of *G. koreanum* for 1 h and then treated with LPS (1 µg/mL) for 24 h.

Cell viability and optical morphological transformation in raw 264.7 cells

To investigate the effects of *G. koreanum* on cell viability, Raw 264.7 cells were cultured in 96-well plates (5 × 10^5 cells/well) and treated with different concentrations (0, 100, and 200 µg/mL) of the extract, followed by co-treatment with LPS (1 µg/mL) for 24 h at 37°C in a 5% CO_2 incubator. Cell viability was measured using a cytotoxicity kit, and the absorbance was measured at 540 nm using a multiscan spectrum (Thermo Scientific, Vantaa, Finland). To observe Raw 264.7 cell morphology transformation, images of the cells were captured by using an inverted microscope (Eclipse, TS200, Nikon, Japan) at fixed ×200 magnification.

Nitric oxide production in raw 264.7 cells

NO production in LPS-induced cells was measured using the Griess reaction. The culture was centrifuged at 2500 rpm for 5 min. Subsequently, 50 µL of the cell culture supernatant was mixed with 50 µL 1% sulfanilamide and 0.1% NED and incubated at 24°C for 10 min. Absorbance at 540 nm was measured using a multiscan spectrum. The amount of nitrite was determined using a sodium nitrite serial dilution standard curve, and subsequently, the nitrite production was measured.

Animal management

The 7-week-old SR rats were handled according to the animal welfare regulation of the Institutional Animal Care and Use Committee (IACUC; CBNU 2017-0078), Chonbuk National University Laboratory Animal Center, South Korea. The rats were maintained in standard rat cages and provided food and distilled water. The temperature (23°C ± 2°C), humidity (35%–60%), and photoperiod (12-h light/dark cycle) were maintained constant over the experimental period.[20]

Acute reflux esophagitis induction

In total, 32 rats were used to establish the model and were fasted for 18 h before the RE induction surgery; however, distilled water was fed. The rats were anesthetized via inhalation anesthesia using an isoflurane. The RE-induced surgery was performed by abdominal incision to expose the stomach, the transitional junction between the fundus of the stomach and the corpus were exposed, and then subsequently ligated with 3–0 silk thread (only sham control rats were not ligated).[21,22]

Following the surgery, the 32 rats were split randomly into four groups as follows: (1) normal control rats, (2) RE control rats, (3) RE rats treated with *G. koreanum* 100 mg/kg body weight, and (4) RE rats treated with *G. koreanum* 200 mg/kg body weight. The rats of the *G. koreanum* groups were treated with 100 and 200 mg/kg *G. koreanum* 90 min before the abdominal surgery. After 5 h, the rats were sacrificed to confirm the degree of esophageal injury. Esophagus samples were collected and maintained at – 80°C for western blotting analysis and immediately fixed with 10% neutral-buffered formalin (NBF) for histological analysis.

Esophageal lesion ratio

The esophagus was cut in a vertical direction from the gastroesophageal junction to the pharynx. The inner esophagus was washed with saline, and the remaining tissue was placed on white paper. Next, the dissected esophagus was photographed using an optical digital camera (Nikon, Tokyo, Japan) and the images were analyzed using the Image J program.[9] The ratio of the mucosal damage ratio was considered as follows:

Mucosal damage ratio (%) = (width of the area with esophageal mucosal damage [mm^2]/width of the total area of the esophagus [mm^2]) × 100.

Histopathological analysis of esophageal mucosa

Esophagus samples were excised for histopathological evaluation. Pieces of the esophagus of each rat were immediately fixed in 10% NBF. The samples were processed using an auto processor (Excelsior ES, Thermo Scientific, USA). Embedded 5-μm sections of the esophagus were stained with hematoxylin and eosin and fixed on glass slides. Following staining, the histological architecture and apoptotic changes in the esophagus were observed. Digital images of the samples were obtained using a Leica DM2500 microscope (Leica Microsystems, Germany) at ×200 magnification. The diameter of the portal vein was measured by using an image measurement software (i Solution DTM).[20]

Preparation of cytosol and nuclear fractions of the esophagus

Esophageal tissues were extracted with ice-cold lysis buffer containing 10 mM HEPES (pH 7.8), 2 mM magnesium chloride ($MgCl_2$), 10 mM potassium chloride (KCl), and 0.1 mM ethylenediaminetetraacetic acid (EDTA), and then 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) mixture solution, and 10% NP-40 were added. After centrifugation (12000 rpm for 2 min at 40°C), the supernatant was separated, and the pellet was washed with distilled water. A nuclear fraction was extracted from the esophagus of each rat. In brief, esophageal tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 1% glycerol, 0.1 EDTA, 1 mM PMSF, and protease inhibitor mixture solution. The extract was then centrifuged at 12000 rpm for 10 min at 4°C. The protein concentration of each sample was measured by using a Bio-Rad protein kit.

Western blot analysis

The proteins were separated by 10 and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred from the gel onto nitrocellulose membranes. The membrane was maintained with 5% skim milk in phosphate-buffered saline solution-1% Tween 20 for 1 h at 25°C–26°C and incubated with primary antibodies (1:1000) overnight at 4°C. Next, the membrane was incubated with secondary antibodies (1:10000) for 2 h. The bands were detected by using western blotting luminal reagent and images were obtained using a ChemiDoc™ MP imaging system (Bio-Rad, USA).

Statistical analysis

Results are expressed as the means ± standard deviation of at least three independent experiments. All statistical analyses were performed with the social sciences (SPSS) software (SPSS Inc., Chicago, IL, USA) by using analysis of variance, followed by Tukey's multiple comparison tests. Value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Constituents of geraniin were analyzed by high-performance liquid chromatography analysis of the *Geranium koreanum* extract

Geraniin, a polyphenolic compound present in various Geranium species, is known to possess anti-nociceptive and anti-inflammatory properties as well as inhibitory effects on osteoclastogenesis and osteoclast functions *in vivo*.[5,15,23-26] Therefore, we identified whether the *G. koreanum* extract contained geraniin by using HPLC analysis. Figure 1 presents the results of the HPLC analysis: the retention times of the *G. koreanum* extract (15.234 min) and standard geraniin (15.298 min) were comparable. The results showed a concentration of 118.82 ± 18.11 mg/g for geraniin in the *G. koreanum* 70% ethanol extract [Figure 1b].

Cytotoxicity and morphological transformation in raw 264.7 cells

The cytotoxicity of the *G. koreanum* extract was evaluated using raw 264.7 macrophages treated with LPS (1 μg/mL) for 24 h using a cytotoxicity assay kit. *G. koreanum* showed no significant cytotoxicity against LPS-induced Raw 264.7 cells at concentrations of 100 and 200 μg/mL compared to that against normal cells [Figure 2b]. Moreover, LPS caused morphological transformation of macrophage Raw 264.7 cells due to the inflammatory reaction. Therefore, we examined the potential effects of *G. koreanum* on LPS (1 μg/mL)-induced transformation of Raw 264.7 cell morphology using a light microscope. LPS-induced inflammation critically transformed the cell morphology compared

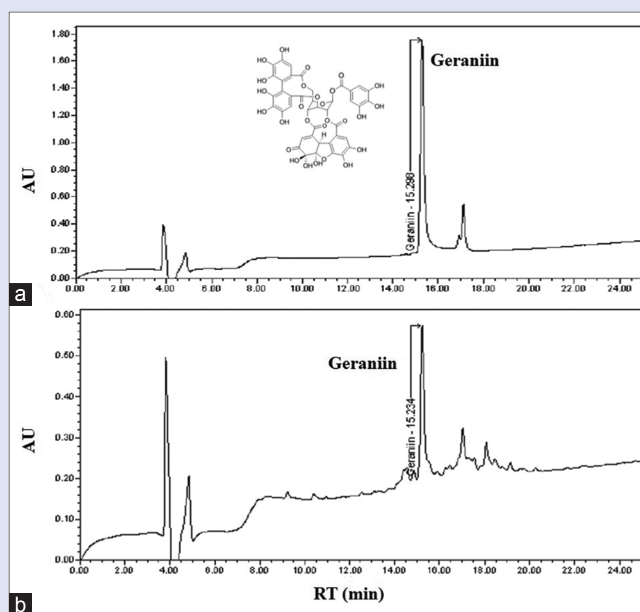

Figure 1: Chromatographic analysis of *Geranium koreanum*. (a) high-performance liquid chromatography chromatogram of the standard solution and geraniin; (b) high-performance liquid chromatography chromatogram of the *Geranium koreanum* 70% ethanol extract

to that of normal cells cultured in the medium alone. Cells pretreated with *G. koreanum* (100 and 200 μg/mL) followed by LPS showed fewer transformed cells than the LPS-treated cells did [Figure 2a].

Nitric oxide production and inducible nitric oxide synthase protein expression in lipopolysaccharide-induced raw 264.7 cells

NO plays a significant role in immune defenses and neuronal signal transduction as an inflammatory mediator and is produced by iNOS during inflammatory responses in the cell or tissue.[1] In this study, *G. koreanum* decreased NO production in LPS-induced Raw 264.7 cells. The cells were pretreated with the extract at concentrations of 100 and 200 μg/mL for 1 h, followed by LPS (1 μg/mL) treatment for 24 h. As shown in Figure 3a, NO production was hardly observed in the normal group compared to that in the LPS-treated group. In the cells pretreated with *G. koreanum* (100 and 200 μg/mL), NO production was inhibited by >50%, and the effects were concentration dependent. iNOS is a pro-inflammatory protein regulated by NF-κB, and it induces inflammation by producing NO. The results showed that iNOS protein expression in LPS-induced Raw 264.7 cells was increased compared to that in the untreated control cells. However, in the *G. koreanum* treatment group, iNOS expression was significantly decreased in a concentration-dependent manner [Figure 3b].

Improvement in esophageal mucosal damage in acute reflux esophagitis rats

We evaluated the improvement in RE in rats orally administered *G. koreanum* (100 and 200 mg/kg body weight) 90 min before the surgery to induce esophageal damage. Next, the rats, except for those in the normal control group, were subjected to laparotomies to ligate the pylorus and the junction between the forestomach and the corpus,[27] and esophageal damage was induced by gastric acid reflux during the ligation. The

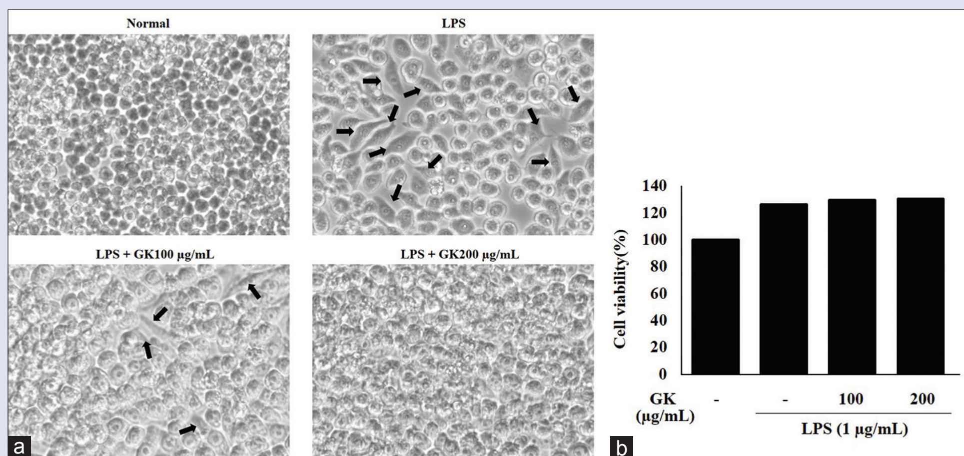

Figure 2: (a) Optical morphological transformation and (b) cytotoxicity of lipopolysaccharidea (1 μg/mL)-induced raw 264.7 cells treated with 100 and 200 μg/mL of *Geranium koreanum* for 24 h. Cell viability was determined by using a cytotoxicity assay kit, and no significant cytotoxicity was observed. The morphology was visualized by using a light microscope at ×200 magnitude

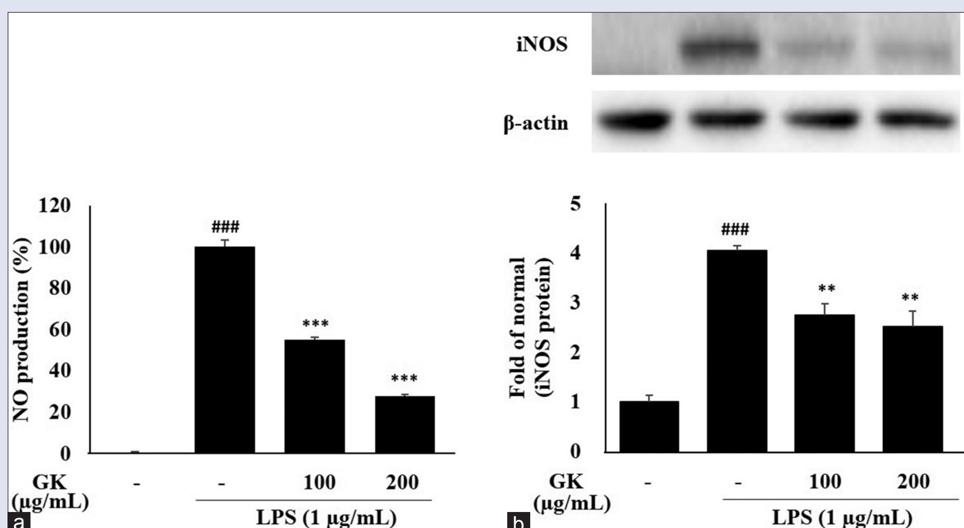

Figure 3: Inhibition of nitric oxide production and inducible nitric oxide synthase protein expression in lipopolysaccharidea (1 μg/mL)-induced raw 264.7 cells pretreated with 100 and 200 μg/mL *Geranium koreanum* extracts for 24 h. (a) Nitric oxide production and (b) inducible nitric oxide synthase protein expression were measured by using the Griess assay and western blot analysis, respectively. Data are means ± standard deviation; ###$P < 0.001$ compared with normal control cells; ***$P < 0.001$ and **$P < 0.01$ compared with lipopolysaccharidea control cells

esophageal tissue of the RE control group (not treated with *G. koreanum*) showed tissue damage and hemorrhage induced by inflammation unlike the normal untreated control group [Figure 4]. The RE + GK100 (100 mg/kg) group showed a decreased incidence of damage and hemorrhage in the esophagus compared to that of the RE control group. In the RE + GK200 (200 mg/kg) group, the esophageal ulcer index of rats decreased by >60% compared to that of the control group. Therefore, the preventive effects of *G. koreanum* against esophageal mucosal damage were mediated by its inflammatory activity in acute RE rats.

Histopathology analysis of esophagus

We investigated the histological changes in the esophageal tissue obtained by performing an abdominal incision after anesthetizing rats in both the untreated control (normal) and the *G. koreanum* co-treated groups. The esophagus tissue of the normal control group did not exhibit mucosal changes, whereas more severe damages such as inflammatory cell infiltration and tissue damage were observed in the mucosa and submucosa of the RE-induced rats. However, we confirmed that the esophagus tissue damage of the RE rats was alleviated to a similar extent to that of the normal group by treatment with 200 mg/kg *G. koreanum*. Furthermore, the *G. koreanum* 100 mg/kg-treated rats also showed reduced esophagus damage [Figure 5].

Inflammation-related protein expression in the esophagus

It is well-known that inflammatory cytokines and stress lead to increased phosphorylation and activation of not only NF-κB but also MAPKs, such as ERK. NF-κB is the main regulator of the production of inflammatory proteins such as iNOS, COX-2, activated by cytokines TNF-α and Il-1 β, and phosphorylated-ERK in the MAPK signaling pathway during immune responses following its dissociation and phosphorylation by IκB-α. In the RE control group, the expression levels of both phosphorylated IκB-α and phosphorylated p65 protein were increased more by mucosal damage compared to that in the normal control group. The result showed that 100 and 200 mg/kg *G. koreanum* improved the esophageal inflammation by inhibiting the expression of cytosolic p-ERK, p-IκB-α, and nuclear p-p65 protein [Figure 6c]. In addition, *G. koreanum* significantly affected the expression levels of iNOS, COX-2, TNF-α, and Il-1 β, especially at 200 mg/kg [Figure 6].

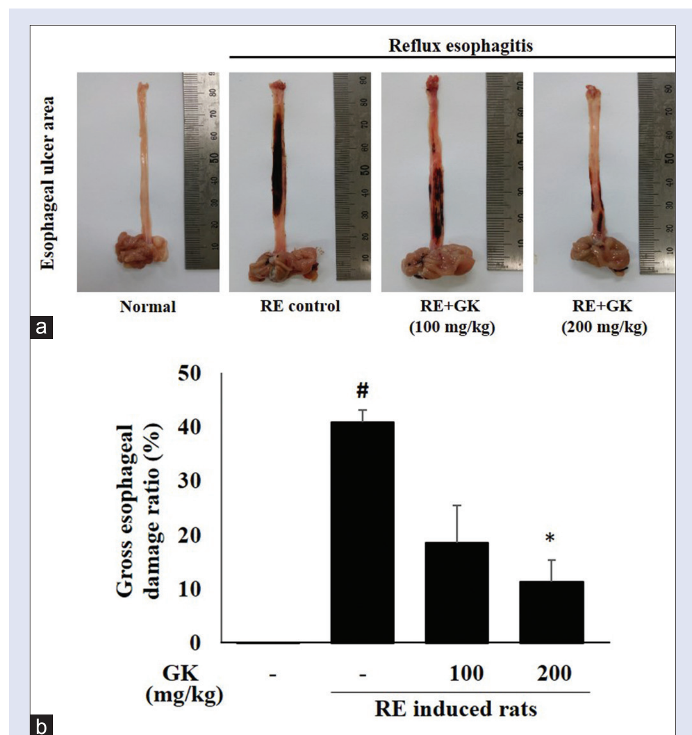

Figure 4: Image of a (a) gross esophageal ulcer and (b) esophageal mucosal ulcer ratio of acute reflux esophagitis rats. Normal, untreated rats; reflux esophagitis control, reflux esophagitis rats; reflux esophagitis + GK100 mg/kg, *Geranium koreanum* 100 mg/kg body weight-treated reflux esophagitis rats; reflux esophagitis + GK200 mg/kg, *Geranium koreanum* 200 mg/kg body weight-treated reflux esophagitis rats. Data are means ± standard deviation; #*P* < 0.05 and **P* < 0.05 compared with normal control and reflux esophagitis control, respectively

DISCUSSION

GERD has been recently recognized as a serious clinical problem with an increasing number of patients being affected by it.[3] Therapeutic agents such as PPIs are available, which improve various acid-related disorders such as peptic ulcer disease, gastroduodenal ulcers, and GERD.[4] However, recent studies have focused on investigating new or improved and safe alternative therapies based on herbal medicines.[2,9,10,28,29] *G. koreanum* is a traditional Korean and Chinese medicinal herb that is usually used to treat various diseases and symptoms such as itching, bruising, shigellosis, enteritis, chronic diarrhea, and liver disorders. Previous animal studies have shown that *G. koreanum* considerably regulates NaAsO2-induced mitochondrial-dependent oxidative damage in the liver.[14] Geraniin, a polyphenolic compound found in Geranium species, has a wide range of beneficial effects and has been reported to have antioxidant and antitumor effects as well as inhibitory effects on NF-κB activity.[17-19] Thus, we conducted the present study to determine whether *G. koreanum* has potential anti-oxidative and anti-inflammatory actions.

Based on the HPLC analysis, geraniin in the *G. koreanum* 70% ethanol extract was found to be present at a concentration of 118.82 ± 18.11 mg/g. LPS is used to induce infection, inflammation, and tissue damage associated with macrophages, which play an important role in inflammatory responses by releasing various pro-inflammatory

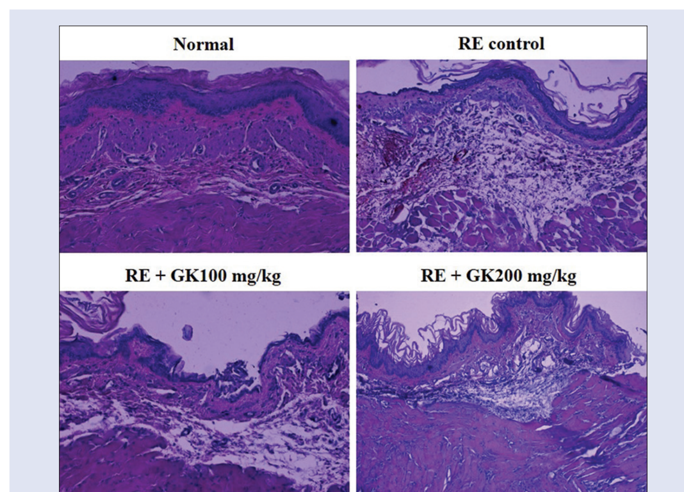

Figure 5: Hematoxylin and eosin-stained sections of esophagus from normal control, reflux esophagitis control, reflux esophagitis + GK100 and reflux esophagitis + GK200 groups. Normal, untreated rats; reflux esophagitis control, reflux esophagitis rats; reflux esophagitis + GK100; *Geranium koreanum* 100 mg/kg body weight-treated reflux esophagitis rats; reflux esophagitis + GK200; *Geranium koreanum* 200 mg/kg body weight-treated reflux esophagitis rats

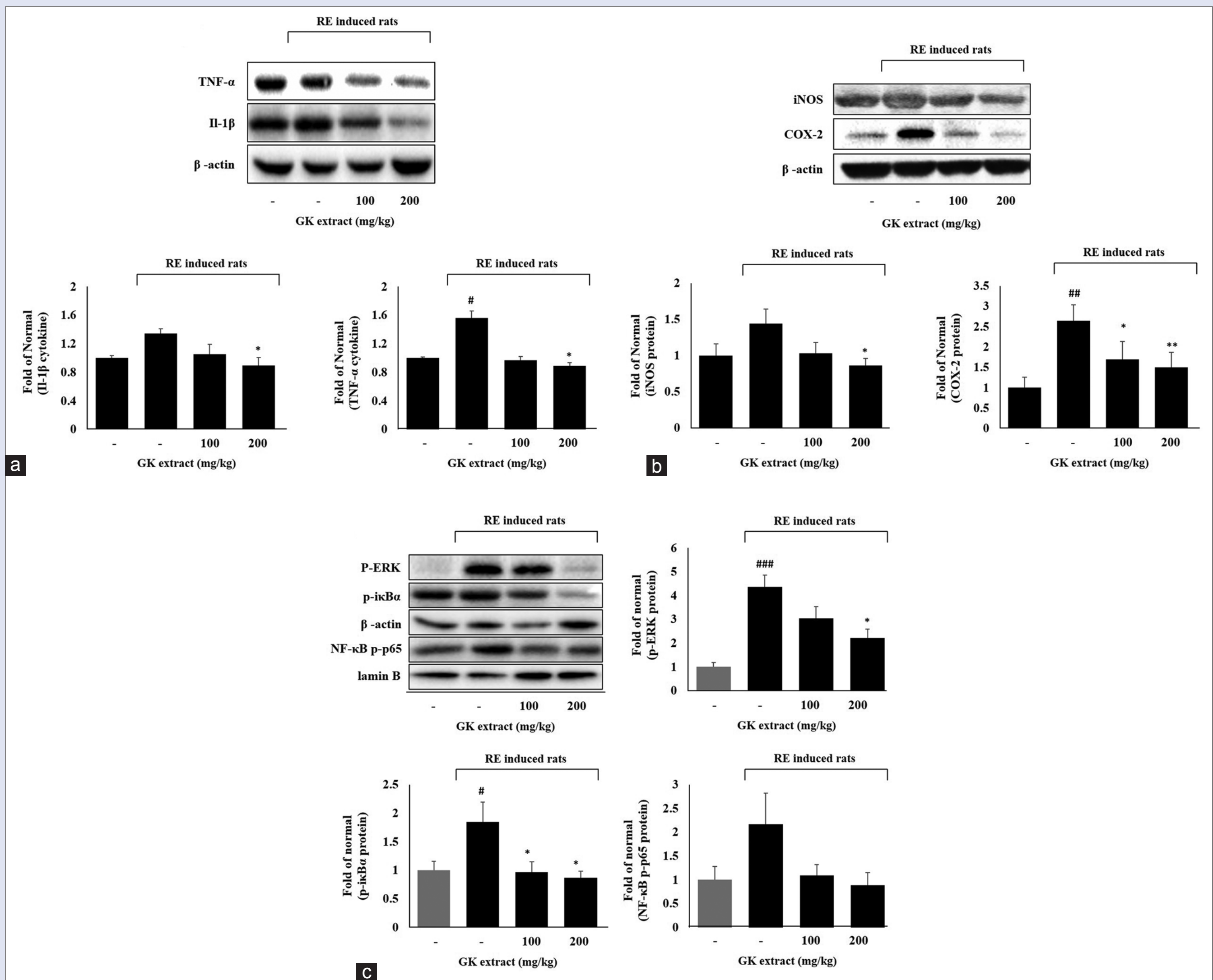

Figure 6: Inhibitory effects of *Geranium koreanum* on inflammatory protein and cytokine expression in esophagus of acute reflux esophagitis-induced rats. Expression of (a) cytokines tumor necrosis factor-alfa and interleukin-1 β; (b) inflammatory protein inducible nitric oxide synthase and cyclooxygenase-2; (c) p-extracellular-signal-regulated kinase, p-IκB-α, and p-p65 proteins. Rats were pretreated with 100 and 200 mg/kg body weight *Geranium koreanum* excluding normal and reflux esophagitis control rats for 90 min before surgery, under inhalation anesthesia. Then, 5 h later, the esophagus was cut, and the abdomen was opened and dissected from each rat. Data are means ± standard deviation. Significance: $^{###}P < 0.001$, $^{##}P < 0.01$ and $^{#}P < 0.05$ compared with normal control; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with reflux esophagitis control

cytokines, nitrogen species, and chemokines in response to the stimulation.[30,31] *G. koreanum* downregulated LPS-induced release of the NO pro-inflammatory factor at 100 and 200 μg/mL, and the decrease was >50% following treatment with 200 μg/mL G. *koreanum*. This result showed that G. *koreanum* remarkably inhibited iNOS protein expression and it appeared to similarly inhibit NO production. We confirmed that the inhibition of NO production was regulated by the iNOS inflammatory protein expression during inflammation in LPS-induced Raw 264.7 macrophages.

In addition, the results of the cell viability assay indicated that pretreatment with G. *koreanum* (100 and 200 μg/mL) showed no significant cytotoxicity against LPS-induced Raw 264.7 macrophages, which showed similar viability to that of normal control cells. Previous studies have shown transformations such as morphological and phenotypic changes in Raw 264.7 cells cultured with LPS (1 μg/mL).[32] In this study, we observed morphological changes such as inflammation in the LPS-induced cells. Cells cultured with *G. koreanum* 100 and 200 μg/mL and LPS (1 μg/mL) showed a dose-dependent decrease in the number of transformed cells compared to that observed in the LPS treatment group. We found that *G. koreanum* had potential anti-inflammatory effects mediated through a decrease in NO production, iNOS protein expression, and morphological transformation in LPS-induced Raw 264.7 macrophages.

RE is the most common cause of decreased function of the lower esophageal sphincter. Therefore, acid secretion is increased in the inner esophagus, leading to mucosal damage or degeneration.[2]

We performed experiments to support the beneficial effects of *G. koreanum* on RE, using a surgically established rat model. The untreated, normal control groups showed no significant indication of esophagus damage such as stenosis, hemorrhage, and edema in the mucosa. On the contrary, the esophagus of the RE controls, which were not treated with *G. koreanum*, exhibited more serious tissue damage and stenosis of the esophagus caused by the inflammation induced by

gastric acid and stomach contents than the normal control rats did. *G. koreanum* decreased the damage to the esophagus compared to that observed in the RE control rats, especially at 200 mg/kg, which decreased the esophageal ulcer index by >60%. We also investigated the effects of *G. koreanum* on histological changes in the esophagus of acute RE rats. The RE-treated groups showed epithelial and muscle destruction and inflammatory cell infiltration in the mucosal and submucosal regions. However, the histological changes in the mucosa were gradually ameliorated by pretreatment with *G. koreanum* 100 and 200 mg/kg. In addition, the tissue structure showed significant improvement following pretreatment with 200 mg/kg, and the morphology was similar to that of the normal controls. Thus, we showed that *G. koreanum* ameliorated the esophagus damage caused by gastric acid reflux and stomach contents in esophagitis-induced rats.

Esophageal damage caused by epithelial destruction and inflammatory cell infiltration is due to the continuous inflammatory response arising from irritation.[2,3,9,13,33] Recent research studies using models of esophagus damage have demonstrated that inflammatory factors such as cytokines and proteins and oxidative stress are involved in RE.[3,27,33-36] NF-κB is activated by pro-inflammatory cytokines, including TNF-α and IL-1 β, which then regulate the synthesis of inflammatory proteins such as iNOS and COX-2 in immune responses by the dissociation and phosphorylation of IκB-α.[27] In the RE control groups, the expression levels of TNF-α, IL-1 β, p-IκB-α, and p-p65 proteins in NF-κB and p-ERK protein in MAPK signaling pathways were increased by esophagus damage compared to the levels in the normal control group. However, production of TNF-α and IL-1 β clearly decreased in the *G. koreanum* pretreated groups, especially at 200 mg/kg. In addition, *G. koreanum* inhibited the expression of p-ERK, p-IκB-α, and p-p65 proteins by over 50%, and then the production of iNOS and COX-2 inflammatory proteins was subsequently inhibited. Therefore, our results confirm that *G. koreanum* has a protective effect against the esophagus from inflammatory tissue damage in acute RE. We anticipate that future studies will identify the active extract fractions and active compounds mediating the mechanisms of the anti-inflammatory effects of *G. koreanum* in RE.

CONCLUSIONS

Esophagus damage causes reflux of gastric acid and stomach contents and is closely associated with inflammatory response. In the study, we demonstrated the anti-inflammatory and protective effects of *G. koreanum* against esophagus tissue damage in acute RE-induced rats. Therefore, *G. koreanum* could be a useful and safe alternative therapeutic source based on an herbal medicine worth future research and development.

Acknowledgement

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kang JW, Lee SM. Protective effects of chlorogenic acid against experimental reflux esophagitis in rats. Biomol Ther (Seoul) 2014;22:420-5.
2. Kim MY, Shin YO, Lee JY, Lee AR, Shin SH, Kwon OJ, *et al.* Improving effect of a combined extract of Rhei Rhizoma and Glycyrrhizae Rhizoma through anti-oxidative stress in reflux esophagitis rats. Korea Assoc Herb 2015;30:37-44.
3. Yoshida N. Inflammation and oxidative stress in gastroesophageal reflux disease. J Clin Biochem Nutr 2007;40:13-23.
4. Eslami L, Nasseri-Moghaddam S. Meta-analyses: Does long-term PPI use increase the risk of gastric premalignant lesions? Arch Iran Med 2013;16:449-58.
5. Wang P, Qiao Q, Li J, Wang W, Yao LP, Fu YJ, *et al.* Inhibitory effects of geraniin on LPS-induced inflammation via regulating NF-κB and Nrf2 pathways in RAW 264.7 cells. Chem Biol Interact 2016;253:134-42.
6. Hossen MJ, Cho JY, Kim D. PDK1 in NF-κB signaling is a target of *Xanthium strumarium* methanolic extract-mediated anti-inflammatory activities. J Ethnopharmacol 2016;190:251-60.
7. Song JH, Kang HB, Park SH, Jeong JH, Park J, You Y, *et al.* Extracts of *Porphyra tenera* (Nori Seaweed) activate the immune response in mouse RAW264.7 macrophages via NF-κB signaling. J Med Food 2017;20:1152-9.
8. Wu Z, Uchi H, Morino-Koga S, Shi W, Furue M. Z-ligustilide ameliorated ultraviolet B-induced oxidative stress and inflammatory cytokine production in human keratinocytes through upregulation of Nrf2/HO-1 and suppression of NF-κB pathway. Exp Dermatol 2015;24:703-8.
9. Kwon OJ, Choo BK, Lee JY, Kim MY, Shin SH, Seo BI, *et al.* Protective effect of Rhei Rhizoma on reflux esophagitis in rats via Nrf2-mediated inhibition of NF-κB signaling pathway. BMC Complement Altern Med 2016;16:7.
10. Choo BK, Roh SS. Berberine protects against esophageal mucosal damage in reflux esophagitis by suppressing proinflammatory cytokines. Exp Ther Med 2013;6:663-70.
11. Han J, Lee JD, Bibbs L, Ulevitch RJ. A map kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994;265:808-11.
12. Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, *et al.* Differential regulation of ikappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A 1998;95:3537-42.
13. Oh JY, Lee KJ, Wei B, Roh JH, Kang M, Cha SY, *et al.* Antibacterial activities of bark extracts from *Fraxinus rhynchophylla* hance and *Geranium koreanum* kom. against clinical strains of *Clostridium perfringens* in chickens. Korea Assoc Herb 2015;55:117-23.
14. Akanda MR, Kim IS, Ahn D, Tae HJ, Tian W, Nam HH, *et al. In vivo* and *in vitro* hepatoprotective effects of *Geranium koreanum* methanolic extract via downregulation of MAPK/Caspase-3 pathway. Evid Based Complement Alternat Med 2017;2017:8137627.
15. Xiao F, Zhai Z, Jiang C, Liu X, Li H, Qu X, *et al.* Geraniin suppresses RANKL-induced osteoclastogenesis *in vitro* and ameliorates wear particle-induced osteolysis in mouse model. Exp Cell Res 2015;330:91-101.
16. Kang KA, Lee IK, Zhang R, Piao MJ, Kim KC, Kim SY, *et al.* Radioprotective effect of geraniin via the inhibition of apoptosis triggered by γ-radiation-induced oxidative stress. Cell Biol Toxicol 2011;27:83-94.
17. Ito H. Metabolites of the Ellagitannin geraniin and their antioxidant activities. Planta Med 2011;77:1110-5.
18. Okabe S, Suganuma M, Imayoshi Y, Taniguchi S, Yoshida T, Fujiki H, *et al.* New TNF-alpha releasing inhibitors, geraniin and corilagin, in leaves of *Acer nikoense*, megusurino-ki. Biol Pharm Bull 2001;24:1145-8.
19. Pan MH, Lin-Shiau SY, Ho CT, Lin JH, Lin JK. Suppression of lipopolysaccharide-induced nuclear factor-kappaB activity by theaflavin-3,3n-digallate from black tea and other polyphenols through down-regulation of IkappaB kinase activity in macrophages. Biochem Pharmacol 2000;59:357-67.
20. Akanda MR, Kim IS, Ahn D, Tae HJ, Nam HH, Choo BK, *et al.* Anti-inflammatory and gastroprotective roles of *Rabdosia inflexa* through downregulation of pro-inflammatory cytokines and MAPK/NF-ation of IkappaBhways. Int J Mol Sci 2018;19. pii: E584.
21. Nakamura K, Ozawa Y, Furuta Y, Miyazaki H. Effects of sodium polyacrylate (PANa) on acute esophagitis by gastric juice in rats. Jpn J Pharmacol 1982;32:445-56.
22. Gweon TG, Park JH, Kim BW, Choi YK, Kim JS, Park SM, *et al.* Additive effects of rebamipide plus proton pump inhibitors on the expression of tight junction proteins in a rat model of gastro-esophageal reflux disease. Gut Liver 2018;12:46-50.
23. Zhu G, Xin X, Liu Y, Huang Y, Li K, Wu C. Geraniin attenuates LPS-induced acute lung injury via inhibiting NF-κB and activating Nrf2 Signaling pathway. Impact J 2017;8:22835-41.
24. Lu C, Gao S, Xu G. Geraniin inhibits TNF-α-induced impairments of osteogenesis through NF-κB and p38 MAPK signalling pathways in bone marrow stem cells. Stroke Vasc Neurol 2017;2:47-52.
25. Küpeli E, Tatli II, Akdemir ZS, Yesilada E. Estimation of antinociceptive and anti-inflammatory

activity on *Geranium pratense* subsp. finitimum and its phenolic compounds. J Ethnopharmacol 2007;114:234-40.

26. Choi HJ, Choi HJ, Park MJ, Lee JY, Jeong SI, Lee S, *et al*. The inhibitory effects of *Geranium thunbergii* on interferon-γ- and LPS-induced inflammatory responses are mediated by Nrf2 activation. Int J Mol Med 2015;35:1237-45.
27. Rieder F, Biancani P, Harnett K, Yerian L, Falk GW. Inflammatory mediators in gastroesophageal reflux disease: Impact on esophageal motility, fibrosis, and carcinogenesis. Am J Physiol Gastrointest Liver Physiol 2010;298:G571-81.
28. Shin MR, Seo BI, Son CG, Roh SS, An HJ. Banhasasim-tang treatment reduces the severity of esophageal mucosal ulcer on chronic acid reflux esophagitis in rats. Biomed Res Int 2017;2017:7157212.
29. Miwa H, Koseki J, Oshima T, Kondo T, Tomita T, Watari J, *et al*. Rikkunshito, a traditional Japanese medicine, may relieve abdominal symptoms in rats with experimental esophagitis by improving the barrier function of epithelial cells in esophageal mucosa. J Gastroenterol 2010;45:478-87.
30. Zong Y, Sun L, Liu B, Deng YS, Zhan D, Chen YL, *et al*. Resveratrol inhibits LPS-induced MAPKs activation via activation of the phosphatidylinositol 3-kinase pathway in murine RAW 264.7 macrophage cells. PLoS One 2012;7:e44107.
31. Huang BP, Lin CH, Chen YC, Kao SH. Anti-inflammatory effects of *Perilla frutescens* leaf extract on lipopolysaccharide-stimulated RAW264.7 cells. Mol Med Rep 2014;10:1077-83.
32. Saxena RK, Vallyathan V, Lewis DM. Evidence for lipopolysaccharide-induced differentiation of RAW264.7 murine macrophage cell line into dendritic like cells. J Biosci 2003;28:129-34.
33. Eden K, Rothschild DE, McDaniel DK, Heid B, Allen IC. Noncanonical NF-κB signaling and the essential kinase NIK modulate crucial features associated with eosinophilic esophagitis pathogenesis. Dis Model Mech 2017;10:1517-27.
34. Yoshida N, Katada K, Handa O, Takagi T, Kokura S, Naito Y, *et al*. Interleukin-8 production via protease-activated receptor 2 in human esophageal epithelial cells. Int J Mol Med 2007;19:335-40.
35. Yoshida N, Imamoto K, Uchiyama K, Kuroda M, Naito Y, Mukaida N, *et al*. Molecular mechanisms involved in IL-8 production by normal human esophageal epithelial cells. Aliment Pharmacol Ther 2006;2:219-26.
36. Yoshida N, Uchiyama K, Kuroda M, Sakuma K, Kokura S, Ichikawa H, *et al*. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. Scand J Gastroenterol 2004;39:816-22.