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Geraniin ameliorate experimental acute reflux esophagitis via NF-κB regulated anti-inflammatory activities in rats

Hyeon Hwa Nam[1], Li Nan[1], Jin Cheon Park[2] and Byung Kil Choo[1*]

Abstract

Repeated reflux of gastric acid and stomach contents into the esophagus leads to esophagus damage, including inflammation, ulcer, and hemorrhage in the epithelium. In this study, we aimed to demonstrate the ameliorating effects of geraniin, a phytochemical in the geraniums, on esophagus damage in an acute reflux esophagitis (RE) rat model. The inflammatory effects of geraniin was measured by nitric oxide (NO) production and pro-inflammatory protein levels in lipopolysaccharide (LPS)-induced RAW 264.7 cells. To evaluate the protective effects of geraniin on damaged esophagus tissue in RE rats, the rats were divided into the following groups: normal control; RE-induced control; RE rats pretreated with geraniin 15 and 30 mg/kg body weight; and RE rats pretreated with ranitidine 30 mg/kg body weight as a positive control. The lesion area of esophagus was determined by the Image J program, and histological changes were examined by hematoxylin and eosin staining of rat esophageal tissue. The expression of pro-inflammatory proteins, cytokines, and tight junction proteins involved in esophagus damages was determined using western blotting of esophageal tissue. Geraniin revealed that anti-inflammatory effects against LPS-induced cells by significantly decreasing NO production and iNOS proteins level. Additionally, the results showed that improvement effects of geraniin on esophagus damages in RE induced rats. The expression of inflammatory proteins involved in nuclear factor NF-kB signaling pathways significantly decreased and tight junction protein (claudin-4 and claudin-5) was increased in esophageal tissue. We found the potential of geraniin as source of replacement therapy products source for inflammatory and reflux esophagitis disease.

Keywords: Acute reflux esophagitis, Anti-inflammatory, Claudin, Esophagus damage, Geraniin, NF-κB

Introduction

The reflux of gastric acid and stomach contents induces epithelial lesion and cell death in the esophagus, which is associated with inflammatory response caused by inflammatory cell infiltration and pro-inflammatory cytokine expression in damaged tissues [1, 2]. The usual therapy includes histamine-2 receptor antagonist, proton pump inhibitors, and antacid, which have been used for gastroesophageal reflux disease (GERD); however, the use of these agents is limited by complications such as high recurrence rate, incomplete recovery of mucosal damages, and esophageal stricture [3].

Claudin, a tight junction protein (TJP) in both endothelial and epithelial cells, has barrier and fence functions and is related to GERD. Barrier dysfunction has been demonstrated in inflammatory bowel disease and gastric tissues, and it is associated with TJP such as claudin, occludin, and ZO-1as well as inflammatory cytokines [4, 5]. In particular, a decrease in claudin-4 protein expression has been observed in epithelial cells in the esophageal mucosa [6–8]. Claudin-5 protein expression has been demonstrated in different types of mucosa and cells, and its expression was changed by the expression of pro-inflammatory cytokines [4, 9].

Herbal medicine from plants has been used in healing various diseases owing to its long history in clinical

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practice as well as therapeutic effects [10, 11]. Recently, the protection effects of Xuanfudaizhetang has been proved in rats with RE via increasing the pH value of the esophagus and reducing inflammation stimulation [7]. *Artemisia campestris* L. extracts have shown protective effects on gastric acid reflux-induced esophageal mucosal injuries [2]. The beneficial effects of *Morinda citrifolia* fruit extract and its biomarker scopoletin on RE and gastric ulcer in rats have been reported [12].

Geraniin, the main polyphenolic compound of geraniums [11, 13], was first isolated from *Geranium thunbergii* [14]. The beneficial effects of geraniin, including anti-inflammatory and anti-cancer effects in LPS-induced macrophages by inhibiting NF-κB and Nrf2 signaling pathways, as well as its antioxidant activities, have been reported [14–16]. However, the ameliorating effects of geraniin on esophageal damages in RE via anti-inflammatory activities have not been reported. In the study, we aimed to investigate the ameliorating effects of geraniin on inflamed esophageal mucosa by regulating pro-inflammatory cytokines, proteins, and TJP in a rat model of RE.

Materials and methods

Chemicals

Geraniin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Dulbeco's modified eagle's media (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin purchased (P/S) from Wel Gene (St. Namchen, Gyeongsan, Korea). Cytotoxicity Kit (EZ-Cytox) bought from Dogenbio (Seoul, Korea). Ranitidine, LPS, dimethyl sulfoxide (DMSO), sulfanilamide, N-(1-naphthyl) ethylene-diamine dihydrochloride (NED), 4-(2-Hydroxyethyl) piperazine-1-ehanesulfonic acid (HEPES), potassium chloride, hematoxylin, eosin and protease inhibitor (PI) purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) protein kit bought from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies lamin B from Cell Signaling (Danvers USA), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), β-actin, tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), claudin-4, claudin-5, phospho-IkB-α and phospho-NF-kB purchased from Santa Cruz (Dallas, TX, USA). The secondary antibodies goat anti-rabbit, goat anti-mouse and western blotting luminal reagent purchased from Santa Cruz (Dallas, TX, USA).

Cell culture

Raw 264.7 macrophage cell line was bought from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM supplemented with 10% FBS and 1% P/S in CO_2 incubator (37 °C, 5%

CO_2) (SANTO, Sakata, Japan). The cells were cultured for 1 week and replaced every 2 days. The cultured cells were pretreated with different concentrations (0, 50 and 100 μM) of geraniin for 1 h and then incubated with 1 μg/mL LPS for 24 h.

Cell viability and optical morphological transformation

We identified the impact of geraniin on Raw 264.7 cells via measurement a cytotoxicity and morphology changes. The cells were plated in 96-well plates (1×10^6 cells/well) and 6-well plates (5×10^5 cells/well) treated with different concentrations (0, 50 and 100 μM), followed by co-treatment with LPS (1 μg/mL) for 24 h. Cell viability was measured by a cytotoxicity assay kit, and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Multiscan Spectrum, Thermo Scientific, Vantaa, Finland). To observation of Raw 264.7 cells morphology transformation, the image of treated cells was captured by an inverted microscope (ECLIPSE TS200, Nikon, Japan) at fixed $200 \times$ magnification.

NO production in Raw 264.7 cells

The cell culture plate conducted centrifugation process at 2500 rpm, 5 min. And then, 50 μL of the cell culture supernatant was mixed with 50 μL 1% sulfanilamide, and 0.1% NED incubated at 24 °C for 10 min. The absorbance at 540 nm was measured in an ELISA reader (Multiscan spectrum). The NO production in the samples was determined using sodium nitrite serial dilution standard curve.

Animal model and management

The 7-week-old SD rats were maintained in 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. Rats were provided a randomly supply of food and distilled water and placed in a controlled environment (temperature, 23 ± 2 °C; humidity, 35–60%; and photoperiod, 12-h light/dark cycle).

Experimental design and acute reflux esophagitis induction

The 40 rats were randomly divided into four groups: (1) normal control rats (n=8), (2) reflux esophagitis control rats (n=8), (3) reflux esophagitis rats treated with geraniin 15 mg/kg body weight (n=8), and (4) reflux esophagitis rats treated with geraniin 30 mg/kg body weight (n=8). (5) positive control: reflux esophagitis rats treated with ranitidine 30 mg/kg body weight (n=8). After 18 h fasting, the rats in the geraniin groups were administrated with 15 and 30 mg/kg geraniin

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90 min prior to the abdominal surgery. The rats were anesthetized using an inhalation anesthesia. A midline laparotomy was performed to expose the stomach, the transitional junction between the forestomach and the corpus were exposed, and then they were subsequently ligated with 3–0 silk thread except to normal control. After 5 h, esophagus samples were collected and kept in −80 ℃ for western blotting analysis and immediately fixed with 10% neutral-buffered formalin (NBF) for histological analysis.

Esophageal lesion ratio
The esophagus was rinsed with saline solution, and the remaining tissue was placed on white paper. Then, the dissected esophagus was take pictures using an optical digital camera (Nikon, Tokyo, Japan) and the images were analyzed using the Image J program. The gross esophagus tissue damage ratio was calculated as follows:

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

Histopathological analysis of esophageal tissue
Esophagus samples of each rat were collected and immediately fixed in 10% (NBF). Samples were processed using an auto processor (Excelsior ES, Thermo Scientific, USA). After embedding, paraffin section (5-μm) were stained with hematoxylin and eosin (H–E) and mounted on glass slides. Digital images of sample were obtained using a Leica DM2500 microscope (Leica Microsystems, Germany) at a fixed 200 × magnification.

Preparation of cytosol and nuclear fraction of esophagus
Tissues were homogenized in 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 (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) mixture solution and 10% NP-40 were added. The nuclear fraction was extracted in 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 PI mixture solution. The protein concentration was measured using a Bio-Rad protein kit.

Western blot analysis
The proteins (8μL) were separated by 10 and 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to nitrocellulose membranes. The membrane was maintained with 5% skim milk in phosphate-buffered saline (PBS) solution-1% Tween 20 for 1 h at room temperature and incubated with the primary antibodies (1:1000) for

overnight at 4 ℃. Then, the membrane incubated secondary antibodies (1:10,000) at 2 h. Bands were detected using western blotting luminal reagent and images were obtained using a ChemiDoc™ MP imaging system (Bio-Rad, USA).

Statistical analysis
The statistical analysis was performed using the statistical package of the social sciences (SPSS) software (SPSS Inc., Chicago, IL) using an analysis of variance (ANOVA), followed by Tukey's multiple comparison tests at $p < 0.05$.

Results
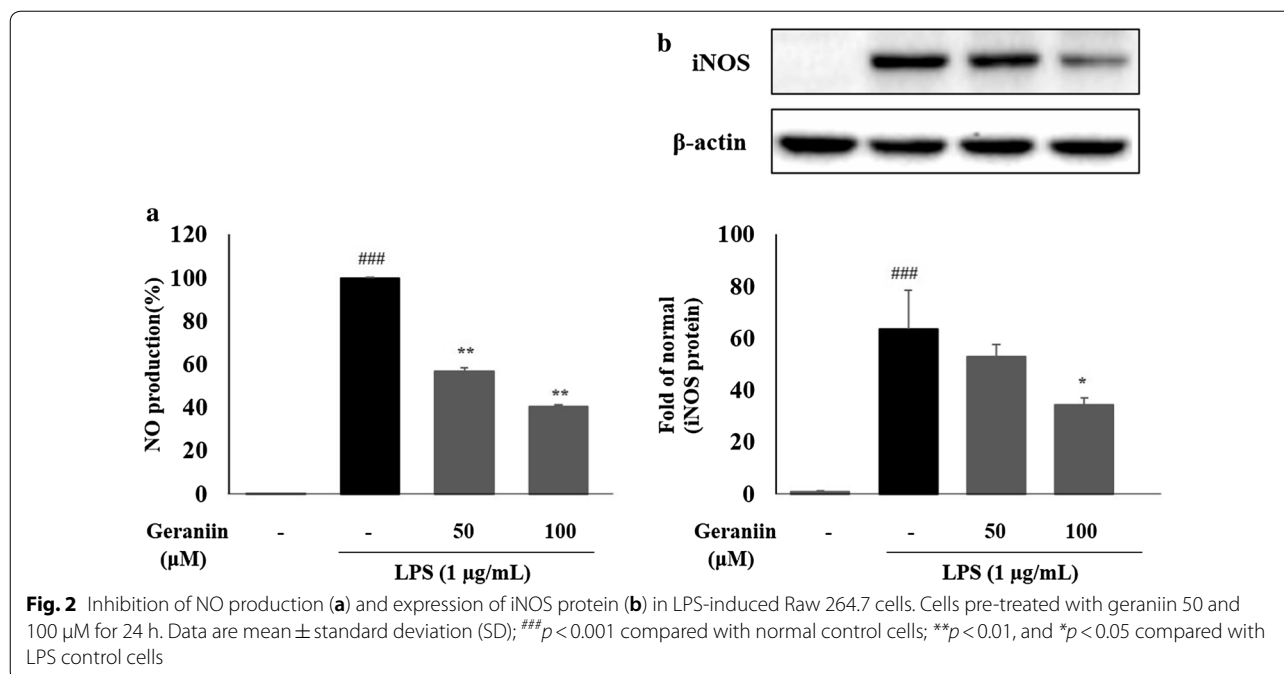### Cytotoxicity and optical morphological transformation in Raw 264.7 cells
The cell viability on LPS (1 μg/mL)-induced Raw 264.7 cells cultured in incubator for 24 h was measured by a cytotoxicity assay kit, to identified the cytotoxicity of geraniin. Geraniin showed does not significant cytotoxicity on Raw 264.7 cells up to concentrations 50 and 100 μM (Fig. 1b). In the view of morphological images, we observed the cell transformation such as a swelling formation and cell wall change by inflammation reaction on LPS (1 μg/mL) induced cells compared with untreated cells. However, the morphological change was decreased on LPS-induced cells pre-treated with geraniin 50 and 100 μM. Also, the result that take a view of morphological transformation of cells showed the inflammation reaction on LPS induced Raw 264.7 cells lead to morphological change. Especially, the cells pre-treated with geraniin 100 μM showed most similar shape to normal (Fig. 1a).

NO production and iNOS expression in LPS-induced inflammation in Raw 264.7 cells
Main pro-inflammatory mediator NO plays a significant role in immune system and it is produced by iNOS during inflammatory responses. Also, iNOS revealed after exposure to pro-inflammatory conditions in the cell and tissue [11]. As shown in Fig. 2, the NO production and iNOS protein expression was hardly observed in the normal group compared to that in the LPS-treated group. In the cells pre-treated with the geraniin (0, 50 and 100 μM), NO production was significantly inhibited compared to LPS treated group and the effects were concentration-dependent. In particular, NO production most decreased by over 50% in cells treated with geraniin 100 μM. In addiction we used western blotting to determine the suppress effect of geraniin in pro-inflammatory protein iNOS. Data showed that geraniin significantly reduced NO production and iNOS protein activity in Raw 264.7 cells.
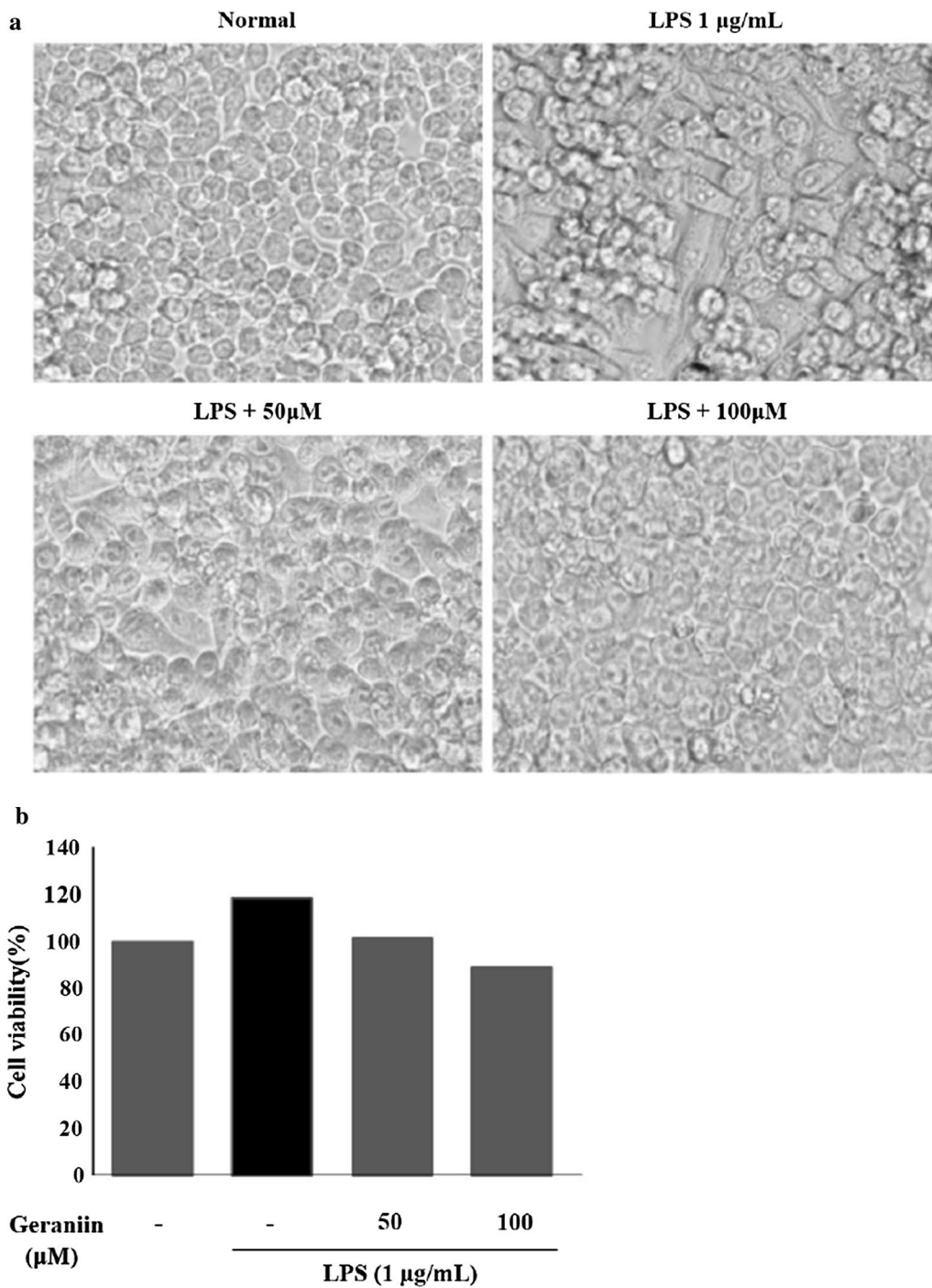
Fig. 1 Morphological transformation (**a**) and cell viability (**b**) on LPS-induced Raw 264.7 cells. Cells treated with the geraniin (0, 50 and 100 μM) for 24 h. The cell viability was determined using a cytotoxicity assay kit and showed not significant cytotoxicity. The morphology was visualized by a light microscope at 200 × magnitude

Fig. 2 Inhibition of NO production (**a**) and expression of iNOS protein (**b**) in LPS-induced Raw 264.7 cells. Cells pre-treated with geraniin 50 and 100 µM for 24 h. Data are mean ± standard deviation (SD); *###p* < 0.001 compared with normal control cells; ***p* < 0.01, and **p* < 0.05 compared with LPS control cells

Changes of esophagus tissue damage in acute RE rats

To evaluated protective effects of geraniin on reflux esophagitis rats, experimental rats were orally administrated with geraniin (15 and 30 mg/kg body weight) and ranitidine 30 mg/kg body weight 90 min before the surgery. Then, 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 [17], and esophageal damage was induced by gastric acid reflux during the ligation. Our data showed that the esophageal tissue of the RE control group showed tissue damages and hemorrhage induced by inflammation compared to the normal control group (Fig. 3). The geraniin 15 (15 mg/kg) group showed a reduced incidence of damages and hemorrhage in the esophagus compared to the RE control group. In the RE + geraniin 30 and ranitidine (30 mg/kg) group, the esophageal erosions area of rats significantly decreased by more than 80% and 90% compared to the RE control group. Therefore, these results indicate that the geraniin has protective effects against esophagus tissue damage in acute RE rats.
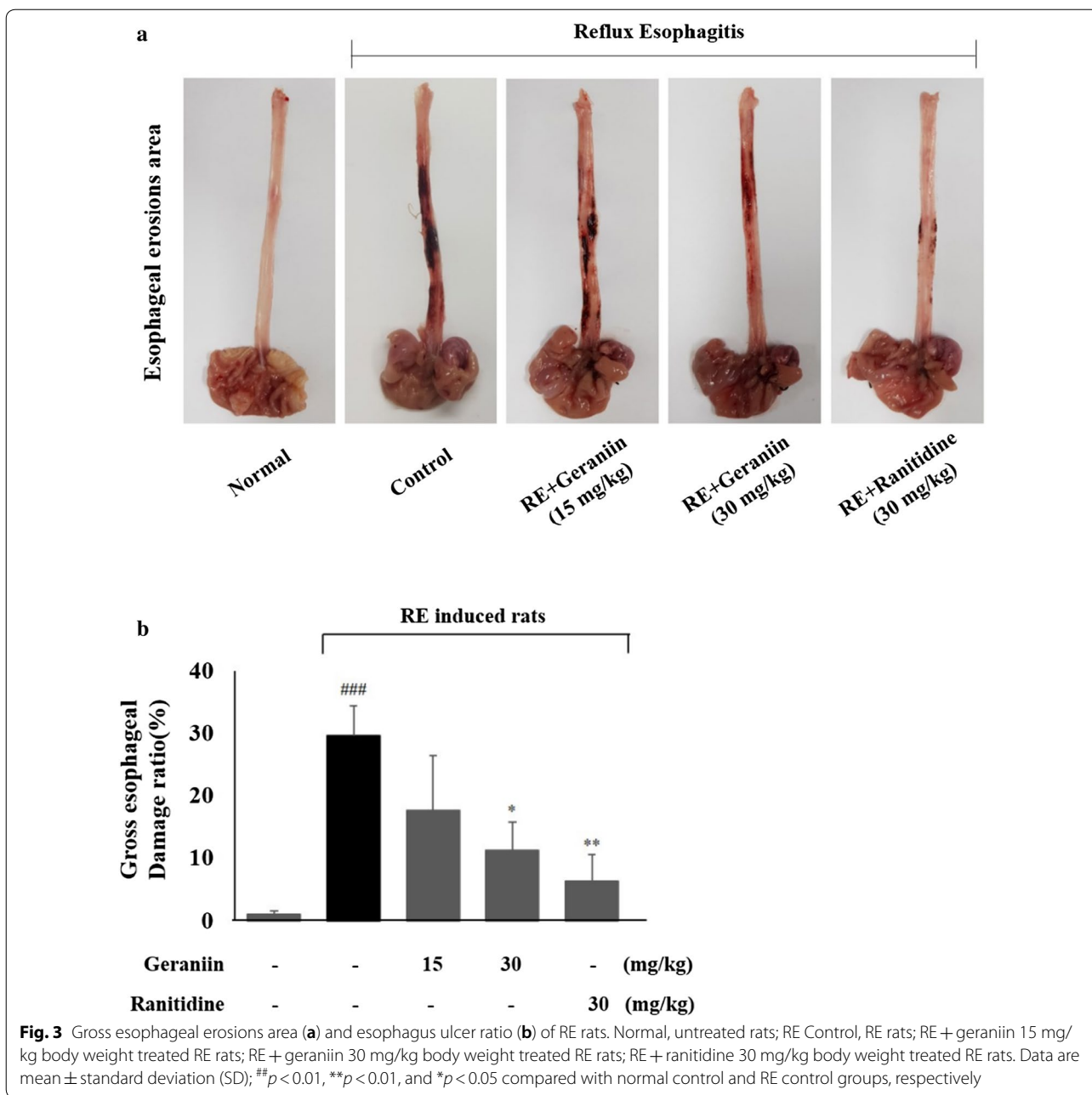
Histopathology analysis of esophagus of RE rats

The esophagus of the normal control group did not exhibit tissue changes while severe damages such as inflammatory cell infiltration and a thin epithelial layer were observed in the mucosa and submucosa of the RE-induced rats. However, we obtained result that the esophagus tissue damage of the RE rats was alleviated to a similar condition to that of the normal group by treatment with geraniin 15, 30 mg/kg and ranitidine 30 mg/kg. The result of histopathology analysis was similar to experimental result which measured a tissue damage area (Fig. 4).

### Pro-inflammatory protein expression in esophagus

Dysregulation of inflammation can induce tissue injuries and wide inflammatory diseases. It is well-known that inflammatory cytokines and stresses lead to increased phosphorylation and activation of activates NF-κB. NF-κB, main regulator of the production of inflammatory proteins such as iNOS, COX-2, activated by cytokines TNF-α and IL-1β and pathway during immune responses following its dissociation and phosphorylation by IκB-α [18]. In the RE control group, the expression level of both phosphorylated-IκB-α (p-IκB-α) and phosphorylated-p65 (p-p65) proteins was increased more by tissue damage than they were in the normal control group (Fig. 5). Geraniin was significantly reduce the p-IκB-α and p-p65 proteins compared with RE control. Effect of geraniin 30 mg/kg treatment was similar to ranitidine 30 mg/kg on damaged tissue in RE rats. In addition, the inflammatory cytokines and enzymes increased by tissue damage. However, the result showed that geraniin and ranitidine improved the esophageal inflammation by inhibiting the expression of cytosolic, COX-2, TNF-α and IL-1β, especially at 30 mg/kg.

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Fig. 3 Gross esophageal erosions area (**a**) and esophagus ulcer ratio (**b**) of RE rats. Normal, untreated rats; RE Control, RE rats; RE + geraniin 15 mg/kg body weight treated RE rats; RE + geraniin 30 mg/kg body weight treated RE rats; RE + ranitidine 30 mg/kg body weight treated RE rats. Data are mean ± standard deviation (SD); $^{\#\#}p < 0.01$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with normal control and RE control groups, respectively

Claudin-4 and claudin-5 protein expression in esophagus

Western blot analysis for measurement TJP claudin-4 and claudin-5 result showed in Fig. 6. The expression of claudin-4 and claudin-5 significantly decreased on esophagus in the RE control. However, reduction of expression showed not significantly in the claudin-5. The expression of the TJP was measured the highest in the normal control. We observed the effects of geraniin on TJP on esophagus tissue in RE rat. In this study, as an esophageal erosions decreased, the expression of claudin-4 and claudin-5 proteins were increased by concentration-dependent manner on esophagus tissue in a RE rat. We found that the expression of claudin-4 and claudin-5 were all increased by concentration-dependent and significantly higher in pre-treated ranitidine and geraniin 30 mg/kg group compared with RE control group.

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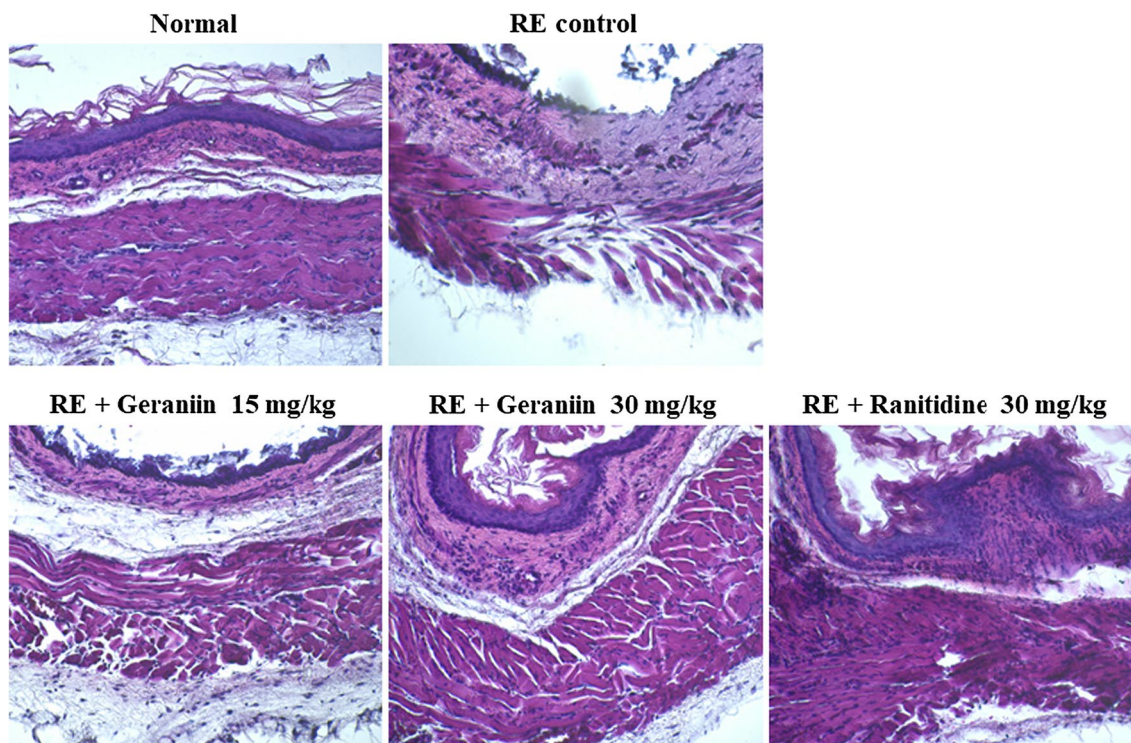
Fig. 4 The image of esophagus stained with Hematoxylin and eosin (H–E) in RE rats. Normal, untreated rats; RE Control, RE rats; RE + geraniin 15 mg/kg body weight treated RE rats; RE + geraniin 30 mg/kg body weight treated RE rats; RE + ranitidine 30 mg/kg body weight treated RE rats

Discussion

Plant secondary metabolites such as phenolic and flavonoid components have a beneficial activity [19]. Geranium has been used form inflammation, diarrhea and skin rash disease and the antiviral, antioxidant and anti-inflammatory components isolated from Geranium has been accomplished [13, 20]. In addition, many studies of traditional medicine showed geraniin, a main phenolic component of Geranium, has a various biology activity such as anti-inflammatory, anticancer and hepaprotective effect [11, 15, 21].

In current study, we investigated the biology activity in vitro and in vivo to identify the protective effects of geraniin. The cytotoxicity was investigated to evaluate the application possibility as a safety replacement therapy. Geraniin has no significantly cytotoxicity and on Raw 264.7 cells up to concentrations 50 and 100 μM. Non-cytotoxic level of geraniin on LPS-induced RAW 264.7 cells and the cytoprotective effect in HepG2 cells have been reported [11, 22]. In the view of morphological images, cell transformation was significantly induced by LPS compared with untreated cells. However, the morphological change was decreased on LPS-induced cells pre-treated with geraniin 50 and 100 μM (Fig. 1).

Inflammatory mediator NO production was hardly increased on LPS treatment. In the cells were pre-treated with geraniin at concentrations of 50 and 100 μM, NO production was significantly inhibited compared to LPS treated group and the effects were concentration-dependent. In particular, NO production most decreased by over 50% in cells treated with geraniin 100 μM. Furthermore, the results indicated geraniin significantly suppressed the expression of inflammatory protein iNOS. All these results, demonstrate a non-cytotoxic and anti-inflammatory effects on LPS-induced Raw 264.7 cells.

(See figure on next page.)

Fig. 5 Inhibitory effects of geraniin on inflammatory protein and cytokine expression in esophagus. Expression of pro-inflammatory protein TNF-α, IL-1β, COX-2, p-IkB-α and p-p65. Rats were pre-treated with geraniin 15 and 30 mg/kg, and ranitidine 30 mg/kg body weight excluding normal and RE control for 90 min before surgery. After 5 h, the esophageal mucosa was cut, and then, the was abdomen open and removed from each rat. Data are mean ± standard deviation (SD). Significance: $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ compared with normal control group; $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with RE control group

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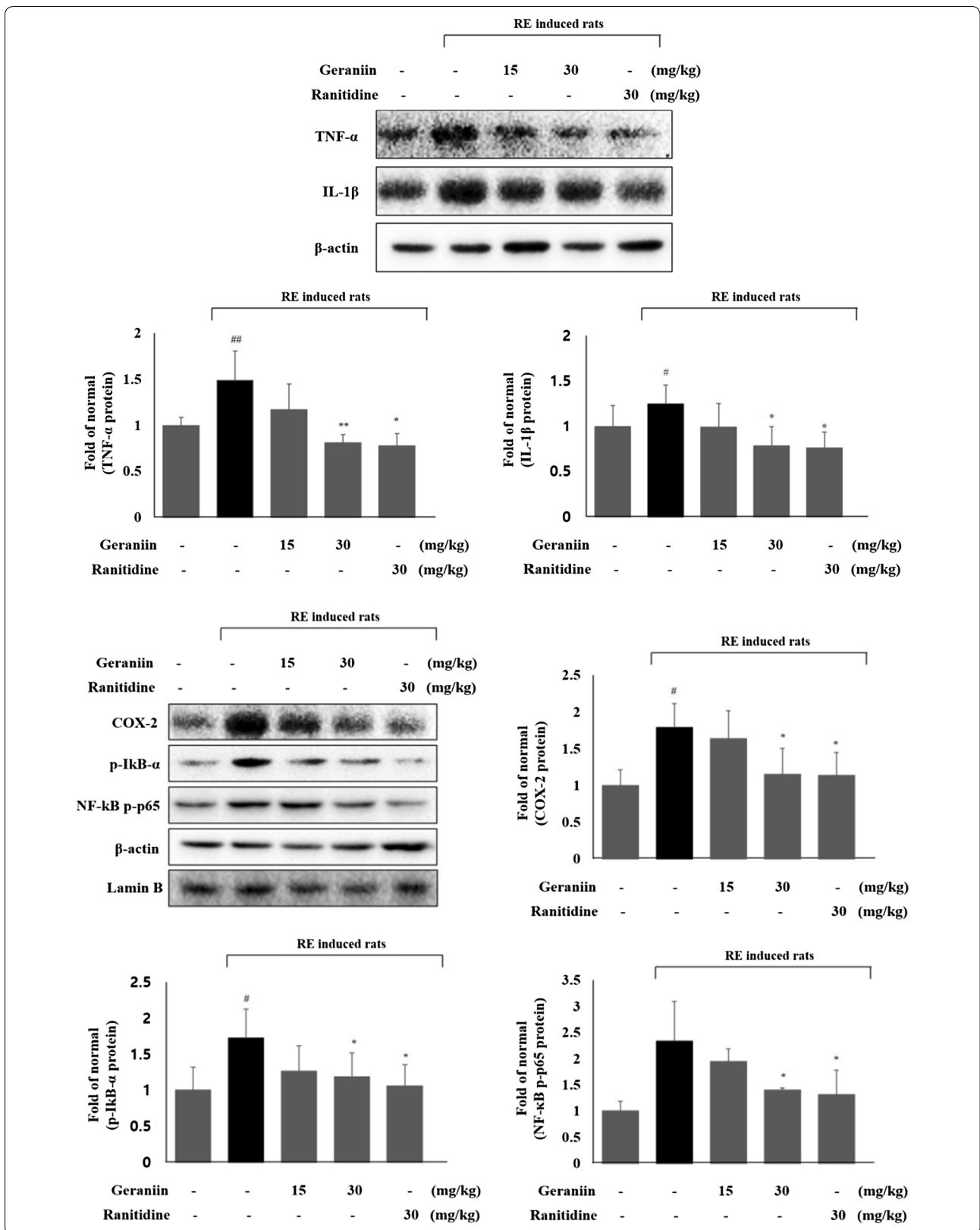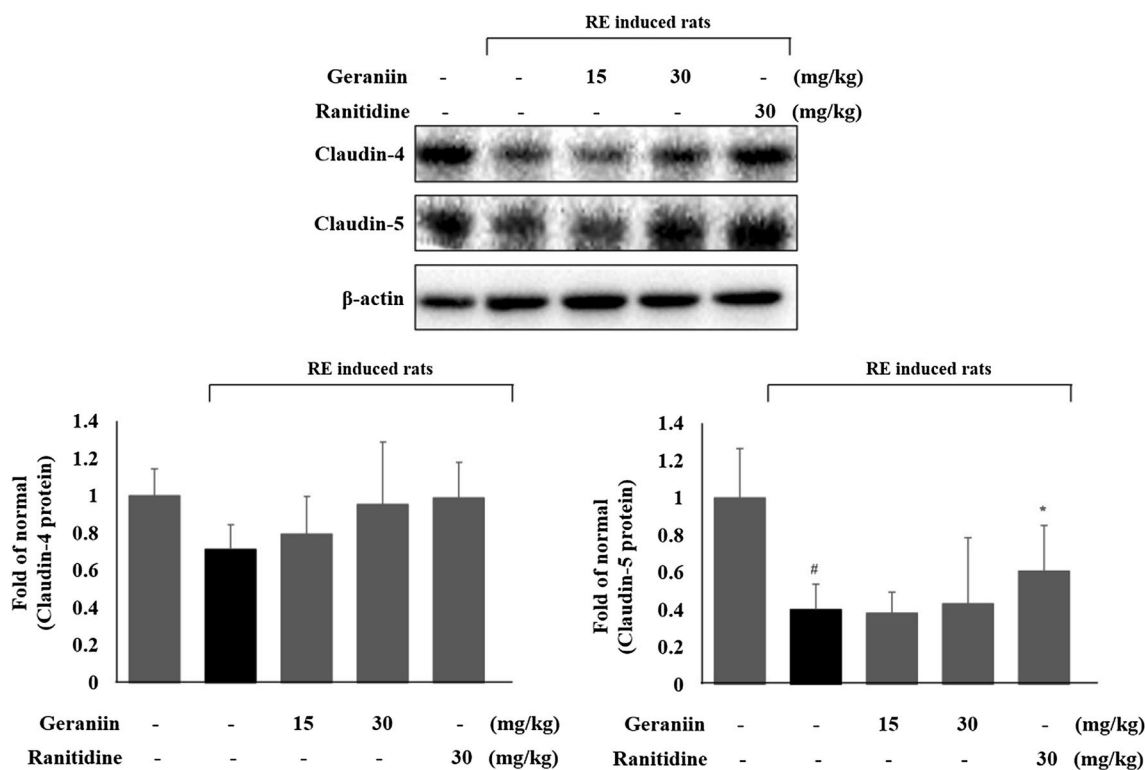
Fig. 6 Effects of geraniin on TJP (claudin-4 and claudin-5) in esophageal damage by RE rats. Rats were pre-treated with 15 and 30 mg/kg body weight geraniin, and 30 mg/kg ranitidine excluding normal and RE control for 90 min before surgery. Then, 5 h later, the esophageal mucosa was cut, and collected from each rat. Data are mean ± standard deviation (SD). Significance: #$p < 0.05$ and *$p < 0.05$ compared with normal control and RE control groups, respectively

The toxic effects of gastric acid and stomach contents lead to epithelial injury and cell death in esophageal mucosa [1, 23]. To evaluated protective effects of geraniin on reflux esophagitis rats, experimental rats were orally administrated with geraniin (15 and 30 mg/kg body weight) and H2 inhibitor ranitidine 30 mg/kg body weight as positive control. Ranitidine, a histamine-2 receptor antagonist, used as a reference drug in study of gastritis and clinical practice [24, 25]. In damage area and histological analysis showed that the esophageal tissue of the RE control group indicated a tissue damages such as hemorrhage, inflammatory cell infiltration and epithelium destruction induced by reflux (Figs. 3, 4). However, these symptoms were markedly decreased by treatment drug ranitidine 30 mg/kg. In the RE + geraniin 30 (30 mg/kg) group, the esophageal erosions area of rats significantly decreased by more than 68% compared to the RE control group and the esophagus tissue damage of the RE rats was alleviated to a similar condition to that of the normal group by treatment with geraniin 30 mg/kg. Therefore, these results indicate that the geraniin has protective effects against esophagus damage in acute RE rats.

In the RE control group, the expression levels of both phosphorylated-IκB-α and phosphorylated-NF-κB protein were increased more by tissue damage than they were in the normal control group. The result showed that the highest inhibitory effects of inflammatory proteins indicated in the ranitidine 30 mg/kg treatment and 15 and 30 mg/kg geraniin improved the esophageal inflammation by inhibiting the expression of cytosolic COX-2, TNF-α and IL-1β, especially at 30 mg/kg (Fig. 5). Several studies have been reported that the expression of pro-inflammatory cytokines and enzymes such as TNF-α, IL-1β, iNOS, COX-2 genes demonstrated in the damage tissue and it was related to degree of damage [24, 26, 27]. Thus, we found that geraniin was protects mucosa membranes via inhibiting pro-inflammatory proteins of NF-κB pathway signal on damaged esophagus in RE rats.

Claudin, one of the TJP in the epithelium and endothelium, was exerts critical barrier function of the tight junction. The expression of claudin-4 and claudin-5 was highest in normal control, in contradistinction to RE control (Fig. 6). Also, as an esophageal erosions decreased, the expression of claudin-4 and claudin-5 proteins were increased by concentration-dependent manner

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on esophagus tissue in a RE rat model. The regulating of claudin proteins in the damaged cells and tissue has been reported in experimental gastro-esophageal reflux disease [8, 28]. We found that the expression of claudin-4 and claudin-5 were all increased by concentration-dependent and significantly higher in pre-treated ranitidine and geraniin 30 mg/kg group compared with RE control group. TJP interact with the intestinal immune system to maintain the mucosal immune system [29]. The study shows that geraniin was ameliorate the esophagus damage by reflux esophagitis via regulating an immunity systems and TJP in tissue.

This study was to demonstrate the ameliorating effects of geraniin on esophagus in RE. Anti-inflammatory effects of geraniin identified by inhibitory activity of NO production and iNOS expression on Raw 264.7 cells. In RE rat model, geraniin showed a protective effect on esophagus damage and decreased inflammatory cell infiltration and epithelial loss in mucosa. In addition, we found that the defense mechanism underlying acid reflux-induced damage involved inflammatory processes, and geraniin protected the esophagus tissue by downregulating on NF-κB pathway signaling. Moreover, the expression of tight junction proteins in the esophagus tissue showed tendency to increase following geraniin treatment in RE rats. In the study, we identified the protective effects of geraniin on RE and can suggested that it could be safe alternative therapeutic source for inflammatory diseases.

Authors' contributions
BKC conceived and designed the experiments. HHN performed most of experiments and wrote the manuscript. LN and JCP conducted secondary experiment and data analysis. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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