

# Ethanol Extract of *Magnolia sieboldii* Buds Ameliorated Esophageal Tissue Injury Induced by Gastric Acid Reflux in Rats via Regulating the Nuclear Factor-κB Signaling Pathway

Li Nan, Hyeon-Hwa Nam, Jin-Cheon Park<sup>1</sup>, Byung-Kil Choo

Department of Crop Science and Biotechnology, Chonbuk National University, Jeonju, <sup>1</sup>Bioenergy Crop Research Institute, Rural Development Administration, National Institute of Crop Science, Bioenergy Crop Research Institute, Jeollanam-do, Republic of Korea

Submitted: 08-02-2019

Revised: 26-06-2019

Published: 11-02-2020

## ABSTRACT

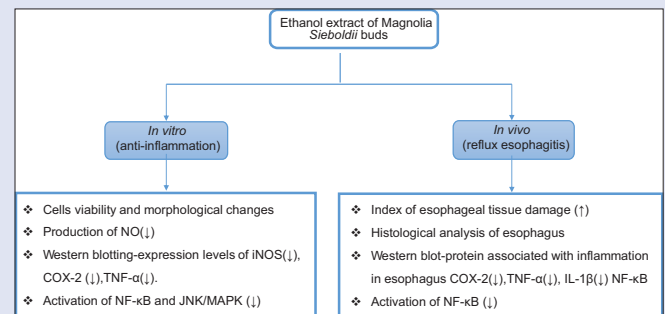
**Background:** Reflux esophagitis (RE) is one of the gastroesophageal reflux diseases that seriously affect the daily life of patients. *Magnolia sieboldii* K. Koch has been used as a traditional Chinese herbal medicine for stomach pain relief and deworming and has various physiological activities. **Objective:** The objective is to investigate the anti-inflammatory and protective effects of the ethanol extract of *M. sieboldii* buds (MsE) on RE. **Materials and Methods:** The different concentrations of MsE were used to determine the anti-inflammatory effects on lipopolysaccharide (LPS)-induced cells. In addition, the treatment of MsE on experimental RE rats were used for determining the protective effects of MsE on RE. **Results:** *M. sieboldii* treatment effectively inhibited LPS-induced nitric oxide (NO) production, protein expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-alpha (TNF-α) by regulating the activation of nuclear factor-κB (NF-κB) and C-Jun N-terminal kinase/mitogen-activated protein kinase in cells. Furthermore, *M. sieboldii* treatment significantly ameliorated esophageal tissue damage caused by gastric acid reflux, as well as inhibited the expression of COX-2, TNF-α, and interleukin-1 beta in esophageal tissues through inhibition the activation of NF-κB. **Conclusion:** We suggested that the ethanol extract of *M. sieboldii* could consider being an alternative medicine material for treating RE. **Key words:** Anti-inflammation, inflammatory cytokines, *Magnolia sieboldii* K. Koch, nuclear factor κB signaling pathway, reflux esophagitis

## SUMMARY

In the present study, we explored the anti-inflammatory effects of ethanol extract of *Magnolia sieboldii* buds (MsE) on lipopolysaccharide-induced RAW 264.7 cells and the protective effects of MsE on experimental reflux esophagitis (RE). We found that the MsE has good anti-inflammatory effects and ameliorated the esophageal tissue injury in rats. We suggest that MsE could consider to be an alternative medicine material for treating the disease of RE.

**Abbreviations used:** RE: Reflux esophagitis; GERD: Gastroenterology gastroesophageal reflux disease; MsE: Ethanol extract of

*M. sieboldii* buds; LPS: Lipopolysaccharide; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase-2; TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor-Kb; IL-1 β: Interleukin-1 beta; MAPK: Mitogen-activated protein kinase; PVDF: Polyvinylidene fluoride; DMEM: Dulbecco's modified eagle's medium; FBS: Fetal bovine serum; NO: Nitric oxide; RIPA: Radio-immunoprecipitation assay; NBF: Neutral buffered formalin; SDS: Sodium dodecylsulfate; HPLC: High-performance liquid chromatography; SPSS: Statistical product and service solutions; JNK: C-Jun N-terminal kinase.



## Correspondence:

Prof. Byung-Kil Choo,  
Department of Crop Science and Biotechnology,  
Chonbuk National University, Jeonju 54896,  
Republic of Korea.  
E-mail: bkchoo@jbnu.ac.kr  
DOI: 10.4103/pm.pm\_57\_19

## Access this article online

Website: www.phcog.com

## Quick Response Code:



## INTRODUCTION

Inflammation occurs when the body is attacked by micro-organisms and physical trauma, among others. Although inflammation is a body protective response, persistent inflammation is harmful, potentially inducing various diseases, such as rheumatoid arthritis, even cancer.<sup>[1]</sup> Macrophages play a vital role in the innate immune response and integrate the innate and acquired immune responses. When inflammation occurs, macrophages proliferate, differentiate, or activate under the stimulation of growth factors.<sup>[2,3]</sup> Excessive activation of macrophages induced by lipopolysaccharide (LPS), generates several pro-inflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1 β), which further aggravate the inflammation progress.<sup>[4]</sup> Nuclear factor-κB (NF-κB), a

transcription factor that binds near the κ light-chain gene in B-cells, can regulate various components of the immune regulatory system, such as pro-inflammatory cytokines (TNF-α and IL-1 β), chemokines and inducible enzymes, including iNOS and COX-2, as well as proteins that

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

**Cite this article as:** Nan L, Nam HH, Park JC, Choo BK. Ethanol extract of *Magnolia sieboldii* buds ameliorated esophageal tissue injury induced by gastric acid reflux in rats via regulating the nuclear factor-κB signaling pathway. Phcog Mag 2020;16:161-8.

regulate various immune responses, including major histocompatibility complexes and costimulatory molecules.<sup>[5,6]</sup>

According to the American Society of Gastroenterology, gastroesophageal reflux disease (GERD) is a modern common gastroesophageal disease caused by esophageal sphincter dysfunction and esophageal motility failure, resulting in reflux of the stomach and duodenal contents into the esophagus, leading to esophageal injury.<sup>[7]</sup> In recent years, its prevalence has increased worldwide. The main symptoms of GERD are heartburn, regurgitation, chest pain, and sleep disturbance.<sup>[8]</sup> Reflux esophagitis (RE) is the most common type of GERD and seriously affects the daily life of patients. Current treatment strategies for RE are mainly through the administration of agents that suppress gastric acid secretion, such as the histamine-2 receptor antagonists and proton-pump inhibitors.<sup>[9,10]</sup> Although the effects of these two drugs are apparent, many patients have experienced recurrence, incomplete mucosal healing, or consequent complications.<sup>[11]</sup> Therefore, investigating the inhibitory effect of active compounds extracted from plants on RE has a positive impact on the development of modern drugs.

*Magnolia sieboldii* K. Koch (Magnoliaceae) is a small deciduous tree widely distributed China, Korea, and Japan. The plant has been used as a traditional Chinese herbal medicine for stomach pain relief and deworming.<sup>[12]</sup> Studies have shown that the pharmacological activities of *M. sieboldii* were anti-inflammatory and antitumor properties.<sup>[13-15]</sup> In addition, the bioactive substance, costunolide, present in this plant or plants of magnoliaceae family, can limit NF- $\kappa$ B activity and thereby inhibit the inflammatory response, induce cancer cell apoptosis and alleviate acute liver and lung injury, by regulating signaling pathway of mitogen-activated protein kinase (MAPK) signaling pathway.<sup>[16-19]</sup> In order to explore the protective effects of the ethanol extract of *M. sieboldii* buds (MsE), first, we determined the anti-inflammatory effects of MsE in LPS-induced RAW 264.7 cells, *in vitro*. In addition, we investigated the *in vivo* protective action of MsE against esophageal tissue damage caused by gastric acid reflux.

## MATERIALS AND METHODS

### Materials

LPS was purchased from Sigma (St. Louis, MO, USA). Luminol reagent and antibodies of iNOS, COX-2, IL-1  $\beta$ , TNF- $\alpha$ ,  $\beta$ -actin, p-I $\kappa$ B $\alpha$ , p-NF- $\kappa$ B, p65, SAPK/C-Jun N-terminal kinase (JNK), and p-SAPK/JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Griess reagent was obtained from Promega (Madison, WI, USA). The cell viability, proliferation, and cytotoxicity assay kit was purchased from DoGenBio Co., Ltd (Korea). Protease inhibitor cocktail was purchased from USB Corporation Cleveland (OH, Cleveland USA). DC<sup>-</sup> protein assay reagent, polyvinylidene fluoride (PVDF) membranes, and bovine serum albumin standard were obtained from Bio-Rad Laboratories (Hercules, California, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin were purchased from Welgene (Namcheon-ro, South Korea).

### Plant collection and ethanol extract of *Magnolia sieboldii* buds preparation

The *M. sieboldii* buds were collected in the Gangwon-do area of South Korea. The dried flower buds were ground into a powder and combined with 70% ethanol at 1:10 w/w for extraction (four times) at 50°C, for 2 h. Then, the MsE was concentrated, frozen and vacuum-dried and stored in an ultra-low-temperature freezer at -80°C until used.

### Determination of costunolide in ethanol extract of *Magnolia sieboldii* buds by high-performance liquid chromatography

One milliliter of 100% methanol was added to 10 mg of MsE and sonicated at room temperature for 30 min (two times). The mixture was filtered through a 0.45  $\mu$ m membrane filter and analyzed by high-performance liquid chromatography (HPLC), using a system equipped with a Waters 1515 binary pump (Milford, MA, USA), Waters 717 autosampler, and Waters 996 photodiode array detector set at a detection wavelength of 210 nm. Chromatographic separations were performed on a Waters Sunfire C<sub>18</sub> analytical column (250 mm  $\times$  4.6 mm). The mobile phase was acetonitrile: 0.05% phosphoric acid in distilled water (58:42, v/v) and was delivered in isocratic mode at a flow rate of 1 ml/min. The injection volume was 20  $\mu$ l. The concentration of costunolide was calculated using a standard curve of costunolide.

### Cell culture

RAW 264.7 cells were grown in DMEM with 10% FBS, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin, in a 5% CO<sub>2</sub> incubator at 37°C. Cells were seeded in 96-well plates or a 60 mm  $\times$  15 mm cell culture dish for 24 h.

### Cell viability and nitric oxide production determination

To detect the effects of MsE on cell viability and the inhibition of NO production, cells were seeded in 96-well cell culture plates at 5  $\times$  10<sup>5</sup> cells/ml. MsE (50, 100 and 200  $\mu$ /mL) was added to the plate. After 1 h, LPS (1  $\mu$ g/mL) was added. Eighteen hours later, the cells were centrifuged at 2500 rpm for 5 min. Then, the supernatant was assayed to determine the inhibitory effect of MsE on NO production (using the Griess reagent), and the precipitated cells were analyzed to determine the cytotoxicity of MsE, according to the protocol of the cell viability, proliferation, and cytotoxicity assay kit.

### Cellular protein extraction and Western blot sample preparation

To extract the protein from the cells, cells were seeded in a 60 mm  $\times$  15 mm cell culture dish at 1  $\times$  10<sup>6</sup> cells/mL. MsE (100 and 200  $\mu$ g/mL) was added to the plate. After 1 h, LPS (1  $\mu$ g/mL) was added to the plate, and the cells left for another 1 or 18 h. Cells were washed with PBS (three times) and centrifuged at 4000 rpm, 4°C for 3 min. The cell pellets were lysed by radio-immunoprecipitation assay lysis buffer with protease inhibitor mixture solution on ice for 15 min. The lysis mixture was centrifuged at 13,000 rpm, 4°C for 15 min. The supernatant was collected for quantification of the total protein of the cells, by the DC<sup>-</sup> protein assay reagent. The cellular protein was mixed with loading buffer (50% glycerol, 10% sodium dodecylsulfate [SDS], 0.1% bromophenol blue, 1 M Tris at pH 6.8 and 2-mercaptoethanol) to prepare the Western blot sample and then stored at -80°C until used.

### Maintenance of rats and experimental reflux esophagitis surgery

Seven-week-old, male Sprague-Dawley rats (body weight 180–220 g) were kept in cages with *ad libitum* access to food and water. In order to adapt rats to the environment, rats were maintained at a temperature of 23  $\pm$  2°C and a humidity of 35%–60% in a 12-h light/dark cycle. After 1 week, the rats were randomly divided into four groups ( $n = 8$ ):

normal group, RE-control group and MsE 100 and 200 mg/kg treatment RE-control groups, respectively. Rats were fasted 18 h before RE surgery. The normal group received no oral administration. The RE-control group and MsE treatment RE-control groups were orally administrated with saline and MsE 100 and 200 mg/kg, respectively, over 2 h. After

respiratory anesthesia, all the rats underwent surgery, to initiate esophageal reflux. Briefly, an opening of about 1.5–2.0 cm was cut around 1 cm below the rat's heart, to expose the stomach. The stomach and duodenum junctions and the forestomach were ligated separately to induce esophageal reflux. After 4.5 h of surgery, all rats were sacrificed. The stomach and esophagus of rats were removed, washed with physiological saline, cut longitudinally to expose the damaged portion of the esophagus, photographed, and the esophageal tissue was then immediately stored at  $-80^{\circ}\text{C}$  for use.

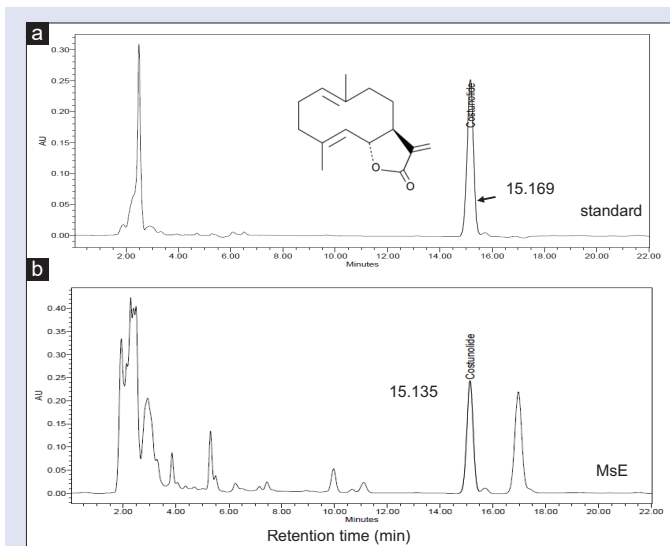
### Esophageal lesion index and histological study

The esophagus was photographed using a digital camera and analyzed using ImageJ software to calculate the esophageal lesion index, using the following formula: esophageal lesion index (%) = (area of esophageal damage [ $\text{mm}^2$ ]/total area of the esophagus [ $\text{mm}^2$ ])  $\times 100$ .

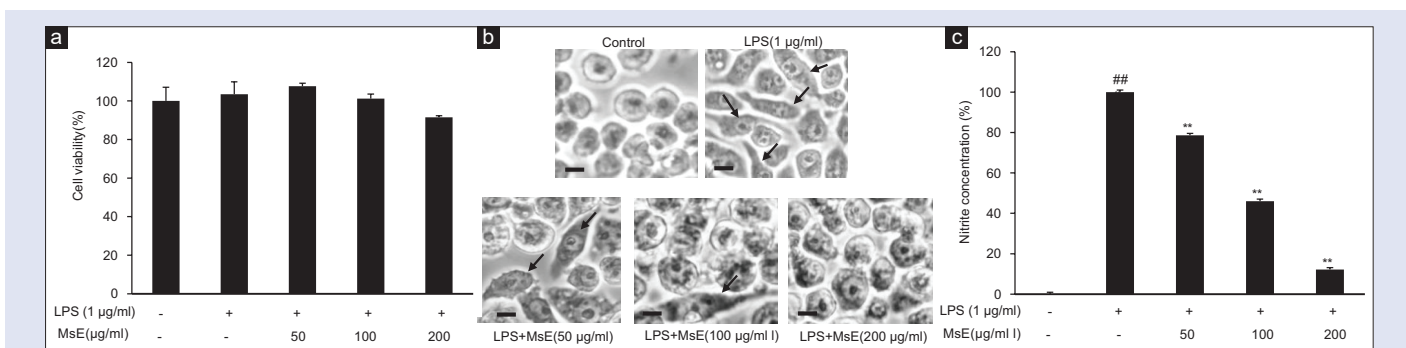
For histological analysis, the esophagus was washed with physiological saline, then cut into small pieces of 2–3 mm and immersed in 10% neutral buffered formalin. The esophageal specimens were rinsed, dehydrated and transparent, dipped in wax, embedded, sectioned ( $5\ \mu\text{m}$ ), and finally stained with hematoxylin–eosin and fixed on a slide. Digital images were captured by using a Leica microscope (Leica Microsystems, Germany) at  $\times 100$ .

### Extraction of esophageal tissue proteins

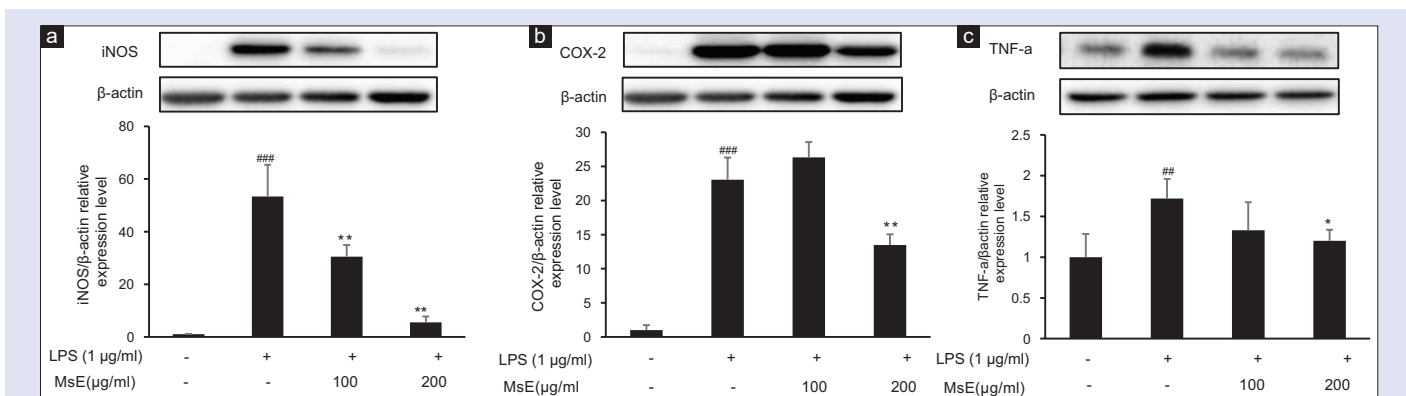
The protein extraction of esophageal tissue was carried out according to the method of Komatsu and colleagues. Briefly, the esophageal tissue was lysed using tissue lysis buffer-containing protease inhibitor cocktail on ice for 30 min and mixed once every 10 min. The lysate was centrifuged



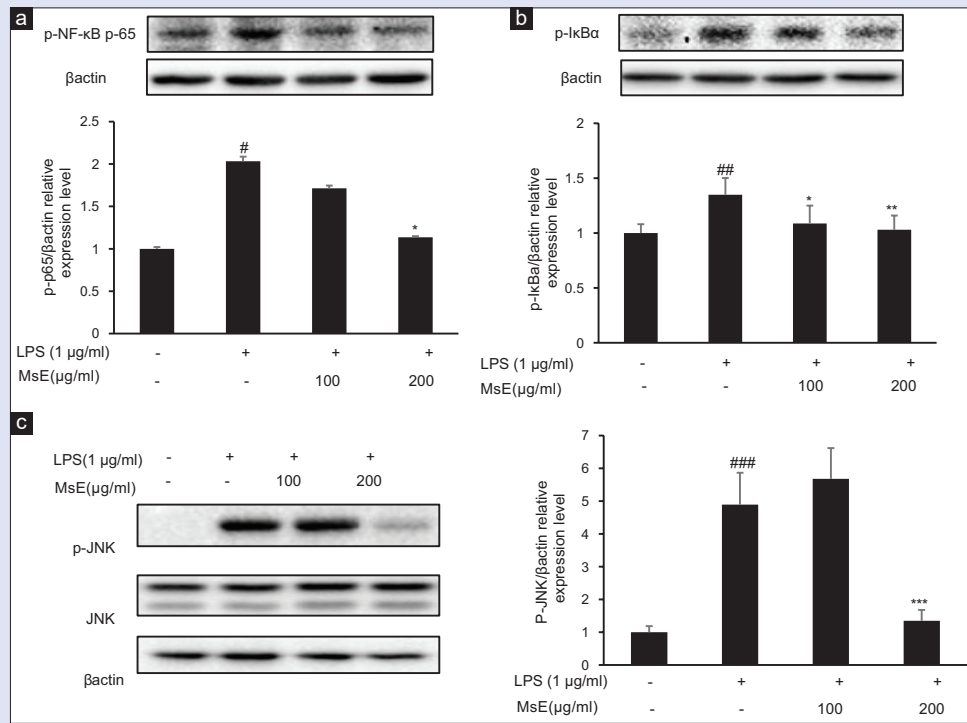
**Figure 1:** High-performance liquid chromatography analysis of standard of costunolide (10 mg/ml) (a) and ethanol extract of *Magnolia sieboldii* buds (10 mg/ml) (b)



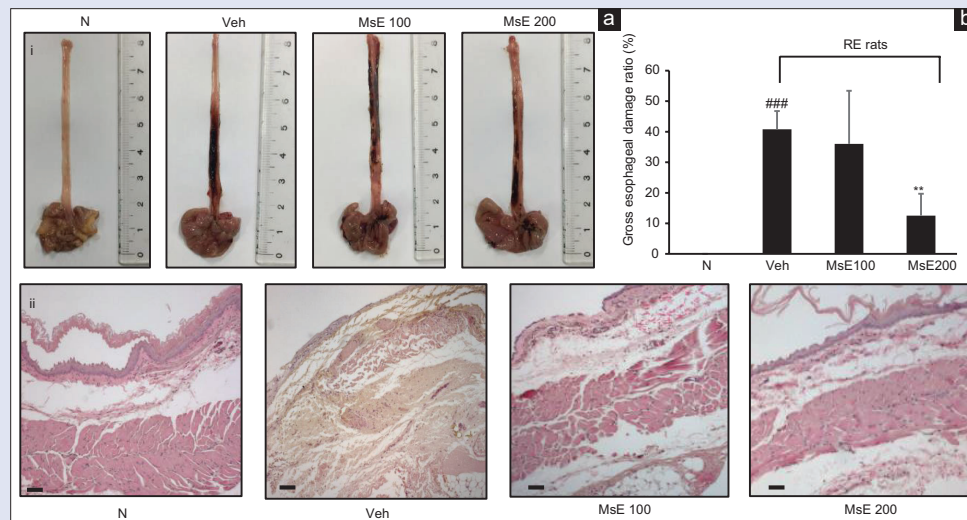
**Figure 2:** Effects of ethanol extract of *Magnolia sieboldii* buds on cell viability (a), morphological change (b) and nitric oxide production (c) measured by cell viability, proliferation and cytotoxicity assay kit and Griess reagent assay



**Figure 3:** Effects of ethanol extract of *Magnolia sieboldii* buds on the expression levels of inducible nitric oxide synthase (a), cyclooxygenase-2 (b) and tumor necrosis factor- $\alpha$  (c) in lipopolysaccharide-induced RAW 264.7 cells were measured by Western blot



**Figure 4:** Effects of ethanol extract of *Magnolia sieboldii* buds on the phosphorylation of nuclear factor-κB (a), IκBα (b) and C-Jun N-terminal kinase/mitogen-activated protein kinase (c) in lipopolysaccharide-induced RAW264.7 cells measured by Western blot



**Figure 5:** Effects of ethanol extract of *Magnolia sieboldii* buds on esophageal reflux induced esophageal mucosal damage in rats. Gross (a-i), microscopic (scale bar 200 μm) (a-ii) and the gross ratio of esophageal mucosal damage (b)

at 13,000 rpm for 3 min at 4°C, and cytoplasmic protein component was collected. The remaining pellet was re-suspended with lysis buffer constituted with protease inhibitor cocktail on ice for 30 min and mixed once every 10 min, followed by centrifugation at 13,000 rpm, 4°C for 15 min. Finally, the nuclear protein components were collected and stored at -80°C until used.

### Western blot analysis

Western blot samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes

were blocked with 5% skim milk at room temperature for 90 min and incubated with primary antibodies at 4°C overnight. After incubating with secondary antibodies at room temperature for 2 h, the bands were scanned by using Bio-Rad imaging software (New York, NY, USA).

### Statistical analysis

All data were expressed as mean ± standard deviation. Analysis of variance and Fisher's least significant difference multiple comparison test were undertaken using statistical product and service solutions 12.0K for

Windows (IBM Crop, Chicago, USA). A statistical significant difference was considered when  $P < 0.05$ .

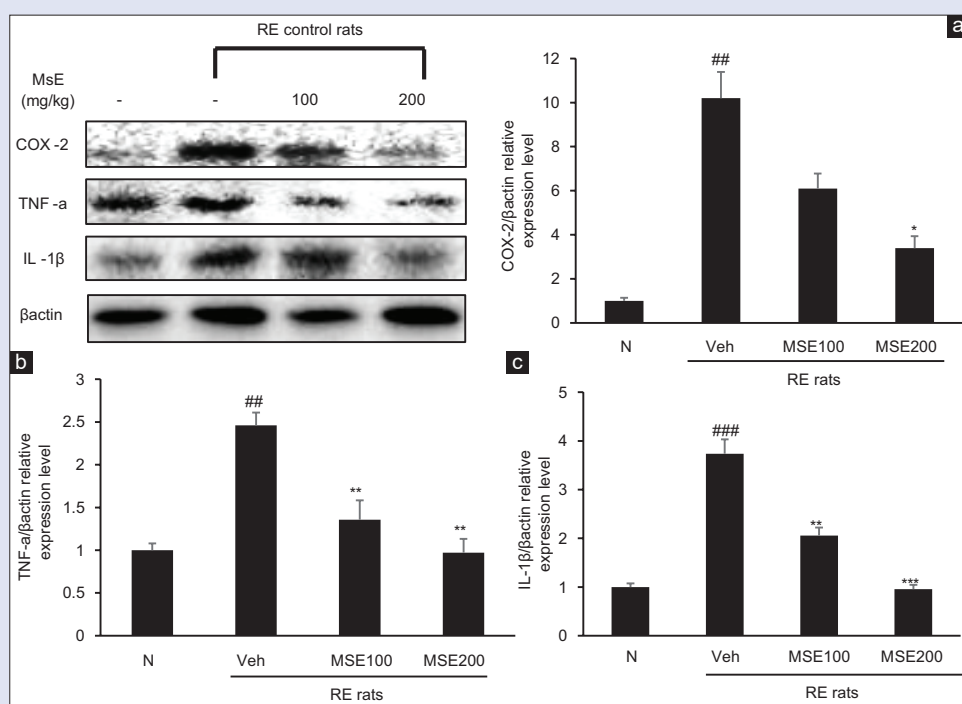
## RESULTS

### Content of costunolide in ethanol extract of *Magnolia sieboldii* buds

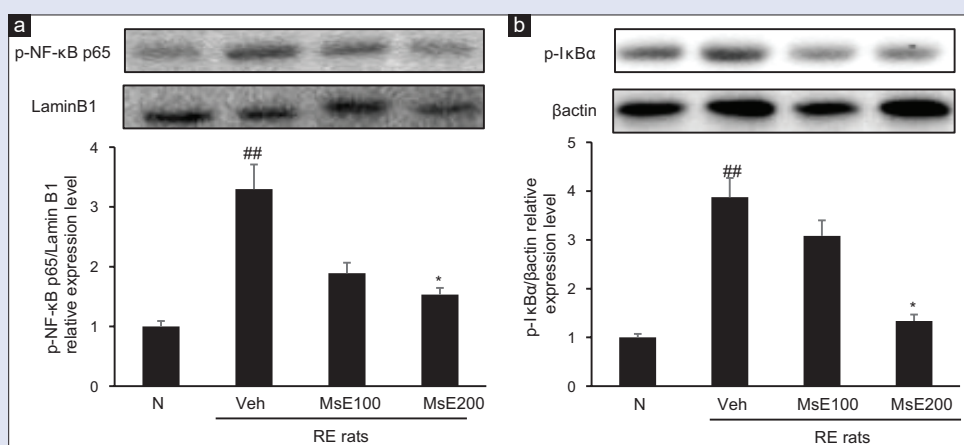
Costunolide was identified in the MsE by comparison of the peak retention time in the HPLC chromatogram for the costunolide standard [Figure 1a] with the MsE chromatogram [Figure 1b]. Based on the standard curve, the content of costunolide in 1 g MsE lyophilized powder was  $9.03 \pm 1.09$  mg.

### Effect of ethanol extract of *Magnolia sieboldii* buds on cell viability and nitric oxide production in RAW 264.7 cells

The effect of MsE on RAW264.7 cells viability was measured by the cell viability, proliferation, and cytotoxicity assay kit, which confirmed that MsE did not affect the cell viability, even at 200  $\mu\text{g}/\text{mL}$  [Figure 2a]. Moreover, as shown in Figure 2b, LPS-induced severe cell morphological changes, which was ameliorated by co-treatment with MsE. The effect of MsE on NO production was measured using the Griess reagent assay. We observed that LPS caused a large amount of NO production, whereas, the MsE co-treatment decreased NO production in a



**Figure 6:** Effects of ethanol extract of *Magnolia sieboldii* buds on the expression levels of cyclooxygenase-2 (a), tumor necrosis factor-alpha (b) and interleukin-1 beta (c) in esophagus were measured by Western blot



**Figure 7:** Effects of ethanol extract of *Magnolia sieboldii* buds on the phosphorylation of nuclear factor- $\kappa$ B (a) and I $\kappa$ B $\alpha$  (b) in esophagus measured by Western blot

concentration-dependent manner. Overall, MsE was not toxic to RAW 264.7 cells, alleviated changes in cell morphology caused by LPS and suppressed the LPS-induced inflammation in RAW 264.7 cells.

### Ethanol extract of *Magnolia sieboldii* buds suppressed the expression levels of inducible nitric oxide synthase, cyclooxygenase-2 and tumor necrosis factor-alpha in lipopolysaccharide-induced RAW 264.7 cells

When an inflammatory reaction occurs in cells, iNOS is produced in large amounts to promote a sharp increase in NO content, which aggravates the inflammatory response.<sup>[20]</sup> Excessive production of COX-2 and TNF- $\alpha$  also exacerbates the inflammatory response. Hence, inhibition of the expression of these proteins in the inflammatory response will play an important role in inhibiting the inflammatory response. According to the results shown in Figure 3, LPS induced an increase in the expression levels of these proteins, which was suppressed by co-treatment with MsE, especially at 200  $\mu\text{g}/\text{mL}$ . These results demonstrated that MsE inhibited NO production, by reducing the expression level of iNOS. At 200  $\mu\text{g}/\text{mL}$ , MsE also down-regulated the inflammatory mediator, COX-2 and the pro-inflammatory cytokine, TNF- $\alpha$ , expression levels, thereby inhibiting the inflammatory response in LPS-induced RAW 264.7 cells

### Ethanol extract of *Magnolia sieboldii* buds suppressed the activation of nuclear factor- $\kappa\text{B}$ and C-Jun N-terminal kinase/mitogen-activated protein kinase in lipopolysaccharide-induced RAW 264.7 cells

The NF- $\kappa\text{B}$  signaling pathway is a classical signaling pathway involved in the inflammatory response.<sup>[21]</sup> The activated NF- $\kappa\text{B}$  transfer to the nucleus where it regulates the gene expression of the target proteins to mediate the inflammatory response.<sup>[22]</sup> Therefore, we explored whether MsE can inhibit the activation of NF- $\kappa\text{B}$  induced by LPS. As a result [Figure 4a], MsE decreased the phosphorylation of NF- $\kappa\text{B}$  p65 compared to the LPS-induced group and also down-regulated the phosphorylation of Inhibitor of Kappa Light Chain Gene Enhancer in B Cells, Alpha I-Kappa-B-Alpha (I $\kappa\text{B}\alpha$ ) [Figure 4b]. MAPK plays an important role in cell differentiation, stress response, and apoptosis. Activated extracellular receptor kinase (ERK), JNK, and p38 MAPKs promote the production of pro-inflammatory factors and inflammatory mediators, which can aggravate the inflammatory response.<sup>[2]</sup> In this study, the phosphorylation of JNK [Figure 4c] was only suppressed by MsE at 200  $\mu\text{g}/\text{mL}$ .

### Ethanol extract of *Magnolia sieboldii* buds alleviated the damage of esophageal tissue and histological changes induced by gastric acid reflux

We observed the protective effect of MsE against experimental RE in rats. As shown in Figure 5a-i, the intact esophagus was observed in the normal (untreated) group, whereas the esophagus of the RE-control group showed a wide range of elongated deep red and black hemorrhagic lesions. However, the extent of the hemorrhagic lesions in the esophagus of the MsE-treated group was significantly reduced, especially at 200 mg/kg and the extent of the lesion was reduced by >50%. The gross ratio of the esophageal mucosal damage is shown in Figure 5b. In addition, histological examination [Figure 5a-ii] revealed that the esophagus of the normal (untreated) group evidenced no microscopic changes, whereas, in the RE-control group, most of the esophageal mucosa was absent and the submucosa and muscle layers showed deformation and looseness. However, these phenomena were alleviated in the MsE treatment group. Especially at the MsE dose of 200 mg/kg, the damage to the esophageal

tissue was significantly improved, and the submucosal and muscular layer tissues were also tightened. These data showed some correlation in the protective effect of MsE against RE.

### Ethanol extract of *Magnolia sieboldii* buds suppressed the expression levels of cyclooxygenase-2, tumor necrosis factor-alpha, and interleukin-1 beta in esophageal tissue

To further investigate the protective effects of MsE against RE, we determined the expression levels of inflammatory mediators and pro-inflammatory cytokines in the esophageal tissues. The protein expression levels of COX-2 [Figure 6a], TNF- $\alpha$  [Figure 6b], and IL-1  $\beta$  [Figure 6c] were up-regulated in the RE-control group, whereas all of these proteins were down-regulated by treatment with MsE. Thus, MsE can suppress the increase in expression of RE-related proteins caused by esophageal reflux.

### Ethanol extract of *Magnolia sieboldii* buds suppressed the phosphorylation of nuclear factor- $\kappa\text{B}$ and I $\kappa\text{B}\alpha$ in esophageal tissue

To explore the possible molecular pathways of the protective effect of MsE against RE, we further ascertained the phosphorylation of NF- $\kappa\text{B}$  and its inhibitory protein, I $\kappa\text{B}\alpha$ . As shown in Figure 7, esophageal reflux increased phosphorylation of NF- $\kappa\text{B}$  and I $\kappa\text{B}\alpha$ , whereas, MsE treatment reduced the phosphorylation of both proteins, particularly at a dose of 200 mg/kg. The results indicated that MsE limits the activation of NF- $\kappa\text{B}$  by inhibiting I $\kappa\text{B}\alpha$  phosphorylation to alleviate RE caused by esophageal reflux.

## DISCUSSION

RE is a disease that is showing a gradually increasing prevalence worldwide. Patients with RE are often plagued by symptoms, such as regurgitation and heartburn and need to take drugs for a long time to inhibit the secretion of gastric acid. Therefore, this disease has a serious burden on patients' lives and economic status.<sup>[23,24]</sup> It is necessary to find an alternative medicine that can effectively treat this disease.

The bark and flower of Magnoliaceae plants are used as a traditional Chinese herbal medicine to treat diseases, such as indigestion, colds and blocked nose.<sup>[25]</sup> It is also demonstrated that magnoliaceae plants have been shown to exhibit various physiological activities, including anti-inflammatory and anti-oxidant effects.<sup>[26,27]</sup> Costunolide, a main active substance present in Magnoliaceae plants, was confirmed to exist in the MsE by HPLC [Figure 1]. Therefore, to evidence whether MsE has protective effects against RE, we performed *in vitro* and *in vivo* experiments, respectively. *In vitro*, we explored the anti-inflammatory effects of MsE on LPS-induced RAW 264.7 cells. *In vivo*, we analyzed the protective effects of MsE against experimental RE in rats.

We found that stimulation of LPS led to an increase in the production of NO and the expression levels of inflammatory mediators (iNOS and COX-2) and pro-inflammatory cytokines (TNF- $\alpha$ ), which are involved in the occurrence and development of the inflammatory response.<sup>[28-30]</sup> However, MsE co-treatment significantly inhibited the production of NO and the expression levels of iNOS, COX-2, and TNF- $\alpha$ , particularly when used at a dose of 200  $\mu\text{g}/\text{mL}$  [Figures 2c and 3]. Furthermore, MsE co-treatment also down-regulated the phosphorylation of NF- $\kappa\text{B}$  and I $\kappa\text{B}\alpha$  [Figure 4a and b]. The MAPK pathway also plays an important role in regulating the gene expression of cytokines and inflammation-related mediators. The three MAPK signaling pathways that have been

extensively studied include p38, ERK and JNK as described.<sup>[31,32]</sup> Our results showed that the JNK/MAPK pathway was activated by LPS and MsE only inhibited JNK phosphorylation at a concentration of 200 µg/mL. Therefore, MsE can significantly inhibit the inflammatory response induced by LPS in RAW 264.7 cells [Figure 4c].

The most standardized and widely used model of experimental RE is the ligation of the pylorus, as well as the limiting ridge of the glandular portion and forestomach.<sup>[11,33,34]</sup> Gastric acid reflux causes symptoms, such as hemorrhagic lesions and esophageal hyperplasia, in the esophageal tissue.<sup>[35]</sup> Histologically, the mucosa of the esophageal tissue is shed, the submucosa and muscle layers are deformed, the submucosal hemorrhages and inflammatory cell infiltration occurs as described.<sup>[36]</sup> To observe the protective effects of MsE against RE, we measured the degree of esophageal tissue damage and conducted a histological analysis. It was found that gastric acid reflux induced a large hemorrhagic lesion in the esophageal tissue while the area of the injury was significantly reduced by MsE treatment [Figure 5a-i]. Concurrent histological observations also showed that MsE treatment significantly improved the changes in the esophageal tissue structure, including the reduction of mucosal shedding and tightening of the submucosa and muscle layer tissue [Figure 5a-ii].

Since NF-κB plays an important role in various diseases, it has become a model of cell, tissue, and organism level responses that are regulated by inducible transcription factors.<sup>[6]</sup> The NF-κB transcription factor family comprises five dimeric complexes, namely, p50:P105 (NF-κB1), p52:P100 (NF-κB2), c-Rel, RelA (p65), and RelB. Among them, only c-Rel, RelA, and RelB have a transcriptional activation region, that is, a domain necessary for gene expression regulation. However, p50 and p52 usually form heterodimers with p65, c-Rel, and RelB.<sup>[37]</sup> In a stable cellular environment, inactive NF-κB is anchored to the cytoplasm by the inhibitory IκB proteins (IκBα, IκBβ, or IκBε). When its signaling pathway is activated, IκBα is phosphorylated and degraded and phosphorylated NF-κB enters the nucleus and binds to the κB enhancer elements of target genes, inducing transcription of pro-inflammatory genes as described, such as iNOS, COX-2, TNF-α, and IL-1 β.<sup>[38]</sup>

To explore the possible molecular mechanism of RE, we performed Western blot analysis of esophageal tissue, which also verified that gastric acid reflux induced an increase in the expression of COX-2, TNF-α, and IL-1 β. However, MsE treatment down-regulated the expression of these proteins, especially at a concentration of 200 mg/kg [Figure 6]. Furthermore, increases in the phosphorylation of NF-κB and IκBα induced by gastric acid reflux were also inhibited by MsE treatment, especially when used at a dose of 200 mg/kg [Figure 7].

## CONCLUSION

Our results indicated that MsE could not only inhibit the inflammation of RAW 264.7 cells induced by LPS but also ameliorate the esophageal tissue damage caused by gastric acid reflux. In summary, MsE promotes amelioration of experimental RE by down-regulating inflammation via regulating the NF-κB signaling pathway, which may be a possible molecular mechanism of RE. Therefore, MsE can be considered as an alternative drug for the treatment of RE. We suggested that the ethanol extract of *M. sieboldii* could consider to be an alternative medicine material for treating RE.

## Acknowledgements

The authors would like to thank the National Research Foundation of Korea (NRF) (NRF-2017R1D1A3B03036020) for the support.

## Financial support and sponsorship

This research was carried out with the support of the National Research Foundation of Korea (NRF-2017R1D1A3B03036020).

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Butterfield TA, Best TM, Merrick MA. The dual roles of neutrophils and macrophages in inflammation: A critical balance between tissue damage and repair. *J Athl Train* 2006;41:457-65.
2. Zhou MM, Zhang WY, Li RJ, Guo C, Wei SS, Tian XM, *et al.* Anti-inflammatory activity of khayandirobilide A from *Khaya senegalensis* via NF-κB, AP-1 and p38 MAPK/Nrf2/HO-1 signaling pathways in lipopolysaccharide-stimulated RAW 264.7 and BV-2 cells. *Phytomedicine* 2018;42:152-63.
3. Cameron B, Landreth GE. Inflammation, microglia, and Alzheimer's disease. *Neurobiol Dis* 2010;37:503-9.
4. Guo H, Zhang Y, Cheng BC, Fu X, Zhu P, Chen J, *et al.* An ethanolic extract of the aerial part of *Siegesbeckia orientalis* L. Inhibits the production of inflammatory mediators regulated by AP-1, NF-κB and IRF3 in LPS-stimulated RAW 264.7 cells. *Biosci Trends* 2018;12:330-7.
5. Moynagh PN. The NF-kappaB pathway. *J Cell Sci* 2005;118:4589-92.
6. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell* 2008;132:344-62.
7. Wang YK, Hsu WH, Wang SS, Lu CY, Kuo FC, Su YC, *et al.* Current pharmacological management of gastroesophageal reflux disease. *Gastroenterol Res Pract* 2013;2013:983653.
8. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R; Global Consensus Group. The Montreal definition and classification of gastroesophageal reflux disease: A global evidence-based consensus. *Am J Gastroenterol* 2006;101:1900-20.
9. Brătucu E, Lucenco L. Gastroesophageal reflux disease – Current trends. *Chirurgia (Bucur)* 2012;107:147-53.
10. 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.
11. Kang JW, Lee SM. Protective effects of chlorogenic acid against experimental reflux esophagitis in rats. *Biomol Ther (Seoul)* 2014;22:420-5.
12. Chen FL, Zu YG, Yang L. A novel approach for isolation of essential oil from fresh leaves of *Magnolia sieboldii* using microwave-assisted simultaneous distillation and extraction. *Sep Purif Technol* 2015;154:271-80.
13. Kimura M, Suzuki J, Yamada T, Yoshizaki M, Kikuchi T, Kadota S, *et al.* Anti-inflammatory effect of neolignans newly isolated from the crude drug "Shin-I" (*Flos magnoliae*). *Planta Med* 1985;51:291-3.
14. Lim SS, Shin KH, Ban HS, Kim YP, Jung SH, Kim YJ, *et al.* Effect of the essential oil from the flowers of *Magnolia sieboldii* on the lipopolysaccharide-induced production of nitric oxide and prostaglandin E2 by rat peritoneal macrophages. *Planta Med* 2002;68:459-62.
15. Park HJ, Kwon SH, Han YN, Choi JW, Miyamoto K, Lee SH, *et al.* Apoptosis-inducing costunolide and a novel acyclic monoterpene from the stem bark of *Magnolia sieboldii*. *Arch Pharm Res* 2001;24:342-8.
16. Koo TH, Lee JH, Park YJ, Hong YS, Kim HS, Kim KW, *et al.* A sesquiterpene lactone, costunolide, from *Magnolia grandiflora* inhibits NF-kappa B by targeting I kappa B phosphorylation. *Planta Med* 2001;67:103-7.
17. Chen J, Chen B, Zou Z, Li W, Zhang Y, Xie J, *et al.* Costunolide enhances doxorubicin-induced apoptosis in prostate cancer cells via activated mitogen-activated protein kinases and generation of reactive oxygen species. *Oncotarget* 2017;8:107701-15.
18. Chen Z, Zhang D, Li M, Wang B. Costunolide ameliorates lipoteichoic acid-induced acute lung injury via attenuating MAPK signaling pathway. *Int Immunopharmacol* 2018;61:283-9.
19. Wang Y, Zhang X, Zhao L, Shi M, Wei Z, Yang Z, *et al.* Costunolide protects lipopolysaccharide/d-galactosamine-induced acute liver injury in mice by inhibiting NF-κB signaling pathway. *J Surg Res* 2017;220:40-5.
20. Hwang JH, Kim KJ, Ryu SJ, Lee BY. Caffeine prevents LPS-induced inflammatory responses in RAW264.7 cells and zebrafish. *Chem Biol Interact* 2016;248:1-7.
21. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001;107:135-42.
22. Liu JX, Tang JS, Zuo YH, Yu Y, Luo P, Yao XS, *et al.* Staurosporine B inhibits macrophage

- activation by inhibiting NF- $\kappa$ B and ERK MAPK signaling. *Pharmacol Res* 2016;111:303-15.
23. Katz PO, Gerson LB, Vela MF. Guidelines for the diagnosis and management of gastroesophageal reflux disease. *Am J Gastroenterol* 2013;108:308-28.
  24. El-Serag HB, Sweet S, Winchester CC, Dent J. Update on the epidemiology of gastro-oesophageal reflux disease: A systematic review. *Gut* 2014;63:871-80.
  25. Poivre M, Duez P. Biological activity and toxicity of the Chinese herb *Magnolia officinalis* Rehder & E. Wilson (Houpo) and its constituents. *J Zhejiang Univ Sci B* 2017;18:194-214.
  26. Rajgopal A, Missler SR, Scholten JD. *Magnolia officinalis* (Hou po) bark extract stimulates the Nrf2-pathway in hepatocytes and protects against oxidative stress. *J Ethnopharmacol* 2016;193:657-62.
  27. Zheng YF, Liu XM, Lai F. Extraction and antioxidant activities of *Mxagnolia kwangsiensis* Figlar & Noot. Leaf polyphenols. *Chem Biodivers* 2019;16:e1800409.
  28. Aktan F. INOS-mediated nitric oxide production and its regulation. *Life Sci* 2004;75:639-53.
  29. Martín Arias LH, Martín González A, Sanz Fadrique R, Vazquez ES. Cardiovascular risk of nonsteroidal anti-inflammatory drugs and classical and selective cyclooxygenase-2 inhibitors: A Meta-analysis of observational studies. *J Clin Pharmacol* 2019;59:55-73.
  30. Zhang JM, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin* 2007;45:27-37.
  31. Ma YL, Chen F, Shi J. Rhein inhibits malignant phenotypes of human renal cell carcinoma by impacting on MAPK/NF- $\kappa$ B signaling pathways. *Onco Targets Ther* 2018;11:1385-94.
  32. Gao W, Wang YS, Hwang E, Lin P, Bae J, Seo SA, *et al.* *Rubus idaeus* L. (red raspberry) blocks UVB-induced MMP production and promotes type I procollagen synthesis via inhibition of MAPK/AP-1, NF- $\kappa$ B and stimulation of TGF- $\beta$ /Smad, Nrf2 in normal human dermal fibroblasts. *J Photochem Photobiol B* 2018;185:241-53.
  33. Jabri MA, Tounsi H, Rtibi K, Marzouki L, Sakly M, Sebai H, *et al.* Ameliorative and antioxidant effects of myrtle berry seed (*Myrtus communis*) extract during reflux-induced esophagitis in rats. *Pharm Biol* 2016;54:1575-85.
  34. Mahattanadul S, Ridditid W, Nima S, Phdoongsombut N, Ratanasuwon P, Kasiwong S. nEffects of *Morinda citrifolia* aqueous fruit extract and its biomarker scopoletin on reflux esophagitis and gastric ulcer in rats. *J Ethnopharmacol* 2011;134:243-50.
  35. Sandhu DS, Fass R. Current trends in the management of gastroesophageal reflux disease. *Gut Liver* 2018;12:7-16.
  36. Jabri MA, Tounsi H, Abdellaoui A, Marzouki L, Sebai H. Protective effects of *Artemisia campestris* extract against gastric acid reflux-induced esophageal mucosa injuries. *Pathophysiology* 2018;25:63-9.
  37. Hayden MS, West AP, Ghosh S. NF- $\kappa$ B and the immune response. *Oncogene* 2006;25:6758-80.
  38. Gilmore TD. Introduction to NF- $\kappa$ B: Players, pathways, perspectives. *Oncogene* 2006;25:6680-4.