

Functional Properties of the Water Extract from *Eriocheir sinensis* (Chinese mitten crab) Via Evaluation of Protective Effects on Reflux Esophagitis Model

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Abstract

Objectives: In the study, we investigated the effects of an *Eriocheir sinensis* water extract on a reflux esophagitis-induced rat model.

Background: The crab has been used as a traditional medicine and food source in many countries worldwide for a long time, and the crab contains various useful substances such as chitin, protein, calcium carbonate, etc.

Materials and Methods: All rats were fasted for 24 h and then orally pretreated with saline, *Eriocheir sinensis* (100 mg/kg), or ranitidine (40 mg/kg). Reflux esophagitis was induced by pylorus and forestomach ligation. Morphological changes in the mucosal damage in the esophagus were analyzed by the ImageJ program. Also, the expression of the inflammatory proteins and cytokine in the esophagus was measured by western blot assay to demonstrate the protective effects of *Eriocheir sinensis* in reflux esophagitis model. Histological analysis of esophagus was performed by hematoxylin & eosin (H&E) staining.

Results: In the reflux esophagitis induction group, the esophageal tissue damage caused by the induction of reflux was 60% of the whole. *Eriocheir sinensis* administration significantly ameliorated esophageal mucosal damage by 40%, also determined by histological evaluation of reflux esophagitis in rats. *Eriocheir sinensis* was also found to downregulate the expression levels of proteins such as cyclooxygenase-2 (COX-2), tumor necrosis factor- α (TNF- α), and tight junction protein (claudin-5) compared to the reflux esophagitis induction group. In addition, *Eriocheir sinensis* markedly attenuated the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the phosphorylation of the inhibitor of NF- κ B (I κ B α).

Conclusion: These results indicated that *Eriocheir sinensis* suppressed the development of esophagitis and modulated inflammation by regulating NF- κ B activation. Based on these findings, we concluded that *Eriocheir sinensis* can protect the esophageal mucosa from reflux esophagitis.

Keywords

Eriocheir sinensis, crab, reflux esophagitis, inflammation

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Introduction

Reflux esophagitis is caused by such things as changes in eating and lifestyle habits, excessive drinking, and obesity. It is a disease that the age-standardized prevalence (ASPR) has increased to

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more than 77% in 2019 compared to the 1990s, and the burden has recently increased worldwide (Watson, 2005; Zhang, 2022). Reflux esophagitis is accompanied the mucosal damage and manifests clinical symptoms such as abdominal pain due to the reflux of stomach contents. The longer the exposure to hydrochloric acid, the more severe the lesion (Lotttrup, 2020). It is known that typical reflux esophagitis is mainly caused by excessive exposure of the esophagus to acid and pepsin, while atypical reflux esophagitis occurs with regular exposure to acid and pepsin (Kellerman, 2017). It is used for various therapies such as neutralizing gastric acid, strengthening the anti-reflux barrier function, and promoting gastrointestinal motility. PPIs and H2 blocking agents were mainly used to ameliorate the clinical symptoms and pain of reflux esophagitis, but it leads to various complications and tolerance in cases of long-term use (Qiu, 2019; Wang, 2022). Additionally, surgical intervention is necessary when patients do not respond to drugs and medical treatments or present with hernias around the esophagus. Surgery and endoscopic treatment are also performed; however, recurrence rates are high and their therapeutic effect is limited (Badillo, 2014). Thus, it needs to find new therapeutic resources from natural products, including traditional medicinal herbs or functional foods, to treat gastro-reflux esophageal disease (GRED).

Eriocheir sinensis (ES) H. Milne Edwards, 1853 (Chinese mitten crab) belongs to *Eriocheir* De Haan, 1835 (family Varunidae H. Milne-Edwards, 1853) and is mainly distributed in Southeast Asia. In addition, the crab has been used as a traditional medicine and a popular food source in many countries worldwide for a long time, and the crab extract contains various useful substances such as chitin, protein, calcium carbonate, selenium, and carotenoids. Therefore, it has excellent nutritional value, making it valuable for food and medicinal purposes (Cho, 1998; Wang, 2019). Previous studies have shown that various biochemical agents present in crabs exhibit anti-diabetic, anti-cancer (Rezakhani, 2017), and anti-inflammatory and antioxidant properties (Yeon, 2008).

“*Donguibogam*” is the most well-known Korean traditional medical book written by Heo Jun, an Eastern physician (1546–1615). In the *Donguibogam*, traditional records on the use of the crab note that it aids digestive function (Heo, 2006). Despite the potential for various effects, there is a lack of verification reports about the various diseases, and in particular, it has been traditionally used for the treatment of digestive diseases, but the bioactivities of crab are unclear. Since crabs have been used for the treatment of digestive diseases in traditional medicine, as well as the anti-inflammatory and antioxidant effects identified by previous studies, we anticipated their potential as a material for the treatment of reflux esophagitis, which is currently increasing in prevalence. In the present study, we aimed to verify our model’s efficacy and study the mechanism underlying reflux esophagitis using an ES water extract (ESE).

Materials and Methods

Preparation of Extract

The sample *Eriocheir sinensis* (ES) were purchased from commercial suppliers, and samples were deposited in the Korean Herbarium of Standard Herbal Resources (Index Herbariorum code KIOM) at the Korea Institute of Oriental Medicine, Naju, Korea. Samples (resource accession number: 2-19-0515) were verified through visual and organoleptic examination (verification ID: 137) and genetic discrimination (verification ID: 97). Extraction was conducted with the lyophilized and ground crabs (930 g) under reflux in 15 L of distilled water at $100 \pm 2^\circ\text{C}$ for 3 h (Lee et al., 2021). The extracted solution was filtered and concentrated using a vacuum concentrator and then lyophilized to obtain the extract (117.81 g, yield 12.67%, w/w) used in the experiment.

Experimental Animals

Twenty-four Sprague Dawley (SD) rats (6-week-old male) were purchased from Han-il (Wanju, Korea) and used in the experiment after being adapted to the nursery environment for one week. All animals were reared in a conventional system at a temperature of $22 \pm 2^\circ\text{C}$, a humidity of $50 \pm 5\%$, and a light-dark cycle adjusted to a 12-hour cycle. Solid feed (Nestle Purina PetCare Company; Bundang-gu, Seongnam, Korea) and water were freely consumed, and all breeding materials were sterilized. The experiment was conducted according to the provisions of the National Institutes of Health, and the experiment was conducted with the approval of the Animal Experimental Ethics Committee of Chonbuk National University based on the International Animal Welfare Act (CBNU 2020-010).

Induction of Reflux Esophagitis in Rats

The experimental group consisted of four groups: the normal control group, the RE control group (saline administration after reflux esophagitis), the *Eriocheir sinensis* water extracts (ESE) treatment group (100 mg/kg administration after induction of reflux esophagitis), and the positive control group (40 mg/kg ranitidine administration after induction of reflux esophagitis). All rats were fasted for 18 hours before surgery for acute reflux esophagitis induction. After induction of respiratory anesthesia using isoflurane, the area 2 cm incision was made below the sternum, and the pylorus of the stomach was ligated to induce reflux (Katada, 2005). The peritoneum and skin were then sutured with silk thread. Esophageal mucosal damages were measured 4 h and 30 min after pyloric and translocation surgery.

Esophageal Mucosal Damages

The esophagus was separated, and the inside of the esophagus was washed with 0.9% sodium chloride (NaCl). For morphological analysis in esophagus, esophageal tissue was placed on a flat surface and photographed using an optical digital camera. Esophageal damage was calculated using the ImageJ program.

Histological Analysis

At the end of the experiment, the experimental animals were anesthetized, and tissues were removed. After fixing the esophageal tissue in formalin, it was embedded in paraffin after dehydration with 75%, 95%, and 100% ethanol and made transparent using xylene. After the paraffin block solidified overnight at room temperature, the tissue was sliced to a thickness of 5 μ m and stained with hematoxylin. The dyed tissue was photographed and analyzed using an image analysis program (i-solution DT) using an optical microscope (Leica DM 2500).

Western Blot Assay

To obtain the cytoplasm of the extracted esophageal tissue, 100 mM Tris-HCl (pH 7.4), 5 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 15 mM $CaCl_2$, 1.5 M sucrose, 0.1 M DTT, and a protease inhibitor cocktail were added. A cytosolic lysis buffer was added, and the sample was crushed with a tissue grinder. Then, 10% NP-40 solution was added, and the mixture was incubated for 30 min on ice. After centrifugation, the supernatant containing the cytoplasm was separated, and 50 mM of the cell nucleus lysis buffer 2-[4-(2-hydroxyethyl)-1-piperazyl]-ethane sulfonic acid (pH 7.9), 50 mM KCl, and

0.3 were added for nuclear extraction. After resuspension using mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol, the mixture was centrifuged, and the supernatant containing the nucleus was obtained and stored at $-80^{\circ}C$ to quantify the existing proteins and to be used as a sample. The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred at 100 V for 75 min. The primary antibody was diluted 1:1000 in PBST and incubated overnight at $4^{\circ}C$, and the secondary antibody was incubated at 1:10000 for 2 h at room temperature, followed by Bio-Rad imaging. Band images were collected using software (Bio-Rad Laboratories, Inc., USA).

Statistical Analysis

The results of all experiments were expressed as mean $\pm$ standard deviation, and statistical significance between all treatment groups was tested using one-way ANOVA. Differences were considered significant only when $p < .05$.

Results

Measurement of Esophageal Mucosal Damage

The observation of the esophageal mucosal damages was adjusted as a represented factor to identify the protective effects on the reflux esophagitis-induced model. No damage was found in the normal control group's mucosa; however, hemorrhagic necrosis, erosion, and ulcer occurred due to mucosal damage caused by irritation of gastric contents and acid in the induced reflux esophagitis group (Figure 1). In the treatment group, where the ESE 100 mg/kg was administered

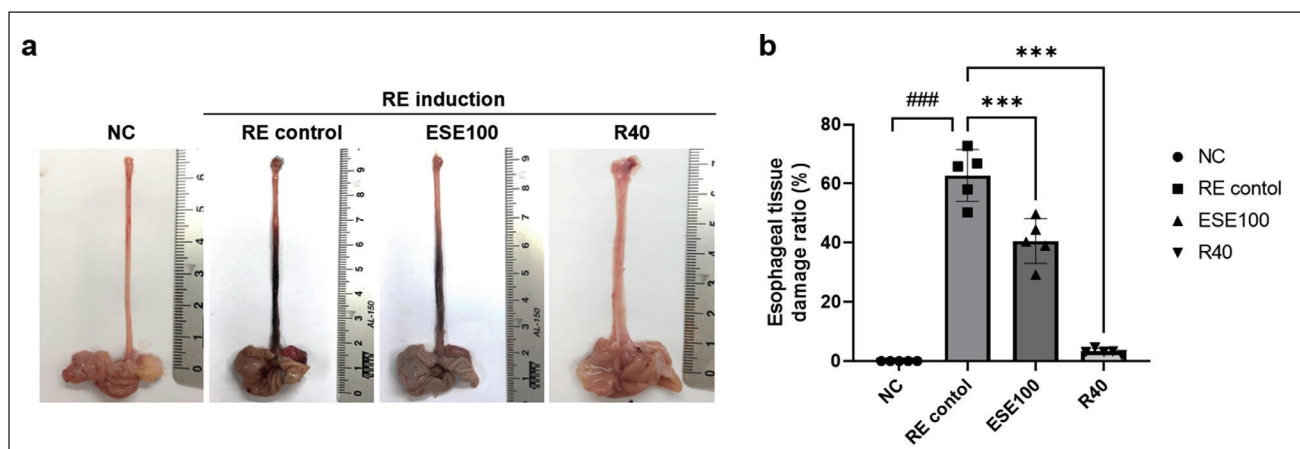


Figure 1. Protective Effects of ESE on the Esophagus of Reflux Esophagitis Induced Rats. (a) The Images and (b) Ratio of the Esophageal Damage Evaluation in Different Groups.

Note: NC: normal control group; RE control: induced esophagitis group; ESE 100: induced esophagitis + crab extract 100 mg/kg group; R40: induced esophagitis + ranitidine 40 mg/kg group. Data are shown as means $\pm$ SEM. ### $p < .001$ vs. normal group; *** $p < .001$ vs. vehicle group.

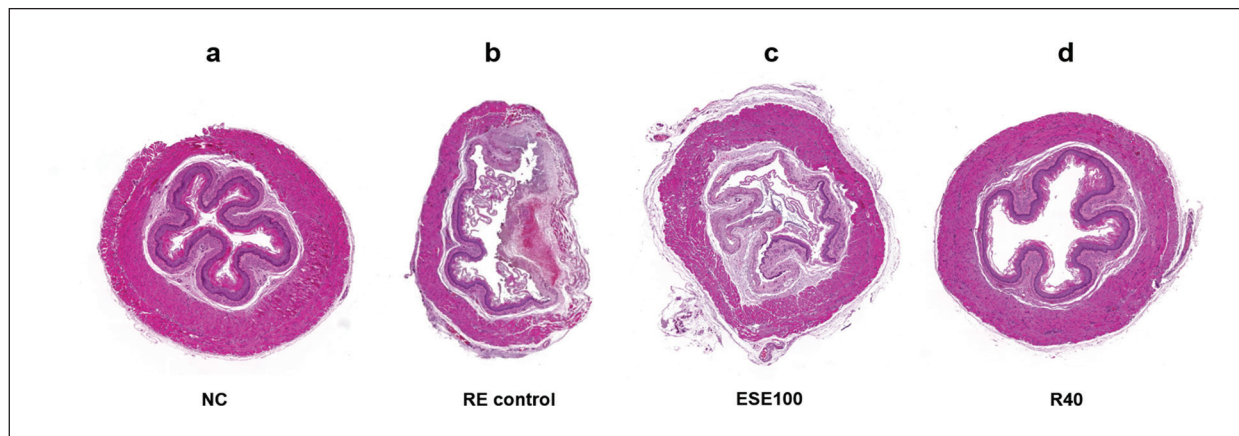


Figure 2. Histological Change Stained with H&E Evaluation in Different Group. (a) NC: Normal Control Group; (b) RE Control: Induced Esophagitis Group; (c) ESE 100: Induced Esophagitis + Crab Extract 100 mg/kg Group; (d) R40: Induced Esophagitis + Ranitidine 40 mg/kg Group.

orally, a 30% significant improvement about main lesions in esophageal mucosa was observed compared to the RE control group.

Histopathological Examination

Histological changes in the esophageal mucosa were not observed in the normal control group; however, inflammatory cell deposit, extensive esophageal detachment, and bleeding were observed in the RE control group (Figure 2). In the ESE treatment group, esophageal epithelial detachment decreased, compared to that in the RE control group. In the ranitidine-positive control group, the epithelial tissue was rarely damaged.

Changes in Tight Junction Protein Expression

The expression of claudin, one of the tight junction molecules, is critical and indication of damage in the barrier function of epithelial (Mönkemüller, 2012). To identify the change in epithelial barrier function, we measured the expression of claudin-5 proteins in esophagus tissue. In the RE control group, tight junction protein expression was decreased by esophagus mucosal damage, while the claudin-5 expression in the esophagus tissue was increased by ESE 100 mg/kg and ranitidine treatments (Figure 3).

Changes in Inflammatory Cytokine Expression

In the RE control group, cyclooxygenase-2 (COX-2) and tumor necrosis factor- α (TNF- α) expression levels were significantly higher when compared to the normal control group, owing to the presence of gastric contents and acid reflux (Figure 4). However, the COX-2 expression levels in

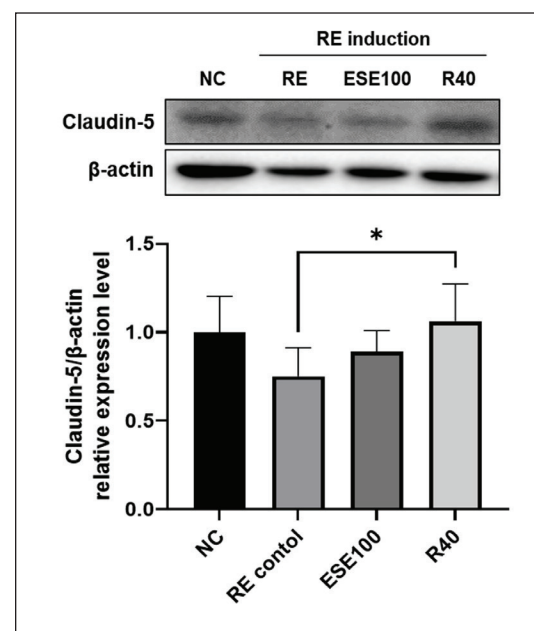


Figure 3. Inhibitory Effects of Crab Extract on the Expression of Claudin-5 in Esophageal Tissues.

Note: NC: normal control group; RE control: induced esophagitis group; ESE 100: induced esophagitis + crab extract 100 mg/kg group; R40: induced esophagitis + ranitidine 40 mg/kg group. Data are shown as means $\pm$ SEM. * $p < 0.05$ vs. vehicle group.

the esophagus mucosa were reduced by 20% in the ESE treatment group and by 60% or more in the ranitidine group when compared to the control group. Compared to the control group, TNF- α expression levels were inhibited by 10% in the ESE treatment group and 14% in the ranitidine group.

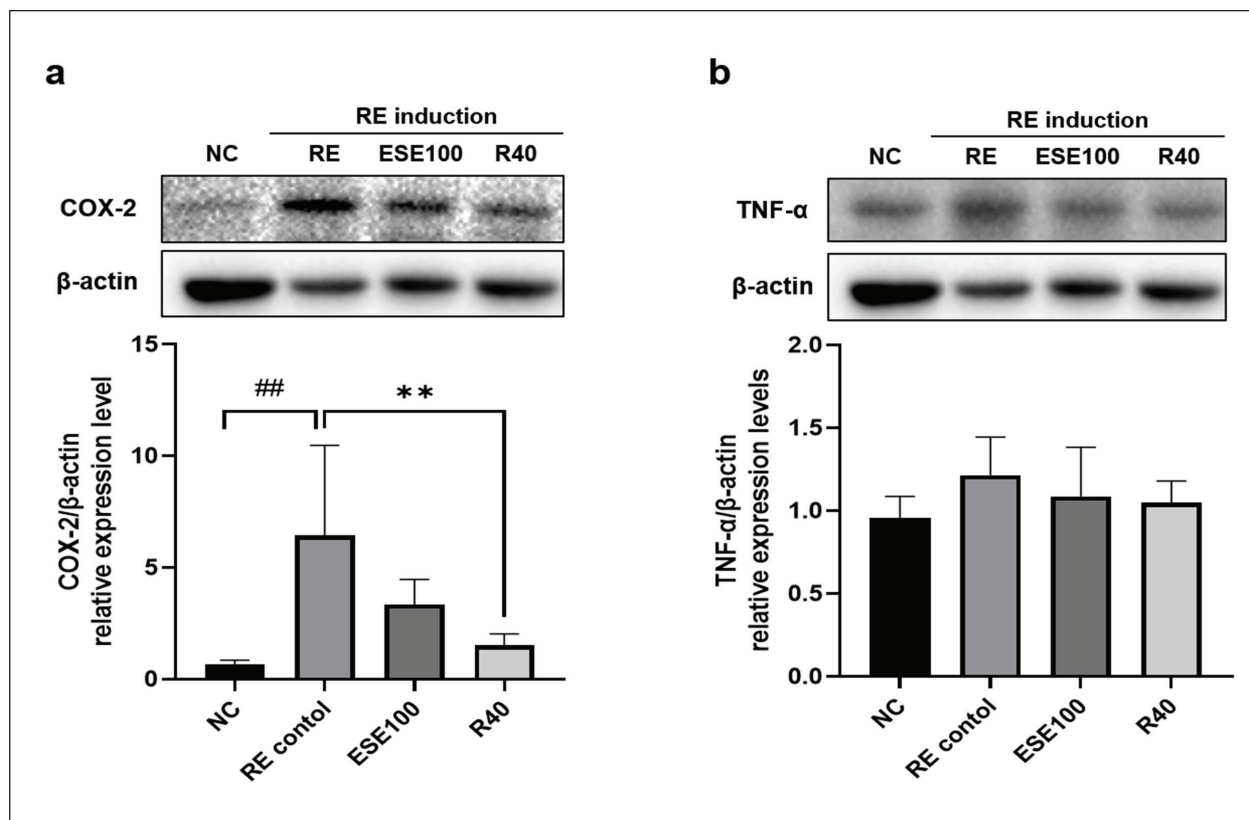


Figure 4. Inhibitory Effects of Crab Extract on the Expression of (a) COX-2 and (b) TNF-α in Esophageal Tissues.

Note: NC: normal control group; RE control: induced esophagitis group; ESE 100: induced esophagitis + crab extract 100 mg/kg group; R40: induced esophagitis + ranitidine 40 mg/kg group. Data are shown as means $\pm$ SEM. $^{###}p < 0.05$ vs. normal group; $^{**}p < 0.01$ vs. vehicle group.

Changes in Inflammatory Proteins Expression

In the control group, it was confirmed that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) subunit p65 activation in the nucleus progressed, owing to activation of the inhibitor of NF- κ B (I κ B α) protein in the cytoplasm due to gastric contents and gastric acid reflux. When the water extract was administered, the protein expression level of I κ B α was inhibited by 39% compared to the control group, and when ranitidine was administered, the inhibition was about 50%. The protein expression level of NF- κ B subunit p65 was inhibited by 39% in the ESE treatment group and by more than 50% in the ranitidine group, compared to the control group (Figure 5).

Discussion

Eriocheir sinensis (ES) H. Milne Edwards is native to China and to the Korean Peninsula, and is useful as a functional food and health resource because it is helpful for various nutritional and digestive functions. Crab meat contains

essential amino acids such as leucine and arginine and various components such as chitin and potassium carbonate, known for their effects on liver disease and their anti-cancer, antioxidant, and anti-inflammatory effects (Rezakhani, 2017; Yen, 2008; Kim, 2009). In addition, studies on chitin and chitosan production and physiological activity in crab shells and studies on the gastrointestinal protective effect of chitosan-containing complexes have suggested their high economic value as a functional food and medicinal material for the treatment of gastrointestinal diseases (Park, 2008; Yadav, 2019; Stenger, 2014).

Reflux esophagitis is a disease that causes esophagus mucosal lesions by repeated reflux of gastric contents and stomach acid due to impeded function of the lower esophageal sphincter and the resulting complications (Watson, 2005; Osadchuk, 2019). Drugs for reflux esophagitis treatment include acid secretion inhibitors, H₂ receptor inhibitors, and proton pump inhibitors (PPIs). However, long-term use reduces their therapeutic effect owing to resistance and may cause side effects such as magnesium deficiency, which necessitates the development of safe therapeutic drugs (Fossmark, 2019).

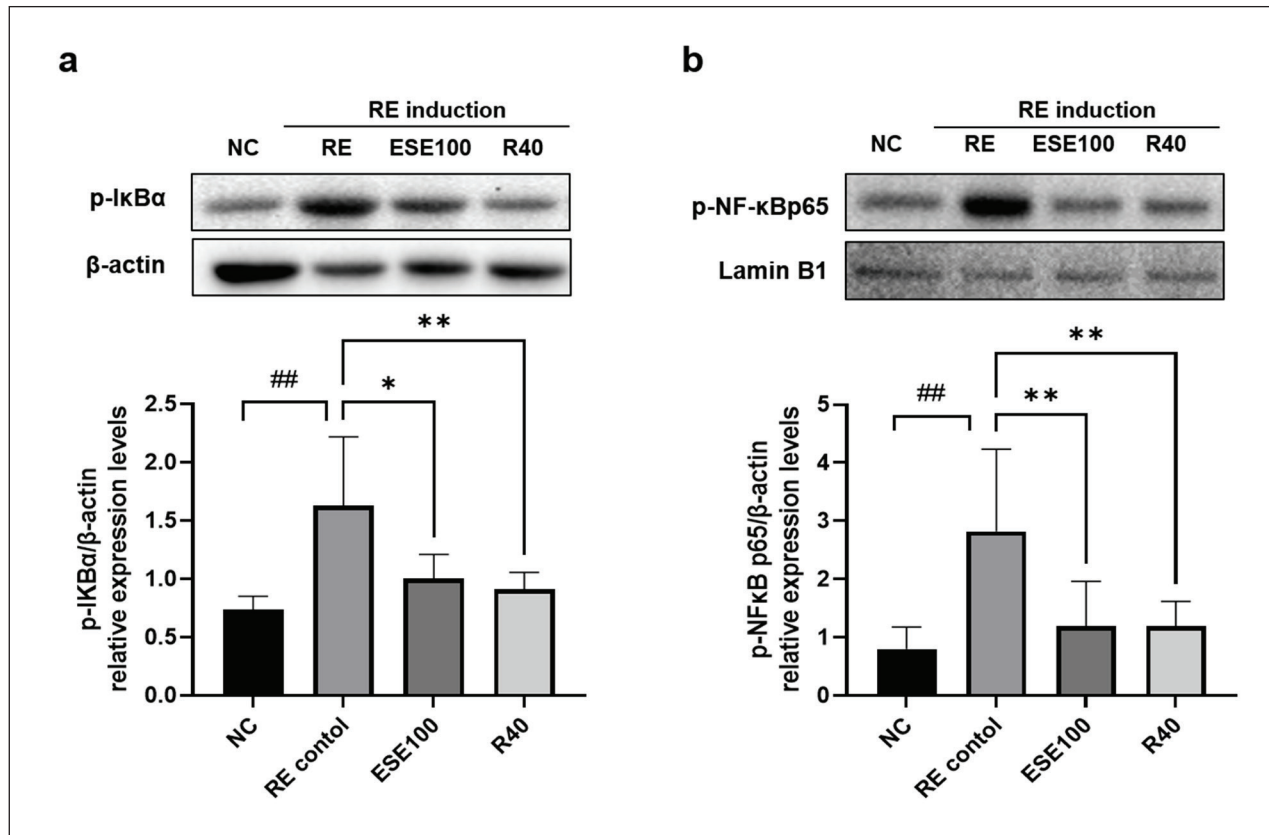


Figure 5. Inhibitory Effect of the Crab Extracts on (a) p-IκBα and (b) p-NF-κBp65 Expression in Esophagus Tissues.

Note: NC: normal control group; RE control: induced esophagitis group; ESE 100: induced esophagitis + crab extract 100 mg/kg group; R40: induced esophagitis + ranitidine 40 mg/kg group. Data are shown as means ± SEM. ###*p* < 0.01 vs. normal group; ***p* < 0.01 and **p* < 0.05 vs. vehicle group.

Reflux esophagitis mainly causes epithelial damage and cell death in the esophageal mucosa (Mitsunaga, 2002). In this study, damage to the esophagus caused by gastric content reflux was clearly observed after reflux esophagitis-inducing surgery. Tissue damage, bleeding, inflammatory cell infiltration, and epithelial destruction were observed when analyzing the esophageal mucosa tissue at the damage site. These symptoms were significantly reduced by ESE and ranitidine administration. The protective effect of ranitidine, an H2 inhibitor, was reported in a previously published study (Koelz, 1986). This effect was confirmed by our study, which used ranitidine as a positive control drug, leading to a significant improvement. In the group treated with 100 mg/kg of ESE, a protective effect on the area of esophagus was also significantly observed.

It is confirmed that tight junction proteins were formed in normal esophagus mucosa, but their expression is reduced in damaged esophagus mucosa in the previously demonstrated in reflux esophagitis-induced models (Nan, 2022). As such, tight junction proteins are an important index that can determine whether or not cell function is damaged in tissue. In the study, we identified the downregulation of claudin-5 expression in

damaged esophageal mucosal, while the expression of claudin-5 was increased by treatments of the ESE and ranitidine. Reflux esophagitis reportedly correlates with changes in the level of tight junction and inflammatory proteins, such as claudin, COX-2 and TNF-α (Nan, 2018; Hatware, 2018; Nam, 2019; Tetreault, 2016, Roh, 2021). In this study, the increase in COX-2 and TNF-α levels was confirmed in the reflux esophagitis model, and a significant decrease in inflammatory cytokine and protein was observed in the group treated with the ESE 100 mg/kg and the ranitidine group.

IκB-α is a factor that mediates the activity of NF-κB, which modulates immune and inflammatory responses and plays a vital role in inflammation and immunity. In particular, it has been reported that IκB-α and NF-κB play an important role in the maintenance of esophageal microenvironment homeostasis (Nam, 2019; Baldwin, 2001). Phosphorylation of IκB-α and NF-κB in the esophageal epithelium has been reported to exacerbate esophageal inflammation, which promotes the secretion of various inflammatory cytokines. In addition, depression of tight junctions via NF-κB phosphorylation was revealed in inflammatory cells (Nan, 2022; Li, 2018). As such, NF-κB plays an important role in

the expression of factors related to inflammation and barrier function of mucosal in esophagus, and the results of this study showed a significant tendency. In this study, it was confirmed that the phosphorylation of two factors was increased in the RE control group. ESE treatment effectively decreased phosphorylation of I κ B- α and NF- κ B led to the expression of pro-inflammation proteins in the esophagus tissue. Our results suggest that ESE ameliorated the esophageal mucosal damage and tight junction proteins by downregulating factors related to inflammation responses.

In this study, the protective effect of ESE was confirmed through visual findings and histopathological examination in a reflux esophagitis animal model. The protection of the tight junctions in the esophageal epithelial and connective tissue and a reduction in various inflammatory factors confirm the anti-inflammatory effect of the ESE. The inhibition of I κ B- α and NF- κ B phosphorylation by the crab water extract is a potential mechanism underlying the resulting protective effect. This study suggests the possibility of ESE as a functional food or new medicinal material for the treatment of esophagus mucosa in reflux esophagitis.

Abbreviations

ES: *Eriocheir sinensis*; ESE: *Eriocheir sinensis* water extract; GRED: Gastro-reflux esophageal disease; H&E: Hematoxylin & eosin; PPIs: Proton-pump inhibitors; NaCl: Sodium chloride; HCl: Hydrochloric acid; MgCl₂: Magnesium chloride; CaCl₂: Calcium chloride; DTT: Dithiothreitol; COX-2: Cyclooxygenase-2; TNF- α : Tumor necrosis factor- α ; NF κ B: Nuclear factor kappa-light-chain-enhancer of activated B; I κ B α : Inhibitor of NF- κ B.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Ethics Approval

The experiment was conducted with the approval of the Animal Experimental Ethics Committee of Chonbuk National University based on the International Animal Welfare Act (CBNU 2020-010).

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