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ORIGINAL ARTICLE

Protective Effect of Fermented *Cudrania tricuspidata* Fruit Extracts on Acute Rat Reflux Esophagitis

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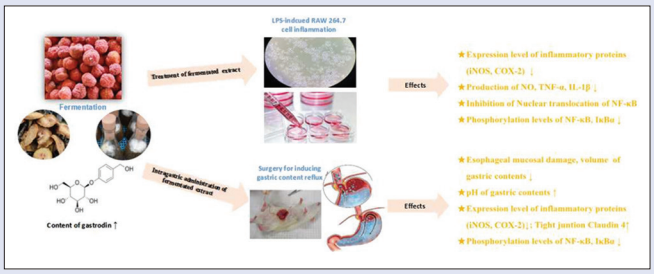
ABSTRACT

Background: Reflux esophagitis (RE) is a type of gastroesophageal reflux disease (GERD) that severely reduces the quality of life. The *Cudrania tricuspidata (C. tricuspidata)* fruit is commonly used in food and has medicinal values such as clearing heat, relaxing muscles, and collaterals. **Objectives:** We evaluated fermented *C. tricuspidata* fruit extract (FCt) in this study. We measured its anti-inflammatory activity by using LPS-induced Raw 264.7 cell model and evaluated its role in improving acute reflux esophagitis in rats. **Materials and Methods:** The gastrodin content before and after fermentation was determined. NO production in cell supernatant was measured using the Griess reagent method, and TNF-α and IL-1β secretion was detected using the Quantikine ELISA kit. Expression level of proteins was measured by western blotting. Immunofluorescence assay was used for confirming the activation of NF-κB. The protective effects of FCt on RE were conducted in the rat RE model by observing esophageal tissue damage and inflammatory proteins expression levels in the esophagus. **Results:** The results showed that the content of gastrodin in *C. tricuspidata* fruit increased upon fermentation. FCt inhibited the production of NO, TNF-α, and IL-1β and the activation of NF-κB. In addition, FCt significantly improved esophageal tissue damage caused by acid reflux and inhibited the expression of inflammatory proteins iNOS and COX-2 in esophageal tissue. The term of tight junction (claudin-4) is increased to maintain the barrier function of cell tissues. **Conclusion:** We suggested that FCt could consider being a candidate medicine material for treating RE.

Key words: Anti-inflammation, *Cudrania tricuspidata*, cytokines, gastroesophageal reflux disease, NF-κB signaling pathway

SUMMARY

- The present study explores the inhibitory effect of the FCt on LPS-induced cell inflammation and the protective effect on acute rat reflux esophagitis. The results showed that FCt inhibited cell inflammation, significantly improved esophageal tissue damage caused by acid reflux, and inhibited the expression of inflammatory proteins in esophageal tissue. Moreover, the barrier function of esophageal tissue cells is also maintained. Our results suggest that FCt may have a role in treating RE or another inflammation-related disease.



Abbreviations used: RE: Reflux esophagitis; **GERD:** Gastroenterology gastroesophageal reflux disease; **FCt:** fermented *C. tricuspidata* fruit extract; **LPS:** lipopolysaccharide; Nitric oxide; **TNF-α:** Tumor necrosis factor alpha; **IL-1β:** Interleukin-1 beta; **iNOS:** Inducible nitric oxide synthase; **COX-2:** Cyclooxygenase-2; **NF-κB:** Nuclear factor (NF)-κB; **IκB:** Inhibitor of NF-κB; **DMSO:** Dimethyl sulfoxide; **DAPI:** 4`,6`-diamidino-2-phenylindole; **PVDF:** Polyvinylidene fluoride; **DMEM:** Dulbecco's modified eagle`s medium; **FBS:** Fetal bovine serum; **NBF:** Neutral buffered formalin; **SDS:** Sodium dodecyl sulfate; **HPLC:** High performance liquid chromatography; **SPSS:** Statistical product and service solutions.

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INTRODUCTION

RE is a gastroesophageal reflux disease caused by the reflux of stomach contents caused by dysfunction of the lower esophageal sphincter, which allows stomach contents to reflux into the esophagus and cause esophageal tissue damage.[1,2] The incidence rate of this disease is about 10%–20% in European and American countries. The incidence rate of the disease in Asian countries increases year by year due to the eating habits and the aging of the population. The prevalence rate is about 5%.[3] The main clinical manifestations of RE include heartburn, chest pain, and sleep disturbances.[1,4] The most basic way to treat this disease is to change one's dietary habits. Drug therapy mainly uses H_2 receptor antagonists and proton pump inhibitors (PPI) to inhibit gastric acid secretion.[5] However, 30%–50% of patients have relapses despite these medications.[6] They may also have incomplete mucosal recovery, lung disease, Barrett's esophagus, esophageal cancer, or other complications.[7,8] Therefore, it is ideal for a patient to improve their symptoms and avoid such medication side-effects by simply eliminating the associated foods from their diet.[9] This idea is of

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great significance for improving the traditional treatments of RE and reducing the medication side effects.

Cudrania tricuspidata (*C. tricuspidata*) is a deciduous shrub or small tree from the *Moraceae* family found in China, South Korea, Japan, and eastern Russia.[10] These trees have a height of 8 m. The wood of *C. tricuspidata* is used to treat diseases such as malaria in traditional Chinese medicine.[11] The fruit is used primarily for food but is also used as a Chinese medicinal material. This plant has the effects of clearing heat, cooling blood, and relaxing muscles.[12,13] According to the results of pharmacological activity studies, the bark contains a large number of active substances such as flavonoids. Therefore, the bark has anti-oxidant and anti-inflammatory physiological activities.[14,15] However, research on the *C. tricuspidata* fruit has mainly focused on separating its components. In contrast, there is a lack of research that addresses physiological activity, such as anti-inflammation. Therefore, it is essential to study the physiological activity of *C. tricuspidata* fruit to define its role as both natural food and restorative material to treat inflammatory diseases and RE.

In recent years, many studies have employed microbial fermentation to improve the physiological activity of materials such as the *Schisandra chinensis* fructus.[16] During the fermentation process, the content of active ingredients increases to improve the material's functionality. *Armillaria mellea* is an edible honey fungus that grows at the base of various tree trunks. This study used *Armillaria mellea* to ferment the *C. tricuspidata* fruit. We measured the content of the active ingredients using HPLC. We explored the inhibitory effect of the fermented extract on LPS-induced cell inflammation and the protective effect on acute rat reflux esophagitis.

MATERIALS AND METHODS

Materials

Antibodies and reagents

β-actin, iNOS, COX-2, p-IκBα, p-NF-κB p65, NF-κB p65, and claudin-4 primary antibodies were purchased from Santa Cruz Biotechnology (Delaware Ave, CA, USA). Cell viability, proliferation, and cytotoxicity assay kits were purchased from DoGenBio Co., Ltd (Guro-gu, Seoul, South Korea). The primary antibody of Lamin B1 was purchased from Cell Signaling Technology (Danvers, MA, USA). Griess reagent was purchased from Promega (Fitchburg, WI, USA). Quantikine ELISA mouse TNF-α kit and human IL-1β/IL-1F2 immunoassay kit were purchased from R&D Systems (MN, USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), 4`,6`-diamidino-2-phenylindole (DAPI), ranitidine hydrochloride, protease inhibitor cocktail was purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Wel-gene (Namcheon-ro, South Korea). Bovine serum albumin (BSA) standard; protein assay reagent A, B, S; and polyvinylidene fluoride membranes (PVDF) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Methods

Fermented C. tricuspidata fruit extract (FCt) preparation

The mature fresh fruit of *C. tricuspidata* was purchased from Sunchang-gun, Jeonbuk, South Korea. It was identified as a ripe fruit of *C. tricuspidata* by botany professor Kim Hong Jun of Jeonbuk National University. *Armillaria mellea* used in the study was provided by Professor Kim Myung Kon of the Food Science Department of Jeonbuk National University. One liter of distilled water was added to 500 g of fresh *C. tricuspidata* fruit. This mixture was mashed and then placed into a 3-L triangular flask and sterilized at 121°C for 30 min. Seventy-five mL of *Armillaria mellea* was inoculated at a rate of 5% and incubated on a shaker at 120 rpm for 4 days at room temperature. The fermented product was freeze-dried. 100 mL of 70% EtOH was added to 10 g of the fermented product powder at 50°C and extracted three times for 2 h. After concentrating the extract, it was re-freeze-dried and stored at −20°C for later use.

High-Performance Liquid Chromatography (HPLC) assay

Ten mL of 70% methanol was added to 0.5 g of FCt. Ultrasonic waves were used to dissolve the FCt, after which it was filtered through 0.45-μm filter paper. Then, high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) was used to analyze the compound.[17] The column was the ZORBAX Eclipse XDB-C18 (250 mm × 4.6 mm, 5 μm; Agilent Technologies, Inc., Santa Clara, CA, USA). The mobile phase was 0.1% formic acid in deionized water (a) and 0.1% formic acid in methanol (b). The gradient condition was as follows: a: b 95:5 (0–5 min), 85:15 (5–10 min), 45:55 (10–25 min), and 95:10 (25–40 min). The flow rate was 0.8 mL/min. A photo-diode-array-detector (DAD) detector was used to compare each component's ultraviolet (UV) profile. Next, the peak area detected at 220 nm was calculated for quantification.

Cell culture

RAW 264.7 Macrophages were purchased from Korean Cell Line Bank (Jongno-gu, Seoul, South Korea). According to laboratory standards, the cells were cultured in DMEM with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The cellular environment included a sterile constant temperature incubator containing 5% CO_2 and a temperature of 37°C.

Nitrite content and cell viability determination

The mouse monocyte macrophage leukemia cells RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10^5 cells/mL. When the cells grew to approximately 80% of the micropore area, FCt was added at 125, 250, and 500 μg/mL concentrations for 1 h. Then, the cells were stimulated with LPS (1 μg/mL) and incubated for 18 h. The plate was centrifuged at 2500 rpm for 5 min. Next, 50 μL of the supernatant was taken to determine the nitrite content according to the Griess reagent method.[18] The absorbance value was measured at 540 nm. The standard curve was drawn using $NaNO_2$ to calculate the nitrite content.

To determine the cell viability, 10 μL of cell viability reagent was added to the cells and then incubated in a 37°C incubator for 40 min. The absorbance value was measured at 450 nm by using an absorbance photometer (Multiscan spectrum, Thermo Scientific). The effect of FCt on cell viability was compared with the blank control cells.[19]

TNF-α and IL-1β content determination

The RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10^5 cells/mL. When the cells grew to approximately 80% of the micropore area, FCt was added at 250 and 500 μg/mL concentrations for 1 hr. The cells were then stimulated with LPS (1 μg/mL) and incubated for 18 h. The plate was centrifuged at 2500 rpm for 5 min. The supernatant was then taken to determine the TNF-α and IL-1β secretion. The Quantikine ELISA mouse TNF-α Kit and human IL-1β/IL-1F2 immunoassay kit were used to measure the content of TNF-α and IL-1β in the cell supernatant according to the manufacturer instructions. When measuring TNF- α, the sample was diluted 500 times.

Immunofluorescence assay

The RAW 264.7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/mL and incubated in a 37°C incubator overnight. FCt was added

at 250 and 500 μg/mL concentrations for 1 h. The cells were then stimulated with LPS 1 μg/mL for another 30 min. The cells were fixed with 4% PFA, after which the cell membrane was permeated with 0.5% Trition-X100. Next, the cells were blocked with 5.0% bovine serum albumin (BSA). Then, the NF-κB p65 primary antibody was diluted at 1:200 with 1% BSA and incubated at 4°C overnight. The m-IgGk BP-FITC (SantaCruz Biotechnology) secondary antibody containing a fluorescent substance was diluted to 1:1000 with 1% BSA and incubated for 2 h at room temperature under dark conditions. Finally, use a sealant containing a nuclear stain (4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, Missouri) was used to seal the slide.[20] Fluorescence images were collected using a super-resolution confocal laser-scanning microscope (magnification, 63×; Carl Zeiss, 73,447 Oberkochen, Deutschland).

Cell total protein extraction and loading sample preparation

To perform western blot analysis, the cells were seeded in a 6-well plate at a density of 5×10^5. When the cells were grown to approximately 80% of the micropore area, the cells were treated with FCt at concentrations of 250 and 500 μg/mL for 1 h. The cells were then stimulated with LPS (1 μg/mL). The cells were collected after 1 and 18 h, respectively. The collected cells were then treated with RIPA cell lysis buffer for extracting total protein. Next, the obtained protein was quantified. A loading sample was made at a ratio of 1:1 with blue loading buffer (50% glycerin, 10% sodium dodecyl sulfate, 0.1% bromophenol blue, 1M Tris HCl, 2-Mercaptoethanol).[21]

Animal maintenance and rat model of acute reflux esophagitis

Thirty-two Sprague–Dawley rats (6-week-old, weighing ~200 g) were fed with sufficient feed and water in a standard animal breeding room for 1 week to allow them to be acclimatized. The environment of the breeding room included 12 h of alternating light and dark, a temperature of 23°C–25°C, and humidity of 35%–60%. This section already states that ethics committee approval has been obtained and an approval number is provided. Thirty-two rats were randomly divided into four groups, each with eight rats. Before the operation to induce reflux esophagitis (RE), the rats fasted for 18 h while given sufficient water. The surgical method to cause acute reflux esophagitis is to ligate the junction between the gastric body and forestomach to reduce the gastric space and ligate the pylorus to prevent gastric contents from entering the duodenum.[22,23] This procedure also included ligation of the pylorus to promote reflux of stomach contents into the esophagus. The first group was the normal control group (Nor). The rats in the normal group did not undergo surgery to induce RE and no oral gavage. The second group was the RE control group (Vehicle, Veh); the rats underwent surgery to induce RE without medications. The third group is the FCt treatment group (Veh + FCt300). The rats in the FCt treatment group were given 300 mg/kg FCt oral gavage 2 h before the operation of acute reflux esophagitis. The fourth group is the positive control group (Veh + R40); rats in this group were given ranitidine 40 mg/kg oral gavage 2 h before the operation of acute reflux esophagitis. The rats were anesthetized with isoflurane (Hana Pharm Co., Ltd., Korea) before and during the operation.

Stomach content collection and gastric acid pH measurement

Rats were sacrificed by intraperitoneal injection of urethane (20%, 4 mL/kg) 4 h 30 min after the operation of RE. The esophagus and stomach tissue of the rats were removed immediately. The gastric contents were collected in a 15-mL tube. Gastric contents' volume and pH value were determined after centrifugation at 1500 rpm for 3 min.[24]

Image acquisition of the rat esophagus and determination of the esophageal tissue damage rate

After the stomach contents were collected, the stomach and esophageal tissues were cleaned with saline. The esophageal tissue was then cut longitudinally to expose the damaged part of the esophagus. The esophagus was photographed using a digital camera. The esophageal tissue damage rate was measured and calculated using the ImageJ program. The esophageal damage rate was calculated as follows:

$$\text{esophageal damage rate (\%)} = \frac{\left[\text{area of esophageal damage}(\text{mm})^2\right]}{\left[\text{total area of esophagus}(\text{mm})^2\right]} \times 100$$

Histological staining analysis

Samples of 2–3 mm of esophageal tissue were fixed in 10% formalin fixative, dehydrated, and cleared to make a paraffin block. The thickness of the tissue sections was 4 μm. These sections were stained using the H and E staining method.[25] The esophageal staining images were collected using a Leica microscope.

Extraction of cytoplasmic and nuclear proteins from esophageal tissue

The tissue protein extraction experiments were all performed on ice. Cold cytoplasmic protein lysis buffer (10 mM HEPES, protease inhibitor mixture solution, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, and 1 mM DTT) was added to grind the esophageal tissue into a homogenate on ice at 13,000 rpm. The mixture was left for 30 min on ice, after which it was centrifuged at 3000 rpm, 4°C for 30 min. The supernatant was obtained as the cytoplasmic protein. The nuclear protein lysis buffer (50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1% (v/v) glycerol, and 1 M DTT) was later added to the remaining esophageal tissue after centrifugation to resuspend it. This mixture was placed on ice for 30 min and then centrifuged at 13,000 rpm and 4°C for 15 min. The supernatant was taken as the nuclear protein.[21]

Western blotting analysis

The prepared loading sample was separated using separating gels of different concentrations according to the size of proteins. Next, the proteins were transferred to the PDVF membrane in the transfer buffer for 1.5 h. After blocking with 5% skimmed milk for 1 h, the membrane was incubated overnight at 4°C with a 1:1,000 iNOS, COX-2, β-actin, p-IκBα, p-NF-κB p65, NF-κB p65, and claudin-4 primary antibodies. Next, the cells were incubated with a 1:10,000 HRP-conjugated secondary antibodies at room temperature for 2 h. A color developer was then added to introduce color, after which a Bio-Rad image software was used for image acquisition.

Statistical analysis

All data are expressed as means ± SD. One-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparison analyses were performed using SPSS 12.0K for Windows (IBM Corporation, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Fermentation treatment increases the content of the active ingredients

Through HPLC analysis and determination, we compared the relative content of the active substances gastrodin and parishin A and B. The ratio of the active substances after fermentation increased by approximately 3-fold compared to that before fermentation [Figure 1].

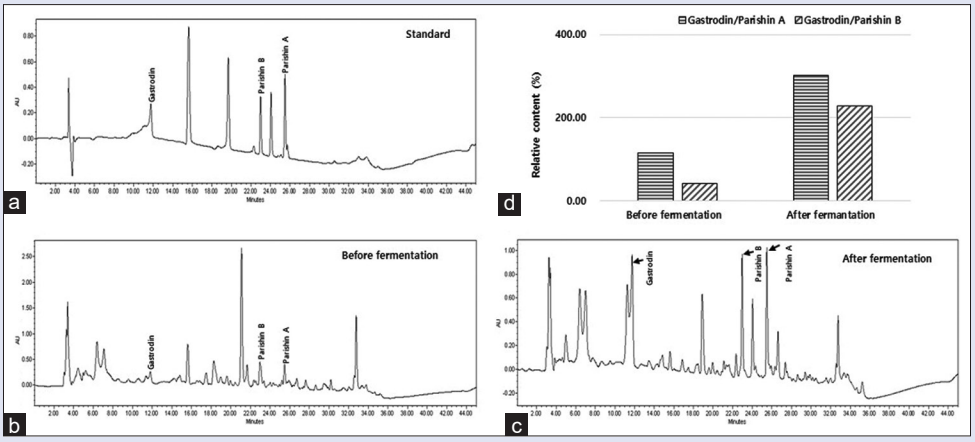


Figure 1: HPLC analysis of standard of mixture of gastrodin, parishin A, and parishin B (a), FCt before fermentation (b) and after fermentation (c). The ratio of gastrodin/parishin A and gastrodin/B in FCt before and after fermentation (d)

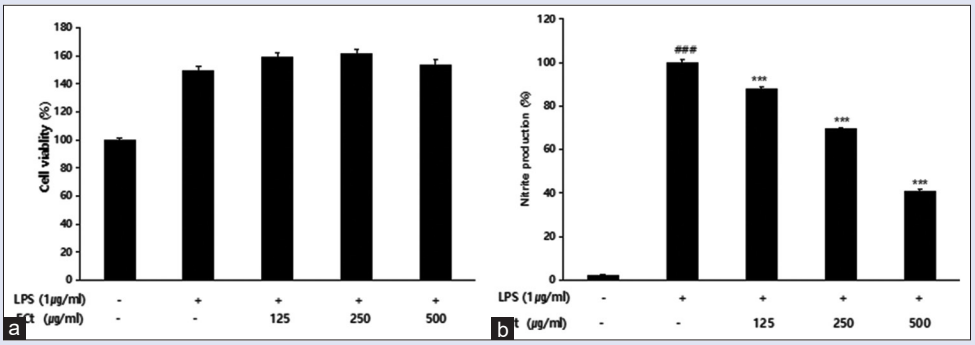


Figure 2: Effects of FCt on cell viability (a) and nitrite concentration (b) in LPS-induced RAW 264.7 cells. Data are expressed as mean ± standard deviation. All experiments were performed in three independent experiments. ###$P < 0.001$ vs. cells without LPS-stimulated; ***$P < 0.001$ vs. cells with LPS-stimulated

I. Inhibitory effect of FCt on LPS-induced cell inflammation

Effect of FCt on cell viability

The results showed that the cell viability of FCt treatment group was higher than that of the control group, indicating that FCT had no cytotoxicity at concentrations of 125, 250, and 500 μg/mL [Figure 2a].

Inhibitory effect of FCt on NO production in cell culture medium

In the LPS treatment group, a large amount of nitrite was produced due to the stimulation of LPS, but FCt treatment significantly reduced the production of nitrite, and the effect was better with the increase of concentration (125 μg/mL, $P < 0.001$). Particularly at a concentration of 500 μg/mL, the NO production inhibition rate reached more than 50% [Figure 2b].

Inhibitory effect of FCt on cytokine secretion in cell culture medium

Excessive cytokine secretion can aggravate inflammation. Therefore, inhibiting the secretion of cytokines plays a decisive role in inhibiting inflammation. LPS stimulation resulted in excessive production of TNF-α and IL-1β; however, FCt treatment significantly inhibited the secretion of IL-1β under low-concentration conditions (250 μg/mL, $P < 0.001$). FCt simultaneously reduced TNF-α under high-concentration conditions (500 μg/mL, $P < 0.001$). This means that FCt can effectively inhibit the production of inflammatory cytokines induced by LPS and has a good anti-inflammatory effect [Figure 3a].

Inhibitory effect of FCt on the expression level of iNOS and activation of NF-κB in LPS-induced RAW264.7 cells

As an inducible protein that regulates NO production, iNOS is synthesized in large quantities under inflammatory conditions to stimulate NO overproduction.[26] Western blotting analysis showed that LPS stimulation induced a large amount of iNOS synthesis. In addition, the pre-treatment of FCt reduced the expression level of iNOS (250 μg/mL, $P < 0.001$) [Figure 3b]. Furthermore, immunofluorescence results showed that LPS stimulation activated NF-κB to cause nuclear transfer and simultaneously increased the phosphorylation levels of NF-κB and IκBα (500 μg/mL, $P < 0.05$) [Figure 4a]. This phosphorylation also leads to the activation of the NF-κB signaling pathway. However, FCt pre-treatment significantly inhibited the activation of NF-κB. The above results indicate that FCt pre-treatment can reduce the excessive production of various inflammatory factors by inhibiting the activation of the NF-κB signaling pathway [Figure 4b and c]. This inhibition ultimately prevents cell inflammation.

II. Improvement effect of FCt on acute reflux esophagitis in rats

Effect of FCt on gastric content volume and gastric acid pH

The measurements of gastric content volume and gastric acid pH are shown in Figure 5. Compared with the RE control group, the FCt

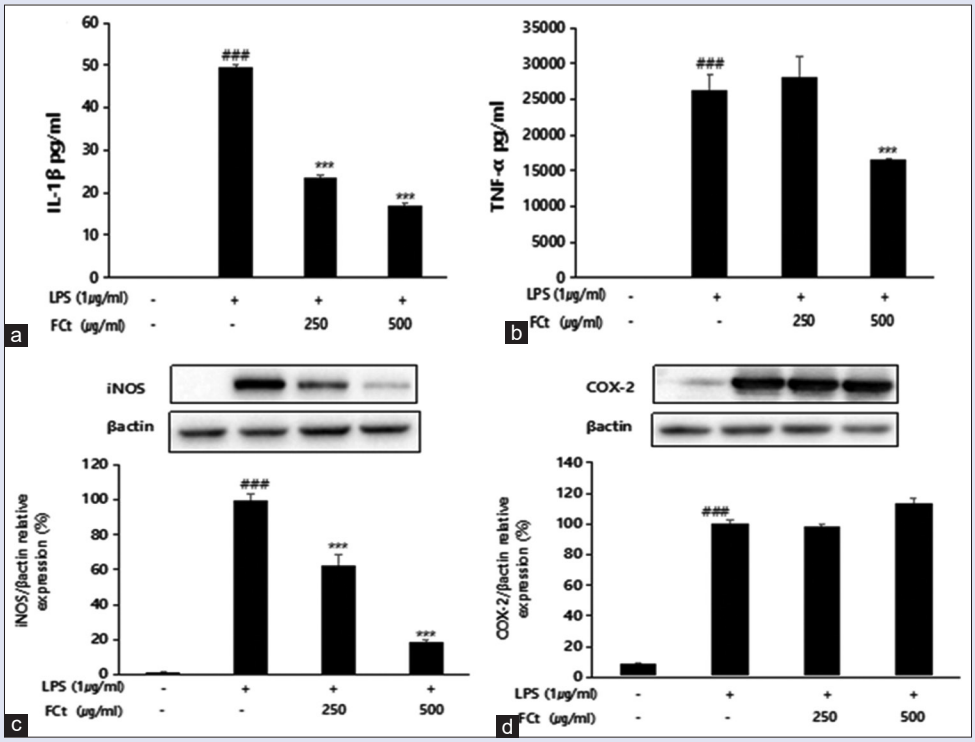


Figure 3: Effects of FCt on the production of TNF-α (a) and IL-1β (b) and expression levels of iNOS (c), COX-2 (d) in RAW 264.7 cells. Data are expressed as mean ± standard deviation. All experiments were performed in three independent experiments. ###$P < 0.001$ vs. cells without LPS-stimulated; ***$P < 0.001$ vs. cells with LPS-stimulated

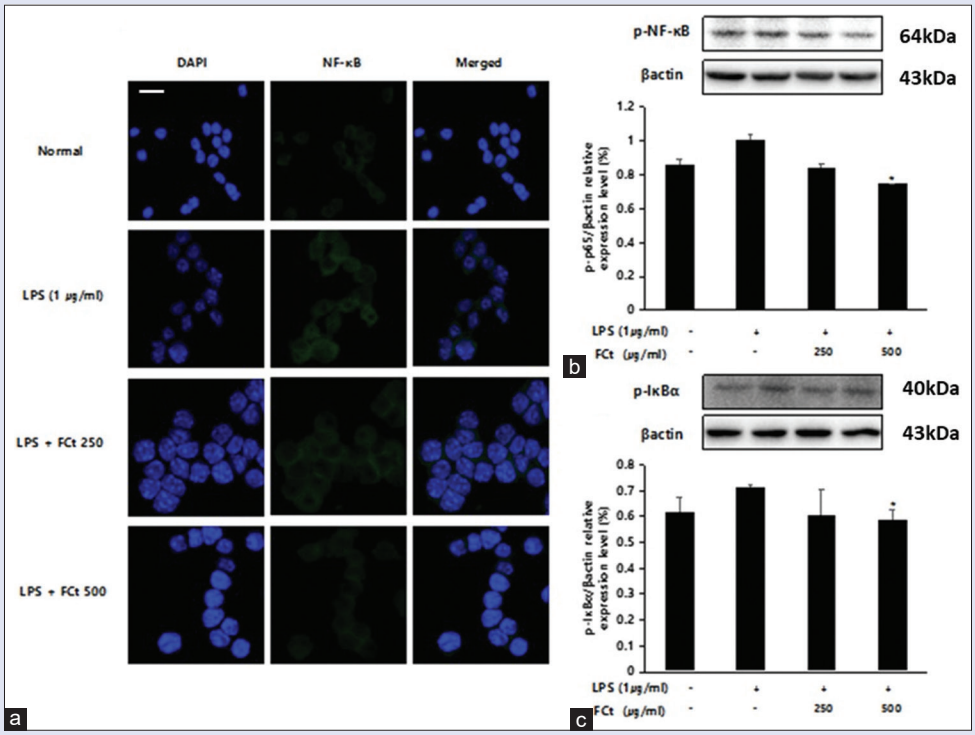
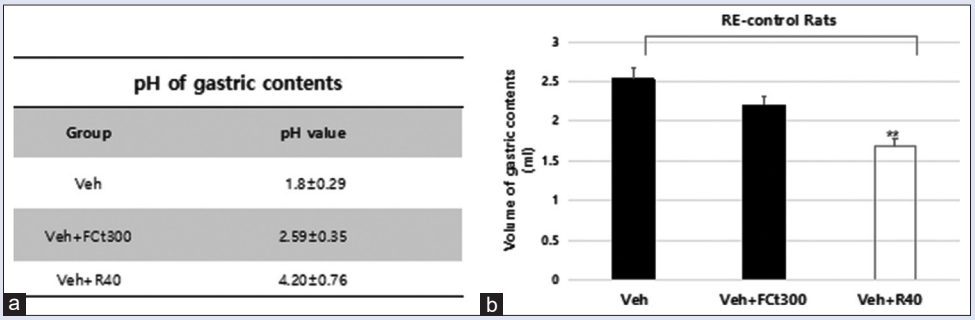


Figure 4: Effects of MsHE on nuclear translocation of NF-κB (a) phosphorylation levels of NF-κB (b), IκBα (c), and in LPS-induced RAW 264.7 cells (scale bar: 200 um). Data are expressed as mean ± standard deviation. All experiments were performed in three independent experiments. *$P < 0.05$ vs. cells with LPS-stimulated

treatment had reduced gastric content volume and increased gastric acid pH, while the effect of ranitidine treatment was more obvious ($P < 0.001$). This indicates that FCt pre-treatment to some extent can inhibit gastric acid secretion and increase the pH value of gastric acid.



pH of gastric contents

Group	pH value
Veh	1.8±0.29
Veh+FCt300	2.59±0.35
Veh+R40	4.20±0.76

a

b

Figure 5: Effects of FCt on the pH of gastric acid (a) and volume of gastric contents induced by gastric acid reflux (b). Veh - RE controlled rats; Veh + FCt300 - RE controlled rats treated with FCt 300 mg/kg; and Veh + R40 - RE controlled rats treated with ranitidine 40 mg/kg. $^{**}P < 0.01$, vs. RE controlled rats. Data are expressed as mean ± standard deviation

Effect of FCt on esophageal damage in RE rats

Repeated gastric acid reflux caused serious damage to the rats' esophageal tissue, including large-scale hemorrhage, changes in tissue morphology, and tissue erosion. Pre-treatment with FCt demonstrated similar results to that of ranitidine treatment. Pre-treatment with either medication significantly reduced the esophageal injury induced by RE, including a reduction in the redness and swelling. No erosions were seen in rats that received FCt pre-treatment. In addition, the calculation results of the esophageal tissue damage rate are shown in Figure 6a and b.

Observations from esophageal staining

A complete and clean esophagus was observed in the normal control group, while the esophageal tissue in the RE-control group was detached with tissue structure lesions (epithelial cell abscission) and inflammatory cell infiltration. However, the esophagus of rats treated with FCt or ranitidine had fewer lesions than did those of the RE-control group [Figure 6c].

Effect of FCt on the expression level of inflammatory proteins in rat esophageal tissue

Western blotting analysis results showed that in the RE control group, gastric acid reflux increased the expression of inflammatory proteins iNOS and COX-2. In contrast, FCt pre-treatment inhibited the expression level of both proteins ($P < 0.05$). The inhibitory effect of FCt on COX-2 was slightly higher than was that of ranitidine [Figure 7a and b].

Effect of FCt on the expression level of tight junction (claudin-4) in rat esophageal tissue

Tight junctions (claudin-4) are proteins that fix cells together and play an important role in cell barrier function.[27] When cells are damaged, their expression of tight junctions decreases. The results of western blotting analysis showed that gastric acid reflux reduced the expression of claudin-4 in the esophagus in the RE control group. However, FCt pre-treatment inhibited this reduction. The effect of FCt was slightly greater than was that of ranitidine in inhibiting the reduction of tight junction expression [Figure 7c].

Effect of FCt on the phosphorylation levels of NF-κB and IκBα in rat esophageal tissue

Acid reflux led to phosphorylation of NF-κB and IκBα in the esophageal tissue. Pre-treatment with FCt inhibited the phosphorylation of NF-κB and IκBα. The above results demonstrated that FCt pre-treatment reduced gastric acid secretion in rats, increased gastric acid pH, inhibited the phosphorylation of NF-κB and IκBα, and inhibited the expression of inflammatory proteins. Overall, FCt treatment lessened esophageal tissue damage caused by gastric acid reflux [Figure 7d and e].

DISCUSSION

RE, one of the gastroesophageal reflux diseases, displays this phenomenon. This disease reduces the quality of life by often necessitating medication and bringing a great economic burden to patients.[28] In addition, long-term medications can cause side effects such as liver damage and intestinal infections. Myrtle berry seed and *Morinda citrifolia* fruit extract have been previously shown to relieve RE.[22]

The LPS-induced macrophage inflammation model is widely used to study the anti-inflammatory effects of certain active substances.[29] LPS is an endotoxin, which is a component of the cell wall of Gram-negative bacteria that is composed of lipids and polysaccharides.[30] When LPS acts on other biological cells such as humans or animals, it exhibits a variety of biological activities.[31] Its physiological role is mediated by Toll-like receptors that are present on the cell membrane surface of host cells. NO plays an important role in signal transfer between cells. However, when the cell undergoes an inflammatory response, the rapid increase in the expression of inducible nitric oxide synthase leads to excessive NO production, which aggravates the inflammatory response.[32] In addition, studies have shown that cytokines such as TNF-α and IL-1β play important roles in the development of inflammation.[33] Inhibiting the excessive secretion of these substances can effectively control inflammation. Studies on the inflammatory response mechanism have shown that activation of the NF-κB signaling pathway may be involved in regulating the occurrence and development of inflammation. Under appropriate stimulation, the inhibitory protein IκBα is phosphorylated and separated from NF-κB.[34] The activated NF-κB transfers into the nucleus and regulates cytokine production. In this study, FCt effectively inhibited the activation of NF-κB and significantly reduced cellular inflammation after FCt treatment.

The rat RE model induces gastric acid reflux through ligation surgery to cause esophageal injury and inflammation.[35,36] Drug pre-treatment is performed to evaluate its effect on RE. This effect is mainly assessed as follows: calculating the damage rate of the esophageal tissue, observing the tissue morphology, measuring the gastric content and the pH value of the gastric acid, and measuring the inflammatory protein expression in the esophageal tissue. In addition, the expression level of claudin-4, 5 and other tight junction proteins are related to esophageal tissue damage.[26] In this study, the intragastric administration of FCt and ranitidine 2 h before surgery effectively improved the esophagus injury in rats caused by gastric acid reflux and inhibited RE development. In addition, FCt inhibited the phosphorylation levels of NF-κB and IκBα. We believe that activation of the NF-κB signaling pathway may regulate RE occurrence. Inhibition activation of NF-κB signaling pathway may

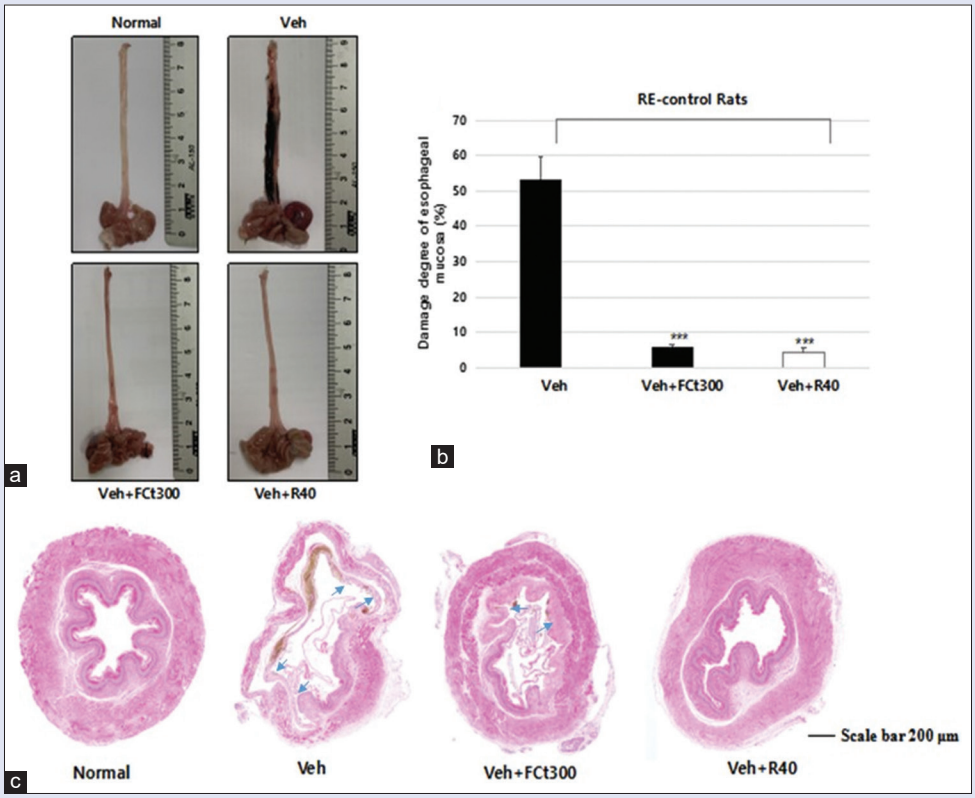


Figure 6: Effects of FCt on esophageal reflux induced esophageal mucosal damage in rats. Gross (a), the ratio of esophageal damage (b) and histological changes of esophagus induced by gastric acid reflux (scale bar: 200 μm) (c). N - Normal rats; Veh - RE controlled rats; Veh + FCt300 - RE controlled rats treated with FCt 300 mg/kg; and Veh + R40 - RE controlled rats treated with ranitidine 40 mg/kg. ***$P < 0.001$, vs. RE controlled rats. Data are expressed as mean ± standard deviation

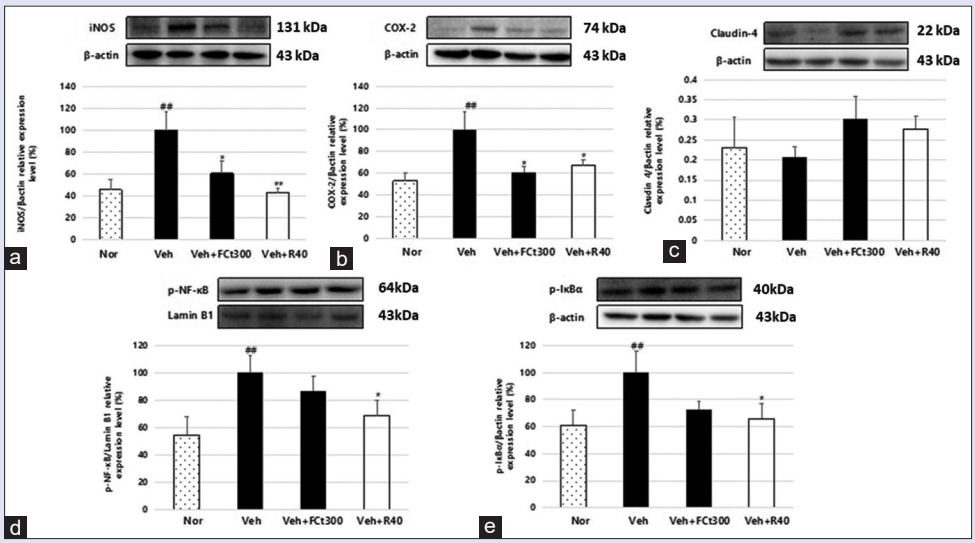


Figure 7: Effects of FCt on the expression levels of iNOS (a), COX-2 (b), Claudin-4 (c), and the phosphorylation of NF-κB (d) and IκBα (e) in esophageal tissue were measured by western blotting. Nor - Normal rats; Veh - RE controlled rats; Veh + FCt300 - RE controlled rats treated with FCt 300 mg/kg; and Veh + R40 - RE controlled rats treated with ranitidine 40 mg/kg. ##$P < 0.01$ vs. Normal rats; **$P < 0.01$, *$P < 0.05$, *$P < 0.01$ vs. RE controlled rats. Data are expressed as mean ± standard deviation

be a possible mechanism of action of FCt in alleviating acute rat reflux esophagitis.

The *C. tricuspidata* fruit contains the active substances parishin A and B. This substance is hydrolyzed during fermentation to form the more active derivative gastrodin.[37] Gastrodin has various known physiological activities such as anti-oxidation and anti-inflammation.[38] Gastrodin also has sound effects in the treatment of neurological diseases.[39] In this study, by comparing the gastrodin/

parishin A and B ratio before and after fermentation, we confirmed that gastrodin (in *C. tricuspidata* fruit) increased after fermentation. The increase of gastrodin content may be why FCt shows good anti-inflammatory activity.

CONCLUSION

The content of gastrodin in *C. tricuspidata* fruit extract was increased by *Armillaria mellea* fermentation, and the fermented *C. tricuspida* fruit extract showed good anti-inflammatory activity and protective effect on esophageal tissue damage. We believe that FCt can be used as a medicine and food material for reflux esophagitis treatment. However, whether this activity is related to the increase of gastrodin content needs further study.

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Conflicts of interest

There are no Conflicts of interest.

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