Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Comparison of Esophageal Mucosa Protective Efficacy of Evodiae Fructus and Toosendan Fructus against Duodenogastroesophageal Reflux Esophagitis

Jin A. Lee, Mi-Rae Shin, Hae-Jin Park[1], Seong-Soo Roh

Department of Herbology, Korean Medicine of College, Daegu Haany University, Deagu, [1]DHU Bio Convergence Testing Center, Gyeongsangbuk-do, Republic of Korea

Submitted: 21-Jun-2021 Revised: 23-Jul-2021 Accepted: 03-Nov-2021 Published: 24-Jan-2022

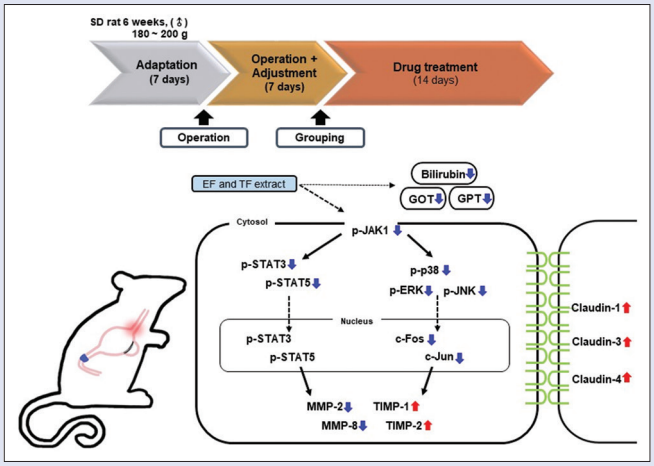
ABSTRACT

Background: Gastroesophageal reflux disease accounts for more than 20% of the Western population and is a disease that is also increasing in incidence in Asian countries. **Objectives:** This work was aimed to assess the impact of Evodiae Fructus and Toosendan Fructus against esophageal mucosal injury with Duodenogastroesophageal Reflux Esophagitis (DGER). **Materials and Methods:** After inducing DGER through surgery, the group was separated (n = 8) and the drug was administered for 2 weeks: normal rats (Normal), Water-treated DGER (Control), Evodiae Fructus 200 mg/kg-treated DGER (EF), and Toosendan Fructus 200 mg/kg-treated DGER (TF). **Results:** The administration of EF and TF significantly protected the esophageal mucosa. Furthermore, the content of bilirubin, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase in serum decreased in EF and TF. In addition, those significantly regulated the protein expression of Janus kinase (JAK)/signal transducer and activator of transcription (STAT), AP-1/MAPK, mtrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP), and tight junction. **Conclusion:** Taken together, the administration of EF and TF significantly protected the esophageal mucosa of reflux esophagitis, and in particular, the esophageal mucosa protective effect was better in EF. Furthermore, EF and TF inhibited the JAK/STAT and AP-1/MAPK pathway, and EF significantly modulated the expression of MMP/TIMP. These results show the potential of EF as a material for DGER by alleviating inflammation and improving esophageal function.

Key words: Duodenogastroesophageal reflux esophagitis, Evodiae Fructus, inflammation Toosendan Fructus

SUMMARY

- Evodiae Fructus and Toosendan Fructus are known to be effective for the digestive system, and published studies have shown that they have protective effects on the esophageal mucosa in chronic reflux esophagitis. The aim of the present study was to determine whether two fruits, Evodiae Fructus and Toosendan Fructus could alleviate esophageal mucosal damage in duodenogastro-oesophageal esophagitis (DGOR). In the present study, EF and TF alleviate damage to the esophagus by protecting the esophageal mucosa from DGOR. Also, they showed the effect of protecting the esophageal mucosa by inhibiting JAK/STAT pathway and AP-1/MAPK pathway, and improving esophageal function by regulating tight junctions. In particular, there was less gross damage to the esophageal mucosa in EF than in TF, and the expression of MMP/TIMP and TJ, which are related to the condition and function of the esophageal mucosa, was significantly regulated. Taken together, these results improve our understanding of the underlying mechanisms of EF and TF in DGOR and suggest potential as novel therapeutic drugs



Abbreviations used: GERD: Gastroesophageal reflux disease DGRE: Duodenogastroesophageal reflux esophagitis; EF: Evodiae Fructus; TF: Tooendan Frucus: JAK: Janus kinase; STAT: Signal transducer and activator of transcription; MMP: Matrix metalloprpteinase; TIMP: Tissue inhibitor of metalloprpteinase; TJ: Tight junction

Correspondence:

Dr. Seong-Soo Roh,
Korean Medicine of College, DaeguHaany University, 136, Shinchendong–ro, Suseong-gu, Deagu 42158, Republic of Korea.
E-mail: ddede@dhu.ac.kr

DOI: 10.4103/pm.pm_277_21

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Gastroesophageal Reflux Disease (GERD) is a gastrointestinal motility disorder in which stomach contents reflux into the esophagus or oral cavity, causing symptoms or complications, the typical symptoms of GERD include heartburn, and pain associated with heartburn is usually due to gastric acid present in the esophagus or bile irritation of the esophagus.[1-4] The most cause of GERD is expected to be the prevalence of obesity due to a fatty diet. Up to 20% of Americans experience symptoms of GERD, and the prevalence of GERD has risen alarmingly

Cite this article as: Lee JA, Shin MR, Park HJ, Roh SS. Comparison of esophageal mucosa protective efficacy of Evodiae Fructus and Toosendan Fructus against duodenogastroesophageal reflux esophagitis. Phcog Mag 2021;17:811-8.

over the past decades.[2] Currently, various therapeutic agents such as proton-pump inhibitors (PPIs) and nonsteroidal anti-inflammatory drugs are being used to treat GERD, but these drugs may also lead to an increase in existing GERD symptoms and signs.[5,6] PPIs are commonly used in patients with GERD, but approximately 30% of patients are refractory to PPIs.[7] Furthermore, nonsteroidal anti-inflammatory drugs are anti-inflammatory drugs that inhibit cyclooxygenase enzymes. However, it has side effects such as exacerbating reflux symptoms of reflux by increasing gastric acid secretion and directly or indirectly damaging the mucous membrane of the digestive tract.[6]

Evodiae Fructus is the dried, unripe fruit of *Evodia rutaecarpa* (Juss.) Benth, and in Korean medicine, Evodiae Fructus has been used to treat abdominal pain, hernia, diarrhea, acid reflux, nausea, and gastrointestinal disorders.[8,9] In a previous publication, Evodiae Fructus showed a gastrointestinal protective effect in ethanol-induced gastric lesions,[8] and it showed a protective effect on the esophageal mucosa in chronic reflux esophagitis.[10] Toosendan Fructus is the dried, unripe fruit of *Melia toosendan* Sieb. et Zucc., which is a Korean medicine used for acute and chronic inflammation.[11] Furthermore, many studies have shown that Toosendan Fructus relieves inflammation and cancer of the large intestine, which is one of the digestive systems.[12,13] In addition, as a result of previous studies, it showed a protective effect on the esophageal mucosa in chronic reflux esophagitis.[14]

Accordingly, the aim of the present study was to determine whether two fruits, Evodiae Fructus and Toosendan Fructus, which had protective effects on the esophageal mucosa in chronic reflux esophagitis, could alleviate esophageal mucosal damage in duodenogastroesophageal reflux esophagitis (DGER), and to compare their efficacy. In addition, we identified the underlying mechanisms for the effects of Evodiae Fructus and Toosendan Fructus on DGER.

MATERIALS AND METHODS

Materials

The protease inhibitor mixture solution and ethylenediaminetetraacetic acid (EDTA) were provided from Wako Pure Chemical Industries, Ltd. The Pierce BCA protein assay kit was provided from Thermo Fisher Scientific, Inc. Enhanced chemiluminescence reagent (ECL), Western blotting detection reagents, and pure nitrocellulose membranes were obtained from GE Healthcare. Janus kinase 1 (JAK1; C-7228), signal transducer and activator of transcription 3 (STAT3; SC-482), signal transducer and activator of transcription 5 (STAT5; SC-835), phospho-p38 mitogen-activated protein kinase (p-p38; SC-7973), extracellular signal-regulated kinase (ERK; SC-514302), phospho-extracellular signal-regulated kinase (p-ERK; SC-7383), c-Jun N-terminal kinase (JNK; SC-571), phosphor-c-Jun N-terminal kinase (p-JNK; SC-6254), matrix metallopeptidase 2 (MMP-2; SC-13595), matrix metallopeptidase 8 (MMP-8; SC-514803), tissue inhibitor of metalloproteinase-1 (TIMP-1; SC-21734), tissue inhibitor of metalloproteinase-2 (TIMP-2; SC-21735), claudin-1 (SC-166338), claudin-3 (SC-517546), claudin-4 (SC-376643), β-actin (SC-47778), and histone (SC-8030) were used antibodies of Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Phosphor-JAK1 (p-JAK1; #3331), phosphor-STAT3 (p-STAT3; #9131), phosphor-STAT5 (p-STAT5; #9351), c-Fos (#4384), c-Jun (#2315), and p38 mitogen-activated protein kinase (p38; #9212) were used antibodies of Cell Signaling Technology, Inc. Goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from GeneTex, Inc. Zoletil®50 was purchased from Virbac Laboratory and Isotroy was purchased from Troikaa Pharmaceuticals, Ltd.

Preparation of the plant material

Evodiae Fructus and Toosendan Fructus were purchased from Ominherb. A voucher herbarium specimen has been deposited at the Herbarium of Daegu Haany University and was identified by Professor S. S. Roh, the herbarium leader of Daegu Haany University. Extracts of Evodiae Fructus (100 g) and Toosendan Fructus (100 g) were obtained by addition of the 1 L of boiled water at room temperature (2 h for each extraction), and the solvent was evaporated in vacuo to obtain powders (Evodiae Fructus; 16%, Toosendan Fructus; 12%). The two prepared powders were stored at −80°C and used after dissolving in water when required.

Experimental animals and treatment

All animal experimental protocols were performed in accordance with the Animal Care and Use Committee of Daegu Haany University (approval no. DHU2021-024). The 6-week-old male Sprague-Dawley rats (body weight, 180–200 g) were obtained from Daehan Biolink and allowed to acclimatize for 1 week. Rats were with a 12-h light/dark cycle at a controlled humidity (50% ± 5%) and temperature (22°C ± 2°C). Rats were fasted for 18 h prior to surgery and maintained with a raised mesh-bottom cage to prevent co-propagation, and water was supplied until surgery. Before surgery, rats were anesthetized using tiletamine and zolazepam (Zoletil®50; 37.5 mg/kg). The general anesthesia of rats was performed using a modified protocol described by Ferrari *et al.*[15] Gastric gland was exposed and tied the transitional junction between the corpus and the forestomach with 2-0 silk thread. In addition, a latex ring (2 mm in thickness; ID, 4 mm, made from 18-Fr Nelaton catheter) was placed in the 1.5–2 cm area of the duodenum. After surgery, dexamethasone and gentamicin sulfate were injected for 4 days to prevent infection, and rats were given water after 24 h and ingested feed after 48 h. After 1 week of adaptation, 32 rats were randomly divided into 4 groups (n = 8 per group) as follows: (i) normal – normal group, (ii) control – DGER-induced rats were treated with distilled water, (iii) EF – DGER-induced rats were treated with Evodiae Fructus (200 mg/kg body weight), and (iv) TF – DGER-induced rats were treated with Toosendan Fructus (200 mg/kg body weight).

We previously reported the protective effect of a mixture containing Evodiae Fructus and Toosendan Fructus on reflux esophagitis.[10,14] Since the mixture exhibited a significant effect at 200 mg/kg body weight, the maximum concentration was set to 200 mg/kg body weight. After group separation, body weight and food intake were measured for 14 days, and drugs were orally administered. On the 15th day, rats were anesthetized by Isotroy inhalation anesthesia (induction, 4% isoflurane; maintenance, 2% isoflurane) for 5–7 min and sacrificed by inhalation anesthesia (isoflurane, Telangana, India), blood and esophageal tissue were collected. Blood was collected from the abdominal vena cava, and serum was separated from the blood and stored at −80°C. Moreover, the esophageal tissue was immediately stored at −80°C.

Esophageal mucosal damage ratio

After sacrificing animals, the rat esophagus was cut from the gastroesophageal junction to the pharynx. The dissected esophagus was imaged using an optical digital camera and then analyzed using the i-Solution Lite software program (Innerview Co.).

The gross mucosal damage ratio as a percentage was calculated as follows:

$$\frac{\text{width of area with esophageal mucosal damage}}{\text{width of the total area of esophagus}} \times 100$$

Measurement of bilirubin, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase levels in serum

Bilirubin was measured using specific assay kit (Cell Biolabs, Inc., CA, USA). Furthermore, hepatic functional parameters, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), were measured using specific assay kits and a microplate fluorescence reader (Asan, Seoul, Korea).

Preparation of cytosol and nuclear fractions

Protein extraction was performed as described by Komatsu[16] with minor modifications. For cytosol sample, esophageal tissues were lysed with buffer A (25 mL; 10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and protease inhibitor mixture solution). The homogenates were incubated, and then 10% NP-40 was mixed. After, centrifugation was performed using a centrifuge (12,000 rpm at 4°C for 2 min). The supernatant (cytosolic fraction) was collected and placed in a clean e-tube. After, the pellet was dissolved with buffer C (300 mM NaCl, 50 mM HEPES (pH 7.8), 50 mM KCl, 1 mM DTT, 0.1 mM PMSF, 0.1 mM EDTA, 1% (v/v) glycerol, and protease inhibitor mixture solution) and incubated at 4° C for 30 min to extract more protein. After centrifugation (12,000 rpm at 4°C for 10 min), the nuclear fraction was prepared to collect the supernatant. Both the cytosolic and nuclear fractions were stored at −80°C prior to the analysis.

Immunoblotting analysis

For the estimation of p-JAK/JAK/p-STAT3/STAT3/p-STAT5/STAT5/c-Fos/c-Jun/p-p38/p38/p-ERK/ERK/p-JNK/JNK/MMP-2/MMP-8/TIMP-1/TIMP-2/claudin-1/claudin-3/claudin-4/β-actin/histone (1:1000), 10 μg of proteins was electrophoresed through 8%–10% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, and then incubated with primary antibodies, respectively, overnight at 4°C. After the blots were washed, they were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody (1:3000) for 2 h at room temperature. Each antigen–antibody complex was visualized using ECL Western blotting detection reagents and detected by chemiluminescence with Sensi-Q 2000 Chemidoc (Lugen Sci Co., Ltd., Gyeonggi-do, Korea). Band densities were measured using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to histone or β-actin. The protein levels of the groups are expressed relative to those of the normal rat (represented as 1).[17]

Table 1: Levels of bilirubin, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase in serum

	Bilirubin (mg/dL)	GOT (IU/L)	GPT (IU/L)
Normal	0.05±0.01	34.29±2.70	8.84±0.32
Control	0.53±0.02$^{###}$	47.40±1.15$^{###}$	11.77±0.40$^{###}$
EF	0.40±0.07	36.07±1.14**	8.89±0.67**
TF	0.46±0.11	41.95±1.38	11.00±0.63

$^{###}P$<0.001 vs. normal, **P<0.01 vs. control. Data are expressed as the mean±SD (n=8). DGER: Duodenogastroesophageal reflux esophagitis; normal: Normal group; control: DGER-induced rats were treated with distilled water; EF: DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight; TF: DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight; SD: Standard deviation; GOT: Glutamate oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase

Statistical analysis

Data are presented as the mean ± standard deviation. Data were compared using a one-way analysis of variance followed by the least significant difference test in SPSS version 26.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Esophageal mucosal damage ratio

As shown in Figure 1, the normal did not exhibit definite damage of the esophageal mucosa, whereas the esophagus in the control showed notable changes, including ulcer and hyperemia. Furthermore, in EF, slight ulcers were found only in the lower part of the esophagus, and in TF, some ulcers were found in the middle and lower parts of the esophagus. It was confirmed that esophageal damage was significantly reduced in EF and TF compared to the control group, and this result was more excellent in EF than in TF.

Levels of bilirubin, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase in serum

Bile produced by the liver and secreted by the duodenum produces bilirubin. Table 1 shows the results of measuring bilirubin in serum. Bilirubin levels in the control were markedly higher compared with the normal group (0.05±0.01 vs. 0.53±0.02 mg/dL, [P<0.001]). In the EF, it decreased by 25% compared to the value in the control, and in the TF, it also decreased by 13%.

In addition, GOT and GPT values used as indicators of liver damage were confirmed. The control showed significantly higher GOT and GPT levels compared with the normal. Specifically, their levels were 38% ($P < 0.001$) and 33% ($P < 0.001$) higher in the control. The increase in these parameters of hepatic function was significantly reduced in the EF ($P < 0.01$).

Expression of the Janus kinase/signal transducer and activator of transcription proteins in esophageal tissue

Expressions of p-JAK1, p-STAT3, and p-STAT5 protein were quantified using Western blotting. As shown in Figure 2, the protein expression levels of p-JAK1, p-STAT3, and p-STAT5 were upregulated by in the control compared with the normal (p-JAK1: 1.26 ± 0.09 [$P < 0.01$], p-STAT3: 1.23 ± 0.06 [$P < 0.01$], and p-STAT5: 1.23 ± 0.19 [$P < 0.05$]), whereas their expressions were markedly reduced in EF and TF to the levels observed in the normal.

Expression of the AP-1/MAPK proteins in esophageal tissue

Expressions of c-Fos, c-Jun, p-p38, p-ERK, and p-JNK protein were quantified using Western blotting [Figure 3]. The protein expression levels in the control group were significantly increased compared with the normal group (c-Fos: 1.26 ± 0.20 [$P < 0.01$], c-Jun: 1.41 ± 0.13 [$P < 0.001$], p-p38: 1.64 ± 0.22 [$P < 0.001$], p-ERK: 1.42 ± 0.19 [$P < 0.01$], and p-JNK: 1.32 ± 0.07 [$P < 0.01$]). Conversely, their expressions were markedly decreased in EF and TF to the levels observed in the normal.

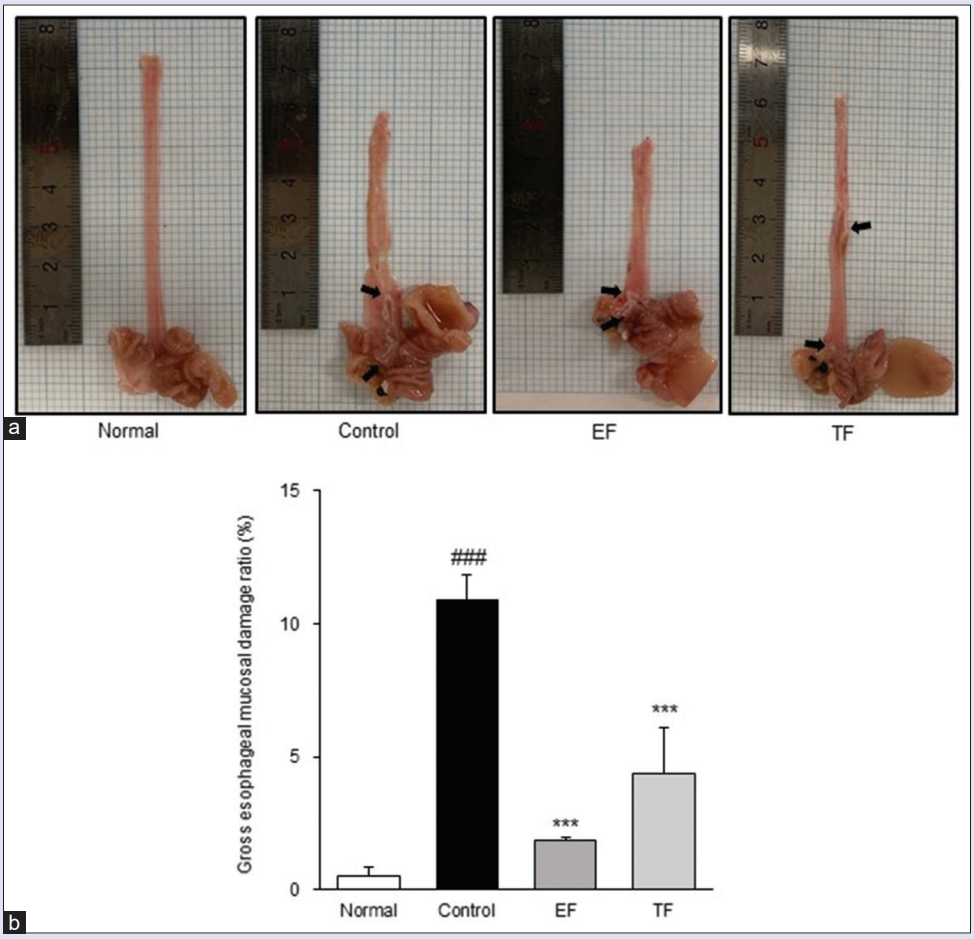


Figure 1: Surgical induction of duodenogastroesophageal reflux esophagitis. Gross images of esophageal mucosal damage (a), gross mucosal damage ratio (b). Data are expressed as the mean ± standard deviation (n = 8). ###P < 0.001 versus normal, ***P < 0.001 versus control. Duodenogastroesophageal reflux esophagitis, DGER; normal group, normal; DGER-induced rats were treated with distilled water, control; DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight, EF; DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight, TF

Expression of the matrix metallopeptidase/tissue inhibitor of metalloproteinase proteins in esophageal tissue

Expressions of MMP-2, MMP-8, TIMP-1, and TIMP-2 protein were quantified using Western blotting. As shown in Figure 4, the protein expression levels of MMP-2 and MMP-8 were upregulated by in the control compared with the normal (MMP-2: 1.40 ± 0.04 [P < 0.001] and MMP-8: 1.42 ± 0.10 [P < 0.001]), whereas their expressions were markedly reduced in EF to the levels observed in the normal. Furthermore, the protein expression levels of TIMP-1 and TIMP-2 were downregulated by in the control compared with the normal (TIMP-1: 0.71 ± 0.07 [P < 0.001] and TIMP-2: 0.51 ± 0.08 [P < 0.001]). Conversely, their expressions were markedly increased in EF and TF to the levels observed in the normal.

Expression of the tight junction proteins in esophageal tissue

The protein expression levels of tight junction (TJ), including claudin-1, claudin-3, and claudin-4, were examined. As shown in Figure 5, the protein expression levels of claudin-1, claudin-3, and claudin-4 were significantly decreased in the esophagus of the control group compared with the normal group (P < 0.001), whereas these decreased levels were significantly increased in the EF and TF. Especially, the EF increased to a value similar to that of the normal.

DISCUSSION

Both Evodiae Fructus and Toosendan Fructus are well documented for their anti-inflammatory effects. Furthermore, as a result of previous studies, they showed a protective effect on the esophageal mucosa in chronic reflux esophagitis.[10,14] The present study compared the effects of Evodiae Fructus and Toosendan Fructus on surgically induced duodenogastroesophageal reflux esophagitis (DGER). Here, the concentration of Evodiae Fructus and Toosendan Fructus was set to 200 mg/kg body weight based on the previously reported experiment. DGER was induced by tying the transitional junction between the corpus and the forestomach with 2-0 silk thread and placing a latex ring in the 1.5–2 cm area of the duodenum. In DGER, EF and TF alleviated damage by protecting the esophageal mucosa from gastric acid and bile, and especially, esophageal damage was significantly reduced in EF. Furthermore, the content of bilirubin, GOT, and GPT in serum decreased in EF and TF. In addition, EF and TF inhibited activation of the JAK/STAT pathway and AP-1/MAPK pathway, and restored the expression of MMP/TIMP and TJ proteins.

According to a study by Cross and Wangensteen and Kauer *et al.*, it was found that the number of gastric and bile reflux patients increased

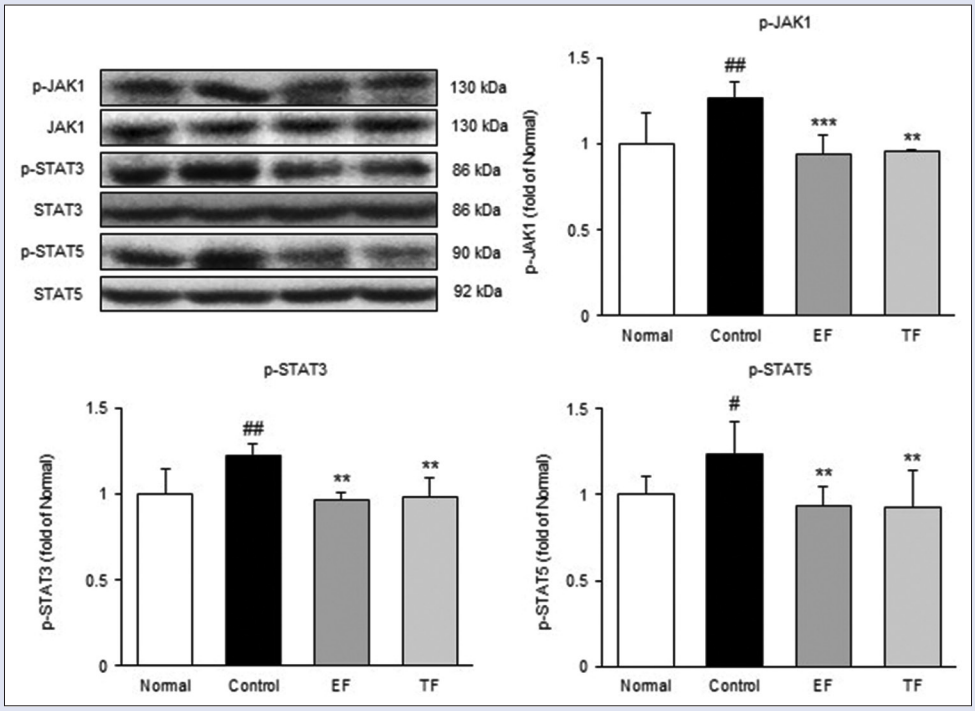


Figure 2: Expression of the Janus kinase/signal transducer and activator of transcription proteins. Data are expressed as the mean ± standard deviation ($n = 8$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Duodenogastroesophageal reflux esophagitis, DGER; normal group, normal; DGER-induced rats were treated with distilled water, control; DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight, EF; DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight, TF

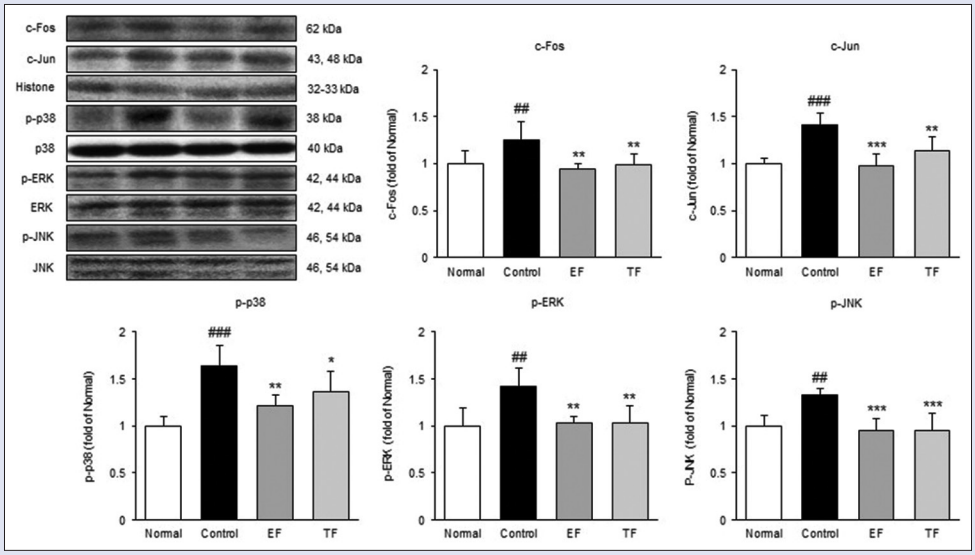


Figure 3: Expression of AP-1/MAPK proteins. Data are expressed as the standard deviation ($n = 8$). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus normal, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Duodenogastroesophageal reflux esophagitis, DGER; normal group, normal; DGER-induced rats were treated with distilled water, control; DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight, EF; DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight, TF

compared to gastric juice alone.[18,19] Bile is one of the digestive enzymes produced by the liver and secreted through the gallbladder into the duodenum.[20] Bile consists of electrolytes, bile salts, bilirubin, etc., of which bilirubin is used as a marker of duodenal juice.[21] When bile refluxes, components of bile such as bilirubin are discharged into the blood, and the discharged bilirubin has a negative effect on the liver, leading to diseases such as jaundice and cholangitis, and in severe cases, tumors can be formed around the pancreas and liver. Therefore, in this study, the concentration of bilirubin, one of the representative components of bile, in serum was analyzed.[22] In the present study, it was confirmed that the concentration of bilirubin in serum was significantly increased in the control; it was confirmed that the concentration of

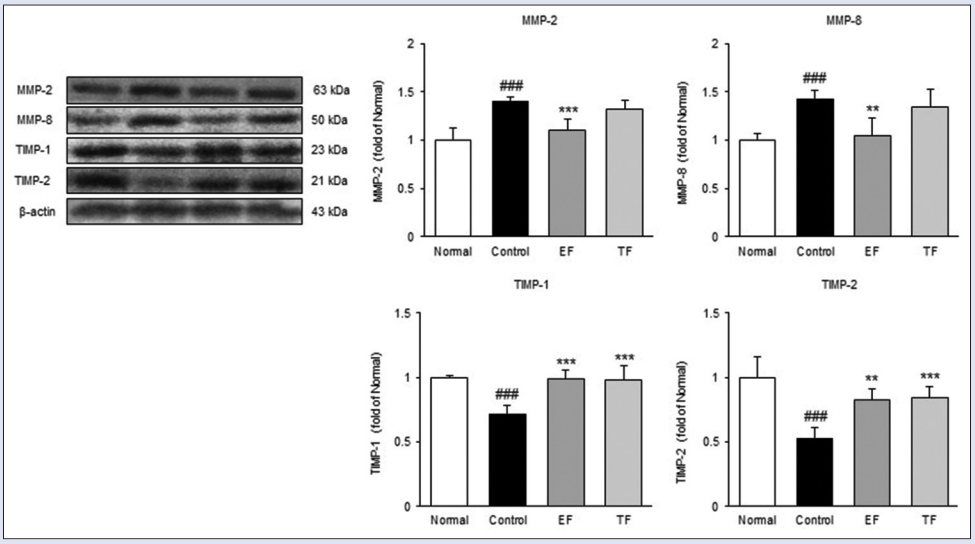


Figure 4: Expression of the matrix metallopeptidase/tissue inhibitor of metalloproteinase proteins. Data are expressed as the mean ± standard deviation (n = 8). $^{###}P < 0.001$ versus normal, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Duodenogastroesophageal reflux esophagitis, DGER; normal group, normal; DGER-induced rats were treated with distilled water, control; DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight, EF; DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight, TF

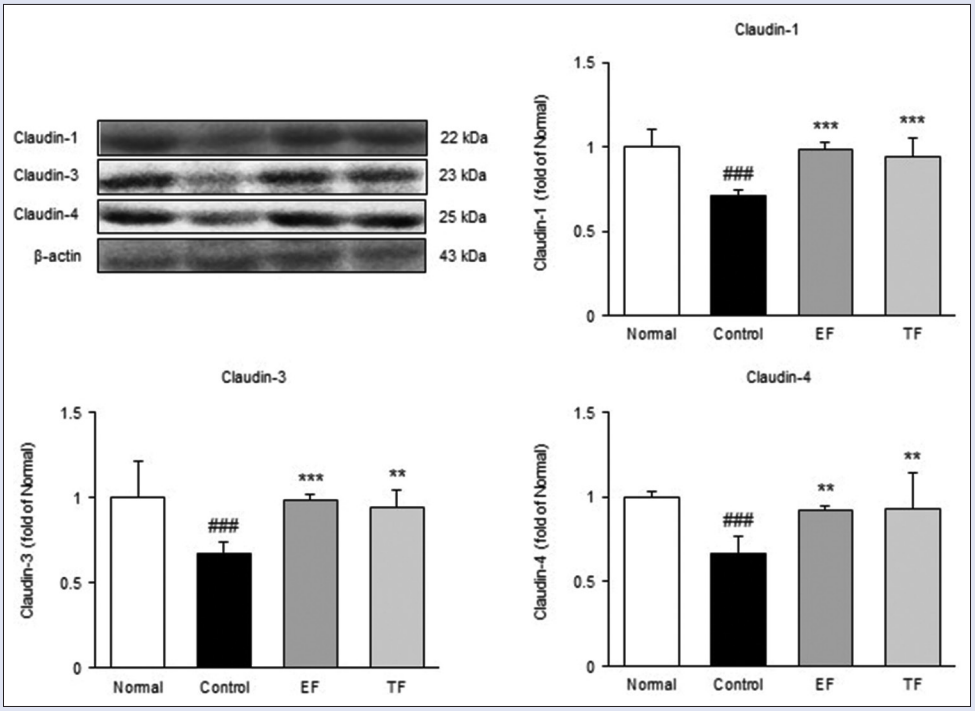


Figure 5: Expression of the tight junction proteins. Data are expressed as the mean ± standard deviation (n = 8). $^{###}P < 0.001$ versus normal, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Duodenogastroesophageal reflux esophagitis, DGER; normal group, normal; DGER-induced rats were treated with distilled water, control; DGER-induced rats were treated with Evodiae Fructus 200 mg/kg body weight, EF; DGER-induced rats were treated with Toosendan Fructus 200 mg/kg body weight, TF

bilirubin in serum decreased in EF and TF. In addition, EF significantly reduced the GOT and GPT levels in serum increased due to DGER. These results show that EF and TF decreased the amount of bilirubin excreted into the blood by DOGR. Furthermore, the levels of GOT and GPT, which are used as indicators of liver damage, decreased, which is thought to be due to a decrease in exposure to bilirubin, which has a negative effect on the liver.

DGER is known to be regulated by many inflammatory mediators, and in reflux esophagitis, inflammatory mediators can reduce the pressure in the esophageal sphincter, helping stomach contents to reflux, impairing the function of the esophageal mucosal barrier.[23,24] The JAK/STAT signaling is involved in a wide range of cellular processes, including inflammation and apoptosis, and inhibition of JAK/STAT signaling suppresses the inflammatory response, reducing the progression

to chronic inflammation. JAK is activated by cytokines to help phosphorylation of STAT, and the phosphorylated STAT is translocated into the nucleus and mediates the inflammatory response by activating gene transcription.[24-,26] Furthermore, activation of the MAPK pathway, another one of the inflammation-mediated pathways, is involved in oxidative stress, DNA damage, and chronic inflammation.[27] p38, one of the MAPKs, induces phosphorylation of numerous transcription factors regulating proinflammatory mediators, and activation of JNK plays an important role in cell survival and apoptosis. This MAPK is known to be involved in the inflammatory response by helping the activation of transcription factors such as c-Fos and c-Jun.[28] Through a previous study, it was confirmed that TF exerts a protective effect on the esophageal mucosa via the MAPK pathway in chronic acid reflux esophagitis.[14] Furthermore, in chronic acid reflux esophagitis, a mixture of EF and EF and Coptidis Rhizoma showed an esophageal mucosal protective effect, and the EF and Coptidis Rhizoma mixture via the MAPK pathway as in the case of TF.[10,29] Based on the results of these studies, the JAK/STAT pathway and the MAPK/AP-1 pathway were identified in this study. As a result, JAK/STAT and AP-1/MAPK proteins were shown to be upregulated in the esophageal epithelium in the control group, whereas EF and TF significantly downregulated the expression of JAK/STAT and AP-1/MAPK proteins. These results suggest that EF and TF relieved esophageal inflammation in DGER also via JAK/STAT and AP-1/MAPK pathways, as in previous studies.

The human epithelium is formed of epithelial cells. Excessive production of matrix metalloproteinases (MMPs) leads to increased expression of fibrotic genes and transformation of epithelial cells.[30] The phosphorylation of ERK, one of the MAPKs, regulates the expression of MMPs through activation of AP-1. MMPs is an enzyme involved in angiogenesis, extracellular matrix (collagen, elastin, and gelatin) degradation, and tissue remodeling and induces tissue remodeling and malignant development.[31-33] TF showed a tendency to downregulate the expression of MMPs up-regulated by DGOR, whereas significantly increased the expression of TIMP-1 and TIMP-2, known as MMPs inhibitors.

Bile acids and gastric acids not only cause greater inflammatory damage to the mucosa and loss of esophageal function, but also interfere with esophageal barrier function by reducing the expression of TJ proteins such as Claudin-3 and -4.[34,35] TJ proteins are the important component for the formation of constitutive barrier function in epithelial cells and gastrointestinal mucosal barrier.[36] TJ consists of junctional adhesion molecule, occludin, and claudins, of which claudins are the major integral membrane proteins. Hashimoto *et al*.[30] demonstrated that the reduction of claudin-3 among claudins reduced the amount of epithelial cells, and a study of Kojima *et al*.[37] reported that claudin-4 was directly regulated in normal epithelial cells or diseases. In addition, a study result was published that claudin-1 knockout mice died within 24 h of birth.[38] Based on these studies, transmembrane proteins such as claudin are considered to be a key factor in barrier function. In the present study, TJs such as claudin-1, claudin-3, and claudin-4 were shown to be decreased in the esophageal epithelium in the control, whereas EF and TF significantly increased the expression of TJ proteins. These results suggest that EF and TF improved the falling of the esophageal epithelium and esophageal barrier function by regulating the TJ protein decreased due to DGER.

CONCLUSION

In the present study, EF and TF alleviate damage to the esophagus by protecting the esophageal mucosa from DGER. Furthermore, they showed the effect of protecting the esophageal mucosa by inhibiting JAK/STAT pathway and AP-1/MAPK pathway, and improving esophageal function by regulating TJ s. In particular, there was less gross damage to the esophageal mucosa in EF than in TF, and the expression of MMP/TIMP and TJ, which are related to the condition and function of the esophageal mucosa, was significantly regulated. Taken together, these results improve our understanding of the underlying mechanisms of EF in DGOR and suggest potential as novel therapeutic drugs, and it is judged that a more in-depth study is needed to determine which components of EF and TF are involved in these results.

Financial support and sponsorship

This work has been supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIT) (No. 2017R1A2B2006858, No. 2018R1A5A2025272, and No. 2019R1I1A1A01064068).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kellerman R, Kintanar T. Gastroesophageal reflux disease. Prim Care 2017;44:561-73.
2. Mikami DJ, Murayama KM. Physiology and pathogenesis of gastroesophageal reflux disease. Surg Clin North Am 2015;95:515-25.
3. Okamoto K, Iwakiri R, Mori M, Hara M, Oda K, Danjo A, *et al*. Clinical systoms in endoscopic reflux esophagitis: Evaluation in 8031 adult subjects. Dig Dis Sci 2003;48:2234-41.
4. Lee SW, Chang CS. Impact of overlapping functional gastrointestinal disorders on the quality of life in patients with gastroesophageal reflux disease. J Neurogastroenterol Motil 2021;27:176-84.
5. Yasawy MI, Randhawa MA. GERD is becoming a challenge for the medical profession: Is there any remedy? Hepatogastroenterology 2014;61:1623-6.
6. Mungan Z, Pınarbaşı Şimşek B. Which drugs are risk factors for the development of gastroesophageal reflux disease? Turk J Gastroenterol 2017;28:S38-43.
7. Labenz J, Labenz G, Stephan D, Willeke F; LOPA-Studiengruppe. Insufficient symptom control under long-term treatment with PPI in GERD – Fact or fiction?. MMW Fortschr Med 2016;158 Suppl 4:7-11.
8. Yu X, Wu DZ, Yuan JY, Zhang RR, Hu ZB. Gastroprotective effect of fructus evodiae water extract on ethanol-induced gastric lesions in rats. Am J Chin Med 2006;34:1027-35.
9. Yu LL, Liao JF, Chen CF. Effect of the crude extract of evodiae fructus on the intestinal transit in mice. Planta Med 1994;60:308-12.
10. Lee JA, Park HJ, Kim SH, Kim MJ, Kim KJ, Shin MR, *et al*. Evaluation of evodiae fructus extract on the chronic acid reflux esophagitis in rats. Kor J Herbol 2016;34:15-23.
11. Xie F, Zhang M, Zhang CF, Wang ZT, Yu BY, Kou JP. Anti-inflammatory and analgesic activities of ethanolic extract and two limonoids from *Melia toosendan* fruit. J Ethnopharmacol 2008;117:463-6.
12. Fan H, Chen W, Zhu J, Zhang J, Peng S. Toosendanin alleviated dextran sulfate sodium-induced colitis by inhibiting M1 macrophage polarization and regulating NLRP3 inflammasome and Nrf2/HO-1 signaling. Int Immunopharmacol 2019;76:105909.
13. Tang XL, Yang XY, Kim YC, Kim SY, Kang BD, Choi DY, *et al*. Protective effects of the ethanolic extract of *Melia toosendan* fruit against colon cancer. Indian J Biochem Biophys 2012;49:173-81.
14. Lee JA, Shin MR, Choi JW, Roh SS. Effect of toosendan fructus on chronic acid reflux esophagitis rats. Kor J Herbol 2021;36:1-8.
15. Ferrari L, Turrini G, Rostello C, Guidi A, Casartelli A, Piaia A, *et al*. Evaluation of two combinations of Domitor, Zoletil 100, and Euthatal to obtain long-term nonrecovery anesthesia in Sprague-Dawley rats. Comp Med 2005;55:256-64.
16. Komatsu S. Extraction of nuclear proteins. Methods Mol Biol 2007;355:73-7.
17. Kim KJ, Kim SH, Shin MR, Kim YJ, Park HJ, Roh SS. Protective effect of S-allyl cysteine-enriched black garlic on reflux esophagitis in rats via NF-κB signaling pathway. J Funct Foods 2019;58:199-206.
18. Cross FS, Wangensteen OH. Roe of bile and pancreatic juice in production of esophageal erosions and anemia. Proc Soc Exp Biol Med 1951;77:862-6.
19. Kauer WK, Peters JH, DeMeester TR, Feussner H, Ireland AP, Stein HJ, *et al*. Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal

reflux disease. Surgery 1997;122:874-81.

20. Hundt M. Basit H, John S. Physiology, bile secretion. In: StatPearls. 2020.
21. Kauer WK, Stein HJ. Bile reflux in the constellation of gastroesophageal reflux disease. Thorac Surg Clin 2005;15:335-40.
22. Jones EA, Bergasa NV. The pruritus of cholestasis. Hepatology 1999;29:1003-6.
23. Hampel H, Abraham NS, El-Serag HB. Meta-analysis: Obesity and the risk for gastroesophageal reflux disease and its complications. Ann Intern Med 2005;143:199-211.
24. di Pietro M, Alzoubaidi D, Fitzgerald RC. Barrett's esophagus and cancer risk: How research advances can impact clinical practice. Gut Liver 2014;8:356-70.
25. Malemud C, Pearlman E. Targeting JAK/STAT signaling pathway in inflammatory disease. Curr Signal Transduct Ther 2009;4:201-21.
26. Seif F, Khoshmirsafa M, Aazami H, Mohsenzadegan M, Sedighi G, Bahar M. The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. Cell Commun Signal 2017;15:23.
27. Chang L, Karin M. Mammalian MAP Kinase signaling cascades. Nature 2001;410:37-40.
28. Liu SQ, Xie Y, Gao X, Wang Q, Zhu WY. Inflammatory response and MAPK and NF-Kb pathway activation induced by natural street rabies virus infection in the brain tissues of dog and humans. Virol J 2020;17:157.
29. Lee JA, Shin MR, Lee JH, Roh SS. Effect of coptidis rhizoma and evodiae fructus mixture on esophageal mucosa in chronic reflux esophagitis. Koe J Pharmacogn 2020;51:349-59.
30. Hashimoto K, Oshima T, Tomita T, Kim Y, Matsumoto T, Joh T, *et al.* Oxidative stress induces gastric epithelial permeability through claudin-3. Biochem Biophys Res Commun 2008;376:154-7.
31. Davelaar AL, Straub D, Buttar NS, Fockens P, Krishnadath KK. Active matrix metalloproteases are expressed early on and are high during the Barrett's esophagus malignancy sequence. Scand J Gastroenterol 2015;50:321-32.
32. Im I, Park KR, Kim SM, Kim C, Park JH, Nam D, *et al.* The butanol fraction of guava (*Psidium cattleianum* Sabine) leaf extract suppresses MMP-2 and MMP-9 expression and activity through the suppression of the ERK1/2 MAPK signaling pathway. Nutr Cancer 2012;64:255-66.
33. Groblewska M, Siewko M, Mroczko B, Szmitkowski M. The role of matrix metalloproteinase (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol 2012;50:12-9.
34. Blevins CH, Iyer PG, Vela MF, Katzka DA. The esophageal epithelial barrier in health and disease. Clin Gastroenterol Hepatol 2018;16:608-17.
35. Chen X, Oshima T, Tomita T, Fukui H, Watari J, Matsumoto T, *et al.* Acidic bile salts modulate the squamous epithelial barrier function by modulating tight junction proteins. Am J Physiol Gastrointest Liver Physiol 2011;301:G203-9.
36. Oshima T, Miwa H. Gastrointestinal mucosal barrier function and diseases. J Gastroenterol 2016;51:768-78.
37. Kojima T, Kohno T, Kubo T, Kaneko Y, Kakuki T, Kakiuchi A, *et al.* Regulation of claudin-4 via p63 in human epithelial cells. Ann N Y Acad Sci 2017;1405:25-31.
38. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, *et al.* Claudin-based tight junctions are crucial for the mammalian epidermal barrier: A lesson from claudin-1-deficient mice. J Cell Biol 2002;156:1099-111.