빈랑자와 천련자 복합물의 만성 역류성 식도염에서 보호 효과

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The protective effect of Areca Semen and Toosendan Fructus mixture in a chronic model of reflux esophagitis

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ABSTRACT

Objective : The aim of present study was to clarify the effect of Areca Semen and Toosendan Fructus Mixture (AT-mix) on chronic reflux esophagitis (CRE) in rats.
Methods : The antioxidant activity of AT-mix was measured through DPPH and ABTS radical scavenging activities *in vitro*. CRE was induced in SD rats (5 weeks, male) by ligating the border forestomach and granular portion with 2-0 silk and the duodenum near the pyloric portion was covered with 2-mm wide piece of 18-Fr Nélaton catheter. And then rats were treated AT-mix 200 mg/kg one daily for 14 days. The anti-oxidant and inflammatory protein levels were evaluated using western blotting.
Results : Gross lesion of esophageal mucosa after AT-mix treatment showed a superior enhancement compared with that of CRE control rats. AT-mix treatment strongly reduced both DPPH and ABTS radical scavenging activities (DPPH, IC_{50} 8.15 ± 0.14 $\mu g/mL$; ABTS, IC_{50} 24.69 ± 0.03 $\mu g/mL$, repspectively). Levels of the NADPH oxidase subunit including NOX4 and $p22^{phox}$ increased in CRE control rats. Otherwise, AT-mix treatment significantly reduced. The activation of Nuclear factor-erythroid 2-related factor 2 (Nrf2) led to significantly the up-regulation of HO-1. The inhibition of $I\kappa B\alpha$ phosphorylation led to NF-κB inactivation. Subsequently, NF-κB inactivation significantly induced the decrease of COX-2, iNOS, TNF-α, and IL-6 protein expressions.
Conclusion : Taken together, these results suggest that AT-mix treatment can attenuate the esophageal mucosal ulcer though inhibiting NF-κB pathway and enhancing Nrf2/HO-1 pathway. Thus, the additional mechanism study about AT-mix would need for the development as a safe herbal therapy for CRE.

Key words : Areca Semen and Toosendan Fructus Mixture, chronic reflux esophagitis, esophageal mucosal ulcer, anti-oxidant, anti-inflammatory

Ⅰ. Introduction

Gastroesophageal reflux disease (GERD), which is characterized by distinguishing symptoms such as dysphagia and heartburn, develops when the reflux of gastric contents toward the esophagus[1]. GERD is recently the most popular gastrointestinal disorder encountered from various ages in gastroenterology practice[2]. GERD

is more highly represented in USA and Europe than in Asia in the past[3]. However, the prevalence of Korea among Asia has been recently increased in parallel with the remarkable growth of obesity associated with westernized or irregular eating habits, lack of exercise, stress, smoking, alcohol consumption, and usage status of health-care utilization[4-6]. The increased prevalence of Korea caused by the economic burden because of requiring long-term use of proton pump inhibitors (PPIs)[7]. Since the introduction of omeprazole in 1989, PPIs for acid suppression are the cornerstone of therapeutic strategy for GERD treatment[8]. As of 2015, there are six PPIs (omeprazone, esomerazole, lansoprazole, dexlansoprazole, pantoprazole, and rabeprazole) approved by the United States Food and Drug Administration (FDA)[9]. Despite their excellent efficacy, PPIs have some limitations related to meal timing (preprandial dosing), an slow expression of medical effect, and their short plasma half-lives[10,11]. Long-term use of high-dose PPIs led to possible risk of fractures of the hip, wrist, and spine. Moreover, it associated with hypomagnesemia, acute interstitial nephritis, and vitamin B12 deficiency[12,13]. Accordingly, the current researches are focused on a new and rational approach about medical herbs or their mixture which can satisfy both the great drug efficacy and safe use of long term[14-16].

Oxidative stress (OS) harmfully affects esophageal mucous membrane through overproduction of the reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide and induces chronic reflux esophagitis[17,18]. OS deteriorates when there is an excessive generation and inefficient scavenging of ROS[19]. Therefore, a redox balance is referred to as the cellular biochemical process for a maintenance of homeostasis. Nuclear factor erythroid 2-related factor 2 (Nrf2), is essential modulator of redox status and cellular detoxification responses is reported to bind promoters of antioxidant genes to promote antioxidant enzymes expression[20-22]. Furthermore, Nrf2 also plays a key role in improving inflammatory response, which is associated with the activation of nuclear factor kappa B (NF-κB) pathway[23]. Hence, Nrf2 has been revealed to be one of possible therapeutic targets for various disorders[24].

Arecae Semen, the fruits of *Areca catechu* L. (Arecaceae), widely distributed in South and Southeast Asia. It has been used as the treatment agent for the improvement of gastrointestinal motor function including functional dyspepsia[25,26]. In addition, it has various pharmacological activities including anti-oxidant, anti-inflammatory, antibacterial, anti-parasitic, antifungal,

analgesic, and anti-allergic effects[27]. However, health disorders the association with repeated and continuous chewing habit of areca nut is a serious issue at present[28]. Toosendan Fructus, the mature fruit of *Melia toosendan* Sieb. et Zucc, has been used as medicinal plant in China and Korea to treat gastritis, stomach ache, and cholecystitis[29,30]. Toosendan Fructus was primally recorded in the Chinese pharmacology 『BEN CAO ZHENG』 and subsequently included in the 『People's Republic of China Pharmacopoeia』[31]. Modern pharmacological studies indicate that Toosendan Fructus has an insecticidal effect and can treat coccidiosis[32]. Toosendan Fructus has anti-oxidant, anti-inflammatory, antibacterial, anti-H1N1 virus, and analgesic effects[33,34]. In addition, Toosendan Fructus can reduce the pain sensitivity[35]. As mentioned above, major effects of Arecae Semen and Toosendan Fructus are considered with the anti-oxidant, anti-inflammatory, and analgesic activities.

Accordingly, Areca Semen and Toosendan Fructus Mixture (AT-mix) may alleviate effectively esophageal mucosal ulcer on chronic reflux esophagitis caused by oxidative stress. However, the protective mechanism of AT-mix treatment in esophageal mucosal ulcer by chronic reflux has not yet been reported. Therefore, we investigated whether AT-mix will exert the protective effect through Nrf2-mediated anti-oxidative function in a chronic model of reflux esophagitis.

Ⅱ. Materials and Methods

1. Experimental materials

The protease inhibitor mixture solution and ethylene diamine tetra acetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phenyl methyl sulfonyl fluoride (PMSF) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The pierce bicin choninic acid (BCA) protein assay kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). ECL Western Blotting Detection Reagents and pure nitrocellulose membranes were supplied by GE Healthcare (Chicago, IL, USA). Rabbit polyclonal antibodies against nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione peroxidase-1/2 (GPx-1/2), p22^{phox}; Goat polyclonal antibodies against tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β); mouse monoclonal antibodies against Nuclear factor-kappa B p65 (NF-κBp65), phosphorylated inhibitor of κBα (p-IκBα),

cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), histone, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-reduced nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) was purchased from LifeSpan BioSciences (Seattle, WA, United States). Rabbit anti-goat, goat anti-rabbit, and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

2. Test material

Areca Semen and Toosendan Fructus were purchased from Ominherb Co. (Youngcheon, Korea). A voucher herbarium specimen has been deposited at the Herbarium of Daegu Haany University and was identified by Seong-Soo Roh, the herbarium leader of Daegu Haany University. Dried Areca Semen (15 g) and Toosendan Fructus (15 g) mixture (AT-mix) boiled with distilled water (300 mL) at room temperature for 2 h and the solvent was evaporated *in vacuo* to give an extract with a yield of 9.7% by weight, of the original AT-mix.

3. DPPH radical scavenging activity

Antioxidant activity determination of AT-mix was performed by the DPPH radical scavenging according to the method of Hatano et al.[36]. In microwells, 100 $\mu\ell$ of an ethanolic solution of the sample (blank: 100 $\mu\ell$ of ethanol) was added to an ethanolic solution of DPPH (60 μm). After mixing gently and leaving to stand for 30 min at room temperature, the optical density was determined using a Microplate Reader, model infinite M200 PRO (Tecan, Switzerland). The mixture was measured spectrophotometrically at 540 nm. The antioxidant activity of each sample was expressed in terms of the IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%) calculated from the log-dose inhibition curve. The radical scavenging activity was calculated as a percentage using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = [1 - (A_{sample}/A_{blank})] \times 100$$

4. ABTS radical scavenging activity

ABTS radical scavenging activity of the different extracts was measured according to the modified method of Re et al.[37]. ABTS stock solution was dissolved in water to a 7.4 mM concentration. The ABTS radical cation (ABTS) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 14 h at room temperature in the dark. The ABTS solution was diluted with ethanol to obtain an absorbance of 0.70 $\pm$ 0.02 at 415 nm. After adding 1.0 mL of diluted ABTS solution (A415 nm = 0.70 $\pm$ 0.02) to 0.01 mL of sample, the mixture was left at room temperature for 30 min in the dark. The absorbance at 415 nm was measured using a microplate reader. The blank was prepared in the same manner, except distilled water was used instead of the sample. The radical scavenging activity was calculated as a percentage using the following equation:

$$\text{ABTS radical scavenging activity } (\%) = [1 - (A_{sample}/A_{blank})] \times 100$$

5. Experimental animals and treatment

Animal experiments were carried out according to the "Guidelines for Animal Experimentation" approved by the Ethics Committee of the Daegu Haany University with certificate number DHU2019-129. Four-week-old male Sprague-Dawley rats (B.W. 135 – 145 g) were purchased from Daehan Bio Link (Eumseong, Korea). Rats were maintained under a 12-h light/dark cycle, housed at a controlled temperature (24$\pm$2°C) and humidity (about 60%). After adaptation (1 week), the rats were fasted for 18 h prior to surgical procedures and kept in raised mesh-bottom cages to prevent coprophagy. And then rats were anaesthetized with an injection of Zoletil at 0.75 mg/kg (Virbac S.A. France). The CRE model was developed by following the methods proposed by Omura et al.[38]. A midline laparotomy was performed to expose the stomach and the transitional region (i.e., limiting ridge) between the fundus and the glandular portion of the stomach was ligated with 2-0 silk thread in order to restrict the compliance of the stomach, which led to the reflux of gastric contents into the esophagus. Additionally, a latex ring (2 mm in thickness; ID, 4 mm, made from 18-Fr Nélaton catheter) was placed around the pyloric sphincter so as to restrict the emptying of gastric contents. Rats were injected with gentamicin sulfate (antibiotic, subcutaneously injection) and dexamethasone (anti-inflammatory agent, subcutaneously injection) for 3 days to prevent infection. After surgery, the rats fasted for a further

48 h but water was provided 24 h after surgery. All animals had an operation adjustment for 7 days after surgery. At 21 days (an operation adjustment period; 7 days + drug treatment period; 14 days) after surgery, rats were sacrificed and the esophageal tissues were obtained for further processing and analysis. Rats divided into three groups. The normal and CRE control groups were given water, while the drug group was orally administered AT-mix at a dose 200 mg/kg body weight daily using a stomach tube for 14 days (*n*=8 in each group). The entire esophagus was removed immediately and examined for gross mucosal injury. The esophageal tissue was immediately frozen in liquid nitrogen and blood samples were collected by vena cava puncture from anesthetized rats. Subsequently, the esophagus and serum were kept at -80°C until analysis.

6. Preparation of cytosol and nuclear fractions

Protein extraction was performed according to the method of Komatsu with minor modifications[39]. Esophageal tissues for cytosol fraction were homogenized with ice-cold lysis buffer A (250 mL) containing 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 1,250 μL protease inhibitor mixture solution. The homogenate incubated at 4°C for 20 min. And then 10% NP-40 was added and mixed well. After centrifugation (13,400×g for 2 min at 4°C) using Eppendorf 5415R (Hamburg, Germany), the supernatant liquid (cytosol fraction) was seperated new e-tube. The left pellets were washed twice by buffer A and discard the supernatant. Next, the pellets were suspended with lysis buffer C (20 mL) containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1% (v/v) glycerol, and 100 μL protease inhibitor mixture solution suspended and incubated at 4°C for 30 min. After centrifugation (13,400 ×g for 10 min at 4°C), the nuclear fraction was prepared to collect the supernatant. Both cytosol and nuclear fractions were kept at -80°C before the analysis.

7. Immunoblotting analyses

For the estimation of Nrf2, NF-κBp65, and histone, 12 μg of protein from each nuclear fraction was electrophoresed through 8-10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, then incubated with primary antibodies (Nrf2, NF-κBp65,

and histone) and overnight at 4℃. After the blots were washed, they were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 1 h at room temperature. In addition, 8 μg proteins of each cytosol fraction of SOD, GPx-1/2, HO-1, COX-2, iNOS, TNF-α, IL-6, IL-1β, NOX4, p22$^{\text{phox}}$, and β-actin were electrophoresed through 8-15% SDS-PAGE. 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).

8. Statistical analysis

The data are expressed as the mean ± SD (or SEM). Significance was assessed by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) method using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). Values of *p* 〈 0.05 were considered significant.

Ⅲ. Results

1. DPPH radical scavenging activity and ABTS radical scavenging activity

Two analysis methods (such as DPPH and ABTS methods) were performed to confirm antioxidant activity of AT-mix. IC_{50} (μg/ mL) represent half maximal concentration of tested compounds to scavenge DPPH and ABTS radical. As shown in Figure 1, the IC_{50} of DPPH radical scavenging activity of AT-mix was found at 8.15±0.14 μg/ mL and the IC_{50} value of ascorbic acid as a positive control was 1.11±0.03 μg/ mL. The calculated IC_{50} value of AT-mix against the ABTS radical was determined to be 24.69±0.03 μg/ mL and the IC_{50} value of ascorbic acid as a positive control was 3.34±0.04 μg/mL.

2. Gross mucosal damage in the esophagus.

Normal rats had no detectable esophageal mucosa lesions, whereas esophageal ulcers were readily apparent in the middle or distal esophagus of CRE control rats. As compared to the normal rats, CRE control rats exhibited basal layer thickening and esophagus length decreased and enlarged. Moreover, esophagus mucosal

ulcer is severe. On the other hand, the AT-mix rats remarkably alleviated an extensive damage (Figure 2).
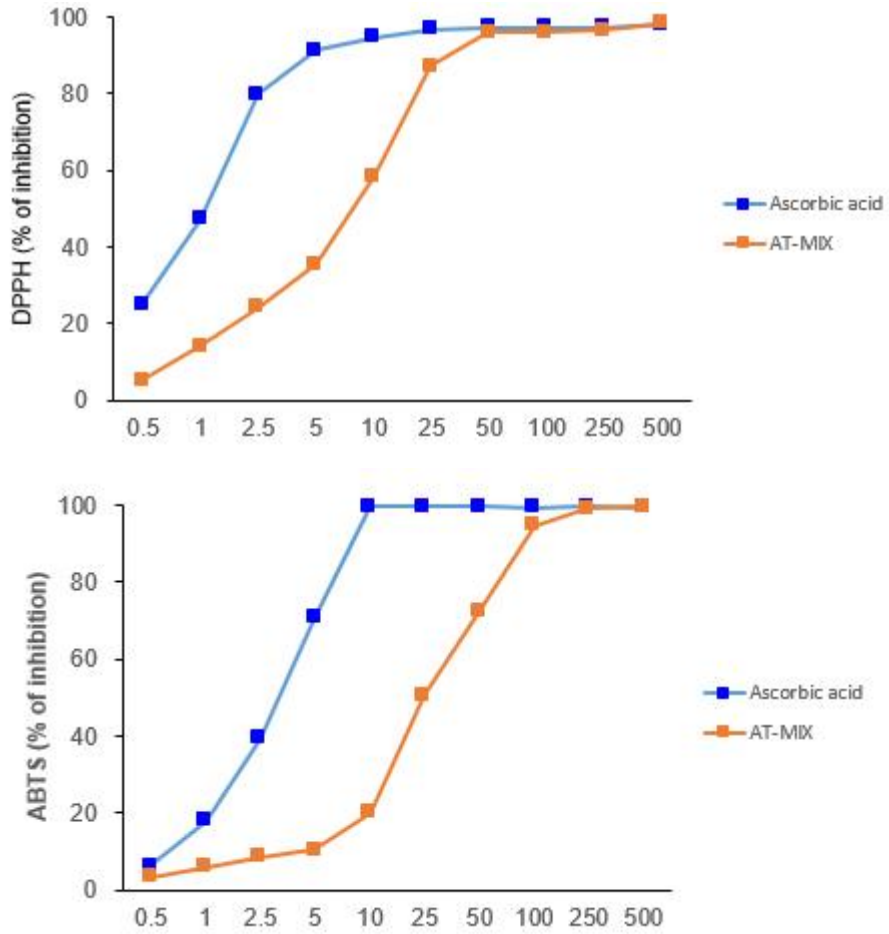

Figure 1. DPPH and ABTS radical scavenging activities.
AT-mix. AT-mix, water extract of Areca Semen and Toosendan Fructus Mixture.
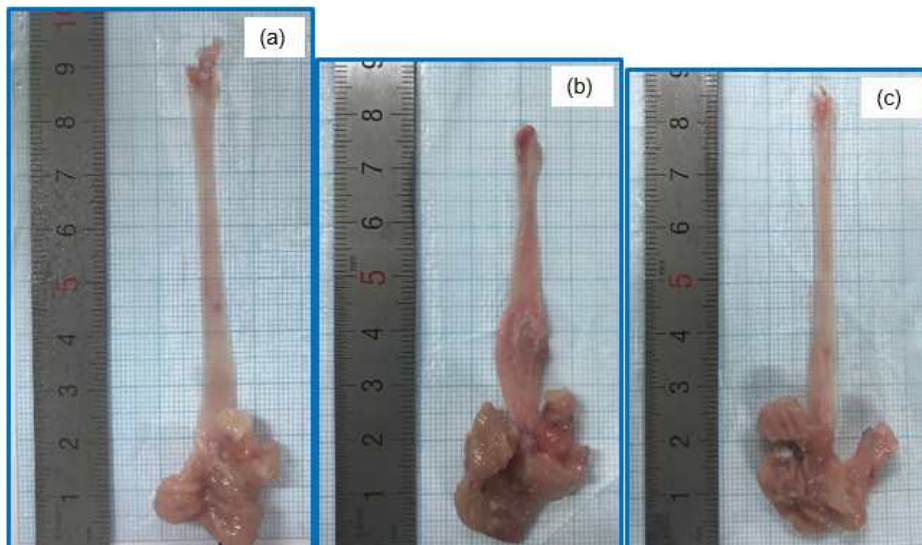Data are mean ± SEM. Each experiment was run in triplicate.

Figure 2. Representative image in each group of esophageal mucosal ulcer after surgical induction of
CRE. A: Normal rat; B: CRE control rat; C: AT-mix-treated rat.

3. Esophageal NOX4 and p22phox protein expressions.

An important cellular source of ROS is the NADPH oxidase family. The protein expressions of both NOX4 and p22phox (the markers of NADPH oxidase activity in the esophageal tissues) were augmented in CRE control rats. However, its change didn't show a significant increase. Otherwise, AT-mix treatment had significantly down-regulated NADPH oxidase (Figure 3).

4. Esophageal Nrf2, HO-1, SOD, Catalase, and GPx-1/2 protein expressions.
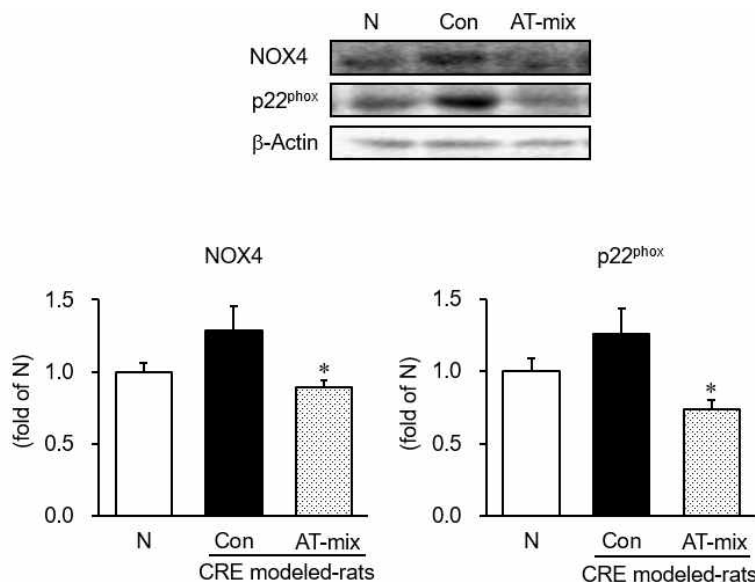
As shown in Figure 4, the Nrf2 protein expression

Figure 3. Esophageal NOX4 and p22phox protein expressions. N, normal rats; Con, CRE control rats; AT-mix, AT-mix 200 mg/kg body weight/day-treated CRE rats. Data are mean ± SD. (n=8) Significance: $^*P < 0.05$ vs. CRE control rats.
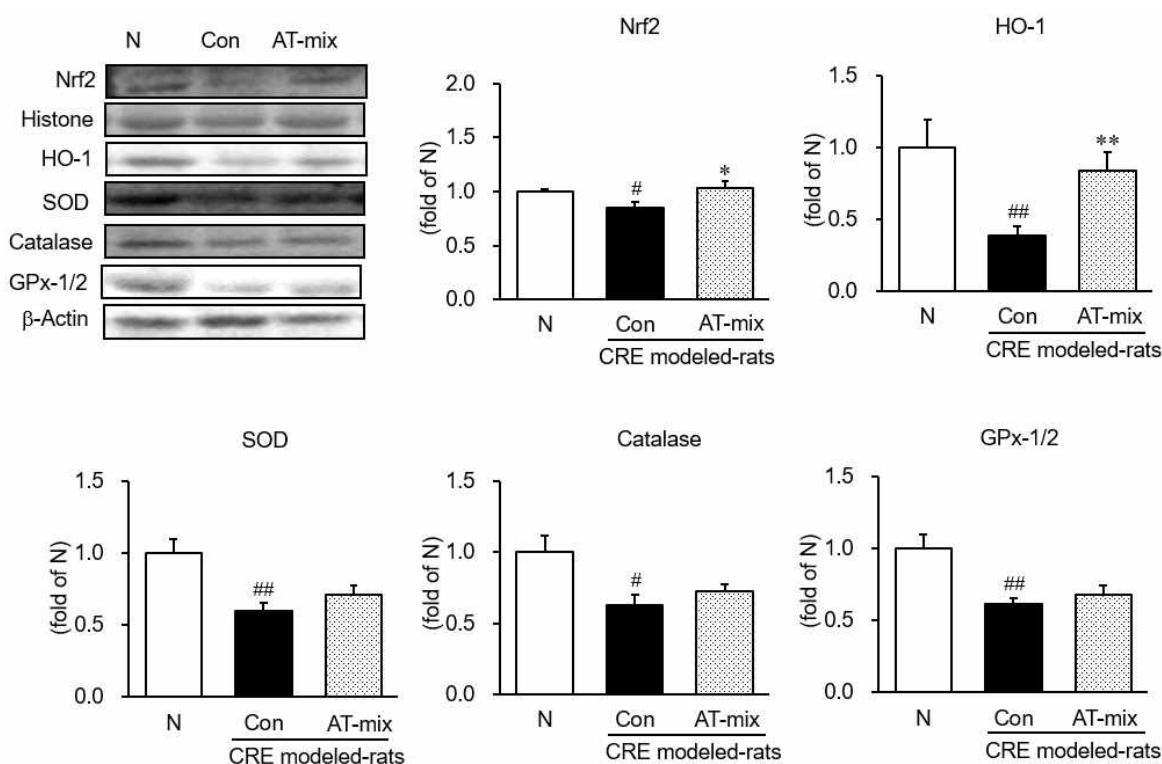

Figure 4. Esophageal Nrf2, HO-1, SOD, Catalase, and GPx-1/2 protein expressions. N, normal rats; Con, CRE control rats; AT-mix, AT-mix 200 mg/kg body weight/day-treated CRE rats. Data are mean ± SD. (n=8) Significance: $^\#P < 0.05$, $^{\#\#}P < 0.01$ vs. normal rats and $^*P < 0.05$, $^{**}P < 0.01$ vs. CRE control rats.
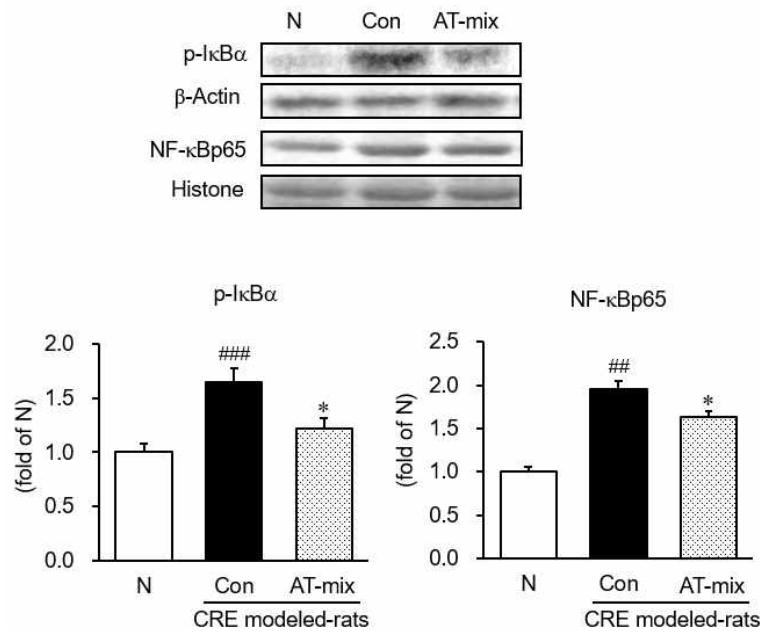
Figure 5. Esophageal p–IκBα and NF–κBp65 protein expressions. N, normal rats; Con, CRE control rats; AT–mix, AT–mix 200 mg/kg body weight/day–treated CRE rats. Data are mean ± SD. (n=8) Significance: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. normal rats and $^{*}P < 0.05$ vs. CRE control rats.

was significantly decreased in CRE control rats compared with normal rats, whereas AT–mix treatment significantly up–regulated Nrf2 expression. CRE control rats significantly showed decreased expressions of HO–1, SOD, Catalase, and GPx–1/2 in esophagus compared with normal rats; however AT–mix administration up–regulated all four antioxidant enzymes compared with CRE control rats. Especially, HO–1 showed a significant increase (Figure 4).

5. Esophageal p–IκBα and NF–κBp65 protein expressions.

The protein levels of the oxidative stress–related proteins, such as p–IκBα and NF–κBp65, were examined. As shown in Figure 5, the protein levels of p–IκBα and NF–κBp65 increased in the esophagus of CRE control rats, whereas these elevated levels significantly reduced in AT–mix treated CRE rats.

6. Esophageal COX–2, iNOS, TNF–α, IL–6, and IL–1β protein expressions.

We quantified COX–2, iNOS, TNF–α, IL–6, and IL–1β protein expressions (Figure 6). The inflammation–related protein expressions in CRE control rats were significantly augmented in the esophagus compared with normal rats. However, treatment with AT–mix suppressed these proteins in the esophagus. Herein, AT–mix supplementation significantly decreased COX–2,

iNOS, TNF–α, and IL–6. In addition, AT–mix administration reduced nearly to normal levels or below in levels of COX–2, iNOS, and IL–6. However, IL–1β didn't exhibit a significant change.

IV. Discussions

GERD is rapidly increasing worldwide and causing a considerable economic impact. Reflux esophagitis (RE), which is known as the early stage of GERD can be principally diagnosed using esophageal pH value and gastroscopy when the abnormalities of upper gastrointestinal motility or the esophageal mucosa is found[40,41]. Inflammatory mediators and other factors can break down the esophageal mucosal barrier, lower the esophageal peristalsis, and reduce the lower esophageal sphincter pressure, ultimately leading to RE[42,43]. The pathogenesis of RE results from an imbalance between a numerous natural defense mechanisms and aggressive factors damaging the esophagus. The fundermental aggressive components of gastric refluxate include acid, pepsin, pancreatic enzymes, and bile cause inflammation, destruction of the normal squamous epithelium of the esophagus, and ulceration. In recent years, a number of studies have detected that the occurrence of esophageal mucosal injury in RE patients are closely associated with oxidative stress factors[44]. Excessive reactive oxygen species (ROS) are produced by mucosal epithelial cells

Figure 6. Esophageal COX-2, iNOS, TNF-α, IL-6, and IL-1β protein expressions. N, normal rats; Con, CRE control rats; AT-mix, AT-mix 200 mg/kg body weight/day-treated CRE rats. Data are mean ± SD. (n=8) Significance: #P〈0.05, ##P〈0.01 vs. normal rats and *P〈0.05, **P〈0.01 vs. CRE control rats.
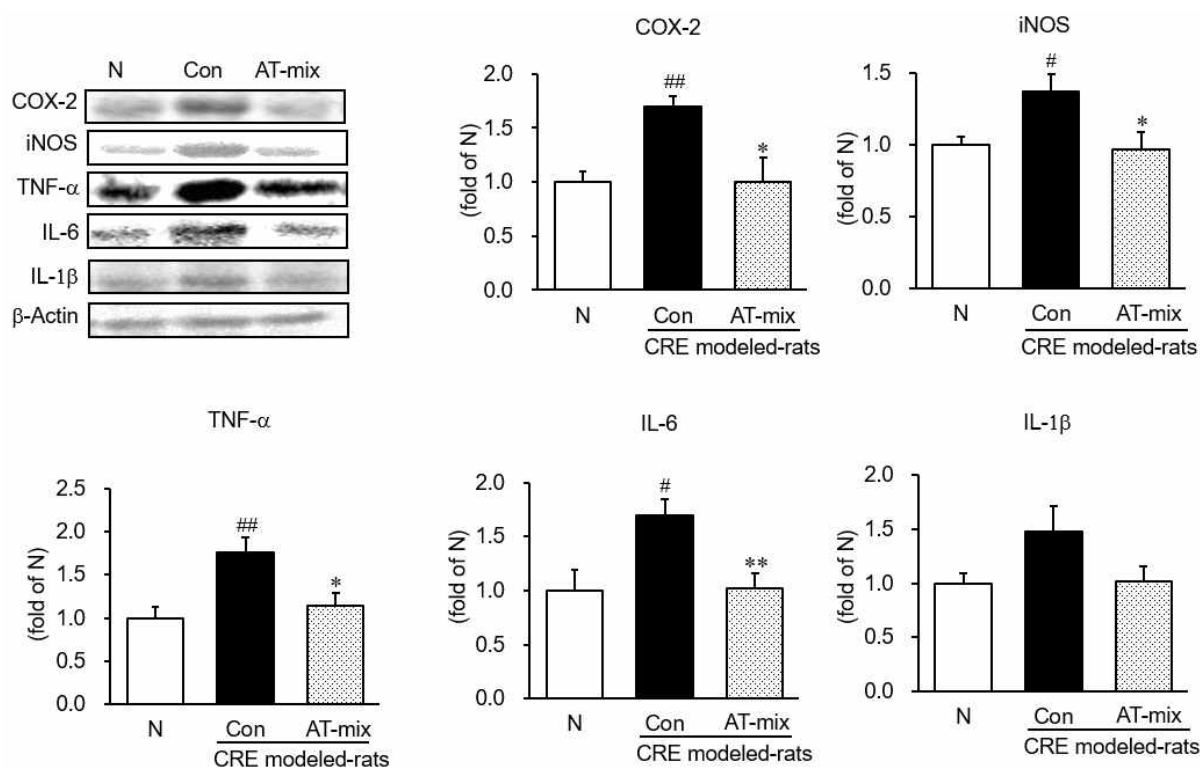
of esophagus under stress due to gastric refluxate[45,46].

Several herbals from Traditional Korea Medicine have alleviated symptoms caused by inflammation. Arecae Semen and Toosendan Fructus contain various pharmacological effects, among those efficacy anti-inflammatory and analgesic effect may exert the protective effect on GERD including reflux esophagitis. Accordingly, mixture of Arecae Semen and Toosendan Fructus may be also possessed effects like anti-oxidant, anti-inflammatory, and analgesic activities. Hence, we investigated the theraputic effect of AT-mix via inhibiting oxidative stress in a chronic model of reflux esophagitis.

Free radicals are a key factor of reflux-caused esophageal damage, since it has been shown that the treatment of the free-radical scavenger inhibited most of free radicals in rats with esophagitis[47]. The in vitro antioxidant assays, such as DPPH and ABTS showed that the supplementation of AT-mix could alleviate CRE-induced oxidative stress in the esophagus of rats (Figure 1). Moreover, the NADPH oxidase family is an important cellular source of ROS. Previous study has reported that pharmacological suppression of NADPH oxidase activity prevents tissue apoptosis[48]. These findings indicate that NADPH oxidases may be involved to apoptosis of esophageal tissue. In the current study, CRE modeled rats showed up-regulation of NADPH

oxidase like NOX4 and p22phox, otherwise these CRE-related changes were reversed by AT-mix administration. That is, the levels of NOX4 and p22phox significantly reduced in AT-mix group (Figure 3)

The Nrf2 pathway, a major cellular defense pathway, is known to regulate expression of enzymes involved in anti-oxidative stress response and detoxification and. Nrf2 in the cytoplasm under normal physiological conditions translocates into nucleus and binds to the antioxidant response elements (ARE) of target genes when cells are exposed to oxidative stress[49]. And then Nrf2 leads to the transcription of antioxidant enzyme including HO-1, SOD, Catalase, and GPx[50]. Many studies suggest that HO-1 which is the inducible isoform of the first and rate-limiting enzyme of heme degradation plays a critical role including cytoprotective and antioxidant, and anti-apoptotic activities in inflammatory diseases of the upper (esophagus and stomach) and lower (intestine) gastrointestinal tract[51]. SOD and Catalase are actually considered to be the first line of defense against free radical attack. SOD interacts with superoxide radical (O_2^-) to form H_2O_2, which is subsequently catabolised by Catalase or GPx to H_2O. In this study, the oral administration of AT-mix significantly increased compared with CRE control rats, otherwise all three of SOD, Catalase, and GPx showed an increased tendancy without a

significance. This suggests that the treatment of AT-mix may effectively scavange ROS though up-regulating the expression of HO-1 via Nrf2 activation. Inflammation promotes ROS generation, and the over-expression of ROS leads to lipid peroxidation, which may cause serious cell damage[52]. ROS can also trigger the NF-κB pathways, as well as promote the expression of inflammatory factors such as NO, TNF-α, and IL-6, thus exacerbate the inflammatory response. The NF-κB pathway is generally considered to be a key inflammatory signaling pathway, which is involved in immune and inflammatory responses in vivo, including apoptosis, cell adhesion, and cytokine production, etc.[53]. Growing evidence suggests that the NF-κB pathway mediates inflammatory processes by regulating the expression of the inflammatory response genes (COX-2 and iNOS) and various pro-inflammatory cytokines[54]. p65, a member of the NF-κB protein family, is activated under ROS stimulation, resulting in nuclear translocation and the production of various pro-inflammatory factors (TNF-α, IL-6, IL-1β, COX-2, and iNOS, etc.), thereby accelerating the inflammatory process[55]. Hence, the expression of p65 can effectively reflect the degree of NF-κB activation, and inhibiting the activation of the NF-κB pathway can exert anti-inflammatory effects[53]. In this study, AT-mix inhibited p65 nuclear translocation though inhibition the phosphorlyation of IκBα. These suggest that anti-inflammatory effects may be exerted by mediating the NF-κB pathway. The present study demonstrated that AT-mix significantly reduce the production of TNF-α and IL-6 as well as the expression levels of COX-2 and iNOS, suggesting that AT-mix may exert an anti-inflammatory effect by inhibiting pro-inflammatory cytokines and mediators. Herein, we found the interaction between Nrf2 and NF-κB. Absence of Nrf2 induces more aggressive inflammation through activation of NF-κB and downstream pro-inflammatory cytokines. On the other hand, activation of Nrf2-mediated anti-oxidative signaling attenuates NF-κB-mediated inflammatory response[56].

A recent study has shown that ROS are one of the potent factors in the pathogenesis of esophageal mucosal damage mediated by oxidative stress in an experimental model of chronic reflux esophagitis. In the present study, the administration of AT-mix reduced the oxidative stress via suppressing NADPH oxidase. These results also showed anti-oxidant effects through HO-1 increase by Nrf2 activation. Furthermore, the anti-inflammatory effect of AT-mix suggested that the inactivation of NF-κB by blocking the phosphorlyation of IκBα, led to the inhibition of the

release of pro-inflammatory mediators and cytokines. Consequently, AT-mix ameliorated esophageal mucosal ulcer in a chronic model of reflux esophagitis.

Ⅴ. Conclusions

In summary, the current study demonstrated that AT-mix may exert anti-inflammatory activity by suppressing the NF-κB inflammatory pathways and activating the Nrf2/HO-1 pathway to reduce the release of inflammatory factors such as COX2, iNOS, TNF-α, IL-6, and IL-1β. To the best of our knowledge, this is the first report of the anti-inflammatory activity of AT-mix in a chronic model of reflux esophagitis. These results can indicate that it is possible to develop AT-mix into an effective anti-inflammatory medicine.

Acknowledgements

References

1. Nakahara K, Fujiwara Y, Tsukahara T, Yamagami H, Tanigawa T, Shiba M, Tominaga K, Watanabe T, Urade Y, Arakawa T. Acid reflux directly causes sleep disturbances in rat with chronic esophagitis. PLoS One. 2014;9(9):e106969.
2. Fujiwara Y, Arakawa T. Epidemiology and clinical characteristics of GERD in the Japanese population. J Gastroenterol. 2009;44:518-34.
3. Savarino E, Marabotto E, Bodini G, Pellegatta G, Coppo C, Giambruno E, Brunacci M, Zentilin P, Savarino V. Epidemiology and natural history of gastroesophageal reflux disease. Minerva Gastroenterol Dietol. 2017;63(3):175-83.
4. Kim O, Jang HJ, Kim S, Lee HY, Cho E, Lee JE, Jung H, Kim J. Gastroesophageal reflux disease and its related factors among women of reproductive age: Korea Nurses' Health Study. BMC Public Health. 2018;18(1):1133.
5. Fock KM1, Talley NJ, Fass R, Goh KL, Katelaris P, Hunt R, Hongo M, Ang TL, Holtmann G, Nandurkar S, Lin SR, Wong BC, Chan FK, Rani AA, Bak YT, Sollano J, Ho KY, Manatsathit S. Asia-Pacific consensus on the management of

gastroesophageal reflux disease: update. J Gastr-oenterol Hepatol. 2008;23(1):8-22.

6. Kim KM1, Cho YK, Bae SJ, Kim DS, Shim KN, Kim JH, Jung SW, Kim N. Prevalence of gastroe-sophageal reflux disease in Korea and associated health-care utilization: a national population-based study. J Gastroenterol Hepatol. 2012;27(4):741-5.

7. Park S, Kwon JW, Park JM, Park S, Seo KW. Treatment pattern and economic burden of refractory GERD patients in South Korea. J Neurogastroenterol Motil. 2019 Nov 5. Available from: DOI: 10.5056/jnm19050.

8. Scarpignato C, Gatta L2,3, Zullo A4, Blandizzi C5; SIF-AIGO-FIMMG Group; Italian Society of Pharmacology, the Italian Association of Hospital Gastroenterologists, and the Italian Federation of General Practitioners. Effective and safe proton pump inhibitor therapy in acid-related diseases - A position paper addressing benefits and potential harms of acid suppression. BMC Med. 2016;14(1):179.

9. U.S. Food and Drug Administration. Proton pump inhibitors: US Food and Drug Administration-approved indications and dosages for use in adults [Internet] Silver Spring: U.S. Food and Drug Administration; 2014. [cited 2019 Dec 07]. Available from: http://www.fda.gov/drugs.

10. Strand DS, Kim D, Peura DA. 25 Years of Proton Pump Inhibitors: A Comprehensive Review. Gut Liver. 2017;11(1):27-37.

11. U.S. Food and Drug Administration. FDA drug safety communication: low magnesium levels can be associated with long-term use of proton pump inhibitor drugs (PPIs) [Internet] Silver Spring: U.S. Food and Drug Administration; 2011. [cited 2019 Dec 07]. Available from: http://www.fda.gov/Drugs/DrugSafety/ucm245011.htm.

12. Chey WD, Mody RR, Izat E. Patient and physician satisfaction with proton pump inhibitors (PPIs): are there opportunities for improvement? Dig Dis Sci. 2010;55:3415-22.

13. Chey WD, Mody RR, Wu EQ, Chen L, Kothari S, Persson B, Beaulieu N, Lu M. Treatment patterns and symptom control in patients with GERD: US community-based survey. Curr Med Res Opin. 2009;25:1869-78.

14. Yunkai Dai, Yunzhan Zhang, Danyan Li, Jintong Ye, Weijing Chen, and Ling Hu. Efficacy and Safety of Modified Banxia Xiexin Decoction (Pinellia Decoction for Draining the Heart) for Gastroesophageal Reflux Disease in Adults: A Systematic Review and Meta-Analysis. Evid Based Complement Alternat Med. 2017;2017:9591319.

15. Kazunari Tominaga, Tetsuo Arakawa. Kampo medicines for gastrointestinal tract disorders: a review of basic science and clinical evidence and their future application. J Gastroenterol. 2013;48(4):452-62.

16. Nan L, Nam HH, Choo BK, Park JC, Kim DG, Lee JH, Moon KH. An Ethanolic Extract of Allium hookeri Root Alleviates Reflux Esophagitis and Modulates NF-κB Signaling. Evid Based Complement Alternat Med. 2018;2018:1834681.

17. Zhou Q, Wu X, Liu Y, Wang X, Ling X, Ge H, Zhang J. Curcumin improves asthenozoospermia by inhibiting reactive oxygen species reproduction through nuclear factor erythroid 2-related factor 2 activation. Andrologia. 2019:e13491.

18. Shin MR, Seo BI, Son CG, Roh SS, An HJ. Banhasasim-Tang Treatment Reduces the Severity of Esophageal Mucosal Ulcer on Chronic Acid Reflux Esophagitis in Rats. Biomed Res Int. 2017;2017:7157212.

19. Kwon O, Choo BK, Lee JY, Kim MY, Shin SH, Seo BI, Seo YB, Rhee MH, Shin MR, Kim GN, Park CH, Roh SS. Protective effect of Rhei Rhizoma on reflux esophagitis in rats via Nrf2-mediated inhibition of NF-κB signaling pathway. BMC Complement Altern Med. 2016;16:7.

20. Cheng Q, Kalabus JL, Zhang J, Blanco JG. A conserved antioxidant response element (ARE) in the promoter of human carbonyl reductase 3 (CBR3) mediates induction by the master redox switch Nrf2. Biochem Pharmacol. 2012;83(1):139-48.

21. Peng D, Lu H, Zhu S, Zhou Z, Hu T, Chen Z, Zaika A, El-Rifai W. NRF2 antioxidant response protects against acidic bile salts-induced oxidative stress and DNA damage in esophageal cells. Cancer Lett. 2019;458:46-55.

22. Wang Y, Sun W, Du B, Miao X, Bai Y, Xin Y, Tan Y, Cui W, Liu B, Cui T, Epstein PN, Fu Y, Cai L. Therapeutic effect of MG-132 on diabetic cardio-myopathy is associated with its suppression of proteasomal activities: roles of Nrf2 and NF-κB. Am J Physiol Heart Circ Physiol. 2013;304(4):H567-H578.

23. Buelna-Chontal M, Zazueta C. Redox activation of Nrf2 & NF-κB: a double end sword? Cell Signal. 2013;25(12):2548-57

24. Negi G, Kumar A, Sharma SS. Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-κB and Nrf2 cascades. J Pineal Res. 2011;50(2):

124-31.

25. Zhang SY, Sun GX, Feng B, and Wu CJ. Study on the effect of different processed betel nut on gastrointestinal function. Zhongguo Zhong Yao Za Zhi. 2010;31:50-2.

26. Zhang S, Yang P, Li X, Wang X, Song J, Peng W, Wu C. Comparative Researches of Semen Arecae and Charred Semen Arecae on Gastrointestinal Motility, Motilin, Substance P, and CCK in Chronically Stressed Rats. Evid Based Complement Alternat Med. 2017;2017:1273561.

27. Peng W, Liu YJ, Wu N, Sun T, He XY, Gao YX, Wu CJ. Areca catechu L. (Arecaceae): a review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. J Ethnopharmacol. 2015;164:340-56.

28. Wu IC, Lu CY, Kuo FC, Tsai SM, Lee KW, Kuo WR, Cheng YJ, Kao EL, Yang MS, Ko YC. Interaction between cigarette, alcohol and betel nut use on esophageal cancer risk in Taiwan. Eur J Clin Invest. 2006;36(4):236-41.

29. Yi HS, Heo SK, Yun HJ, Kim BW, Park SD. Anti-oxidant and anti-inflammatory effect of Melia toosendan in mouse macrophage cells. Kor J Herbol. 2008;23(4):121-34.

30. 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.

31. Pharmacopoeia Commission of People's Republic of China. China Pharmacopoeia Chemical Industry Press: Beijing. 2005;29.

32. ZB Li, Kong DJ, Dong Y, Zi B, He XL. Study on treat coccidiosis efficacy of Toosendan Anim. Husb. Vet. Med. 2010;29:9-13.

33. Xu L, Yu JQ, Wang XY, Xu N, Liu JL. Microwave extraction optimization using the response surface methodology of Fructus Meliae Toosendan polysaccharides and its antioxidant activity. Int J Biol Macromol. 2018 ; 118(Pt B):1501-10.

34. Zhang Q, Shi Y, Liu XT, Liang JY, Ip NY, Min ZD. Minor limonoids from Melia toosendan and their antibacterial activity. Planta Med. 2007;73: 1298-303.

35. Xiang XX, Tang DX, Xiong JY, Liang YJ, Mu DH, Yang XW, Hang M, Tan ZH. Effect of meliae toosendan fructus on nerves system and its mechanism. Zhong Yao Cai. 2013;36(5):767-71.

36. Hatano T, Edamatsu R, Hiramatsu M, Mori A, Fujita Y, Yasuhara T, Yoshida T, Okuda T. Effects of the interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. Chem Pharm Bulletin. 1989;37(8):2016-21.

37. Re R, Pellegrini N, Proteggente A. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine. 1999;26(9-10):1231-7.

38. Omura N, Kashiwagi H, Chen G, Suzuki Y, Yano F, Aoki T. Establishment of surgically induced chronic acid reflux esophagitis in ratsScand J Gastroenterol. 1999;34(10):948-53.

39. Komatsu S. Extraction of nuclear proteins. Methods in Mole Biol. 2007;355:73-7.

40. Tsai J, Blinman TA, Collins JL, Laje P, Hedrick HL, Adzick NS, Flake AW. The contribution of hiatal hernia to severe gastroesophageal reflux disease in patients with gastroschisis. J Pediatr Surg. 2014;49:395-8.

41. Deng Y, Pan L, Qian W. Associations between the severity of reflux esophagitis in children and changes in oxidative stress, serum inflammation, vasoactive intestinal peptide and motilin. Exp Ther Med. 2019;18(5):3509-13.

42. 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.

43. 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.

44. Pawlik MW, Kwiecien S, Pajdo R, Ptak-Belowska A, Brzozowski B, Krzysiek-Maczka G, Strzalka M, Konturek SJ, Brzozowski T. Esophagoprotective activity of angiotensin-(1-7) in experimental model of acute reflux esophagitis. Evidence for the role of nitric oxide, sensory nerves, hypoxia-inducible factor-1alpha and proinflammatory cytokines. J Physiol Pharmacol. 2014;65(6):809-22.

45. Giri AK, Rawat JK, Singh M, Gautam S, Kaithwas G. Effect of lycopene against gastroesophageal reflux disease in experimental animals. BMC Complement Altern Med. 2015;15:110.

46. Deng Y, Pan L, Qian W. Associations between the severity of reflux esophagitis in children and changes in oxidative stress, serum inflammation, vasoactive intestinal peptide and motilin. Exp Ther Med. 2019;18(5):3509-13.

47. Wetscher GJ, Hinder RA, Bagchi D, Hinder PR, Bagchi M, Perdikis G, McGinn T. Reflux esophagitis in humans is mediated by oxygen-derived free

radicals. Am J Surg. 1995;170(6):552-7.

48. Shin MR, An HJ, Seo BI, Roh SS. Anti-apoptotic effect of banhasasim-tang on chronic acid reflux esophagitis. World J Gastroenterol. 2017;23(25):4644-53.

49. Sagai M, Bocci V. Mechanisms of Action Involved in Ozone Therapy: Is healing induced via a mild oxidative stress?. Med Gas Res. 2011;1:29.

50. Giri AK, Rawat JK, Singh M, Gautam S, Kaithwas G. Effect of lycopene against gastroesophageal reflux disease in experimental animals. BMC Complement Altern Med. 2015;15:110.

51. Chang M, Xue J, Sharma V, Habtezion and A. Protective role of hemeoxygenase-1 in gastrointestinal diseases. Cellular and molecular life sciences. 2015;72(6):1161-73.

52. Ihsan AU, Khan FU, Khongorzul P, Ahmad KA, Naveed M, Yasmeen S, Cao Y, Taleb A, Maiti R, Akhter F, Liao X, Li X, Cheng Y, Khan HU, Alam K, Zhou X. Role of oxidative stress in pathology of chronic prostatitis/chronic pelvic pain syndrome and male infertility and antioxidants function in ameliorating oxidative stress. Biomed Pharmacother. 2018;106:714-23. Ihsan AU, Khan FU, Khongorzul P, Ahmad KA, Naveed M, Yasmeen S, Cao Y, Taleb A, Maiti R, Akhter F, Liao X, Li X, Cheng Y, Khan HU, Alam K, Zhou X. Role of oxidative stress in pathology of chronic prostatitis/chronic pelvic pain syndrome and male infertility and antioxidants function in ameliorating oxidative stress. Biomed Pharmacother. 2018;106:714-23.

53. Yoon WJ, Moon JY, Song G, Lee YK, Han MS, Lee JS, Ihm BS, Lee WJ, Lee NH, Hyun CG. Artemisia fukudo essential oil attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW264.7 macrophages. Food Chem Toxicol. 2010;48(5):1222-9. Yoon WJ, Moon JY, Song G, Lee YK, Han MS, Lee JS, Ihm BS, Lee WJ, Lee NH, Hyun CG. Artemisia fukudo essential oil attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages. Food Chem Toxicol. 2010;48(5):1222-9.

54. Yodkeeree S, Ooppachai C, Pompimon W, Limtrakul P. O-Methylbulbocapnine and Dicentrinesuppress LPS-induced inflammatory response by blocking NF-κB and AP-1 activation through inhibiting MAPKs and Aktsignaling in RAW264.7 macrophages. Biol Pharm Bull. 2018;41(8):1219-27.

55. Li Y, Liu H, Xu QS, Du YG, Xu J. Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-κB and endothelial inflammatory response.

Carbohydr Polym. 2014;99:568-78.

56. Chen H, Fang Y, Li W, Orlando RC, Shaheen N, Chen XL. NFκ B and Nrf2 in esophageal epithelial barrier function. Tissue Barriers. 2013;1(5):e27463.