





SR-5, the specific ratio of Korean multi-herbal formula: An evaluation of antiulcerogenic effects on experimentally induced gastric ulcers in mice

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Abstract

Purpose: Previously, we demonstrated that the specific ratio of Korean multi-herbal formula (SR-5) exhibits hepatoprotective properties against ethanol-induced hepatic damage in rats. Chronic and excessive alcohol consumption is a major etiological factor involved in gastric disease and ulcer development induced by the inflammatory response and oxidative stress.

Methods: The present study evaluated the gastroprotective effects of SR-5 (100, 150, and 200 mg/kg) against hydrochloride acid/ethanol (HCl/EtOH)-induced and indomethacin/hydrochloride acid (INDO/HCl)-induced gastritis in a mouse model and the mechanisms involved.

Results: All the tested doses of SR-5 significantly inhibited gastric lesions in the HCl/EtOH-induced ulcer model mice. Similarly, all the tested doses of SR-5 significantly inhibited gastric lesions in the INDO/HCl-induced ulcer model mice. Furthermore, mice pretreated with SR-5 had significantly increased gastric levels of enzymatic and nonenzymatic antioxidants, namely, catalase (CAT) and glutathione (GSH), with concomitant reductions in malondialdehyde (MDA) and reactive oxygen species (ROS) levels compared with those in the HCl/EtOH or INDO/HCl group. SR-5 suppressed the expression of nuclear factor-kappa B (NF- κ B)/p65, inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), and cyclooxygenase-2 (COX-2) to their normal values.

Conclusion: These findings are the first to demonstrate the powerful protective effect of SR-5 against gastric injury development and provide hope for clinical application.

Keywords

Bioactivity, gastric ulcer, antioxidant, anti-inflammation, indomethacin, animal model

Introduction

Gastric ulcers are one of the most common problems affecting approximately 5% of the population. There are several causes of gastric ulcers. The main cause is the imbalance between aggressive and intrinsic defense factors.¹ The principal etiological factors associated with gastric ulcers are alcohol, nonsteroidal anti-inflammatory drug (NSAID) abuse, stress, smoking, and *Helicobacter pylori* infection.²⁻⁵ Among these risk factors, alcohol consumption can directly interfere with gastric motility and metabolism. This action leads to mucosal

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damage and ulceration of the stomach.⁶ The second most common aggressive factor is NSAIDs, which account for 25% of peptic ulcer cases.³ Indomethacin (INDO) is commonly used to induce peptic ulcers experimentally.⁷ INDO has a higher potential to induce gastric damage than other commonly used NSAIDs.^{8,9}

Although effective anti-ulcer drugs are available, most of these drugs produce many adverse effects and toxicities; thus, there is a need to focus attention on the search for new alternatives.¹⁰ Finding a safe and effective treatment strategy for peptic ulcers is critical. Accordingly, the introduction of a safe drug of natural origin to be used for prophylaxis or management of gastric ulcers without side effects is the main goal. Since ancient times, nature has provided several herbal phytochemicals that benefit humans. Most recent studies on the treatment of gastrointestinal disorders have focused on the potential role of natural medicine due to its availability, better protection capacities, lower cost, and lower toxicity.¹¹⁻¹³

Traditional Korean medicine has been used for several centuries in Korea and is a rich source of therapeutics. For example, many herbal medicines are used for the treatment of digestive system diseases. Potent anti-ulcer agents used in traditional Korean medicine approaches that have strong antioxidant effects, such as *Artemisia asiatica* extract (Stillen™, Dong-A Pharmaceuticals, Korea), are also commercially available.¹⁴

Based on ethnopharmacological information, our previous hepatoprotective experiments,¹⁵ and the reported antioxidant and anti-inflammatory effects and safety data, we selected 12 Korean medicinal herbs: soybean sprouts,^{16,17} *Oenanthe javanica* (Blume) DC.,¹⁸ immature *Citrus reticulata* Blanco peel,¹⁹ *Hovenia dulcis* Thunb.,^{20,21} immature *Rubus coreanus* Miq.,^{22,23} *Artemisia capillaris* Thunb.,²⁴ *Chaenomeles sinensis* (Dum. Cours.) Koehne,²⁵⁻²⁷ *Peucedanum japonicum* Thunb.,^{28,29} *Chrysanthemum indicum* L.,³⁰ *Ziziphus jujuba* var. *inermis* (Bunge) Rehder,^{31,32} *Pueraria montana* var. *chinensis* (Ohwi),³³ and *Glycyrrhiza uralensis* Fisch.³⁴ for gastroprotective evaluation. The 12 selected Korean medicinal herbs are a traditional medicinal food in Korea based on Donguibogam, a Korean traditional medical encyclopedia written by Jun Heo.³⁵ The effects of germination on the antioxidant activities of soybean sprouts have been reported in diverse conditions¹⁶ and different areas.¹⁷ Phytochemical studies have revealed that *Z. jujuba* var. *inermis* contains various constituents, including triterpenoid acids, flavonoids, cerebrosides, amino acids, phenolic acids, polysaccharides, and lignans.³¹ Extracts and triterpenoids from *Z. jujuba* var. *inermis* have anti-inflammatory activities through effects on NO production, and anticancer activities.^{31,32} *Chaenomeles sinensis* (Dum. Cours.) Koehne, a member of the Rosaceae family, is used as an herb in Korea for the treatment of throat diseases, anaphylaxis, viral infection, and neurodegenerative disease.²⁵ *Chaenomeles sinensis* (Dum. Cours.) Koehne has also been reported to have antipruritic and anti-inflammatory activities and to inhibit oxidative damage resulting from free

radicals.²⁵⁻²⁷ A previous study also showed that *Hovenia dulcis* Thunb. extract had anti-inflammatory activity by suppressing the activation of the MAPK, AP-1, JAK2/STAT, and NF-κB signaling pathways.^{20,21}

The use of herbal medicine, as one element of complementary and alternative medicine, is increasing worldwide and it has been shown that the selected 12 Korean medicinal herbs have antioxidant and anti-inflammatory activities. Furthermore, a specific Korean multi-herbal formula (SR-5) has been used as an anti-hangover medicine in traditional Korean medicine and has demonstrated good clinical effects. Alcohol consumption has a stimulatory effect on the gastric mucosa via the cholinergic system. Alcohol stimulates parietal cells and increases the levels of cAMP and histamine release, leading to increased gastric and mucosal secretions.³⁷ Administration of alcohol is thought to cause direct damage to the gastric mucosa, leading to gastric lesions, edema, inflammation, hemorrhage, and congestion of blood vessels.^{1,2,38,39} Inhibition of mucosal prostaglandin synthesis by NSAIDs such as indomethacin is thought to be a major mechanism of gastrointestinal mucosal injury caused by NSAIDs.^{3,40} Inflammation and oxidative stress underlie the majority of peptic ulcer diseases, which contribute to the global burden of disease.⁴¹ Although many gastroprotective drugs have been used to prevent and treat gastric ulcer disease, side effects such as cardiovascular disease, bone fractures, hypomagnesemia, bacterial gut infections, chronic kidney complication disease, and risk of gastric cancer development have also been reported.⁴²⁻⁴⁴ Thus, finding a safe and effective gastroprotectant for peptic ulcer prevention is imperative. However, the gastroprotective activity of each of the 12 Korean medicinal herbs and SR-5 has not yet been studied. Hence, the aim of the present study was to evaluate the gastroprotective activity of SR-5 in a hydrochloride acid/ethanol (HCl/EtOH)-induced gastritis and indomethacin/hydrochloride acid (INDO/HCl)-induced gastric ulcer model in mice. Moreover, we examined the underlying molecular mechanisms of SR-5.

Materials and methods

Reagents

Aluminum phosphate colloidal gel was purchased as Gelfos M® from Boryung Pharmaceutical Co., Ltd., Seoul, Republic of Korea. DA-5204 (Stillen™ 2X) was obtained from Dong-A Pharmaceuticals Co., Ltd., Yongin, Republic of Korea. All other chemicals were of analytical reagent grade.

Preparation of the herbal formula (SR-5)

The major ingredients in the herbal formula were obtained from 12 Korean medicinal herbs (Table 1). This new herbal formula originated from Korean traditional medicine as a Danbang prescription (single-herb formulae)³⁵ and has been shown to be effective due to its hepatoprotective and

Table 1. Components of SR-5.

No	Genus Species	Common Names	Plant Part	Content (%)
1	<i>Glycine max</i> (L.) Merr	Soybean	Bean sprouts	20
2	<i>Oenanthe javanica</i> (Blume) DC.	Water Celery	Leaves	15
3	<i>Citrus reticulata</i> Blanco	Mandarin	Immature fruit peel	13
4	<i>Hovenia dulcis</i> Thunb	Japanese raisin tree	Fruits	9
5	<i>Rubus coreanus</i> Miq	Korean Bramble	Immature fruits	9
6	<i>Artemisia capillaris</i> Thunb	Yin Chen Hao	Leaves	8
7	<i>Chaenomeles sinensis</i> (Dum. Cours.) Koehne	Chinese quince	Fruits	5
8	<i>Peucedanum japonicum</i> Thunb	-	Leaves	5
9	<i>Chrysanthemum indicum</i> L	Ye ju	Flower	5
10	<i>Ziziphus jujuba</i> var. <i>Inermis</i> (Bunge) Rehder	Daechu	Fruits	4
11	<i>Pueraria montana</i> var. <i>Chinensis</i> (Ohwi)	Kudzu	Root	3.5
12	<i>Glycyrrhiza uralensis</i> Fisch	gan cao	Root	3.5
Total content (%)				100

antioxidant functions.¹⁵ The extraction of SR-5 and the determination of the yield of the extraction were conducted according to our previous report.¹⁵ Briefly, 12 dried Korean medicinal herbs were purchased at the Kyung-Dong oriental herbal market (Seoul, Republic of Korea) in December 2020 and authenticated by professor C-S Na at the College of Korean Medicine, Dongshin University, South Korea. The specific ratio of Korean multi-herbal formula was extracted using 20 volumes of distilled water at 100°C for 6 h as described in Table 1. The extracted solution was then filtered, concentrated with an evaporator under vacuum, and freeze-dried. The dry matter content of the lyophilized samples was determined by drying at 105°C to a constant mass. 26.4 g of dried powder was obtained from 100 g of the 12 Korean medicinal herbs. All doses were expressed in terms of dried residues obtained and prepared immediately before use by dissolving in sterile normal saline solution.

Animals

Specific pathogen-free (SPF)-grade healthy male six-week-old ICR mice weighing 25 ± 5 g each were purchased from Samtako Co. (Osan, Republic of Korea). Animals were maintained in a constant room temperature of $22 \pm 2^\circ\text{C}$ with a humidity level of $50 \pm 5\%$ and with free access to water and food under a 12:12 h light:dark cycle (lights on at 8:00 a.m.). The animals were acclimatized for 1 week before the beginning of the experiments. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiment was conducted according to the International Guidelines for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of the Bioresources and Technology (B&Tech, Gwangju, Korea) Co., Ltd., Republic of Korea (approval number: BT-002-2020). When the experiment began, all mice were anesthetized with isoflurane and were subsequently sacrificed by cervical dislocation in accordance with the IACUC guidelines.

Acute oral toxicity

An acute toxicity study was carried out in accordance with the Organization for Economic Cooperation and Development (OECD) guideline No. 423. Six-week-old male and female ICR mice weighing 25 ± 5 g were selected and kept in a constant room temperature of $22 \pm 2^\circ\text{C}$ with a humidity level of $50 \pm 5\%$ and with free access to water and food under a 12:12 h light:dark cycle (lights on at 8:00 a.m.). Animals were divided into four groups of three animals, including a control group (male and female mice) and groups treated with 2000 mg/kg SR-5 (male and female). Before treatment with SR-5, the mice were fasted for 12 h with free access to water. After a single dose of 2000 mg/kg SR-5, mice were maintained under observation, continuously during the first 30 min and with special attention given during the first 4 h for the next 24 h and then daily thereafter for 14 days. Changes in weight and wellness parameters were compared with those of the control animals.

HCl/ethanol-induced gastric ulcer model and dosing

Induction of the HCl/EtOH-induced gastric ulcer model was performed according to a previously reported method,³⁶ with some modifications. The mice were randomly divided into six groups ($n = 7/\text{group}$). SR-5 (100, 150, and 200 mg/kg) at .5 mL/mouse, dissolved in physiological saline solution, was orally administered using a stainless oral sonde (Jungdo-BNP, Seoul, South Korea), and the control mice received the same volume of saline solution. The dosing was performed as follows: the normal control group (CTL) received .5 mL of distilled water, the ulcer model control group received a mixture of 150 mM HCl and 60% ethanol solution orally (.5 mL/mouse), the positive control group (PCTL) received aluminum phosphate gel (1.5 g/kg), the low-dose SR-5 group received extract of SR-5 (100 mg/kg), the middle dose SR-5 group received extract of SR-5 (150 mg/kg), and the high dose SR-5 group received extract of SR-5 (200 mg/kg). The SR-5-

treated groups were intragastrically administered pretreatment for 3 days. On day 3, all mice were fasted for 12 h before oral dosing. After 1 h, the control and orally treated groups received a mixture of 150 mM HCl and 60% ethanol solution orally (.5 mL/mouse) for ulcer induction, as shown in Figure 1. After 2 h, the animals were anesthetized with isoflurane and sacrificed. The entire stomach was removed immediately and examined for gross mucosal injury. The stomach was subsequently stored at -80°C .

Indomethacin/HCl-induced gastric ulcer model and dosing

Induction of the INDO/HCl-induced gastric ulcer model was performed according to a previously reported method,⁷ with some modifications. After adaptation for 1 week, 6-week-old male ICR mice were divided randomly into the following six groups of equal numbers ($n = 7$), avoiding intergroup differences in body weight: the normal control group (CTL), the ulcer model control group, positive control group (PCTL), and the SR5 100, 150, and 200 mg/kg treated groups. The mice were restricted from food for 24 h and from water for 12 h prior to induction of gastric ulcers. The normal control group and ulcer model control groups were given distilled water, and the positive control group was given 36 mg/kg DA-5204 (Stillen 2X) by oral administration. The SR-5 groups were pretreated for 3 days with 100, 150, and 200 mg/kg SR-5 extract by oral administration. Every 2 h beginning 1 h after the pretreatment, 30 mg/mL indomethacin (200 μL /mouse) was orally administered four times for ulcer induction, as shown in Figure 1. Two hours after the final indomethacin administration, all groups were given 200 μL of 200 mM HCl. Animals were sacrificed by anesthetization with isoflurane 30 min after treatment with HCl. The entire stomach was removed immediately and examined for gross mucosal injury. The stomach was subsequently stored at -80°C .

Measurement of gross mucosal damage area and the ulcer index

The inner mucous was washed away with cold phosphate buffered saline (PBS), and the remaining tissue was laid out on paper. Thereafter, the stomach was photographed with a CMOS camera (Sony, Tokyo, Japan). Images captured were imported into ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) for measurement and determination of ulcer parameters. The total ulcer area was determined by first calibrating ImageJ software with a known distance in millimeters (mm) using an e-ruler. Thereafter, the stomach mucosa images of each animal in each group were imported into ImageJ, and with the help of free-hand tools, the ulcerated areas were mapped, the data were generated, and the total ulcer area for each animal was calculated.

The index of gastric lesions, with scores from 0 to 3, was divided into six lesions. The scoring is shown in Table 2. The ulcer index for each animal was expressed as the mean ulcer score.⁴⁵

The gross mucosal ulcer inhibition ratio was calculated as follows:

The gross mucosal ulcer inhibition ratio (%) = $\{[\text{CTL lesion index (mm}^2\text{)} - \text{Sample lesion index (mm}^2\text{)}] / \text{CTL lesion index (mm}^2\text{)}\} \times 100$.

ROS and MDA levels measurements

The specific weight of the tissue was homogenized with 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA cooled with ice, and the supernatant was collected to measure ROS and MDA levels. The reactive oxygen species (ROS) level of the stomach was measured using the method reported by Ali et al.⁴⁶ The cell-permeant reagent 2',7'-dichlorofluorescein diacetate (DCFH-DA) was employed to assess the ROS levels. Fluorescence was detected using excitation at

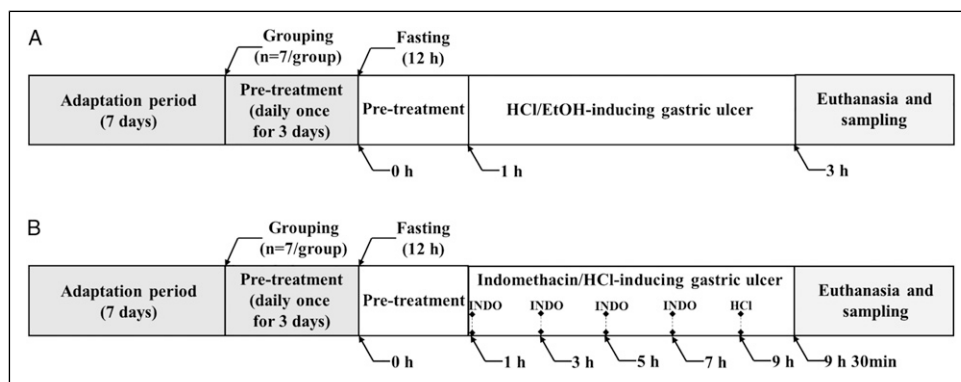


Figure 1. Time course of experimental schedule. The gastric ulcer was induced using HCl/EtOH (A). The animals received SR-5 (100, 150, and 200 mg/kg) or aluminum phosphate (1.5 g/kg) for 3 days by oral administration. Then, absolute HCl/ethanol (.5 mL/mouse) was orally administered to all animals (except for the control group). Mice were sacrificed 2 h later. Gastric ulcers were induced using indomethacin and HCl (INDO/HCl) (B). The animals received SR-5 (100, 150, and 200 mg/kg) or DA-5204 (36 mg/kg) for 3 days by oral administration. Then, indomethacin (30 mg/kg) was orally administered to all animals (except for the control group). Mice were sacrificed 8 h later.

Table 2. Scoring of Gastric Lesions.

Ulcer Score	Gastric Lesions
0	Normal stomach
0.5	Red coloration
1	Spot ulcers
1.5	Hemorrhagic streaks
2	Deep ulcers (ulcer >3 mm but <5 mm)
3	Perforation (ulcers >5 mm)

485 nm and emission at 535 nm. Lipid peroxidation was estimated by the determination of malondialdehyde (MDA) levels using a thiobarbituric acid-reactive substances (TBARS) assay kit (DoGen Bio Co, Seoul, Korea) according to the manufacturer's instructions. The results are expressed as mM MDA per mg of protein.

Determination of oxidative status

Catalase (CAT) activity in stomach tissue was determined using a commercially available Catalase Activity Assay Kit (BioVision, No. K773-100, Mountain View, CA94043, USA). In this assay, catalase first reacts with H₂O₂ to produce water and oxygen. The unconverted H₂O₂ reacts with the OxiRed™ probe to produce a product, which can be measured by a colorimetric method. Briefly, stomach tissues were homogenized in cold assay buffer and centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatants were collected for the assay. The assay was performed in 96-well microplates. The rate of decomposition of H₂O₂ was measured spectrophotometrically at 570 nm using an absorbance microplate reader. The CAT activity is expressed as μmol/mg protein.

Glutathione (GSH) activity in stomach tissue was determined using a Glutathione Colorimetric Assay Kit (BioVision, No. K261-100, Mountain View, CA94043, USA). Stomach tissues were homogenized in 5% sulfosalicylic acid. The lysate was then centrifuged at 8000 × *g* for 10 min. Supernatants were assayed for GSH by mixing with glutathione reductase and GSH reaction buffer. The absorbance was determined at 415 nm using a microplate reader, and the results are expressed as μmol/mg protein.

Protein extraction and immunoblot assays

Stomach samples were washed three times with cold PBS before being lysed in radioimmunoprecipitation (RIPA) lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 1% NP-40; .1% sodium deoxycholate, .2% SDS, 150 mmol/L NaCl, and 1 mmol/L EDTA) supplemented with 1× protease inhibitor cocktail (Thermo, Fremont, CA, USA) on ice. The separated proteins were transferred onto a nitrocellulose membrane. Anti-NF-κB/p65 (1:1000, #8242) and anti-COX-2 (1:1000, #12282) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The monoclonal anti-iNOS antibody

(1:1000, ab136918), anti-TNF-α antibody (1:500, ab6671), and the secondary antibodies (1:10,000) were obtained from Abcam (Cambridge, MA, USA). Immunoreactive protein bands were visualized using a ChemiDoc XRS+ System (BioRad) and quantified with Gel Pro Analyzer software (Silk Scientific, Inc., Orem, UT, USA). The internal control, β-actin (1:2000, #sc-47778, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), was used to normalize differences due to loading variations.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Data were statistically evaluated using Student's *t*-test or one-way analysis of variance (ANOVA) with GraphPad Prism 5 version 5.01 for Windows (GraphPad, Inc., San Diego, CA, USA) software programs. Statistical significance was indicated when *p* < .05.

Results

Acute oral toxicity

In our previous study, we evaluated the cytotoxicity of 12 herbal plant extracts or mixed extracts on liver cells using the MTT assay and found no cytotoxicity.¹⁵ Furthermore, we also reported an *in vivo* safety level of 150 mg/kg for SR-5 in Sprague-Dawley (SD) rats.¹⁵ Importantly, no liver or kidney toxicities were observed with chronic intake of SR-5. In the present acute toxicity study, no mortalities were recorded in animals treated with a single dose of 2000 mg/kg SR-5. There were no adverse clinical symptoms in the eyes or mucus membranes, skin and fur, respiratory rate, and there were no autonomic effects, circulatory signs, or central nervous system effects at an SR-5 dose of 2000 mg/kg. Therefore, the approximate lethal dose in the experimental mouse is higher than 2000 mg/kg. According to the Organization for Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, an LD₅₀ dose of >300–2000 mg/kg is categorized as category 4; hence, SR-5 can be considered safe. Thus, 100–200 mg/kg SR-5 was applied in this study in the *in vivo* experiments. In addition, we verified the optimal safety and efficacy concentration at 150 mg/kg in the previous study,¹⁵ and in this study, a concentration range of 100–200 mg/kg was established and studied.

Gastroprotective effect of SR-5 on HCl/ethanol-induced gastric lesions

As shown in Figure 2A, mice in the acute acidified ethanol-induced gastric mucosal damage group presented with hemorrhage and hyperemia, which was not present in the normal control group mice. Animals pretreated with SR-5 (100, 150, and 200 mg/kg) or aluminum phosphate (1.5 g/kg) showed considerably fewer gastric lesions. Quantitative analysis

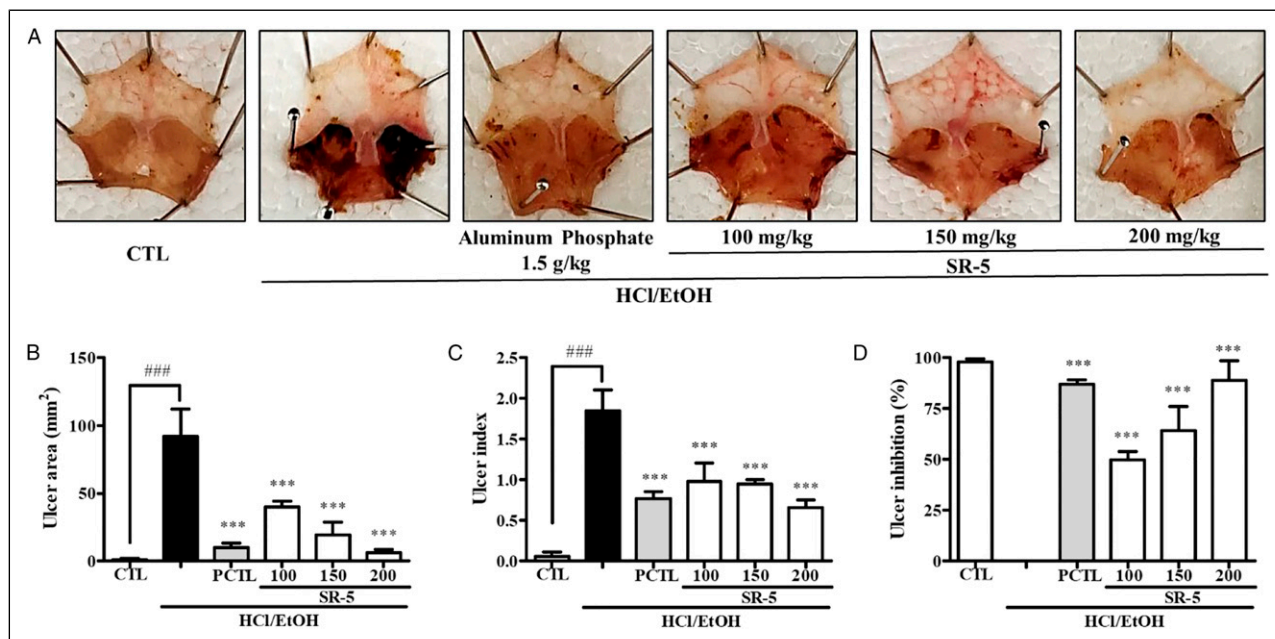


Figure 2. Protective effect of SR-5 on HCl/EtOH-induced gastric mucosal damage in ICR mice. Gross morphology of HCl/EtOH-induced gastric lesions (A) and quantitative analysis (B). Protective effect of SR-5 on the ulcer index (C) and ulcer inhibition ratio (%) (D) against HCl/EtOH-induced gastric ulceration experimentally in mice. Each bar represents the mean \pm SD for 7 mice. ###Significant difference at $p < .001$ compared to the control group. ***Significant difference at $p < .001$ compared to the HCl/EtOH-induced gastric mucosal injury group.

showed that mice in the SR-5 pretreatment groups receiving doses of 100, 150, and 200 mg/kg had significantly reduced ($p < .001$) ulcer areas of 39.88 ± 3.46 , 19.37 ± 4.24 , and 6.01 ± 1.08 mm², respectively, in comparison with mice in the HCl/EtOH-induced ulcer control group (91.92 ± 9.13 mm²) (Figure 2B). As shown in Figure 2C, all tested doses of SR-5 also significantly ($p < .001$) reduced the gastric ulcer index compared to that observed in the HCl/EtOH-treated group. These results show that SR-5 ($.66 \pm .09$) significantly reduces the ulcer index at a high dose (200 mg/kg) compared to that observed in the HCl/EtOH-treated group ($1.85 \pm .25$). The improvement in the gastric ulcer index was similar in the SR-5 (200 mg/kg)-pretreated group to the aluminum phosphate-treated group ($.77 \pm .08$). The percent ulcer inhibition by 200 mg/kg SR-5 was $88.78 \pm 9.81\%$, while that by 100 mg/kg was $49.80 \pm 4.20\%$ and that by aluminum phosphate was $86.99 \pm 2.31\%$ (Figure 2D). The 200 mg/kg treatment was found to be more effective against HCl/EtOH-induced gastric ulcers than the 100 mg/kg treatment.

Gastroprotective effect of SR-5 on indomethacin-induced gastric lesions

The gastric mucosa of the treated groups is shown in Figure 3. INDO/HCl treatment induced considerable damage to the gastric mucosa, as evidenced by ulcerated and hemorrhagic lesions. However, preventive oral administration of SR-5 (100, 150, and 200 mg/kg) or DA-5204 (36 mg/kg)

distinctly reduced the area of the INDO/HCl-induced gastric mucosal lesions (Figure 3B). Moreover, quantitative analysis showed that pretreatment with SR-5, irrespective of dose level, offered significant protection ($p < .001$) against INDO/HCl-induced gastric ulcers in mice, as demonstrated by the ulcer index (Figure 3C). These results show that SR-5 ($1.24 \pm .20$) significantly reduces the ulcer index at a high dose (200 mg/kg) compared to that observed in the INDO/HCl-treated group ($2.03 \pm .11$). The percent ulcer inhibition by 200 mg/kg SR-5 was $77.26 \pm 7.05\%$, while that by 100 mg/kg was $62.57 \pm 13.01\%$ and that by DA-5204 (36 mg/kg) was $62.95 \pm 13.88\%$ (Figure 3D). The 200 mg/kg treatment was found to be more effective in protecting against INDO/HCl-induced gastric ulcers than the 100 mg/kg treatment.

Increased antioxidant activities in mice pretreated with SR-5 on HCl/ethanol-induced ulcers

Redox reactions and redox signaling have been demonstrated to be involved in maintaining the balance of gastric mucosal homeostasis.⁴⁷ Our results showed that MDA levels were significantly increased in the gastric mucosal injury groups ($1.62 \pm .57$ μ M/mg, $p < .01$) as a result of HCl/EtOH in the gastric tissue (Figure 4A). Compared with the HCl/EtOH injury groups, all the SR-5 pretreatment groups displayed significantly suppressed MDA production. Similarly, pretreatment with 150 or 200 mg/kg SR-5 decreased the ROS level ($p < .05$ and $p < .01$, respectively), as shown in

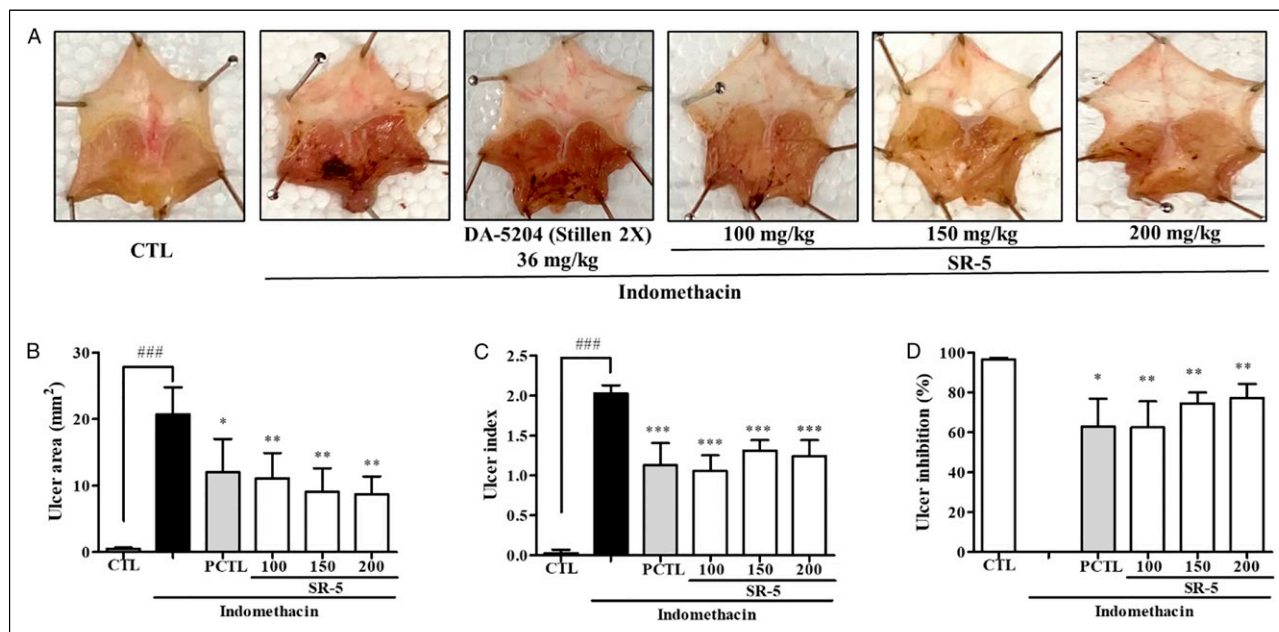


Figure 3. Protective effect of SR-5 on indomethacin/HCl (INDO/HCl)-induced gastric mucosal damage in ICR mice. Gross morphology of INDO/HCl-induced gastric lesions (A) and quantitative analysis (B). Effect of INDO/HCl, SR-5, and DA-5204 on the ulcer index (C) and ulcer inhibition ratio (%) (D) against INDO/HCl-induced gastric ulceration experimentally in mice. Each bar represents the mean \pm SD for 7 mice. ###Significant difference at $p < .001$ compared to the control group. *Significant difference at $p < .05$, ** at $p < .01$, and *** at $p < .001$ compared to the IND-induced gastric mucosal injury group.

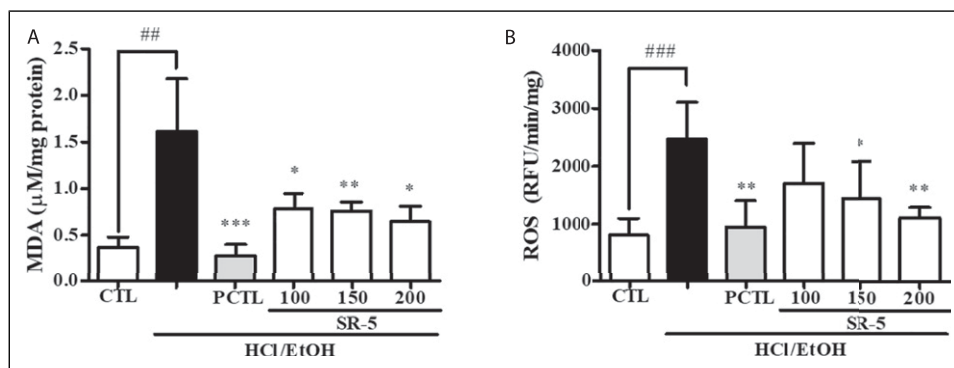


Figure 4. Effects of SR-5 on gastric MDA concentration (A) and ROS contents (B) of gastric tissues exposed to HCl/EtOH. Each bar represents the mean \pm SD for 7 mice. ##Significant difference at $p < .01$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$, ** at $p < .01$, and *** at $p < .001$ compared to the HCl/EtOH-induced gastric mucosal injury group.

Figure 4B. However, treatment with the lower dose (100 mg/kg) did not produce a significant reduction in ROS levels compared to those observed in the HCl/EtOH-treated group.

In contrast to the MDA and ROS responses, the administration of HCl/EtOH significantly reduced GSH levels by $9.32 \pm 6.82 \mu\text{M/mg}$ ($p < .001$) compared with the normal control values ($48.23 \pm 14.21 \mu\text{M/mg}$) (Figure 5A). In addition, the antioxidant enzyme CAT was significantly decreased in the HCl/EtOH injury groups ($p < .01$) compared

with that in the control groups (Figure 5B). On the other hand, the administration of SR-5 (150 and 200 mg/kg) significantly increased the level of GSH by $32.73 \pm 17.29 \mu\text{M/mg}$ ($p < .05$) and $38.17 \pm 24.19 \mu\text{M/mg}$ ($p < .05$), respectively, compared with that observed in the HCl/EtOH-induced ulcerated control group. Moreover, a similar effect on CAT activity upregulation was observed in the SR-5 (150 and 200 mg/kg)-treated groups compared with that observed in the HCl/EtOH-induced ulcerated group. However, GSH levels and CAT activities in the

group treated with a low concentration of SR-5 (100 mg/kg) did not differ from those in the HCl/EtOH-induced ulcerated control group.

Increased antioxidant activities in mice with INDO/HCl-induced ulcers pretreated with SR-5

As shown in Figure 6A, MDA levels were significantly increased in the gastric mucosal injury groups ($1.47 \pm .09 \mu\text{M}/\text{mg}$, $p < .001$) as a result of INDO/HCl in the gastric tissue. Compared with the INDO/HCl injury groups, all the SR-5 pretreatment groups displayed significantly suppressed ($p < .001$) MDA production. Furthermore, INDO/HCl-induced gastric lipid peroxidation was significantly suppressed after oral application of SR-5 (200 mg/kg), which was similar to the inhibition observed by treatment with DA-5204 (36 mg/kg). Similarly, pretreatment with 150 or 200 mg/kg SR-5 decreased the ROS level, as shown in Figure 6B. However, treatment with the lower dose (100 mg/kg) did not produce a significant

difference in ROS levels in comparison with those observed in the INDO/HCl-treated group.

In contrast to the MDA and ROS responses, the administration of INDO/HCl significantly reduced GSH levels and CAT activities by $21.84 \pm 4.79 \mu\text{M}/\text{mg}$ ($p < .01$) and $7.94 \pm 1.73 \mu\text{M}/\text{mg}$ ($p < .001$), respectively, compared with the normal control values (97.35 ± 34.36 and $14.74 \pm 1.68 \mu\text{M}/\text{mg}$, respectively) (Figures 7A and B). On the other hand, the administration of SR-5 (100, 150, and 200 mg/kg) significantly increased the level of GSH by 38.89 ± 11.16 ($p < .05$), 42.24 ± 16.27 ($p < .05$), and $44.94 \pm 18.20 \mu\text{M}/\text{mg}$ ($p < .05$), respectively, compared with that observed in the INDO/HCl-induced ulcerated control group (Figure 7A). Similarly, SR-5 pretreatment (150 and 200 mg/kg) significantly increased the CAT level by 14.79 ± 3.65 ($p < .01$) and $12.22 \pm 2.12 \mu\text{M}/\text{mg}$ ($p < .01$), respectively, compared with that observed in the INDO/HCl-induced ulcerated control group. However, CAT activities in the low SR-5 (100 mg/kg) concentration group did not differ from those in the INDO/HCl-induced ulcerated control group (Figure 7B).

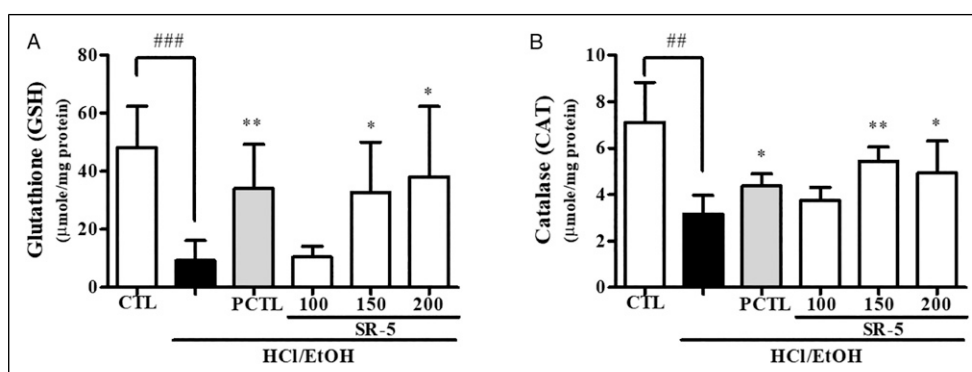


Figure 5. Effects of SR-5 on gastric glutathione (GSH) contents (A) and catalase (CAT) activities (B) of gastric tissues exposed to HCl/EtOH. Each bar represents the mean \pm SD for 7 mice. ##Significant difference at $p < .01$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$ and ** at $p < .01$ compared to the HCl/EtOH-induced gastric mucosal injury group.

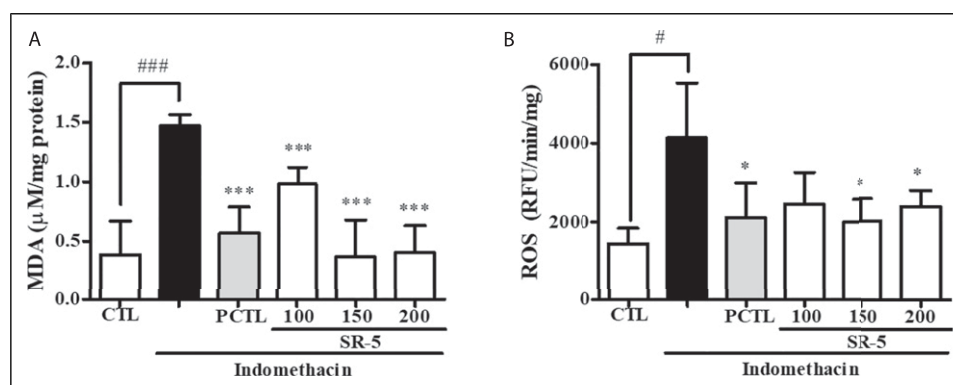


Figure 6. Effects of SR-5 on gastric MDA concentration (A) and ROS contents (B) of gastric tissues exposed to indomethacin/HCl (INDO/HCl). Each bar represents the mean \pm SD for 7 mice. #Significant difference at $p < .05$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$ and *** at $p < .001$ compared to the INDO/HCl-induced gastric mucosal injury group.

Effect of SR-5 on NF κ B/p65, iNOS, COX-2, and TNF- α in HCl/ethanol-induced ulcers

Redox imbalance causes a gastric mucosal inflammatory response and contributes to disease processes.⁴⁸ The Western blots of NF κ B/p65, iNOS, COX-2, and TNF- α expression in the gastric tissue of the normal groups, ulcerated groups, SR-5 groups, and positive drug-treated mice are shown in Figure 8 and Figure 9. The expression of NF κ B/p65 was assessed by determining the level of activated subunit p65 in stomach tissue.

As shown in Figure 8, mice exposed to HCl/EtOH showed a significant increase in NF κ B/p65, iNOS, COX-2, and TNF- α contents to 1.9-, 1.3-, 1.6-, and 2.0-fold, respectively, in comparison to the levels observed in the normal control group. The middle dose (150 mg/kg) of SR-5 pretreatment significantly decreased NF κ B/p65 and iNOS expression to 28.7 and 27.8%, respectively, of the levels observed in the HCl/EtOH-induced gastric ulcer control group. Low-dose (100 mg/kg) SR-5 did not significantly affect NF κ B/p65 and iNOS expression, but significantly inhibited COX-2 and TNF- α expression level to 39.3 and 15.6%, respectively, of the

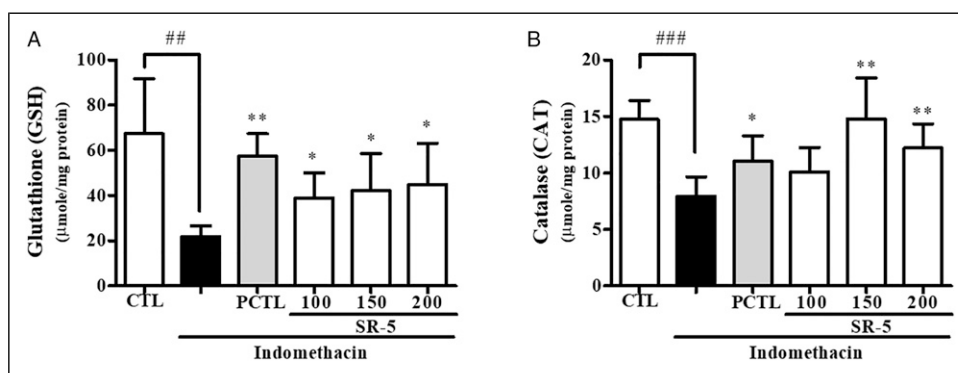


Figure 7. Effects of SR-5 on gastric glutathione (GSH) contents (A) and catalase (CAT) activities (B) of gastric tissues exposed to indomethacin/HCl (INDO/HCl). Each bar represents the mean \pm SD for 7 mice. ##Significant difference at $p < .01$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$ and ** at $p < .01$ compared to the INDO/HCl-induced gastric mucosal injury group.

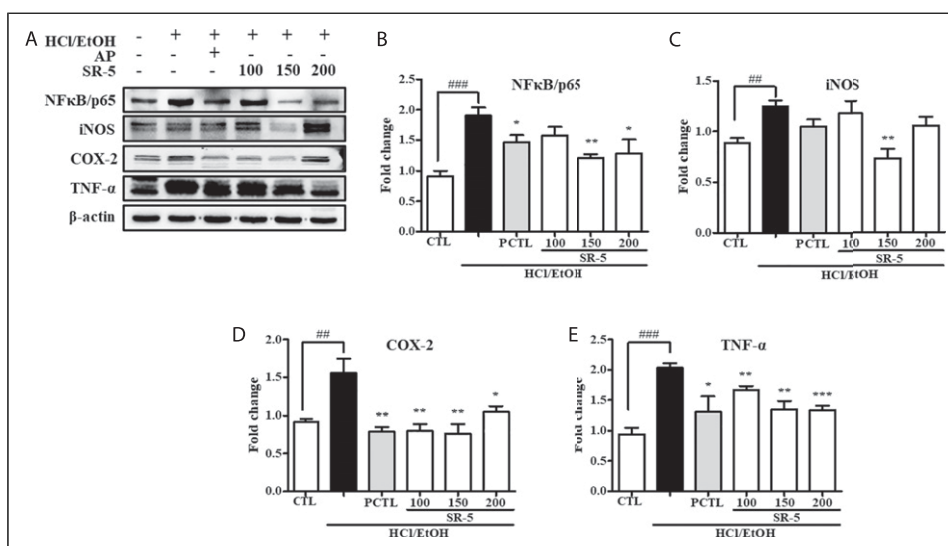


Figure 8. Effect of SR-5 on NF κ B/p65, iNOS, COX-2, and TNF- α protein expression in HCl/EtOH-induced gastric mucosal injury mice. The Western blots are representative of NF κ B/p65, iNOS, COX-2, and TNF- α proteins in mouse gastric tissue (A). The results are shown in the histogram of NF κ B/p65 (B), iNOS (C), COX-2 (D), and TNF- α (E) proteins expressed as the ratio of the relative intensity of the level of expression of each protein to β -actin. Each bar represents the mean \pm SD for 7 mice. ##Significant difference at $p < .01$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$, ** at $p < .01$, and *** at $p < .001$ compared to the HCl/EtOH-induced gastric mucosal injury group.

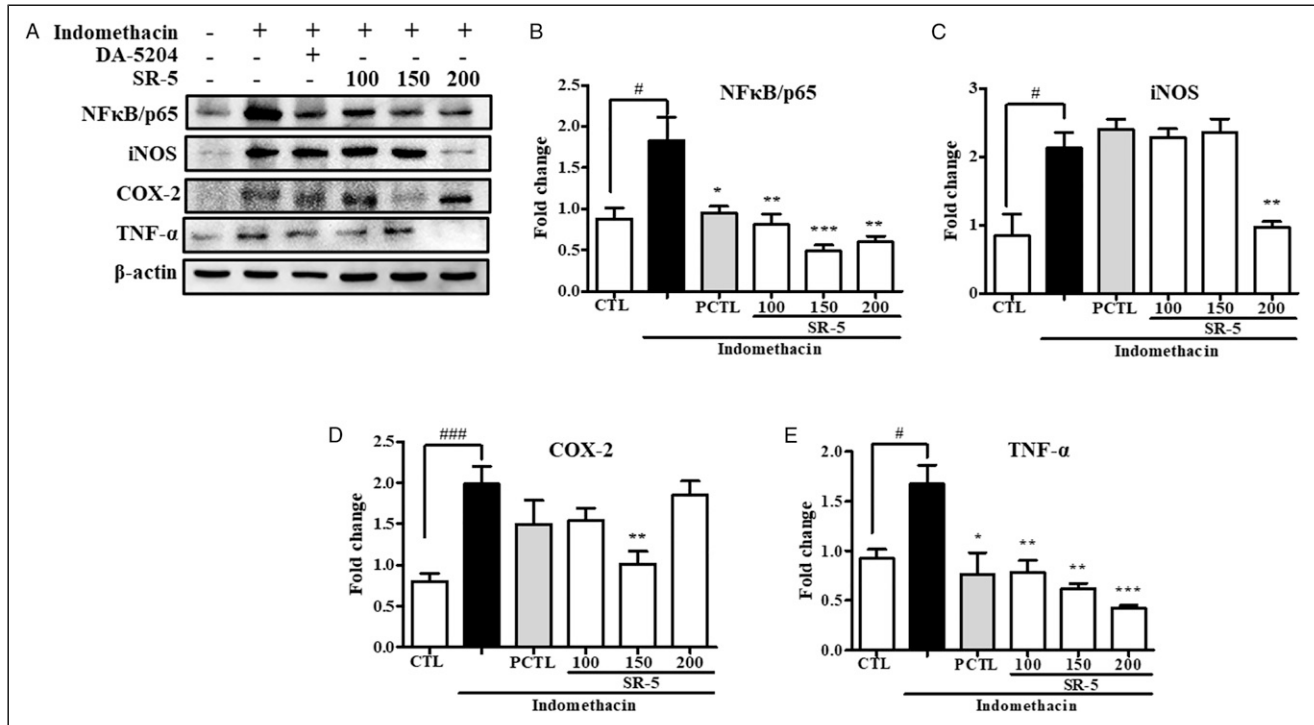


Figure 9. Effect of SR-5 on NFκB/p65, iNOS, COX-2, and TNF-α protein expression in INDO/HCl-induced gastric mucosal injury mice. The Western blots are representative of NFκB/p65, iNOS, COX-2, and TNF-α proteins in mouse gastric tissue (A). The results are shown in the histogram of NFκB/p65 (B), iNOS (C), COX-2 (D), and TNF-α (E) proteins expressed as the ratio of the relative intensity of the level of expression of each protein to β-actin. Each bar represents the mean ± SD for 7 mice. #Significant difference at $p < .05$ and ### at $p < .001$ compared to the control group (CTL). *Significant difference at $p < .05$, ** at $p < .01$, and *** at $p < .001$ compared to the INDO-induced gastric mucosal injury group.

expression levels observed in the HCl/EtOH-induced gastric ulcer control group.

Effect of SR-5 on NFκB/p65, iNOS, COX-2, and TNF-α in INDO/HCl-induced ulcers

As shown in Figure 9, administration of INDO/HCl caused gastric inflammation manifesting as a marked elevation in the levels of NFκB/p65 (1.8-fold, $p < .05$), iNOS (2.1-fold, $p < .05$), COX-2 (2.0-fold, $p < .001$), and TNF-α (1.7-fold, $p < .05$) compared to those in the control group. Pretreatment with DA-5204 markedly diminished the elevated levels of NFκB/p65 and TNF-α by 39.0% ($p < .05$) and 41.1% ($p < .05$), respectively, compared those in the INDO/HCl group. Similarly, the high dose of SR-5 (200 mg/kg) significantly decreased NFκB/p65, iNOS, and TNF-α expression levels by 58.6% ($p < .01$), 43.0% ($p < .01$), and 69.7% ($p < .001$), respectively, compared with those in the INDO/HCl group. The low dose of SR-5 (100 mg/kg) also significantly decreased NFκB/p65 and TNF-α levels by 52.2% ($p < .01$) and 45.3% ($p < .01$), respectively, compared to those in INDO/HCl-treated mice. However, the middle dose of SR-5 (150 mg/kg) only significantly decreased COX-2 expression (35.5%, $p < .01$) compared with that in the control group.

Discussion

For the first time, we explored the effect of SR-5 on HCl/EtOH and INDO/HCl-induced gastric ulcers in mice. We showed that SR-5 at three dose levels (100, 150, and 200 mg/kg) exerted gastroprotective effects that were comparable in most aspects to the well-known gastroprotective drug DA-5204 (Stilten™) or to aluminum phosphate gel. Our findings not only show the gastroprotective effects of SR-5 but also outline the involved mechanisms of this effect.

The following findings were obtained: (1) SR-5 ameliorated the structural derangements of the gastric mucosa and improved the ulcer index; all doses exerted potent effects in reversing the structural derangement; (2) SR-5 reduced lipid peroxidation and ROS and increased the gastric GSH levels and CAT activities; and (3) In addition to the previously demonstrated anti-inflammatory actions of each of the 12 Korean medicinal herb single extracts, this study showed, for the first time, a modulatory action of SR-5 on NFκB/p65, iNOS, COX-2, and TNF-α signaling as a contributing mechanism to its gastroprotective potential.

In the present study, we evaluated the effect of SR-5 on HCl/EtOH- and INDO/HCl-induced gastric mucosal injury through *in vivo* experiments. To investigate the pathogenesis and pathophysiology of human gastric ulcers, a large number

of gastric ulcer animal models have been established.⁴⁹ High alcohol consumption is the greatest cause of gastric mucosal damage.^{38,39} Thus, the most common type of animal model is established by ethanol, which penetrates quickly into the gastric mucosa, inducing damage to gastric tissue. Such lesions are characterized by extensive subnormal edema, hemorrhage, desquamation of epithelial cells, and infiltration of inflammatory cells, which are typical characteristics of alcohol injury in humans.^{50,51} Through its direct action, acidified ethanol penetrates the gastric mucosa, rapidly causing damage to gastrointestinal mucosa cells and membranes.⁵²

Indomethacin, a common NSAID, also damages the integrity of the gastric mucosa, resulting in submucosal edema, inflammatory cell infiltration, and ulcer formation due to the inhibition of prostaglandin E2 (PGE2) synthesis.⁵³ Therefore, indomethacin became the best choice for the creation of an experimental gastric ulcer model because of its higher ulcerogenic potential than other NSAIDs.⁹ In addition, indomethacin generates harmful ROS that are involved in the pathogenesis of gastric ulcers.⁵⁴ These radicals in particular appear to play an important role in ulcerative and erosive lesions of the gastrointestinal tract as they attack and damage many biological molecules.⁵⁵ Therefore, treatment with antioxidants and free-radical scavengers can decrease gastric mucosal damage.⁵⁶ Our *in vivo* results using these gastric ulcer models demonstrated that pretreatment with SR-5 (100, 150, and 200 mg/kg) increased gastric wound repair and antioxidant production and decreased inflammation-associated protein expression. Pretreatment of mice with SR-5 significantly reduced the ulcer index at all doses compared to the ulcer indices observed in the HCl/EtOH- or INDO/HCl-induced ulcer model mice. Moreover, ulcerated animal pretreatment with SR-5 (200 mg/kg) induced a similar reduction in the ulcer index to the aluminum phosphate (1.5 g/kg) and DA-5204 (36 mg/kg) positive control drugs, indicating that SR-5 could be valuable for healing gastric ulcers.

Another factor that has been found to contribute to mucosal lesion formation is oxidative stress. In the present study, HCl/EtOH-induced ulcer model mice had significant decreases in gastric GSH ($p < .001$) and CAT ($p < .01$) levels and typically showed significant increases in MDA ($p < .01$) and ROS ($p < .001$) levels compared to those in the normal group. On the other hand, pretreatment with SR-5 at a dose of 200 mg/kg significantly increased CAT and GSH contents and significantly decreased MDA and ROS levels compared to those in the HCl/EtOH group. Pretreatment with SR-5 at a dose of 200 mg/kg also resulted in significant improvement in the expression of all antioxidant markers, with the highest improvement levels observed in the INDO/HCl-induced ulcer model. MDA, ROS, GSH, and CAT levels were improved ($p < .001$, $p < .05$, $p < .05$, and $p < .01$, respectively) by 200 mg/kg SR-5 compared with those observed in the INDO/HCl-treated group.

The primary product of free radical-mediated lipid peroxidation is a complex mixture of peroxides, which are broken down to produce carbonyl compounds such as MDA that form a characteristic chromogenic adduct with 2-thiobarbituric acid (TBA), a widely accepted reaction for measuring the extent of lipid peroxidation.⁵⁷ Furthermore, the increase in MDA levels is associated with increased tissue damage and is an important cause of gastric damage associated with EtOH⁵⁸ and indomethacin.⁵⁹ The present study showed that all tested doses of SR-5 markedly lowered lipid peroxidation (MDA content) compared with that observed in the HCl/EtOH control group and the INDO/HCl control group. SR-5 (200 mg/kg) also decreased the MDA level in a similar manner as aluminum phosphate (1.5 g/kg) or DA-5204 (36 mg/kg).

Similarly, acidified ethanol can also augment the production of ROS.⁶⁰ ROS react with cellular lipids and form lipid peroxides. Subsequently, ROS accumulation depletes the protective antioxidant defense mechanisms (such as GSH and CAT activity) and increases lipid peroxidation. GSH and other antioxidants play a crucial role in free radical degradation, which would otherwise result in lipid peroxidation.⁵⁶ In the present study, SR-5 increased GSH levels in the mucosa of the stomach, indicating that the antioxidant action of these herbal factors was responsible, at least partially, for the anti-ulcer action of SR-5. Additionally, aluminum phosphate or DA-5204 significantly increased GSH levels. These results suggest that the increase in the GSH level is a possible mechanism for the protective effect of SR-5 in the HCl/EtOH- and INDO/HCl-induced ulcer models. StillenTM had a protective effect on peptic ulcers and gastroesophageal reflux disease in ethanol- and indomethacin-induced animal models.⁶¹ In particular, sodium taurocholate (TCA)-induced chronic reflux gastritis in animals and StillenTM normalized GSH and MDA levels.¹⁴ Antioxidant compounds have been demonstrated to protect the gastric mucosa from ulceration.⁶²⁻⁶⁴ It has also been reported that soybean sprouts, the main component of SR-5, have higher *in vitro* antioxidant activities than soybean and soybean oil.^{16,17} The present study showed that pretreatment with SR-5 significantly protected the gastric mucosa from HCl/EtOH- and INDO/HCl-induced ulceration by restoring the depleted GSH level and CAT activity, together with reducing the level of MDA and ROS. Thus, the protective effect of SR-5 against ulcers could be partly attributed to its inhibitory effect on oxidative stress and lipid peroxidation.

It has been reported that oxidative stress can induce inflammatory responses, which significantly participate in the pathogenesis of ulceration. The use of anti-inflammatory agents to suppress gastric ulceration showed promising results.^{48,65} Furthermore, the inflammatory response is one of the characteristics of gastric ulcers that promotes gastric mucosal injury through the migration of macrophages and leukocytes into the ulcerated and surrounding areas.⁶⁵ NF- κ B, a principal transcriptional regulator of several genes involved in inflammation, is activated by ROS production.⁶⁶ NF- κ B is

activated in response to ulceration. It induces the production of several proinflammatory cytokines, such as TNF- α . TNF- α is a major proinflammatory cytokine released by migrated macrophages during inflammation.^{66,67} It stimulates neutrophil infiltration in gastric inflamed areas,⁶⁸ suppresses the gastric microcirculation around ulcerated mucosa, and delays gastric ulcer healing.⁶⁹ Thus, the NF- κ B/p65 subunit is considered a marker for NF- κ B activation and a perfect target for a molecular approach to alleviating gastric ulcers. The present data indicate that HCl/EtOH or INDO/HCl administration induces inflammatory responses, as evidenced by the marked increase in gastric tissue levels of TNF- α and activation of NF- κ B/p65 in the treated groups compared with those in the control group. On the other hand, SR-5 pretreatment (150 or 200 mg/kg) significantly decreased the TNF- α and NF- κ B/p65 expression levels in the treated groups compared with those in the ulcer control groups, which may be attributed to the anti-inflammatory effect of SR-5. Interestingly, the gastro-protective effect of SR-5 was comparable to that of aluminum phosphate or DA-5204, a reference drug currently prescribed for gastric ulcers, emphasizing the efficacy of SR-5 for attenuating HCl/EtOH- and INDO/HCl-induced gastric damage associated with NF- κ B/p65 expression.

Additionally, NF- κ B and proinflammatory cytokines are known to induce the expression of COX-2.⁷⁰ Consistently, the present data reveal that the administration of HCl/EtOH or INDO/HCl upregulates the inflammatory response as manifested by the marked overexpression of NF- κ B/p65 and COX-2. Conversely, pretreatment with SR-5 significantly suppressed NF- κ B stimulation by reverting the protein expression level of p65 to normal in gastric tissue and decreasing the expression of the proinflammatory enzyme COX-2.

The physiologically important free radical NO, produced during arginine catabolism by NOS, plays dual roles in gastric mucosal defense and injury. The low concentration of NO produced by endothelial NOS (eNOS), one of the constitutive NOS isoforms, promotes wound healing by increasing blood flow⁷¹ and angiogenesis⁷² in the damaged gastric mucosa. However, the enhanced generation of NO by iNOS may contribute to the pathogenesis of various gastroduodenal disorders, including peptic ulcers.⁷³ Thus, the status of eNOS vs iNOS expression in gastric tissue is crucial for maintaining its integrity. Experimental evidence indicated that the upregulation of NF- κ B also induces the transcriptional activation of iNOS.⁷⁴ In the current study, we confirmed that HCl/EtOH- or INDO/HCl-induced gastric ulceration increased mucosal iNOS expression. In contrast, SR-5 administration effectively suppressed iNOS expression. According to the results of several studies, the expression level of iNOS in the gastritis animal model is highest after 3–6 h.^{75,76} In the INDO/HCl-induced gastritis model, an increase in iNOS expression by longer-term treatment with indomethacin has been reported.⁷⁷ In the present study, for determination of the iNOS level, gastric tissue was sampled 2 h after HCl/EtOH-administration or 8 hours after INDO/HCl-administration; thus, the expression

level of iNOS in the gastric tissue was not maximally increased and could be further increased by HCl/EtOH- or INDO/HCl-induced gastric ulcers. Therefore, this may be the reason for the significant reduction of iNOS levels in the SR-5 150 mg/kg-treated mice compared to those in the HCl/EtOH-induced gastric ulcer model mice or in the 200 mg/kg-treated mice compared to in the INDO/HCl-induced gastric ulcer model mice. Du et al.⁷⁸ and Li et al.⁷⁹ reported a significant increase in TNF- α levels in the gastric mucosa of animals with EtOH-induced gastric ulcers after 1–4 h. Regarding the effect of SR-5 on TNF- α levels, a significant increase was observed in mice treated with all SR-5 doses (100–200 mg/kg) mice compared with those in HCl/EtOH- or INDO/HCl-induced gastric ulcer model mice. These findings suggest that SR-5 suppresses the inflammatory response in gastric tissue through the inhibition of NF- κ B and the suppression of proinflammatory cytokines such as TNF- α . SR-5 also reduced COX-2 and iNOS expression.

This study utilized HCl/EtOH and INDO/HCl-induced mouse gastric ulcer models, which are analogous to gastric mucosal damage caused by alcohol consumption and excessive NSAID use, respectively, in humans. Since *H. pylori* infection is also a major cause of gastric ulcers,⁵ a future study is warranted to investigate the effects of SR-5 on *H. pylori*-induced gastric ulceration. In addition, additional research is needed to identify the most effective substance and its active compounds in SR-5, which has a gastritis protective effect.

Conclusion

In conclusion, the results of the present study strongly indicate that SR-5 exerts gastroprotective and ulcer-healing effects in ethanol- or indomethacin-induced gastric ulcer model animals. Intra-gastric administration of SR-5 effectively protected the gastric mucosa from ethanol or indomethacin damage in a dose-dependent manner. The ameliorating effect of SR-5 against gastric ulcers might be attributed to the observed antioxidative and anti-inflammatory properties. Based on these findings, SR-5 is a potential preventive and therapeutic agent for the treatment of gastric ulcers. However, further studies are warranted to examine the gastroprotective efficacy of SR-5 in a clinical setting.

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Author Contributions

Conceptualization, SK; data curation, KK; formal analysis, EK and WK; funding acquisition, SK; methodology, MJ, HC, LH, and KM-H; project administration, SK; resources, JK and C-SN; writing—original draft, SK; writing—review and editing, C-SN. and SK. All authors have read and agreed to the published version of the manuscript.

Declaration of Conflicting Interests

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