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The Treatment Effect of Ulcerative Colitis of Supercritical Heat-Treated Radish Extracts

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Abstract

With the recent rapid improvement in the standards of life and westernization of dietary lifestyles, the consumption of high-calorie diets such as high-fat and high-protein red meat and instant foods has increased, while less vegetables containing dietary fiber are consumed. In addition to that, stress, erroneous dietary behaviors, and contaminated environments are linked to the risk of developing ulcerative colitis, which is on the rise. Another cause of ulcerative colitis is that involve laxative abuse, including repeated, frequent use of laxatives, and include such conditions as deteriorated bowel function, irritable bowel syndrome, diarrhea, intestinal inflammation, etc. The present study aimed to investigate the comparative evaluation of pharmacological efficacy between sulfasalazine alone and combination with herbal medicine on dextran sodium sulfate (DSS)-induced UC in mice. Balb/c mice received 5% DSS in drinking water for 7 days to induce colitis. Animals were divided into five groups (n = 9): group I-normal group, group II-DSS control group, group III-DSS + sulfasalazine (30 mg/kg), group IV-DSS + sulfasalazine (60 mg/kg), group V-DSS + sulfasalazine (30 mg/kg) + Radish Extract mixture (30 mg /kg) (SRE). DSS-treated mice developed symptoms similar to those of human UC, such as severe bloody diarrhea and weight loss. SRE supplementation, as well as sulfasalazine, suppressed colonic length and mucosal inflammatory infiltration. In addition, SRE treatment significantly reduced the expression of pro-inflammatory signaling molecules through suppression both mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) *signaling pathways, and prevented the apoptosis of colon. Moreover, SRE administration significantly led to the up-regulation of anti-oxidant enzyme including SOD and Catalase. This is the first report that Radish extract mixture combined with sulfasalazine protects against experimental UC via the inhibition of both inflammation and apoptosis, very similar to the standard-of-care sulfasalazine.*

Keywords*: Supercritical heat-treated radish, Ulcerative colitis, Dextran sodium sulfate, Sulfasalazine, Mesalamine*

1. Introduction

The radish is a vegetable of the family Cruciferae containing volatile sulfur-compounds that cause its unique spiciness. The radish contains a greater larger amount of free amino acids, sugars, calcium, phosphorus, etc.

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than other vegetables. The root of radishes contains sugar components like glucose and fructose and other ingredients, such as coumaric acid, caffeic aid, ferulic acid, phenylpyruvic acid, gentidin acid, hydroxyl benzoic acid, and a variety of amino acids. Particularly, it has the content of vitamin C amounting up to 20 to 25 mg, which is an important source of vitamin in winter and becomes an important source of vitamin C in winter. According to the ancient medicinal records, the root of radish, nabok, has the curative effects on phlegm, coughing, dysentery, etc. and eliminates food poisoning associated with fish, shellfish, and noodles. Diastase contained in the radish is used to promote digestion, neutralize the effects of food poisoning, and ease a hangover, and rapine is known as an antibiotic component against germs, fungus, parasites, etc [1-3].

Meanwhile ulcerative colitis is a chronic recurrent disease that causes inflammation or ulcers in the colon mucosa. The cause is not yet known, but excessive immune response of the human body to bacteria normally present in the intestine, along with environmental and genetic factors, is primarily considered to cause the disease. Since the surgical method for the cure of ulcerative colitis is very complicated and a large variety of sequelae caused by the surgical operation are persisting, it is in principle to treat ulcerative colitis with drug therapy instead of surgery as much as possible [4]. There is still no drug treatment that can cure ulcerative colitis, and most commonly used are anti-inflammatory drugs such as sulfasalazine and mesalamine and treatments such as corticosteroid drugs. In addition to these drugs, immunosuppressants or antibiotics are appropriately selected and used depending on the patient's condition. Out of these drugs, sulfasalazine has been used for decades as a standard treatment for ulcerative colitis, but high dose and long-term use of sulfasalazine can cause oxidative stress and side effects, such as nausea, heartburn, headache, dizziness, anemia, and skin rash, and infrequently hepatitis, pancreatitis and pneumonia [5,6]. Moreover, ulcerative colitis with pathological changes only in the rectum can be cured merely with temporary medication solely, but ulcerative colitis with pathological changes in the areas above the rectum has a high recurrence frequency and possibly leads to complications such as intestinal perforation or toxic giant colon, or colon cancer [7,8]. So, the present study was conducted to evaluate the treatment of ulcerative Colitis with supercritical heat-treated radish extracts.

2. Experiment Materials and Methods

2.1. Preparation of Processed Extract

Korean radish (Cheongwoon Mu) was purchased from an agricultural and fishery wholesale market in Korea and washed without removal of its skin and green tops. The whole radish specimen was put into a container, which was then placed in an external container filled with a defined amount of water and heated at a defined temperature for a defined period of time using heat treatment equipment (Jisco, Seoul, Korea) specially designed to resist the pressure of 10 kg/cm$_2$ or above. The equipment and method for the heat treatment were devised to prevent carbonization of the specimen from a direct heat transfer and allow the radish steamed with water vapor during the heat treatment process. The heat treatment was conducted at temperatures of 110 ºC, 120 ºC, 130 ºC, 140 ºC, or 150 ºC separately for 6 hours. After completion of the heat treatment, the radish was dried out in an airy space for 24 hours and ground as fine as 200 mesh or less. The ground radish was placed in a supercritical fluid extractor and subjected to a supercritical CO_2 extraction using butylene glycol as a co-solvent at 40 to 80 ºC under pressure of 200 to 500 bar. The extracted liquid was captured, freeze-died and then put into use for the experiment materials.

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2.2. Preparation of Experimental Animals and Induction of Ulcerative Colitis

Eight-week-old male Balb/c mice weighing 22–24 g were purchased from were purchased from Orient (Gyeonggi-do, Korea). Mice were maintained under a 12 hours light/dark cycle, housed at a controlled temperature (24±2°C) and humidity (about 60%). After adaptation (1 week), acute colitis was induced by oral

administration of 5.0 % (w/v) DSS dissolved in drinking water, for 7 days. For each experiment, the mice were divided into 5 experimental groups and 36 colitic mice were arbitrarily allocated into 4 groups (n = 9/group). Normal mice received drinking water without DSS throughout the entire experimental period. Sufasalazine was used as a positive reference agent and it was given at 30 or 60 mg/kg/day. The entire colon was removed immediately and examined for gross mucosal injury. The colon tissue was immediately frozen in liquid nitrogen and blood samples were collected by cardiac puncture from anesthetized mice [9,10].

2.3. Measurement of Expression of Reactive Oxygen Species (SOS) and NADPH Oxidases in Serum

According to the previously reported clinical data, ROS are increased in patients with colitis, causing oxidative cell damage and cancer. The main producers of ROS are NADPH oxidase enzymes, including NOX4, p47phox, and Rac. Hence, the animal model with colitis was evaluated in regards to the expression of ROS and the related NADPH oxidases in serum. Blood samples were collected by cardiac puncture from anesthetized mice during the sacrificial process of the mice. Serum was stored and used at -80 ºC for analysis. The level of ROS in serum was measured according to the method of Ali et al. (J. Exp. Clin. Cancer Res. 2007 26(3), 395-404). Briefly, 25 mM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes) was added to the serum, which was then incubated for 30 minutes and measured in regards to the level of ROS by way of the change in the fluorescence value under the conditions of an excitation wavelength of 486 nm and an emission wavelength of 530 nm. In order to evaluate the expression level of NADPH oxidases, proteins were extracted from colon tissue according to the method of Komatsu et al. (Plant Proteomics 73-77, Humana Press Inc., 2007). The colon tissue for cytosol fractionation was homogenized with 205 ml of a cold lysis buffer A containing a mixed solution (Wako Pure Chemical Industries) of 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgC12, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), and 1,250 µl protease inhibitor. The resultant homogenate was incubated at 4 ºC for 20 minutes and then mixed with 10% NP-40. A centrifugation was then conducted at 13,400xg for 2 minutes using an Eppendorf 5415R (Hamburg, Germany) at 4 ºC. The supernatant (cytosol fraction) was separated and set aside. Then, the residue was washed twice with buffer A, suspended in 20 ml of a lysis buffer C containing a mixed solution of 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 of protease inhibitor, and incubated at 4 ºC for 30 minutes. A centrifugation was conducted at 13,400xg for 10 minutes, and the supernatant were separated to prepare a nucleus fraction. Both the cytosol fraction and the nucleus fraction were stored at -80 ºC for use. The cytosol fraction was used to evaluate the expression of NADPH oxidase according to the immunoblotting using anti-NOX4 (LifeSpan BioSciences), anti-p47phox (1:1,000, SC-14015, Santa Cruz Biotechnology, Inc.) and anti-Rac (1:1,000, SC-217, Santa Cruz Biotechnology, Inc.).

2.4. Evaluation of Expression of Pro-Inflammatory Cytokaines

The expression levels of pro-inflammatory mediators such as iNOS and COX-2 and pro-inflammatory cytokines such as IL-1 and TNF-α in serum were measured by immunoblotting.

2.5. Evaluation of Effect on Apoptosis

Apoptosis is known to interfere with the over-accumulation of nonfunctional cells in tissuees. In the inflammaory conditions, continuous exposure of the intestinal mucosa to ROS results in an increase in the apoptosis of epithelial cells, altering the integrity of the intestinal barrier and causing intestinal damages. Bcl-2 is regarded as an anti-apoptotic molecule, and Bax binds and antagonizes Bcl-2 to exhibit anti-apoptotic actions. The activation of Cascase-3 plays an important role in cell dealth. Accordingly, apoptosis-related proteins in serum were analyzed by immunoblotting [11].

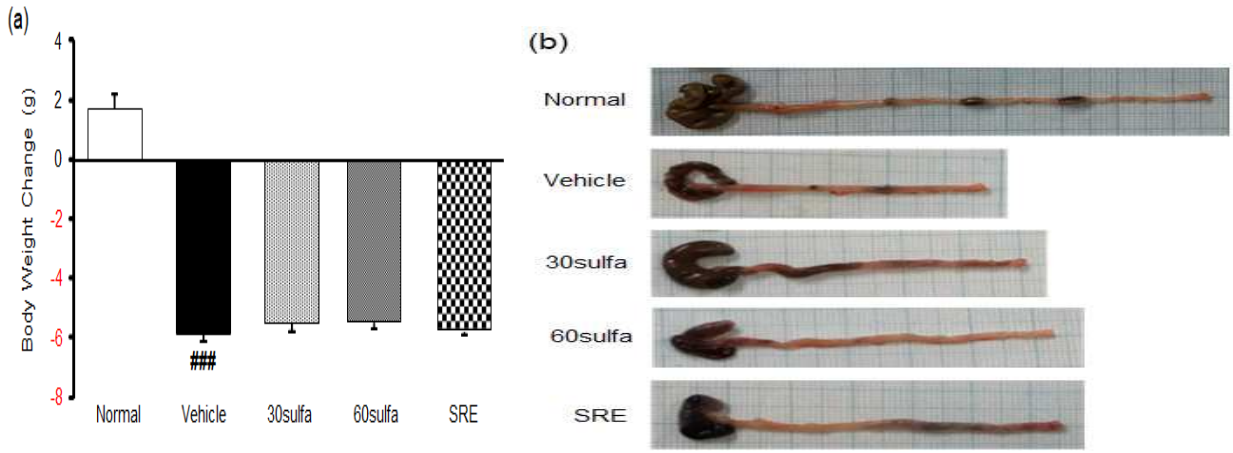
2.6. Statistical analysis

The data are expressed as the mean ± S.E.M. Significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered significant.

3. Result and Discussion

3.1. Effects of Ulcerative colitis (UC) and anti-inflammatory

Ulcerative colitis (UC), chronic and complex autoimmune inflammatory disorders, is associated with a diverse dysfunction led to overproduction of inflammatory cells and cytokine. Especially, the continuous progression of UC increases a risk for development of colorectal cancer (CRC). Accordingly, new complement remedy of sulfasalazine, which mainly used in the treatment of UC, however, had various side effects can be alleviated a deterioration of UC symptoms and use for a long-term in safety [12]. The present study revealed, for the first time, comparative evaluation of a pharmacological efficacy between sulfasalazine combined with Radish Extract mixture (SRE) and sulfasalazine alone in a mouse model of UC. As shown in Figure 1, DSS control group significantly increased body weight loss and decreased colon length ($P < 0.001$) in comparision with normal group. Results in previous studies had showed that length of colon was negatively correlated with severity of experimental colitis. Sulfasalazine or SRE administration led to anti-inflammatory effects, including reduced body weight loss and less shortening of the colon length, whereas there were few significance in body weight change and colon length among the DSS-treated groups (Figure 1a, c). Colonic inflammation involves the disruption of the apparatus of colonic mucosa and ulceration, resulting in the infiltration of inflammatory cells such as inflammatory monocytes and macrophages and thickening of the lamina propria. To investigate mucosal inflammation, we performed H/E staining. Colons in normal group exhibited with normal crypt morphology, abundant goblet cells, no signs of mucosal thickening, and complete absence of ulceration. On the contrary, microscopic damage was lower in with SRE or sulfasalazine group than those in DSS control group (Figure 1d).



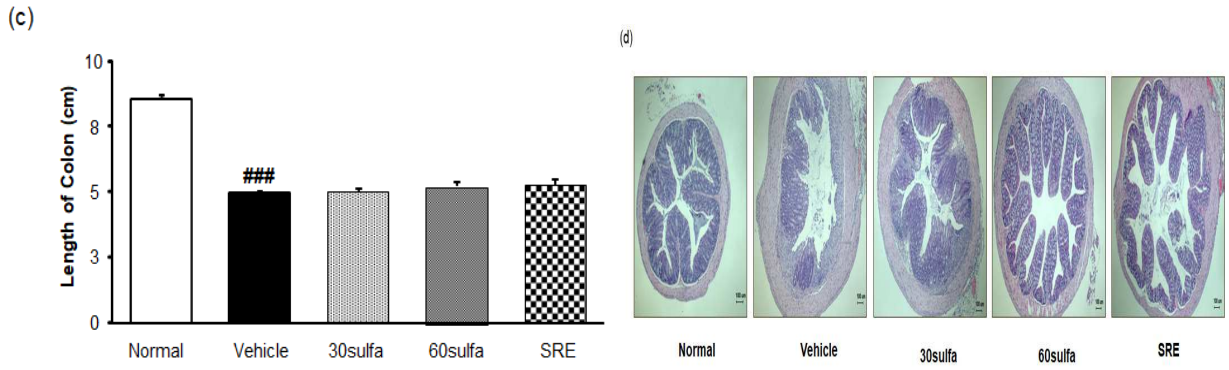


Figure 1. SRE alleviated dextran sodium sulfate-induced experimental colitis. (a) Body weight changes after induction of colitis by dextran sodium sulfate (DSS); (b) Macroscopic appearance; and (c) Length of colon (d) H/E staining of colon magnification×40. Data are mean ± S.E.M. (n=7). Significance: ###P <0.001 versus normal rats.

3.2. Effect of supercritical heat-treated radish extract(SRE) on the Expression of ROS and NADPH Oxidase in Serum.

Reactive oxygen species (ROS) are generated as part of normal oxidative metabolism, yet cell damage is induced by their excess formation. Moreover, redox-active sulfur species, which are the wildly known pathway of free radical generation by oxygen species, have been characterized as part of a sulfate assimilation pathway. This reactions also involve the metabolism of sulfinic and sulfonic acids that are oxidized sulfur molecules. Since DSS is so highly sulfated, we estimate that it may lead a sulfate load on cells and that this is associated with an elevation of the noticeable ROS, leading to acceleration of an inflammatory cascade. The reported clinical data show that ROS increases in colitis patients, causing oxidative cellular damage and promoting carcinogenesis. Previous studies indicated the importance of ROS-induced oxidative stress in the development of UC. Besides, the key producers of ROS are NADPH oxidase enzymes including NOX4, p47phox, and Rac 1. Overproduction of ROS via NADPH oxidase has been implicated in tissue damage observed in chronic inflammatory disorders and play vital roles in various biological activities, including host defense, cell growth and differentiation, stimulation of pro-inflammatory genes, and cell death[13]. In present study, the DSS injury was markedly higher than those of normal group ($P < 0.001$), whereas, the elevated level of serum ROS were significantly decreased lower to the levels of normal group both SRE and sulfasalazine ($P < 0.001$) (Figure 2a). The protein expressions of both NOX4 and p47phox , the markers of NADPH oxidase activity, in the colon were augmented in the DSS control group (vs. normal group, $P < 0.05$, $P < 0.01$, resp.). However, SRE-treated group had significantly down-regulated NADPH oxidase, whereas sulfasalazine-treated group decreased without significance. Otherwise, Rac 1 expression showed a tendency to a decrease (Figure 2b). In general, ROS are known to be neutralized by the endogenous antioxidant enzymes. SOD converts O^{2-} to H_2O_2, which is subsequently neutralized to water by Catalase and GPx-1/2. The activity of enzymic antioxidants such as SOD, Catalase, and GPx-1/2 was decreased in DSS-induced group. Herein, SRE administration significantly increased the activity of SOD and Catalase except for GPx-1/2 (without significance) (Figure 3). These findings indicated that SRE treatment of colitis may be reducing the extent of colonic injury by its antioxidant effect. Especially, SRE supplementation was superior to those when sulfasalazine alone (Figure 3).

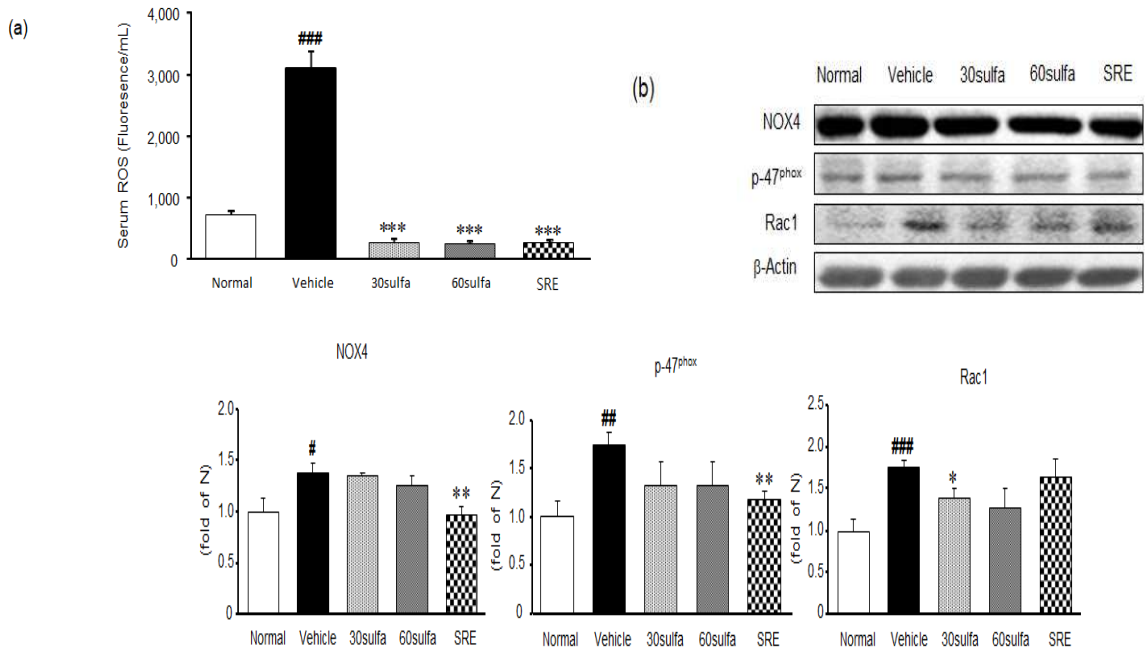


Figure 2. Effect of supercritical heat-treated radish extract(SRE) on the Expression of ROS and NADPH Oxidase in Serum. (a) serum ROS (b) NOX4, p47phox, and Rac 1 protein expressions. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: #P <0.05, ##P <0.01, ###P <0.001 versus normal mice and **P <0.01, ***P <0.001 versus DSS control mice.

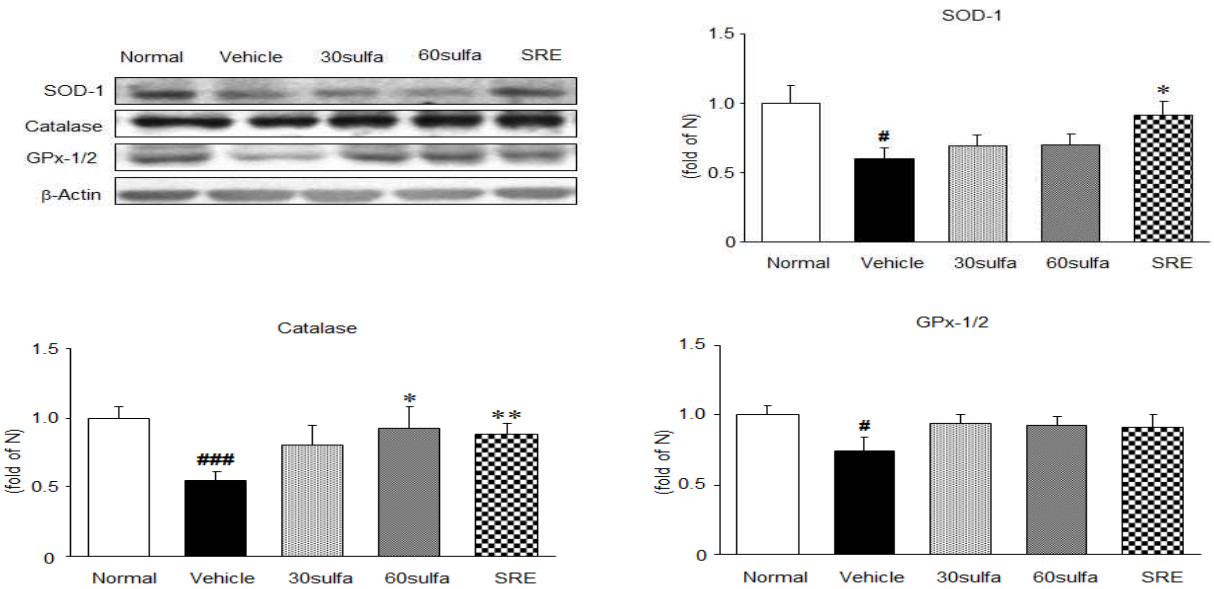


Figure 3. Effect of supercritical heat-treated radish extract(SRE) on the expression of endogenous antioxidant enzymes in serum. SOD, Catalase, GPx-1/2 protein expressions. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: ##P <0.01, ###P <0.001 versus normal mice and *P <0.05, **P <0.01 versus DSS control mice.

3.3. ROS overexpression activates MAPK including p-p38 and p-ERK and NF-κB

ROS overexpression activates MAPK including p38 and p-ERK. The MAPK cascades on p38 and p-ERK are proving to play major roles in the regulation of intracellular metabolism and gene or protein expression in many parts, including disease, apoptosis, and cellular responses to external stresses. Furthermore, the phosphorylation of p-p38 and p-ERK, MAPK are also implicated by leading to the activation of nuclear transcriptions factors[14,15]. In this study, increased expressions of p-ERK and p-p38 were observed in colon of DSS control group ($P < 0.05$). As expected, SRE and sulfasalazine treatment were decreased via inhibition of their upstream c-Fos protein expression. Herein, SRE supplementation significantly attenuated activation of not p-ERK but p-p38 ($P < 0.05$) (Figure 4). As an important nuclear transcription factor, NF-κBp65 controls several important physiological processes, as well as immune and inflammatory responses. Prior to activation, NF-κB p65 is complexed with IκBα, an inhibitory protein keeping NF-κBp65 inactive state in the cytoplasm. Induced by various stimuli, NF-κBp65 is released and translocates from cytoplasm into the nucleus due to IκBα phosphorylation, ubiquitinylation, and degradation. Attempts to control mucosal inflammation through the use of agents that suppress the NF-κBp65 pathway have achieved some success in mouse models. Similarly, SRE treatment has been shown to suppress the activation of NF-κBp65 by inhibition of IκBα phosphorylation. Above all, SRE supplementation was much lower than sulfasalazine alone. ($P < 0.01$) (Figure 5).

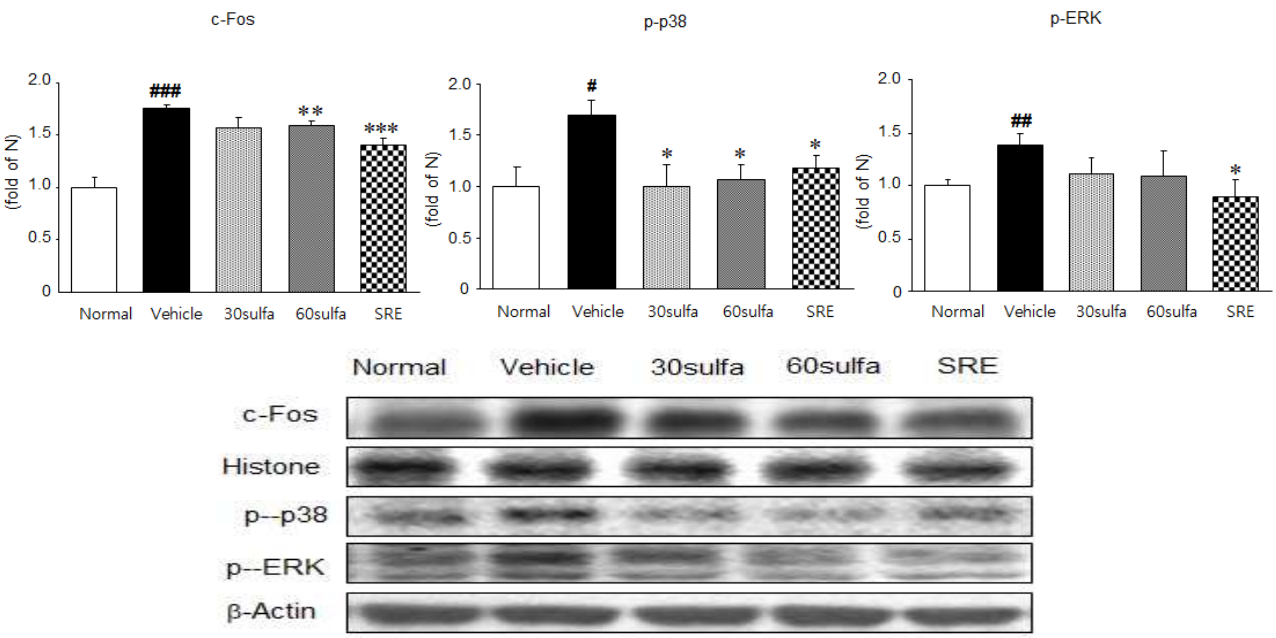


Figure 4. c-Fos, p-p38, and p-ERK protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: #P <0.05, ###P <0.001 versus normal mice and *P <0.05, ***P <0.001 versus DSS control mice.

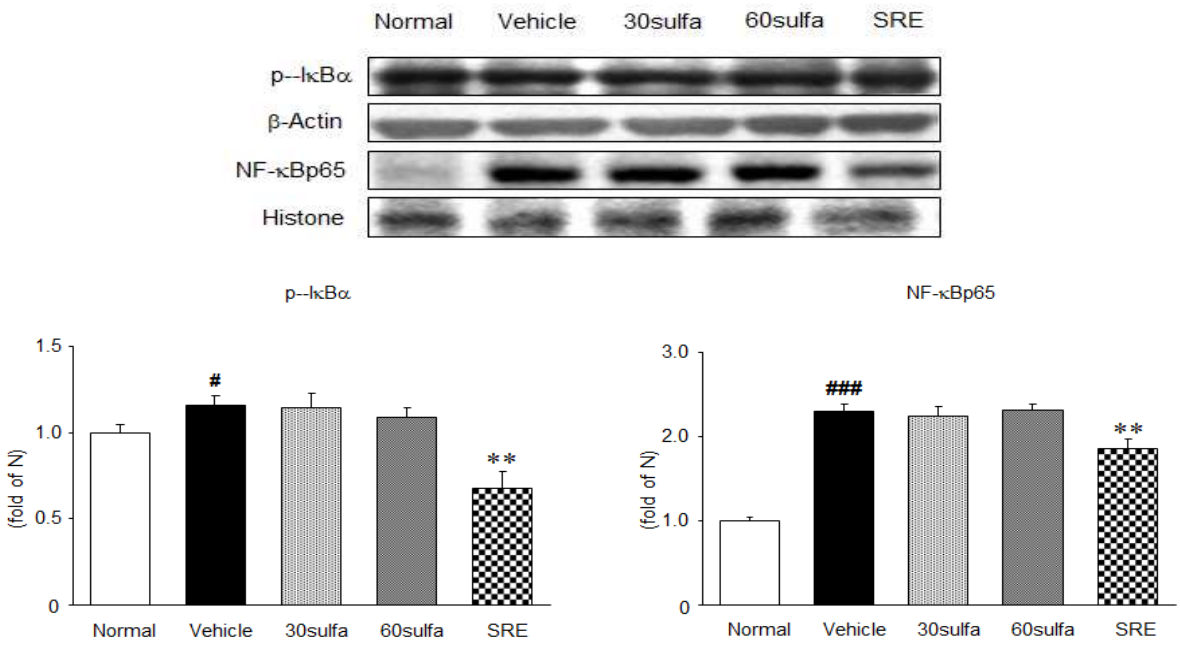


Figure 5. p- IκBα and NF-κBp65 protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: #P <0.05, ###P <0.001 versus normal mice and **P <0.01 versus DSS control mice.

3.4. Pro-inflammatory

NF-κB participates in controlling the activation of various pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) and cytokines such as IL-1, and tumor necrosis factor-α (TNF- α), supporting a critical role in the pathogenesis of UC. As the result, the activation of NF-κB results in disruption of the oxidant/antioxidant balance. TNF-α is crucial in recruiting immune cells at the sites of damaged tissues and in the pathogenesis of UC. TNF- α and IL-1β as well as COX-2 and iNOS were noticeably amplified in DSS control group. Our results also indicate that SRE significantly inhibits the induction of COX-2 and iNOS expressions and the production of pro-inflammatory cytokines such as TNF-α and IL-1β. These protein levels were down-regulated to nearly normal levels (Figure 6). MCP-1 promotes monocyte infiltration into inflamed tissues and elevated levels of MCP-1 can be found in the intestinal mucosa of IBD patients. Accordingly, reduced MCP-1 by SRE treatment might reduce the attraction of inflammatory cells into the intestine and thereby decrease inflammatory responses (Figure67). Several studies have reported that TNF- α causes an increase in endothelial permeability and then leads to neutrophils recruitment to the gut in part through stimulating the synthesis of intracellular adhesion molecule (ICAM). ICAM-1 is up-regulated at sites of inflammation. Similar to this study, DSS control group significantly increased compared with normal group, whereas SRE treatment showed a tendency to a decrease.

3.5. Apoptosis

Apoptosis is considered to prevent excessive accumulation of non-functional cells in the tissue. Excessive exposure of intestinal mucosa to ROS under inflammatory conditions increases epithelial cell apoptosis, which is likely to change epithelial barrier integrity and contributes to intestinal damage. Bcl-2 is regarded as a pro-survival molecule, whereas Bax is a pro-apoptotic molecule as it binds to and antagonizes the effects of Bcl-

2. Thus, Caspase-3 activation is an important event in cell death. SRE showed a substantial down-regulation of pro-apoptotic genes, such as Bax and Caspase 3 ($P < 0.001$, $P < 0.05$, resp). Meanwhile, the Bcl-2 protein expression during UC didn't show a marked difference as only a mild increase (Figure 7).

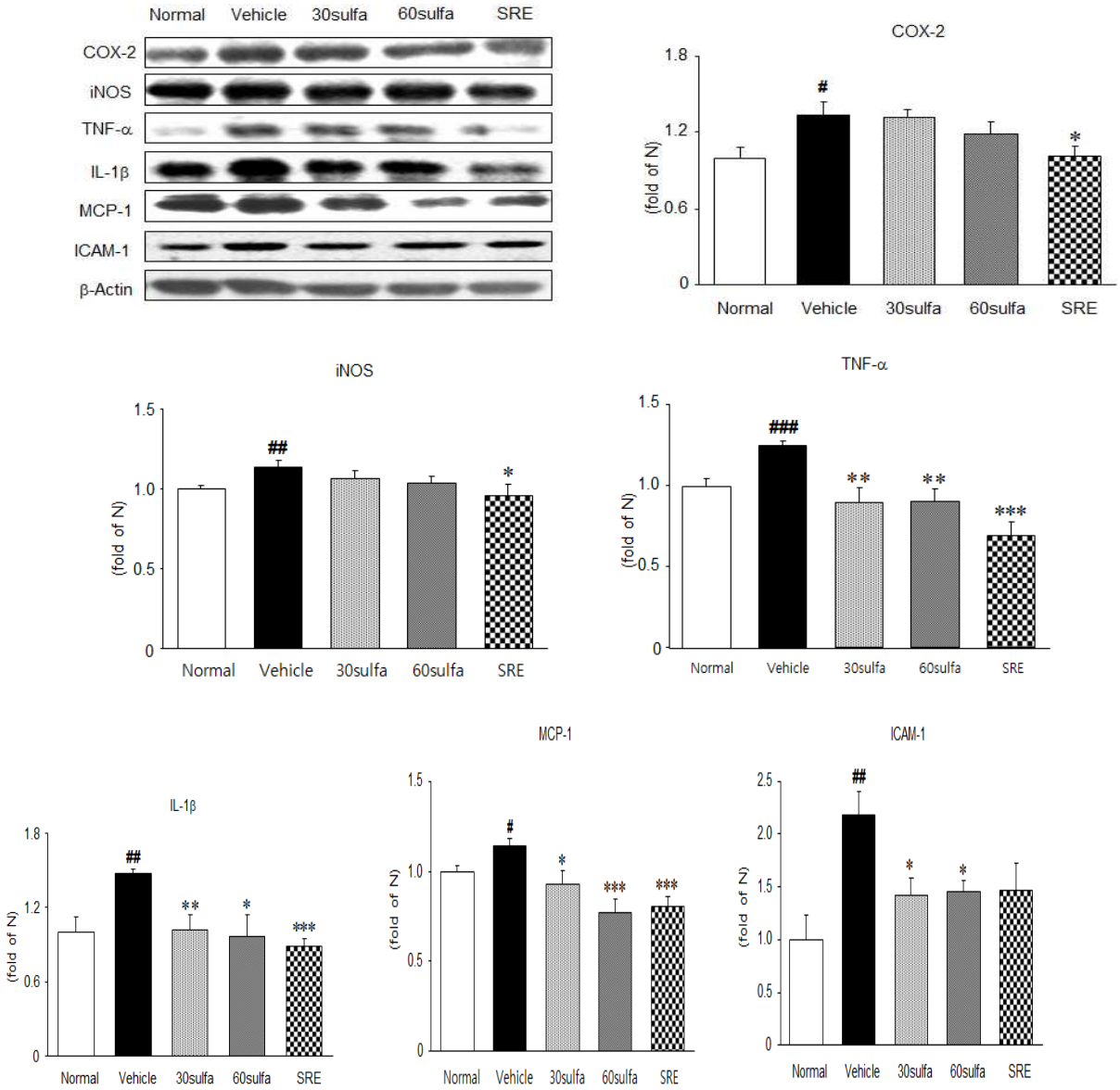


Figure 6. COX-2, iNOS, MCP-1, ICAM-1, TNF-α, and IL-1β protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: ##P <0.01, ###P <0.001 versus normal mice and *P <0.05, ***P <0.001 versus DSS control mice.

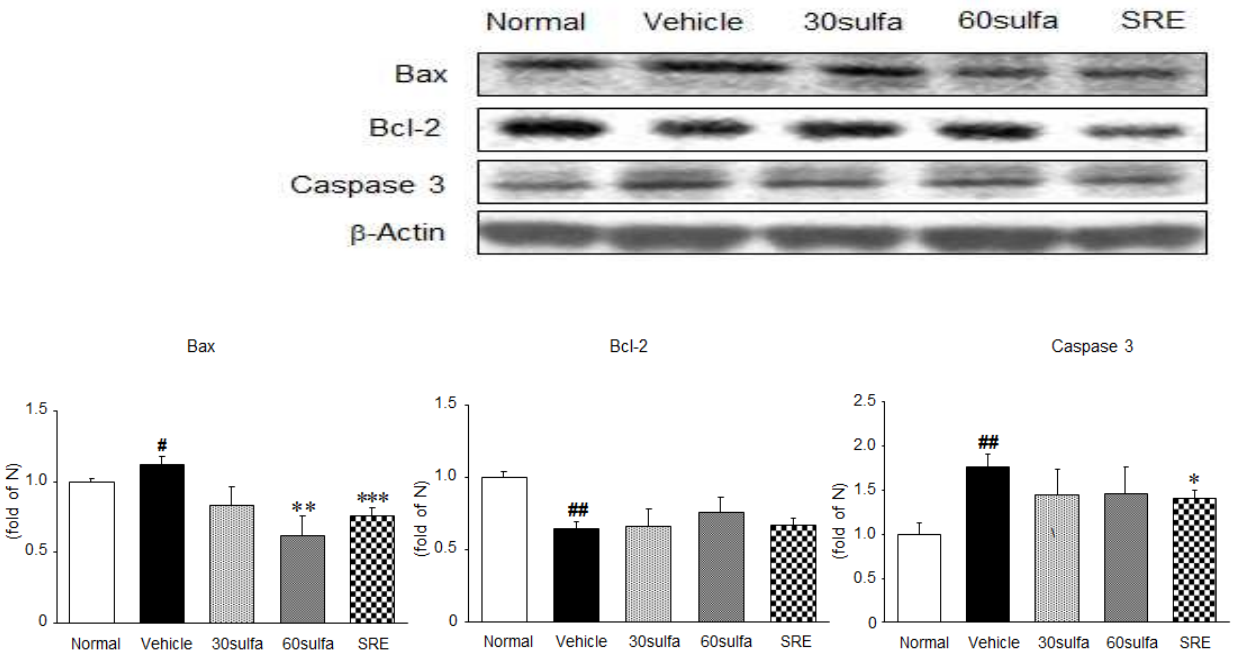


Figure 7. Bax, Bcl-2, and Caspase 3 protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: #P <0.05, ##P <0.01 versus normal mice and *P <0.05, ***P <0.001 versus DSS control mice.

5. Conclusion

To achieve the object of the research, there is provided a pharmaceutical composition for Ulcerative Colitis that contains a supercritical heat-treated radish extracts as an active ingredient. The supercritical heat-treated radish is more preferably subjected to a heat treatment prior to extraction. A preliminary experiment revealed that the heat treatment of radish prior to extraction made an effect to strengthen the physiological activities of the radish, which remarkably increased with an increase in the temperature for the heat treatment.

DSS-treated mice developed symptoms similar to those of human UC, such as severe bloody diarrhea and weight loss. Supercritical heat-treated radish extracts, as well as sulfasalazine, suppressed colonic length and mucosal inflammatory infiltration. In addition, supercritical heat-treated radish extracts treatment significantly reduced the expression of pro-inflammatory signaling molecules through suppression both mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF- B) signaling pathways, and prevented the apoptosis of colon. Moreover, supercritical heat-treated radish extracts administration significantly led to the up-regulation of anti-oxidant enzyme including SOD and Catalase. The supercritical heat-treated radish extract findings suggest that effective inhibitor of DSS-induced colitis in mice. The administration of the supercritical heat-treated radish extracts to mice treated with DSS attenuated acute inflammation and apoptosis in the colon. Above all, the supercritical heat-treated radish extract may exert the similar protective effect by sulfasalazine alone. Accordingly, the supercritical heat-treated radish extract may be a promising herbal formula combined with sulfasalazine in the treatment of ulcerative colitis field.

In accordance with the experimental results, there is also provided a health function food composition for preventive effects of colon cancer and inflammatory bowel disease that contains a supercritical heat-treated radish extract s as an active ingredient.

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