

The Preventive Effects of Colon Cancer and Inflammatory Bowel Disease of Supercritical Heat-Treated Radish Extracts

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Abstract

There is a strong connection between the diet rich in antioxidants and the decreased incidence of inflammatory bowel disease and cancerous diseases. Diets that are rich in anti-oxidants particularly include fruits and vegetables containing the high amounts of vitamin A-E, carotenoids, and minerals. The supercritical heat-treated radish extracts of the research result had an inhibitory effect on the development of aberrant crypt foci (ACF), namely, preneoplastic lesions having a potential to become cancer cells and reduced the number of the aberrant crypt foci (ACF) consisting of four or more aberrant crypts (AC) having high risk to become tumors by about half. The supercritical heat-treated radish extracts can reduce the incidence of preneoplastic lesions having a high risk of developing cancer by about 28 %. 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- κ 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.

Keywords: Anti-colon cancer, Inflammatory bowel disease, Supercritical heat-treated radish, Ulcerative colitis, Dextran sodium sulfate

1. Introduction

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 colon cancer, which is on the rise. Another cause of colon cancer is colon-related disorders 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. According to the report released by Statistics Korea, the mortality rates for colon cancer markedly increased between 2001 and 2016 by 73 % from 9.5 to 16.5 per 100,000 men and women per year and exceeded those for stomach cancer in 2016 for the first time since 1983, at which time the cancer mortality survey started in Korea.

Cancer development, carcinogenesis, involves the transformation of a normal cell into a cancer cell, which is a multistage process consisting of initiation, promotion, and progression. The first stage, initiation, is an irreversible genetic alteration where cellular genome undergoes mutations induced by exposure of DNA to cancer-causing substances (carcinogens), while the cell merely has the potential to become a cancer cell unless under prolonged exposure to tumor promoters. The next stage, promotion, is a relatively prolonged, creeping process that involves proliferation of the initiated cell induced to create colonies of cells in the form of preneoplastic lesions. Unlike the initiation stage, promotion is reversible to the previous stage when the causes are completely removed. The final stage, progression, is a process that benign lesions become malignant to show progressively increased invasiveness and develop the ability to metastasize.

Aberrant crypt foci (ACF) are clusters of abnormal tube-like glands in the lining of the colon and rectum and considered as one of the preneoplastic lesions that can progress to colorectal cancer (CRC). Colonic preneoplastic lesions can be induced by the administration of chemical carcinogens such as DMH (1,2-dimethylhydrazine) or AOM (azoxymethane) to rodents in which DMH or AOM forms cancer-causing methylcarbonium ions reactive to DNA through the metabolism in the liver. In the rodents' colonic mucosa, aberrant crypts (AC) appear distorted with large overlapping nuclei and pericryptal zones at least twice or three times larger than normal crypts, and collections of the distorted aberrant crypts form preneoplastic lesions called aberrant crypt foci (ACF). Prolonged proliferation of the preneoplastic lesions raises the risk of developing colon cancer[1-2]..

Ulcerative colitis (UC) with high incidences worldwide, commonly referred as chronic inflammatory bowel diseases (IBD) and characterized by a uncontrolled inflammatory condition of the intestinal mucosa. The main symptoms of UC are abdominal pain, mucous and bloody diarrhea, weight loss, and anemia. Generally, recommended therapies for UC patients include anti-inflammatory drugs (corticosteroid; prednisolone and aminosalicylic acid; sulfasalazine), immunosuppressants (thiopurines; azathioprine, 6-mercaptopurine and 6-thioguanine), antibiotics (metronidazole, ciprofloxacin) [3-5]. Herein, sulfasalazine, a composed of 5-aminosalicylic acid and sulfapyridine, has been used as a standard-of-care in UC for decades, however, it generates excessive oxidative stress after high dosage and long term intake, resulting in severe adverse symptoms, such as blood disorders, hepatotoxicity, hypospermia, and male infertility [6, 7]. Accordingly, a sole treatment of sulfasalazine is not entirely satisfactory. Therefore, therapeutic strategy for UC has a need to focus on replace current therapy, in addition, the new therapy has to act locally at the site of inflammation to maximize efficacy, to increase convenience, and to minimize side effects [8].

The radish(*Raphanus sativus* L.) is a vegetable of the family Cruciferae containing volatile sulfur-compounds that cause its unique spiciness. The spiciness peculiar to the radish is caused by the production of thiocyanate and isothiocyanate released enzymatically from the thioglucoside contained in the radish as a result of the enzyme glucosidase activity when the radish is cut to break cells. The radish contains a larger amount of free amino acids, sugars, calcium, phosphorus, etc. than other vegetables. The root of radishes contains sugar components like glucose and fructose and other ingredients, such as coumaric acid, caffeic acid, 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 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. The supercritical heat-treated radish extracts had been widely used as herbal medicine for treating cold related disorder such as common cold and influenza [9, 10]. Besides, a variety of pharmacological effects have been suggested: anti-inflammatory action, anti-bacterial activity, anti-platelet aggregation, sedative effect, improvement of blood circulation, and inhibition of cancer metastasis [11]. So, the present study was conducted to evaluate the pharmacological preventive effect of colon cancer and inflammatory bowel disease 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/cm² 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 °C, 120 °C, 130 °C, 140 °C, or 150 °C 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 CO₂ extraction using butylene glycol as a co-solvent at 40 to 80 °C under pressure of 200 to 500 bar. The extracted liquid was captured, freeze-died and then put into use for the experiment materials.

2.2 Assessment of inhibitory efficacy on colon cancer

50 male F344 rats (SLC Japan, Tokyo, Japan) aged 4 weeks were obtained for the measurement of preneoplastic lesions (aberrant crypt foci, ACF) for colon cancer. The animals were housed three per polycarbonate cage in a temperature-controlled room (24±0.5°C) with a relative humidity 55±5%, a 12 hours light/12 hours dark cycle, and a luminance intensity of 150 to 300 Lux. They were maintained on a CRF-1 basic diet (Charles River Japan Inc., Kanazawa, Japan) and purified water ad libitum. 1,2-Dimethylhydrazine (DMH), a carcinogen for colon cancer, was purchased from Fluka (Switzerland). DMH (20 mg/kg body weight) was dissolved in sodium citrate buffer before use, and rats were given subcutaneous injections of DMH in the head and neck regions twice a week for 2 weeks (four administrations in total). To determine the inhibitory effect on colon cancer, the radish extract (HRE) prepared by steaming under pressure according to the Example 1 was added to food (CRF-1 basic diet) to a concentration of 0.32% w/v and fed to the rats.

2.3 Examination of preneoplastic lesions for colon cancer

The aberrant crypt foci (ACF) and aberrant crypts (AC) in the rat's colonic mucosa were methylene blue-stained as described by Bird et al. and observed for visual inspection. More specifically, the rats were sacrificed at twelve weeks of the start of the experiment. With the lumen swollen in a mixed solution of physiological saline and 10% neutral buffered formalin (1:1), colon tissues were cut out along the mesenteric taenia from colon to anus and fixed flat on a filter paper. After fixing of colon tissues with 10% buffered neutral formalin, the colon tissues were divided into three segments and stained with a 0.3% solution of methylene blue for 1 to 2 min for ACF and AC counting. Under a light microscope (x40, x100), the stained colon tissues were carefully

examined to determine the numbers of aberrant crypt foci (ACF) and aberrant crypts (AC) in the colonic mucosa.

2.4 Experimental animals and induction of colitis

Eight-week-old male Balb/c mice weighing 22–24 g were purchased from Orient (Gyeonggi-do, Korea). Mice were maintained under a 12 hours light/dark cycle, housed at a controlled temperature ($24\pm 2^{\circ}\text{C}$) 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 ($n = 9/\text{group}$). 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 [12-14].

2.5 Measurement of ROS level in the serum

Serum ROS level was measured by employing the method of Ali et al. [15-17]. 25 mM DCFH-DA was added to the serum. After incubation for 30 min, the changes in fluorescence values were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm.

2.6 Preparation of cytosol and nuclear fractions

The Protein extraction was performed according to the method of Komatsu with minor modifications [18]. Colon tissues for cytosol fraction were homogenized with ice-cold lysis buffer A (250 mL) containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2mM 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 ($13,400 \times g$ for 2 min at 4°C) using Eppendorf 5415R (Hamburg, Germany), the supernatant liquid (cytosol fraction) was separated into 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 ($13,400 \times g$ 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.

2.7 Immunoblotting analyses

For the estimation of c-Fos, NF-Bp65, and histone, 12g of protein from each nuclear fraction was electrophoresed through 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 hours, and then incubated with primary antibodies (c-Fos, NF-Bp65, and histone) and overnight at 4°C . 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, 7.5g protein of each cytosol fraction of NOX4, p47phox, Rac1, Bax, Bcl-2, Caspase 3, SOD, Catalase, GPx-1/2, HO-1, COX-2, iNOS, TNF-IL-1, and β -actin was electrophoresed through 8-12% 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 mouse[18].

2.8 Hematoxylin and eosin (H/E) stain of colon tissue

For microscopic evaluation, intestine tissue was fixed in 10% neutral-buffered formalin and, after embedding in paraffin, cut into 2m sections and stained using hematoxylin and eosin (H/E) for microscopic evaluation. The stained slices were subsequently observed under an optical microscope and analyzed using the i Solution Lite software program (Innerview Co.).

2.9 Statistical analysis

The data are expressed as the mean \pm 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 Clinical sign, body weight and water intake

The food and water intakes of the rats during the administration sessions were measured in order to determine the effects of drug administration on the body weight of the rats. The measurement results were as presented in Table 1. As can be seen from the Table 1, the groups were all similar in food intake (13.5 to 14.5 g/day) and water intake (20.8 to 22.1 mL) with no significant difference. Throughout the experiment, none of the rats showed any specific clinical sign considered resulting from the test substances.

Table 1. Clinical sign, body and water intake.

Table 1. Feed and water consumption by the rats treated with DMH alone or in combination with supercritical heat-treated radish extracts.

	Food intake (g/day)	Water intake (ml/day)
Control DMH	14.5 \pm 0.5	21.3 \pm 1.5
DMH	13.5 \pm 1.0	21.5 \pm 1.9
DMH + HRE	13.8 \pm 0.9	20.8 \pm 1.5
DMH \rightarrow HRE	14.3 \pm 1.5	22.1 \pm 1.8
HRE 12 wk	15.3 \pm 0.8	22.5 \pm 2.5
HRE 9 wk	14.8 \pm 1.2	21.9 \pm 2.1

3.2 Body weight and organ weight

The rats' organs (liver, spleen, and kidney) were inspected for damage in order to determine the effects of the test substance on the internal organs. Macroscopic examinations in the necropsy found no lesions and nothing significant to report. As can be seen from Table 2, there was no significant difference found in the comparative analysis on the relative organ weights with respect to the absolute organ weights and the body weight in the groups. In relation to the DMH group treated with DMH alone, the DMH+HRE and DMH \rightarrow >HRE groups treated with DMH and the radish extract had slight reductions of body weight and organ weight, but with no significance.

Table 2. Absolute and relative organ weights of rats treated with DMH and or supercritical heat-treated radish extracts.

	Weight (g)			Relative Weight (%)		
	Liver	Spleen	Kidney	Liver	Spleen	Kidney
Control	11.46±0.57	0.78±0.04	2.67±0.26	3.27±0.21	0.22±0.01	0.76±0.05
DMH	10.08±0.86	0.79±0.04	2.65±0.24	2.78±0.19	0.22±0.01	0.73±0.05
DMH+HRE	9.57±0.82	0.75±0.04	2.46±0.22	2.77±0.13	0.22±0.01	0.71±0.05
HRE 12wk	8.89±1.07	0.73±0.05	2.44±0.20	2.60±0.15*	0.22±0.01	0.72±0.02
DMH→HRE	9.43±1.19	0.74±0.03	2.55±0.23	2.82±0.23	0.22±0.01	0.76±0.03
HRE 9wk	9.18±0.58	0.74±0.04	2.41±0.08	2.69±0.15	0.22±0.01	0.71±0.02

3.3 Examination of preneoplastic lesions for colon cancer

Figure 1 presents enlarged images of aberrant crypt foci of the colonic mucosa in the DMH group: ACF consisting of 2 AC in (A); ACF consisting of 3 AC in (B); ACF consisting of 6 AC in (C); different types of ACF in (D). The results of the ACF and AC counting are summarized in FIG. 3 and Table 4.

According to the experiment, the supercritical heat-treated radish extracts had an inhibitory effect on the development of aberrant crypt foci (ACF), namely, preneoplastic lesions having a potential to become cancer cells and reduced the number of the aberrant crypt foci (ACF) consisting of four or more aberrant crypts (AC) having high risk to become tumors by about half. In conclusion, the supercritical heat-treated radish proved to be effective in remarkably reducing the risk of developing colon cancer.

The supercritical heat-treated radish extracts is particularly available for the prophylaxis of colon cancer induced by chemical carcinogens. The chemical carcinogens react to DNA to cause genomic aberration, and the aberrant cells proliferate to form preneoplastic lesions. The preneoplastic lesions are lesions having high potential to become cancer. The supercritical heat-treated radish extracts greatly reduces the development of the preneoplastic lesions. As the formation of preneoplastic lesions is a reversible process, it is considered that the supercritical heat-treated radish extracts promotes the progress of conversion from preneoplastic lesions into normal cells. Accordingly, The supercritical heat-treated radish extracts are particularly available to the people with a high risk of exposure to chemical carcinogens for the sake of prophylaxis of colon cancer. Especially, The supercritical heat-treated radish extracts are more effective in the prophylactic aspect when used before exposure to chemical carcinogens and useful for the prophylactic purpose due to its nontoxicity proved by the dietary uses of radish from old times. Even if used for the prophylaxis purpose after exposure to chemical carcinogens, The supercritical heat-treated radish extracts can reduce the incidence of preneoplastic lesions having a high risk of developing cancer by about 28 %. In conclusion, the supercritical heat-treated radish extracts are also available for use after exposure to carcinogens.

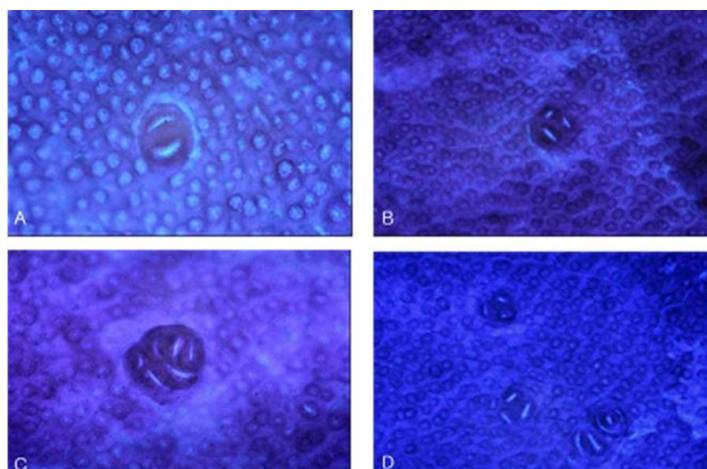


Figure 1. ACF is observed in the colonic mucosa of F344 rats treated with DMH(methylene blue staining($\times 40$)). A: ACF with 2AC, B: ACF with 3AC, C: ACF with 6AC, D: diverse ACF.

Table 3. Effect of supercritical heat-treated radish extracts on the colonic aberrant crypt foci formation induced by DMH in F344 rats

Treatment	Number of rats	Number of total ACF		Total ACF/colon
		$\sum \leq 3\text{ACF}$	$\sum \geq 4\text{ACF}$	
Control	6	-	-	-
DMH alone	12	221.8 \pm 67.2	75.3 \pm 19.8	297.0 \pm 79.2
DMH+HRE (12wk)	12	158.0 \pm 57.9	40.7 \pm 18.4*	208.7 \pm 70.2*
DMH->HRE (9wk)	12	201.7 \pm 55.9	53.8 \pm 22.2	255.5 \pm 71.2
HRE 12wk	4	-	-	-
HRE 9wk	4	-	-	-

Value are means \pm S.D.

(*: Significantly different from DMH alone at * $P < 0.05$, ** $P < 0.01$)

No ACF was found in the F344 rats of the control and HRE 12wk and HRE 9wk groups treated with HRE alone. Yet, ACFs were induced in the rats of the DMH group treated with DMH alone. As for the number of crypts (AC) per ACF, about 75% ACF had one, two or three crypts and about 25% ACF had four or more crypts. The administration of the supercritical heat-treated radish extract(HRE) prepared in the Experiment effectively inhibited the development of aberrant crypts (AC). The inhibitory effects of supercritical heat-treated radish extract(HRE) on AC formation was more significant in the DMH+HRE group treated with DMH and HRE simultaneously than in the DMH->HRE group treated with HRE after exposure to DMH. Particularly, the DMH+HRE group had almost half the number of ACF consisting of four or more aberrant crypts (AC) having high potential to become tumors in the DMH group. As can be seen from Figure 2., the DMH+HRE group showed a reduction of the total number of AC by about 35 % and the total number of ACF by about 30 %. The DMH->HRE group, as compared with the DMH group, had a decrease in the ACF consisting of four or more aberrant crypts (AC) by about 28 % and in the total number of ACF by about 14 %.

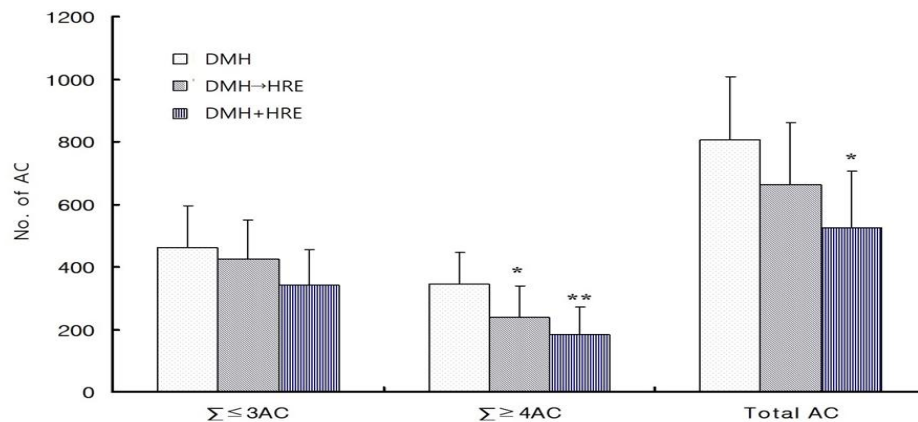


Figure 2. Effect of supercritical heat-treated radish extracts on colonic aberrant crypt(AC) formation induced by DMH in F344 rats.

*,** : Significantly different from DMH alone at * $P < 0.05$, ** : $P < 0.01$.

3.4 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 widely 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. 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 3a). 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 3b). 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 4). 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 4).

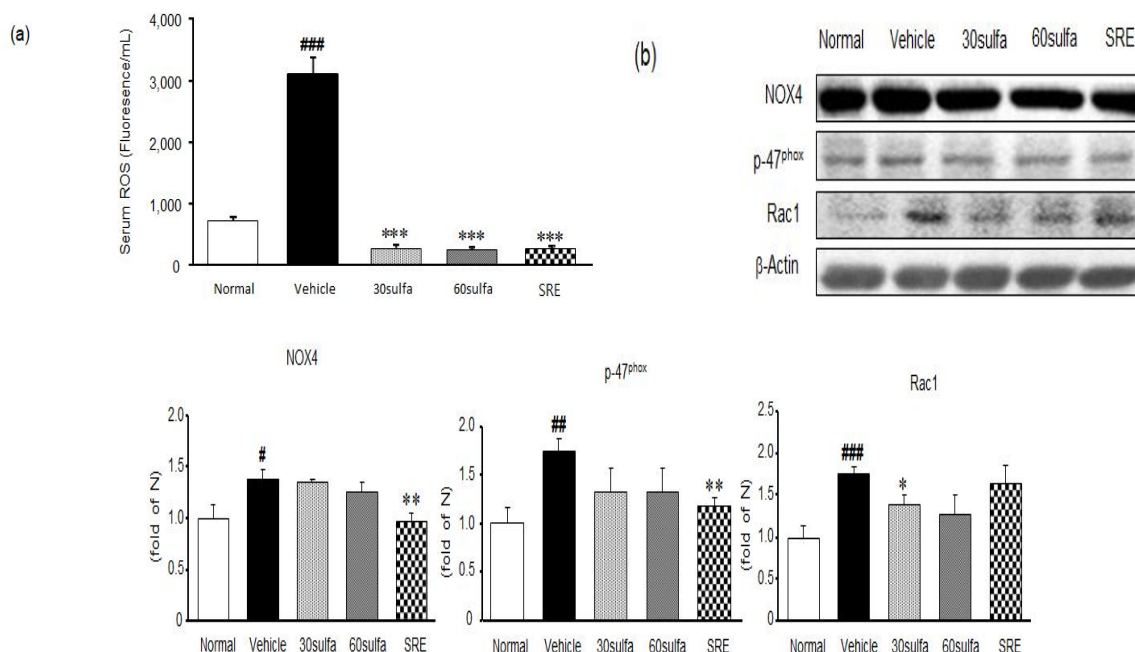


Figure 3. Effect of supercritical heat-treated radish extract(SRE) on the Expression of ROS and NADPH Oxidase in Serum. (a) serum ROS (b) NOX4, p47phox, and Rac 1 protein expressions. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean \pm 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.

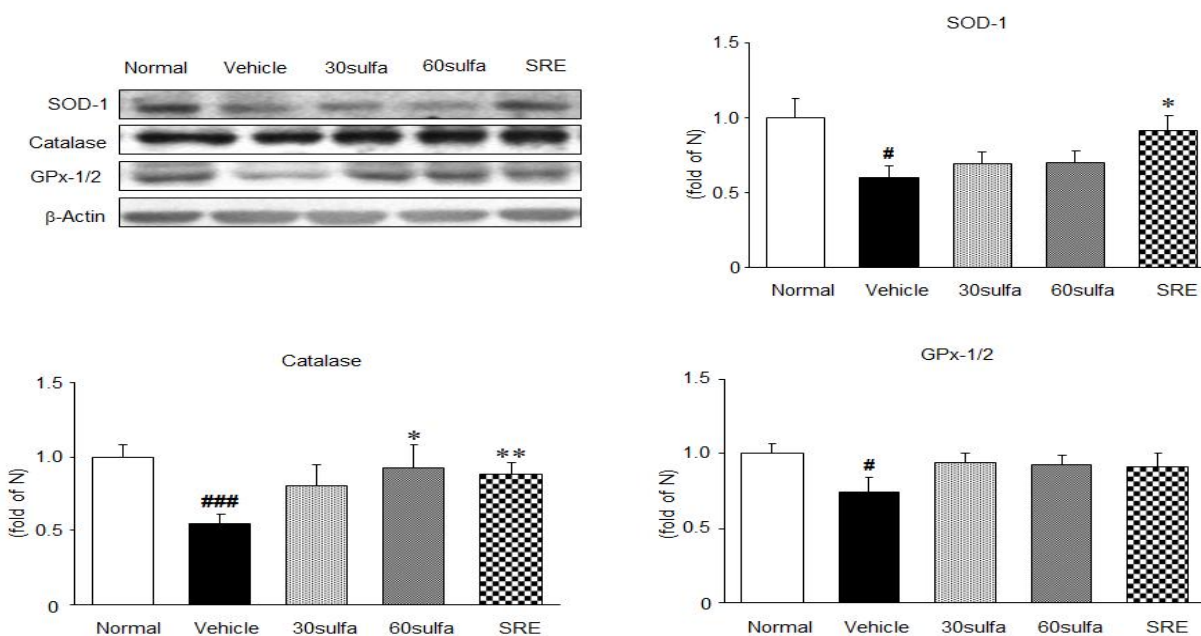


Figure 4. Effect of supercritical heat-treated radish extract(SRE) on the expression of endogenous antioxidant enzymes in serum. SOD, Catalase, GPx-1/2 protein expressions. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean \pm 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.

3.5 ROS overexpression activates MAPK including p-p38 and p-ERK and NF- κ B

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. 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 5). As an important nuclear transcription factor, NF- κ Bp65 controls several important physiological processes, as well as immune and inflammatory responses. Prior to activation, NF- κ Bp65 is complexed with I κ B α , an inhibitory protein keeping NF- κ Bp65 inactive state in the cytoplasm. Induced by various stimuli, NF- κ Bp65 is released and translocates from cytoplasm into the nucleus due to I κ B α phosphorylation, ubiquitinylation, and degradation. Attempts to control mucosal inflammation through the use of agents that suppress the NF- κ Bp65 pathway have achieved some success in mouse models. Similarly, SRE treatment has been shown to suppress the activation of NF- κ Bp65 by inhibition of I κ B α phosphorylation. Above all, SRE supplementation was much lower than sulfasalazine alone. ($P < 0.01$) (Figure 6).

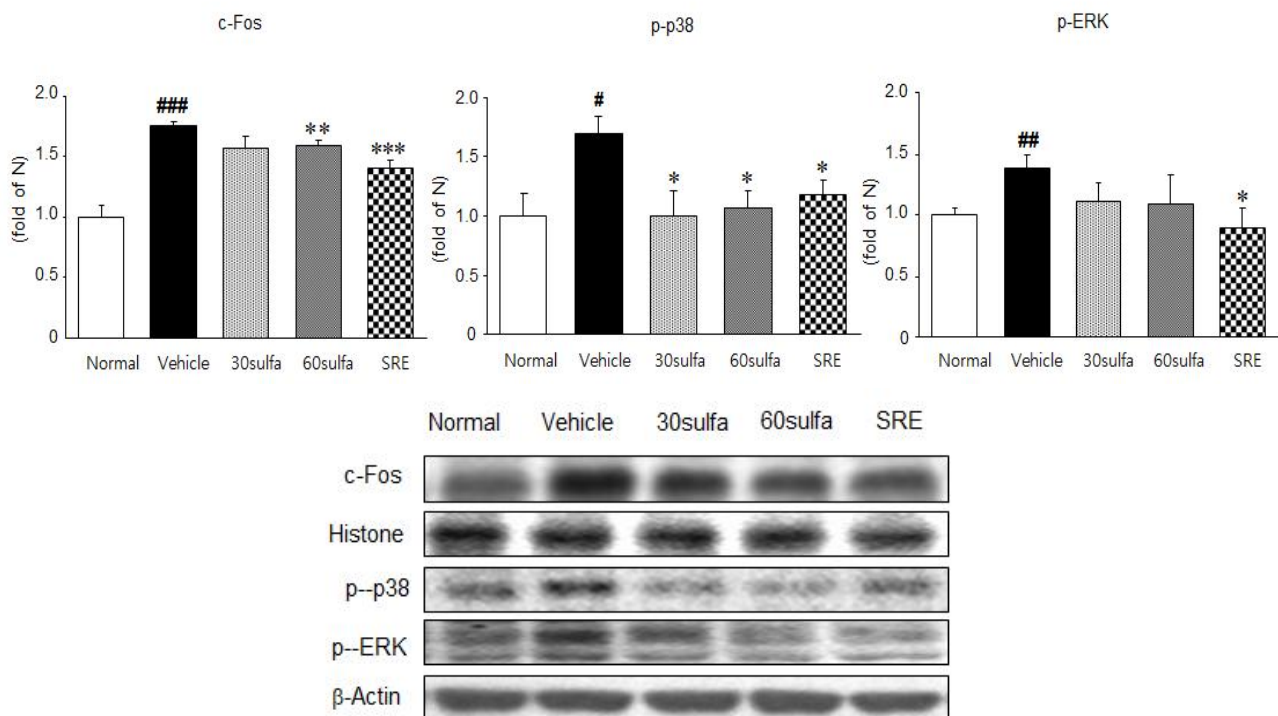


Figure 5. c-Fos, p-p38, and p-ERK protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean \pm S.E.M. (n=7) Significance: # $P < 0.05$, ### $P < 0.001$ versus normal mice and * $P < 0.05$, *** $P < 0.001$ versus DSS control mice.

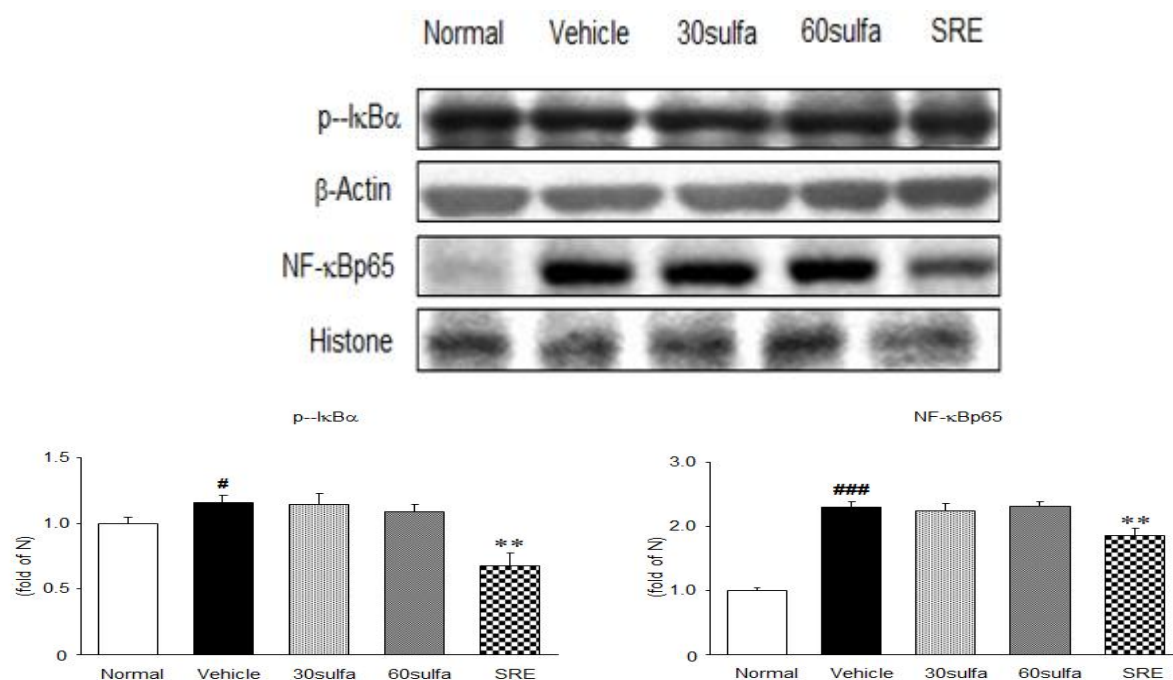


Figure 6. p-IκBα and NF-κBp65 protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60 mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: [#]P <0.05, ^{###}P <0.001 versus normal mice and ^{**}P <0.01 versus DSS control mice.

3.6 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 7). 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 (Figure 7). 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.

3.7 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 8).

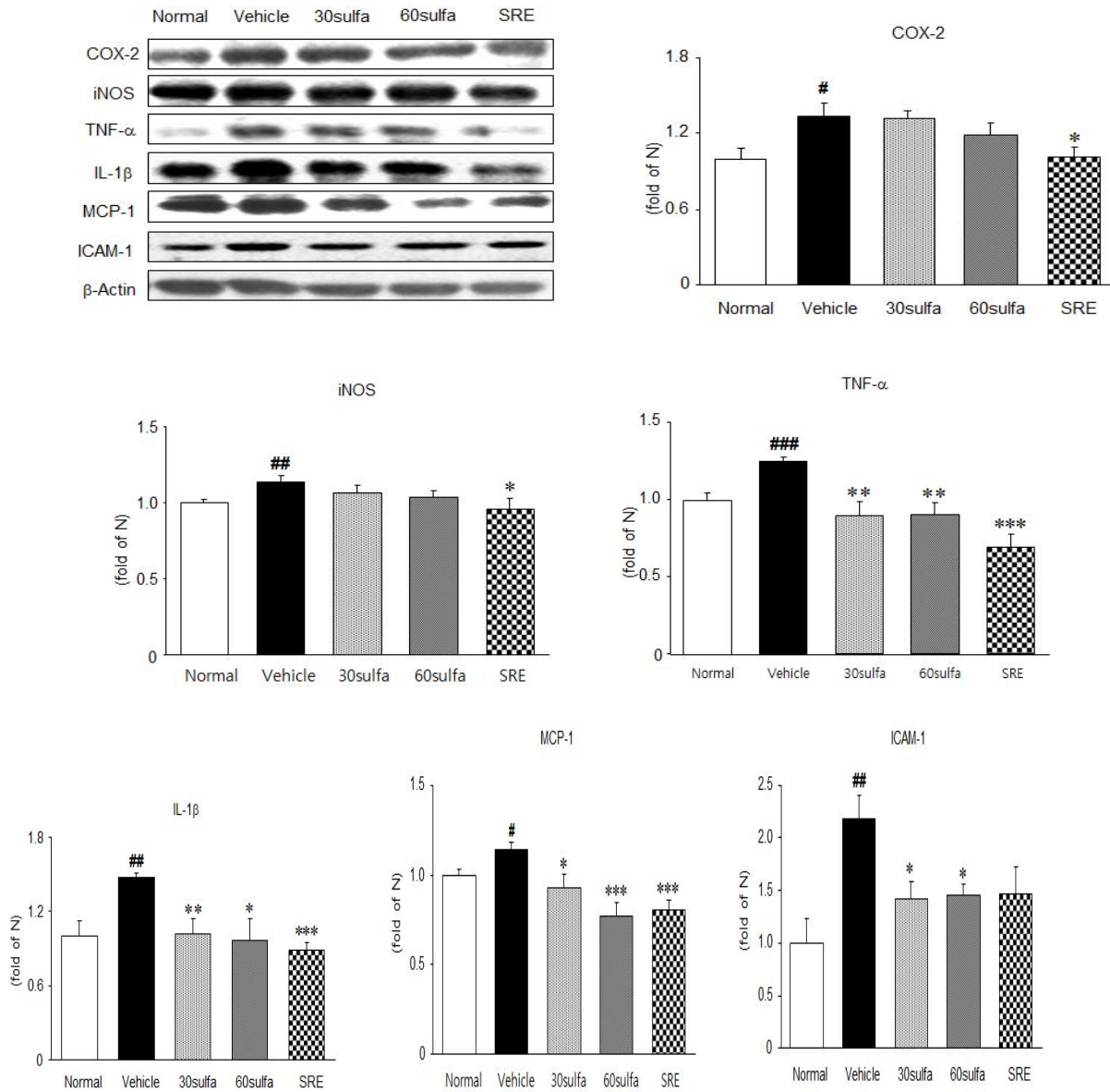


Figure 7. COX-2, iNOS, MCP-1, ICAM-1, TNF-α, and IL-1β protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa, sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean ± S.E.M. (n=7) Significance: ###P < 0.01, ###P <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

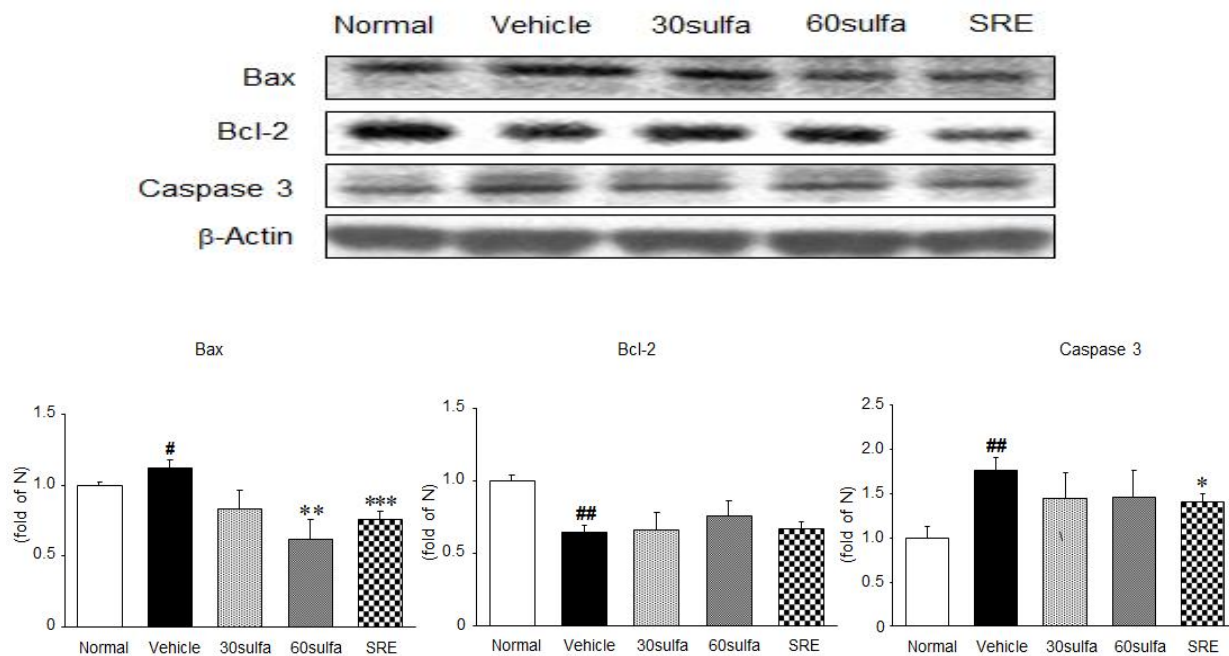


Figure 8. Bax, Bcl-2, and Caspase 3 protein expressions in DSS-induced colitis. Normal, normal mice; Vehicle, DSS control mice; 30sulfa; sulfasalazine 30 mg/kg-treated mice; 60sulfa, sulfasalazine 60mg/kg-treated mice. Data are mean \pm S.E.M. (n=7) Significance: #P <0.05, ##P <0.01 versus normal mice and *P <0.05, **P <0.01, ***P <0.001 versus DSS control mice.

5. Conclusion

There is a strong connection between the diet rich in antioxidants and the decreased incidence of inflammatory bowel disease and cancerous diseases. Diets that are rich in anti-oxidants particularly include fruits and vegetables containing the high amounts of vitamin A-E, carotenoids, and minerals. To achieve the object of the research, there is provided a pharmaceutical composition for preventive effects of colon cancer and inflammatory bowel disease 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.

The supercritical heat-treated radish extracts of the research result had an inhibitory effect on the development of aberrant crypt foci (ACF), namely, preneoplastic lesions having a potential to become cancer cells and reduced the number of the aberrant crypt foci (ACF) consisting of four or more aberrant crypts (AC) having high risk to become tumors by about half. In conclusion, the radish extract proved to be effective in remarkably reducing the risk of developing colon cancer.

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- κ 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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