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Protective effect of decursin and decursinol angelate-rich *Angelica gigas* Nakai extract on dextran sulfate sodium-induced murine ulcerative colitis

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ABSTRACT

Objective: To investigate the anti-inflammatory effects of decursin and decursinol angelate-rich *Angelica gigas* Nakai (AGNE) on dextran sulfate sodium (DSS)-induced murine ulcerative colitis (UC).

Methods: The therapeutic effect of an AGNE was analyzed in a mouse model of UC induced by DSS. Disease activity index values were measured by clinical signs such as a weight loss, stool consistency, rectal bleeding and colon length. A histological analysis was performed using hematoxylin and eosin staining. Key inflammatory cytokines and mediators including IL-6, TNF-α, PGE_2, COX-2 and HIF-1α were assayed by enzyme-linked immunosorbent assay or western blotting.

Results: Treatment with the AGNE at 10, 20, and 40 mg/kg alleviated weight loss, decreased disease activity index scores, and reduced colon shortening in mice with DSS-induced UC. AGNE inhibited the production of IL-6 and TNF-α in serum and colon tissue. Moreover, AGNE suppressed the increased expression of COX-2 and HIF-1α and the increased production of PGE_2 in colon tissue were observed in mice with DSS-induced UC. Additionally, histological damage was also alleviated by AGNE treatment.

Conclusions: The findings of this study verified that AGNE significantly improves clinical symptoms and reduces the activity of various inflammatory mediators. These results indicate the AGNE has the therapeutic potential in mice with DSS-induced UC.

1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease, has been defined as relapsing inflammation in the digestive system. The course of IBD is unpredictable and its pathogenesis is not fully known [1]. UC, the chronic inflammation of the colon, is affected by factors including abnormal immune system of microbiota, a disorder of the cell wall caused by antibodies in the intestine, and or pathogenesis of epithelial cell of colon [2]. The primary symptoms of UC are abdominal pain, rectal bleeding, and diarrhea mixed with blood [3]. The wall of the colon consists of four sections: the mucosa, submucosa, muscular layer, and serous membrane. UC develops from pathological changes to the mucosa and submucosa, in which neutrophilic and eosinophilic leukocytes infiltrate the colon and cause the surrounding blood vessels and crypt abscess to become inflamed, causing ulcer formation [4]. Cytokines secreted from intestinal epithelial cells are important markers of the intestinal immune system, and regulate the inflammatory response in UC. Elevated levels of cytokines, including interleukin (IL)-6, IL-8, IL-12, and tumor necrosis factor-alpha (TNF-α), in serum and mucosal tissue samples have been reported in patients with IBD [5]. Cyclooxygenase (COX) has been implicated the progression of inflammation. Among them, COX-2 is not expressed under normal conditions in healthy tissues and cells, but is expressed under inflammation conditions. COX-2 is known to stimulate prostaglandins involved in the mediation of

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inflammation. In particular, prostaglandin E_2 (PGE_2) and COX-2 are expressed in the inflamed tissues of UC patients [6]. Hypoxia inducible factors (HIFs), which are hypoxia-mediated gene-regulating transcription factors, link inflammatory pathways. Among the HIFs, HIF-1 is used as a marker of inflammatory disease [7]. Expression of HIF-1α in the colon tissue of patients with UC has been reported to be elevated [8].

Therefore, the regulation of inflammatory cytokines and enzymes in the colon tissues is an important target for colitis treatment. Drugs containing aminosalicylates, such as sulfasalazine (SSZ) and mesalazine, are commonly used for IBD, but these drugs are associated with side effects, including gastrointestinal upset, headaches, and myocarditis [9]. Several phytonutrients that were used in traditional medicine demonstrated anti-inflammatory effects [10–12]. Of those studies, kolaviron, curcumin, and resveratrol were reported to prevent gastrointestinal mucosal damage [13–15]. Therefore, there has been growing interest in recent years in identifying herbal medicines that provide therapeutic benefits without undesirable side effects.

Angelica gigas Nakai (AGNE) is a Korean traditional herbal medicine and is one of the most popular herbal medicines used in Asian countries, including Korea, Japan (*Angelica acutiloba* (*A. acutiloba*)), and China (*Angelica sinensis*). AGNE has been studied extensively and found to contain various substances, including coumarins [16]. Coumarins comprise decursin and decursinol angelate (D/DA), which have been used as a traditional medicine for the treatment of anemia, as a sedative, and as an anodyne or a tonic agent. It was previously reported that the oral acute and subacute toxicity and the genotoxicity of extracts containing approximately 95% D/DA [17,18]. AGNE has been widely used for the treatment of dysmenorrhea, amenorrhea, menopausal syndromes, abdominal pain, injuries, migraine headaches, and arthritis [19,20]. AGNE is also known to exert anti-bacterial and anti-amnesic effects as well as to inhibit acetylcholinesterase, depress cardiac contraction, and activate protein kinase C [21,22]. In addition, D/DA from AGNE has been evaluated for anti-inflammatory effects [23–25]. The therapeutic effects of D/DA in colonic inflammation have not yet been documented, despite the reported anti-inflammatory effect of D/DA. IBD is usually assessed by using mice as animal models of dextran sulfate sodium (DSS)-induced UC. The IBD in these animal models is similar to acute UC in humans, thus, these models are frequently used in colitis research [26]. In the present study, the authors investigated the effects of AGNE on colonic inflammation in a mouse model of UC induced by DSS.

DSS-induced UC in mice is characterized by bloody stools, the mucosal infiltration of inflammatory cells, and ulceration [27]. Therefore, the aims of this study were: 1) to analyze the main bioactive components derived from AGNE; 2) to evaluate inhibition of inflammatory-gene expression of AGNE in mice with DSS-induced UC; 3) to examine clinical signs such as weight loss, reduction of colon length, diarrhea, and bloody feces.

2. Materials and methods

2.1. Animals and reagents

Male ICR mice (4 week old) were obtained from Daehan Biolink Animal Facility (Chungbuk, Korea). The mice were housed in a pathogen-free environment for at least 1 week to allow them to adapt to the environmental changes and were euthanized by using CO_2 inhalation at the end of the study. All animal studies were carried out in accordance with regulations issued by the Institutional Review Board of Daegu Haany University (confirmation number: DHU2016-045). DSS (molecular weight: 36 000–50 000) was purchased from MP Biomedicals (Solon, OH, USA). Anti-mouse TNF-α/IL-6, recombinant TNF-α/IL-6, and biotinylated TNF-α/IL-6 were purchased from BD Bioscience (San Diego, CA, USA). Specific antibodies against COX-2, HIF-1α, and β-actin were obtained from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). SSZ and other chemical reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Preparation and high performance liquid chromatography (HPLC) analysis of AGNE

Fresh AGNE was purchased from the Ginbu GAP Farming Corporation (Pyeongchang, Gangwon, Korea) in 2015. AGNE was air-dried and stored at room temperature. Dried AGNE (4 kg) was extracted with 20 L of 95% ethanol at 80 °C for 4 h in non-woven fabric. The extract was collected and filtered to remove precipitates. The filtrate was concentrated again and the 330 g of concentrate obtained was designated AGNE. The main components present in AGNE were analyzed using an Agilent HPLC system (1100 series, Agilent Technology, Santa Clara, CA, USA) with an Agilent Zorbax SB-C18 column (250.0 mm × 4.6 mm, 5 μm). Signals were detected at 329 nm using a UV monitor (1100 series, Photo-Diode Array UV/Vis detector, Agilent Technology). The D/DA contents of AGNE were quantified using a calibration curve established by injecting dilutions of each standard (62.5–250.0 μg/mL) into the HPLC system (correlation coefficient $\geq$ 0.996; standard curve formula: y = 20.928 28x + 38.319 67). Standards (D/DA) for HPLC analysis were obtained from Chengdu Biopurity Phytochemicals Ltd. (Chengdu, China).

2.3. Induction of UC by DSS and experimental procedures

UC in mice was induced by adding 5% (w/v) DSS to drinking water, which was provided *ad libitum* for 7 d. The mice were examined daily for weight loss, stool consistency, and the presence of gross bleeding. The mice were randomized into 5 groups (n = 7 per group). They received AGNE (10, 20, 40 mg/kg), SSZ (300 mg/kg) as a positive control, and saline as a negative control. AGNE and SSZ were diluted with saline (150 μL) and orally administered once daily starting at day 0 of DSS treatment. The mice were euthanized and assessed after 7 d of DSS administration [28].

2.4. Assessment of disease activity index (DAI)

DAI values were assessed by scoring clinical signs such as a weight loss, stool consistency, and rectal bleeding to evaluate the efficacy of AGNE on DSS-induced rat model [29,30]. In the present study, the values were calculated using the following formula: DAI = (weight loss score) + (stool consistency score) + (occult blood/gross bleeding score). Each variable was expressed in number from 0 to 4 depending on the three scores. Each DAI score was characterized as follows: for body

weight loss, 0 = none, 1 = 1%–5%, 2 = 6%–10%, 3 = 10%–20%, and 4 > 20%; for stool consistency, 0 = normal, 2 = loose stools, and 4 = watery diarrhea; and for blood in the stool, 0 = normal, 2 = slight bleeding, and 4 = gross bleeding. The clinical parameters were chosen to represent the subjective clinical symptoms observed in human UC.

2.5. Assessment of histological score

All trimmed rectums were fixed in 10% neutral buffered formalin. After paraffin embedding, 4-μm-thick sections were prepared. Representative sections were stained with hematoxylin and eosin (H&E) for examination under a light microscope. Histological score was assessed by inflammation severity. Each score was defined as follows: for histological changes, 0 = normal colonic mucosa, 1 = crypt damage less than 1/3, 2 = crypt damage less than 1/3–2/3, 3 = mucosal erosion, and 4 = mucosal erosion or ulcer with significant infiltration of inflammatory cells [28].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-6 and TNF-α in serum and colon tissue were measured using an ELISA as previously described [28]. Briefly, 96-well plates were coated with 100 μL of anti-mouse monoclonal antibody (1 mg/mL and pH 7.4 in phosphate-buffered saline) and incubated overnight at 4 °C. After removal of the antibody, 100 μL of sample was added and incubated at room temperature for 2 h. After washing, 100 μL of biotinylated anti-mouse antibody (0.2 μg/mL) was added, followed by incubation with avidin-peroxidase 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) substrate was added and the optical density was measured at 405 nm with a microplate reader (BioTek, Winooski VT, USA). Standard curves were prepared using serial dilutions of recombinant antibodies. Protein concentrations were measured using bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) [28].

2.7. Western blot analysis

The distal colon lysate was prepared by homogenization with lysis buffer (Thermo Fisher Scientific) and centrifugation (14500 r/min, for 5 min). After protein quantification of the lysate with bicinchoninic acid assay reagent, the sample (20 μg of protein) was separated with 10% SDS-PAGE and transferred to nitrocellulose membranes (Merck Millipore, Millerica, MA, USA). The membranes were then blocked with primary antibodies against COX-2 and HIF-1α and washed 3 times with phosphate-buffered saline with tween 20. Blots were incubated with secondary antibodies for 1 h at room temperature and antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham Corp., Piscataway, NJ, USA). Membranes were analyzed with the bio-imaging program of the High Resolution *In Vivo* Imaging System (Davinch-K, Jeju Island, Korea) [28,31].

2.8. PGE_2 assay

PGE_2 levels were measured using a commercially available kit (R&D System, Minneapolis, MN, USA). Tissue protein extraction reagents were added at a proportion of 1:10 and mixed in phosphate-buffered saline. After blending, the mixtures were centrifuged at 5000 r/min for 5 min and the supernatants were tested for PGE_2 levels. PGE_2 concentrations were determined using the standard curve [31].

2.9. Statistical analysis

The results were presented as mean ± SEM of at least three independent experiments. Results were analyzed by one-way analysis of variance followed by the Student–Newman Keuls test. P values< 0.05 were considered statistically significant.

3. Results

3.1. D/DA was an active component of AGNE

AGNE has been cultivated in Korea and used as a medicinal herb. AGNE contained pyranocoumarin compounds as major components, including D/DA. D/DA exhibited various biological activities, such as aldose inhibition, neuroprotection, and antiplatelet, antibacterial, and anticancer activities [32–35]. Recently, *A. acutiloba* Kitagawa extract exhibited protective effects against intestinal inflammation [36], but there was no information about the exact content of D/DA in *A. acutiloba* Kitagawa extract. To identify the active components of AGNE that could exhibit effects on DSS-induced UC, HPLC analysis was performed. The main components in 5 mg of AGNE were decursin (1.32 mg, 26.3%) and decursinol angelate (1.14 mg, 22.7%). A chromatogram of AGNE along with the standard decursin and decursinol angelate are shown in Figure 1.

3.2. AGNE alleviated clinical signs in DSS-induced UC

Among the various physical or clinical signs (*e.g.* loss of body weight, colon length, diarrhea, occult blood, and bloody feces) [37], the authors first measured the effects on weight change to verify any improvements on UC in the DSS-induced animal models. Clinical signs were checked in mice induced with acute colitis by administering 5% DSS for 7 d along with saline, AGNE (10, 20, 40 mg/kg), or SSZ (300 mg/kg). As shown in Figure 2A, the mice treated with saline, AGNE (10, 20 mg/kg), and SSZ developed acute colitis and a decreased body weight was observed starting on day 5 compared with the control group with just saline. However, an

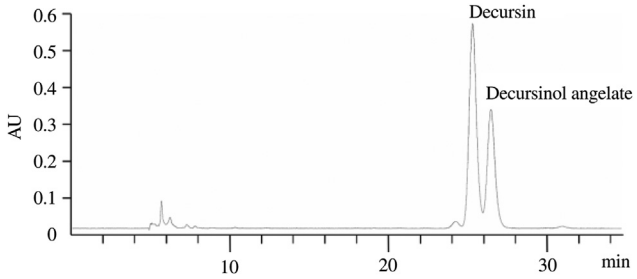

Figure 1. Representative high-performance liquid chromatography profile of AGNE.

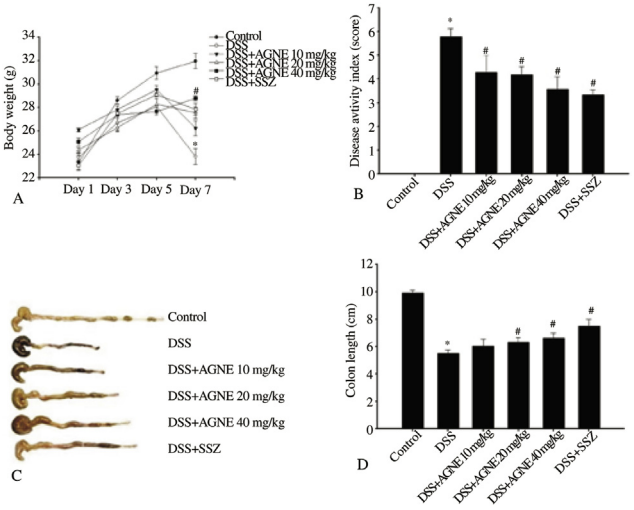

Figure 2. Effect of AGNE on clinical signs in DSS-induced mice.
(A) Body weights. (B) Disease activity index. (C) Colon lengths. (D) Relative colon lengths. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* DSS alone.

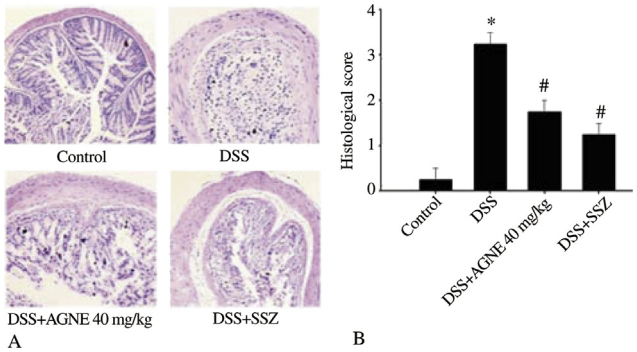

Figure 3. Effect of AGNE on epithelial injury in colonic tissues of DSS-induced mice.
(A) H&E result (10× magnification). (B) Histological score. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* DSS alone.

increased body weight was observed in the AGNE treatment group (40 mg/kg) compared with that in the control group starting at day 5. Weight loss tended to occur in all the groups except in the control group and AGNE treatment group (40 mg/kg). Compared with the DSS group, the AGNE treatment group showed a dose-dependent loss of body weight according to the progression of UC. The effect of AGNE treatment group (40 mg/kg) on weight loss was superior to those of the positive control (SSZ). DSS-induced UC models exhibited clinical signs characterized by loss of body weight, diarrhea, and bloody feces. The effects of AGNE on these symptoms were measured using the DAI. The DAI score was remarkably increased in mice in the saline treated DSS group compared with that in the control group. The AGNE (10, 20, 40 mg/kg) and SSZ treatment groups displayed a significant reduction in DSS-elevated DAI score (Figure 2B). The effect of AGNE treatment group (40 mg/kg) was not superior to those of positive control. Shortening of the colon was a marker that reflected the severity of colorectal inflammation. The mice in the saline treated DSS had a significantly shortened colon length compared with that in the control group. The groups that were administered AGNE (10, 20, 40 mg/kg) and SSZ had a significantly concentration-dependent longer colon length than that of the saline-treated DSS group (Figure 2C and D).

3.3. Histological appearance of colonic tissues of mice with DSS-induced UC were reduced by administration of AGNE

The results of the present study indicated that AGNE displayed a concentration-dependent effect. Therefore, a histological analysis was performed using H&E staining, on colonic tissue from mice treated with AGNE 40 mg/kg because this was the dose that most effectively alleviated UC induced by DSS. As indicated in Figure 3, DSS induced epithelial injury compared with the control group. However, administration of AGNE significantly reduced the number of infiltrating cells and extent of mucosal injury. The severity of the inflammation was scored as indicated in the Materials and methods section. Tissue from mice with UC induced by DSS exhibited higher histological scores than that from mice in the control group. Histological scores were significantly improved in mice treated with AGNE 40 mg/kg.

3.4. AGNE suppressed levels of IL-6 and TNF-α in mice with DSS-induced UC

The results indicated that serum IL-6 and TNF-α levels were significantly increased in all mice administered DSS compared with those in the control group. The serum IL-6 and TNF-α levels of mice treated with AGNE (10, 20, 40 mg/kg) and SSZ were lower than those in the saline-treated DSS group (Figure 4A and B). In particular, the AGNE 40 mg/kg group exhibited lower IL-6 production in serum than that of the SSZ group. In addition, IL-6 and TNF-α levels in the colon tissue of the saline-treated DSS group were significantly higher than those in the control group. The colon tissue IL-6 and TNF-α levels of mice treated with AGNE (10, 20, 40 mg/kg) and SSZ were concentration-dependently lower than those of the mice in the saline-treated DSS group (Figure 4C and D).

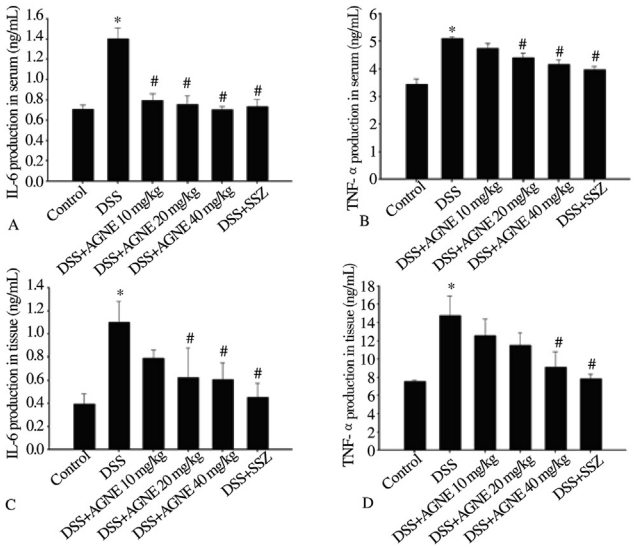

Figure 4. Effect of AGNE on IL-6 and TNF-α levels in DSS-induced mice.
(A, B) Serum IL-6 and TNF-α. (C, D) Tissues IL-6 and TNF-α level. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* DSS alone.

3.5. AGNE suppressed COX-2 expression and PGE_2 production in colon tissue of mice with DSS-induced UC

COX-2 has been reported to mediate the development of inflammatory diseases such as UC [38]. COX-2 was preeminently expressed in colon tissue by DSS compared with that in control, but COX-2 induction was concentration-dependently decreased by AGNE and SSZ administration (Figure 5A). Mice treated with AGNE 40 mg/kg and SSZ exhibited significantly decreased COX-2 levels compared with those in the saline-treated DSS group. Figure 5B demonstrated the effect of AGNE on PGE_2 levels in colon tissue because PGE_2 biosynthesis was catalyzed by COX-2. PGE_2 level of the saline-treated DSS group were significantly higher than it was in the control group. However, the levels of PGE_2 that were administered AGNE (10, 20, 40 mg/kg) and SSZ decreased significantly in a concentration-dependent manner. The results indicated that PGE_2 levels were enhanced following DSS treatment and inhibited significantly by AGNE and SSZ.

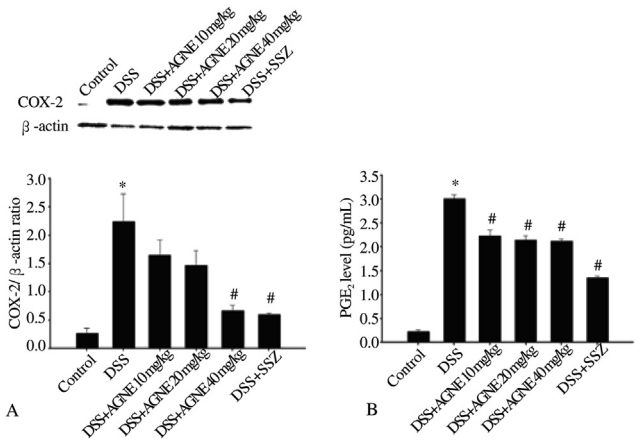

Figure 5. Effect of AGNE on COX-2 and PGE_2 levels in colonic tissues of DSS-induced mice.
(A) COX-2/β-actin ratios. (B) PGE_2 level. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* DSS alone.

3.6. AGNE suppressed HIF-1α expression in colon tissue of mice with DSS-induced UC

HIF-1, a transcription factor related with inflammation, was over expressed in colon tissue of mice with DSS-induced UC [39]. As shown in Figure 6, HIF-1α expression was increased in colon tissue of all mice treated with DSS compared with that in controls, but it was reduced in mice treated with AGNE (10, 20, 40 mg/kg) and SSZ compared with that in mice in the saline-treated DSS group. The relative expression levels of HIF-1α in the mice treated with AGNE 20 and 40 mg/kg were similar to that in the mice treated with SSZ as a positive control.

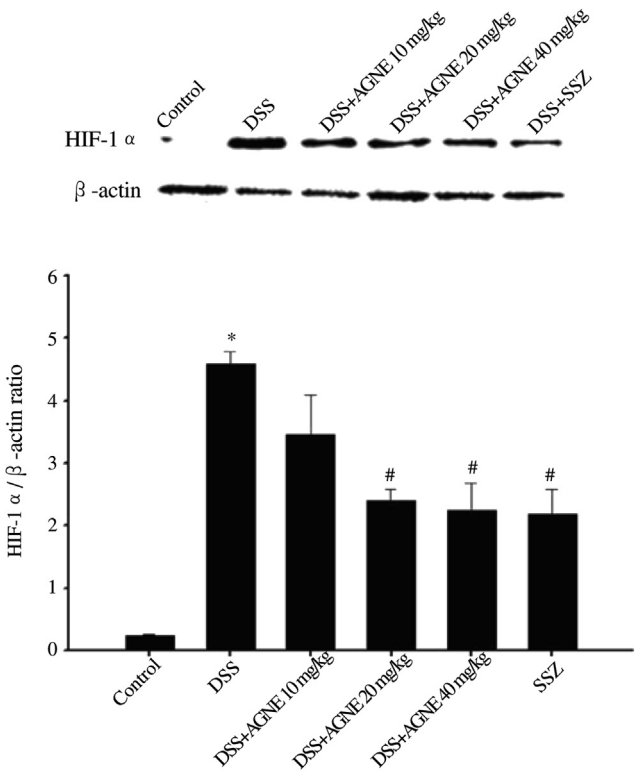

Figure 6. Effect of AGNE on HIF-1α in colonic tissues of DSS-induced mice.
$^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* DSS alone.

4. Discussion

UC used to be more common in the West, where meat is the staple food, but its incidence has been increasing in South Korea in recent years owing to the westernization of dietary habits in the country. UC becomes chronic as defects in the immunomodulatory functions cause immune cells to aggregate at the inflammation site [40]. To date, no clear molecular pathology of UC has been identified. UC is treated with steroids and immunosuppressants to mitigate inflammation, but long-term use of these agents is associated with side effects such as nausea, vomiting, indigestion, headache, and skin rashes [41]. In response to this, the development of natural therapeutic agents that control UC with minimal side effects has gained traction. Accordingly, this study aimed at investigating the efficacy of decursin and decursinol angelate-rich AGNE as an agent that has been used as a folk remedy to treat headache, dizziness, menstrual irregularity, stomachache, distortion, trauma, and diarrhea, for UC.

In this study, the authors examined how AGNE improved UC by using DSS-induced acute UC mouse models, which modeled acute and chronic UC in humans. DSS-induced UC animal models are appropriate to study UC in humans because of the similarity of symptoms such as weight loss, reduced colon length, bloody feces, and ulcer formation in the mucosa of colon epithelial cells [26,27]. The findings of this study showed that the DSS-induced UC group lost weight, with the peak weight loss on day 7. However, weight loss was significantly inhibited in the group that received an AGNE (40 mg/kg) injection. Patients with colitis characteristically show clinical symptoms such as weight loss, diarrhea, and colon bleeding, which are comprehensively represented by the DAI [42]. The DSS-induced UC group had a high DAI, while the AGNE treatment group showed a dose-dependent improvement of clinical symptoms. Furthermore, a decrease in colon length, which is a typical symptom of colitis, was significantly inhibited in the AGNE treatment group in comparison with the DSS-induced UC group. In this study, the authors compared the AGNE treatment group with a positive control, and the improvements of the clinical symptoms in the AGNE treatment group were similar to those in the SSZ group. The authors also observed tissue and cellular changes in the epithelial mucosal cells in the site with active UC in the normal,

DSS, AGNE treatment group (40 mg/kg) and SSZ groups via histochemical staining. The DSS-induced UC group showed notable inflammatory cell infiltration, mucosal erosion, and loss of crypts, epithelial cells, and goblet cells, all of which were alleviated in the AGNE and SSZ groups. These results support that AGNE effectively improves the clinical symptoms of UC [43].

UC is a chronic disease that undergoes cycles of improvement and exacerbation, and various inflammatory mediators are over expressed in the UC site. Elevated secretions of the pro-inflammatory cytokines IL-6 and TNF-α further stimulate inflammatory responses. Hence, inhibiting the production of inflammatory mediators such as cytokines would hinder UC from becoming chronic. IL-6 and TNF-α act in the earlier stages of the immune response. IL-6 increases the body temperature by inducing the production of acute-phase response proteins, while TNF-α promotes the collection of inflammatory immune cells to the inflammation site by increasing the permeability of the vascular epithelial cells [44–46]. Thus, elevations of IL-6 and TNF-α expression levels promote chronic IBD, while their inhibition improves chronic IBD. In this study, the authors measured the IL-6 and TNF-α expression levels within the serum and colon tissues in the DSS-induced UC models. The AGNE treatment group showed a significant dose-dependent reduction of IL-6 and TNF-α production, and their inhibitory patterns were similar to those of the positive control (SSZ).

Arachidonate, which is produced at the phospholipid membrane, is converted to PGE_2 via the COX pathway. COX exists in two subtypes, COX-1 and COX-2. The former is expressed in normal colon cells for normal mucosal functions, while the latter is secondarily expressed by an inflammatory stimulus by NF-kB. This induces diarrhea, inflammatory responses, and release of various cytokines, ultimately making the inflammation chronic. Furthermore, COX-2 and PGE_2 expression levels are known to increase proportionately to the degree of inflammation in IBD [47,48]. We measured the effects of AGNE in the synthesis of COX-2 and PGE_2 in DSS-induced UC models. Compared with the normal group, the DSS group showed marked increases but the AGNE treatment group showed significant declines in COX-2 and PGE_2 expression levels.

HIF-1α is a transcription factor activated in a hypoxic state [49]. In hyperoxia, NF-kB is activated and gene expressions are regulated to relieve oxygen-induced stress. That is, the expression levels of pro-inflammatory cytokines such as IL-6 and TNF-α are elevated. A recent report suggested that HIF-1α was stabilized in relation to the NF-kB expression. Although the correlation between NF-kB and HIF-1α has not been revealed, an increase in HIF-1α expression level is known to activate inflammatory responses [50]. This study measured the HIF-1α expression level within colon tissues in DSS-induced UC models and found that it was reduced in the AGNE treatment group in comparison with the DSS group. This is similar to the production of IL-6, TNF-α, COX-2, and PGE_2, suggesting the NF-kB-mediated anti-inflammatory effects of AGNE at the molecular level.

The findings of this study verified that AGNE significantly improved clinical symptoms and reduced the activity of various inflammatory mediators. These findings substantiated the potential of AGNE as a therapeutic agent for UC.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgments

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