Prophylactic effects of *Acanthopanax senticosus* Harms on the development of colitis in mice

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Abstract. Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis (UC), comprises chronic relapsing inflammatory disorders of the gastrointestinal tract. Although the acute dextran sodium sulfate (DSS)-induced murine colitis model does not reproduce chronic human IBD, it reflects acute colonic epithelial injury and superficial mucosal inflammation, which are more characteristic of UC than of Crohn's disease. Therefore, the present study investigated whether the root extract of *Acanthopanax senticosus* Harms (ASH) exerts a prophylactic effect in an acute DSS-induced experimental colitis model in mice. Mice were fed a chow diet containing 5% ASH for 28 days, followed by water containing 3% DSS for 7 days to induce acute colitis. After DSS administration, mice fed the ASH diet exhibited significant inhibition of colon shortening and a reduction in clinical disease severity compared with the control group. Furthermore, the elevated levels of pro-inflammatory cytokines and oxidative stress markers in the serum were mitigated. Mechanistically, ASH pretreatment was associated with reduced NADPH oxidase 2 expression, relative restoration of nuclear factor erythroid 2-related factor 2 expression and increased Cu/Zn-superoxide dismutase expression in the colon. These findings suggested that ASH pretreatment attenuated acute DSS-induced experimental colitis by alleviating mucosal inflammation and restoring redox homeostasis.

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Introduction

Inflammatory bowel disease (IBD) encompasses two main disorders: Crohn's disease and ulcerative colitis. These chronic conditions cause gastrointestinal (GI) tract inflammation. Although the exact cause of IBD is unknown, it involves a combination of genetic (1), environmental (2), and immune factors (3). In Crohn's disease, inflammation can occur anywhere along the GI tract, from the mouth to the anus, and it often affects the deeper layers of the bowel (4). By contrast, ulcerative colitis is confined to the colon and rectum, and the associated inflammation is typically restricted to the innermost lining of the colon (5). The symptoms of IBD vary depending on the severity and location of inflammation; however, they commonly include abdominal pain, diarrhea, rectal bleeding, weight loss, and fatigue. Besides, the disease can lead to complications such as strictures and fistulas and an increased risk of colorectal cancer. Diagnosis typically involves a combination of endoscopic procedures, imaging studies, and laboratory tests to assess inflammation and exclude other conditions. The management of IBD focuses on reducing inflammation, controlling symptoms, and achieving and maintaining remission. Treatment options include anti-inflammatory drugs, immune system suppressors, biologics, and lifestyle modifications such as diet changes and stress management (6). In severe cases, surgery may be

necessary to remove the damaged portions of the GI tract. Recent experimental studies have reported anti-inflammatory effects of several compounds in colitis models. Isoliquiritin, a flavonoid glycoside, attenuated TNBS-induced colitis through suppression of the caspase-3/HMGB1/TLR4-dependent pathway (7). A novel bipyrazole derivative, TMNB, reduced disease activity and inflammatory mediators in experimental colitis (8). In addition, Artepillin C has been proposed as a candidate intestinal anti-inflammatory and antitumor compound, potentially through modulation of PAK1/NF-κB and PPAR-γ-related signaling (9). With proper treatment and monitoring, many patients with IBD can lead active and fulfilling lives.

Ezoukogi (*Acanthopanax senticosus* Harms, ASH), also known as Siberian ginseng or Eleuthero, is a deciduous shrub native to the cold northern regions of Japan, China, and Hokkaido (10). ASH is widely used as a traditional herbal medicine and has recently been marketed as a dietary supplement in Japan and Western countries. It contains several active ingredients, including phytochemicals, which have shown therapeutic effects in diabetes (11), allergies (12), gastric ulcers (13), neurodegenerative diseases (14), and cancers (15). In addition, ASH roots are known to inhibit inflammation and oxidative stress (16) in a mouse model of arthritis, and the aforementioned effects on IBD have been suggested to be beneficial.

Among experimental models of colitis, the DSS-induced model is one of the most widely used because of its simplicity and reproducibility. Acute DSS-induced colitis is generally considered to more closely resemble UC than Crohn's disease, particularly with respect to colon-restricted epithelial injury, diarrhea, rectal bleeding, crypt damage, and superficial mucosal inflammation. In the present study, we investigated whether ASH pretreatment attenuates acute DSS-induced experimental colitis in mice.

Materials and methods

Animals. Six-week-old C57BL6/6J female mice were purchased from HOKUDO (Hokkaido, Japan). The mice were housed in groups of four per cage under controlled conditions (23˚C temperature, a 12:12 h light-dark cycle, and a relative humidity of 50-55%), with free access to food and water. The study protocol was approved by the Animal Experiments Committee of Health Sciences University of Hokkaido (approval number: 21-016) and performed in compliance with the guidelines of the Committee of Animal Care and Use of Health Sciences University of Hokkaido and ARRIVE guidelines.

Experimental design of DSS-induced colitis. The experiments were designed as shown in Fig. 1. The mice were fed with either chow diet alone (chow diet) or chow diet supplemented with 5% ASH (ASH diet). The ASH used in this study was prepared and supplied by Sun Chlorella Corp. (lot no. 5152). The processed diets were prepared by Orient Yeast Corporation (Tokyo, Japan). After 4 weeks feeding, the mice were administered with either H_2O water or 3% (w/v) DSS (MW 36,000-50,000; MP Biomedicals, Santa Ana. CA, USA) in autoclaved drinking water for additional 7 days. Under deep anesthesia with 5% isoflurane (VITAS Pharmaceuticals, Inc. Tokyo, Japan), blood was collected from the inferior vena cava. Mice were then euthanized by cervical dislocation, and death was confirmed by cessation of respiration and heartbeat. Colon tissues were collected immediately thereafter. During DSS administration, all mice were monitored daily for body weight and clinical signs. Predefined humane endpoints were as follows: >20% loss of baseline body weight, inability to access food or water, and moribund appearance. Mice that met any humane endpoint criterion were humanely euthanized.

Assessment of colitis in mice. During DSS treatment, the severity of colitis was evaluated using the disease activity index (DAI), as previously described by Cooper *et al* (17). The DAI includes body weight loss (0, none; 1, 1-5%; 2, 5-10%; 3, 10-20%; 4, >20%), stool consistency (0, normal; 2, loose stool; 4, diarrhea), and rectal bleeding (0, normal; 2, hemoccult; 4, gross bleeding). After DSS treatment for 7 days, the length of the colon was measured following separation of the colon and cecum from the small intestine at the ileocecal junction to the anus. Thereafter, the colon was fixed in 10% buffered neutral formalin solution (MUTO PURE CHEMICALS, Tokyo, Japan), processed for paraffin embedding, and sectioned into 4 μm sections. Each section was deparaffinized, rehydrated, and stained with H&E. The histological degree of colitis was evaluated using the scoring system previously described by Williams *et al* (18), which included inflammation severity (0, none; 1, mild; 2, moderate; 3, severe), extent of inflammation (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), and crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, crypt lost and surface epithelium present; 4, loss of crypt and surface epithelium). The histological score means the sum of these three parameters.

Measurement of serum cytokine. Mice blood samples were collected from the inferior vena cava, and their sera were separated via centrifugation at 3,000 rpm at 4˚C for 10 min and stored at -80˚C until measurement. Cytokine concentrations were measured using Bio-Plex Pro Mouse Cytokine 23-Plex Assay kits (Bio-Rad) according to the manufacturer's protocol. This kit measures the concentration of 23 cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MCP-1α, MCP-1β, RANTES, and TNF-α).

dROM and BAP measurements. The degree of oxidation and antioxidant defense capacity, dROMs and BAP, in mice sera were measured by Wismerll Co., Ltd. (Tokyo, Japan).

Immunostaining of NOX2. Mice colon sections were incubated with anti-NOX2 antibody (19013-1-AP, 1:1,000 dilution, Proteintech, Wuhan, China) or anti-rabbit IgG (30,000-0-AP, 1:1,000 dilution, Proteintech, Wuhan, China) overnight at 4˚C. The following day, the sections were visualized with diaminobenzidine chromogen and counterstained with Mayer's hematoxylin under a microscope (NIKON CORPORATION, Tokyo, Japan). NOX2 expression was evaluated by their staining pattern: weak (1), moderate (2), diffuse (3) and intense (4) according to previously reported (19).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was isolated from mice colons using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA concentration was measured using NanoDrop spectrophotometer (NanoDrop

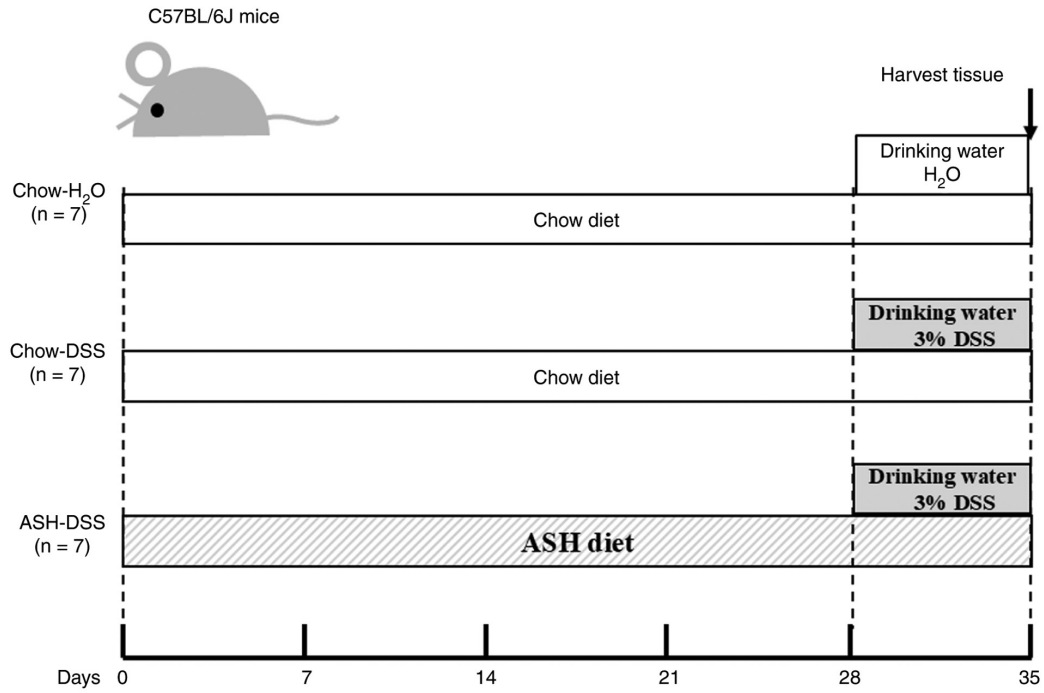

Figure 1. Experimental design of dietary treatment and DSS-induced colitis. DSS, dextran sulfate sodium; ASH diet, chow diet supplemented with 5% *Acanthopanax senticosus* Harms.

Technologies, Wilmington, DE, USA). cDNA was synthesized from 500 ng of total RNA using SuperScript IV First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. RT-qPCR was performed in triplicate using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, USA). PCR amplification protocol was as follows: 95˚C for 3 min; 40 cycles of amplification (at 95˚C for 10 sec, 60˚C for 30 sec, and 72˚C for 5 sec). Changes in the NOX2 gene between cDNA samples were determined using the $2^{-\Delta\Delta Cq}$ method (20). The primers were as follows: NOX2 Forward primer; 5'-TCGAAAACTCCTTGG GTCAGC-3', Reverse primer; 5'-GTGCAATTGTGTGGATGG CG-3', Cu/Zn-superoxide dismutase (SOD) Forward primer; 5'-AACCAGTTGTGTTGTCAGGAC-3', Reverse primer; 5'-CCACCATGTTTCTTAGAGTGAGG-3', nuclear factor erythroid 2-related factor 2 (Nrf2) Forward primer; 5'-TCTTGG AGTAAGTCGAGAAGTGT-3', Reverse primer; 5'-GTTGAA ACTGAGCGAAAAAGGC-3', and GAPDH Forward primer; 5'-GGCTGCCCAGAACATCAT-3', Reverse primer; 5'-CGG ACACATTGGGGGTAG-3', and 18S ribosome Forward primer; 5'-AGTCCCTGCCCTTTGTACACA-3', Reverse primer; 5'-CGATCCGAGGGCCTCACTA-3'.

Statistical analysis. Statistical analyses were performed using statistical analysis software (GraphPad Prism version 9.4.1). Comparisons among the Chow-H_2O, Chow-DSS and ASH-DSS groups were conducted using one-way ANOVA followed by Tukey's post hoc test. For ordinal data, including histological scores and clinical scores, statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. For body weight, statistical comparisons were performed among groups at each timepoint, and no within-group comparisons across different timepoints were conducted. Statistical significance was defined as $P<0.05$.

Results

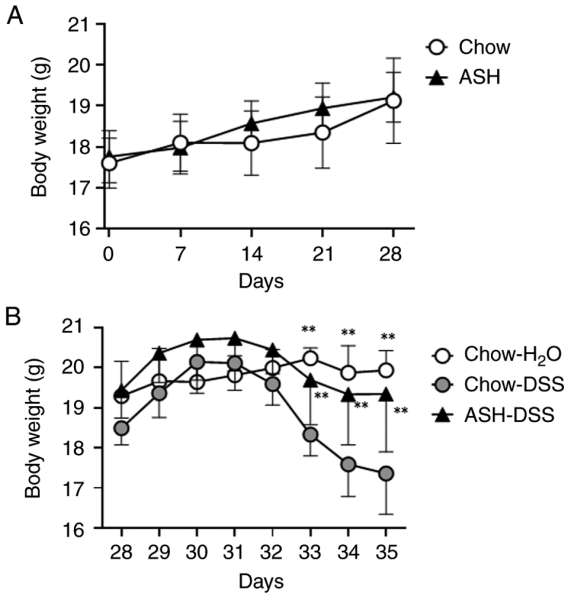

Figure 2. Changes in body weight. (A) Body weight of mice fed with either chow diet alone or chow diet supplemented with 5% ASH for 28 days (day 0-28). (B) Body weight of mice administered either H_2O or 3% (w/v) DSS in water for 7 consecutive days (day 28-35). One-way ANOVA and Tukey's post hoc test were used to analyze statistical significance. Results are presented as the mean ± standard deviation (n=7). $^{**}P<0.01$ vs. chow-DSS. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms.

Inhibitory effect of ASH diet on body weight loss caused by dextran sulfate sodium (DSS)-induced colitis. To examine the effect of ASH on mice weight, we measured body weight of mice fed both the chow and the ASH diet for 28 days respectively. No significant change between mice fed the chow and

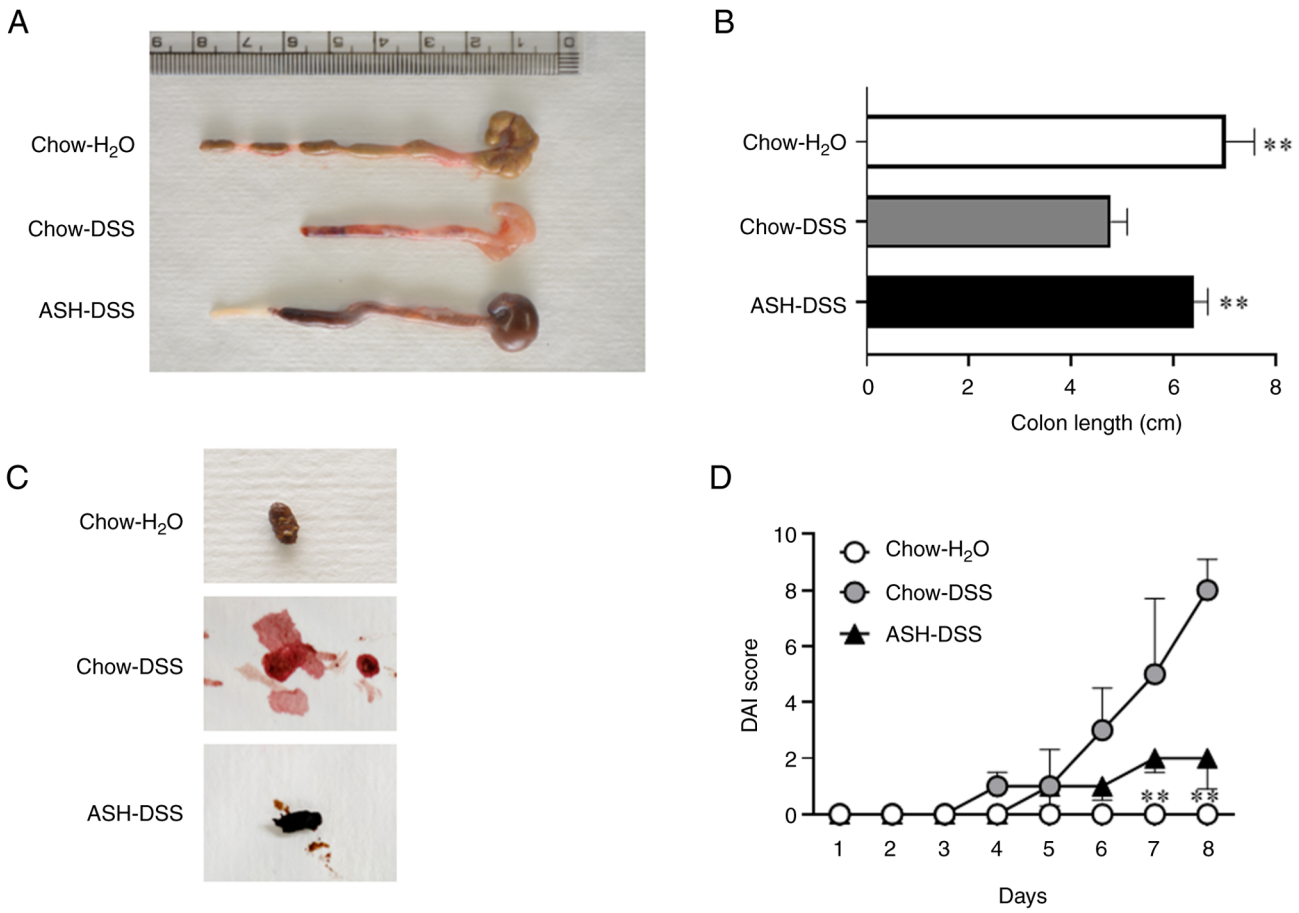

Figure 3. ASH diet attenuates DSS-induced intestinal inflammation in mice. (A) Representative macroscopic appearance of mice colons from each experimental group. (B) Colon length in each group of mice. (C) Representative feces from each group of mice. (D) DAI score of each group of mice during DSS treatment. **P<0.01 vs. chow-DSS. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms; DAI, disease activity index.

ASH diet was observed (Fig. 2A). After administration of 3% DSS, body weight loss was gradually observed in mice fed the chow diet (Fig. 2B). However, the mice fed the ASH diet showed the attenuation of body weight loss, which was similar to the mice fed the chow diet and H_2O water only (Day 35; Chow-H_2O: 19.9±0.5 g, ASH-DSS: 19.3±1.4 g, Chow-DSS: 17.4±1.0 g).

Inhibition of colon length shortening and exacerbation of the disease activity index (DAI) in mice fed the ASH diet. After 3% DSS treatment for 7 days, the colon length in mice fed the chow diet was significantly shortened [Chow-H_2O: 7.0±0.6 cm, Chow-DSS: 4.8±0.3 cm; P<0.01] (Fig. 3A and B). Meanwhile, the mice fed the ASH diet with DSS treatment showed a significant inhibition of colon length shortening [ASH-DSS: 6.4±0.3 cm, P<0.01 vs. Chow-DSS]. The feces of mice fed the chow diet with DSS (Chow-DSS) treatment showed loose and gross bleeding (Fig. 3C). The color of feces in mice fed the ASH diet with DSS treatment (ASH-DSS) was dark brownish black, reflecting the original color of ASH. Similar to the feces in mice fed the chow diet without DSS treatment (Chow-H_2O), fewer bloody and solid feces were observed in mice fed the ASH diet with DSS treatment. Afterward, we assessed the DAI score of colitis for 7 consecutive days (Day 28-35) during DSS treatment (Fig. 3D). The DAI score in mice fed the chow diet with DSS treatment (Chow-DSS) gradually increased from day 31, whereas the increase in DAI score in mice fed the ASH diet with DSS treatment (ASH-DSS) tended to be attenuated compared with that in the Chow-DSS group, suggesting that the ASH diet may mitigate the symptoms of DSS-induced colitis.

Histological improvement in colitis in mice fed the ASH diet. Representative hematoxylin and eosin (H&E)-stained images of mice colons are shown in Fig. 4. The colon tissues of DSS-treated mice fed the chow diet (Chow-DSS) showed strong inflammatory cell infiltration, crypt damage, and a thickened edematous submucosal layer. However, these findings were less observed in the colon tissues of mice fed the ASH diet with DSS treatment (ASH-DSS). According to these morphological findings, the histological score in mice fed the ASH diet with DSS treatment tended to be lower than that in mice fed the chow diet [Chow-DSS: 7.57±1.72, ASH-DSS diet: 3.57±2.76; Table I]. These results indicated that the ASH diet had a preventive effect against DSS-induced colitis in mouse models.

Decreased inflammatory cytokine production and oxidative stress in mice fed the ASH diet. To elucidate the mechanism of the effect of ASH on improvement in colitis, the concentrations of 23 inflammatory cytokines were

Table I. Histological assessment of DSS-induced colitis.

Parameter	Chow-H_2O	Chow-DSS	ASH-DSS
Score	0.00±0.00[a]	7.57±1.72	3.57±2.76
Range	(0,0,0,0,0,0,0)	(5,6,7,8,8,9,10)	(0,0,3,4,5,6,7)

Score data are presented as the mean ± standard deviation. [a]P<0.01 vs. chow-DSS. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms.

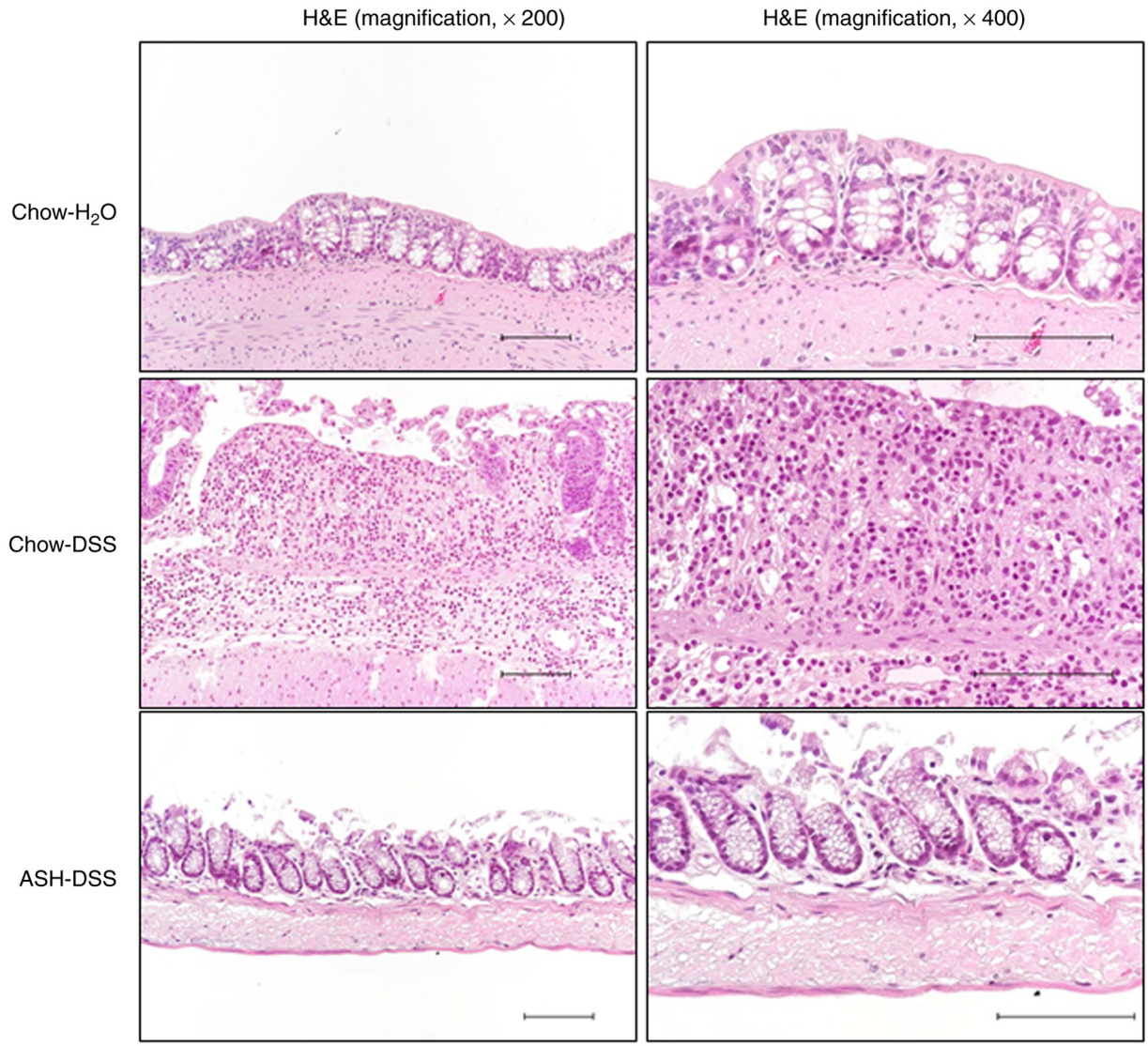

Figure 4. ASH diet induces histopathological improvement of DSS-induced colitis. Paraffin sections of the colon tissue were stained with H&E and observed under a microscope. Scale bar, 100 μm. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms.

measured in mice sera. Among them, five cytokines, IL-2, IL-9, Eotaxin, GM-CSF, and MCP-1α, were undetectable in all three mice groups (Table II). All measurable cytokines, except IL-4, and RANTES, showed decreased concentrations in mice fed the ASH diet (ASH-DSS) compared with that in DSS-treated mice fed the chow diet (Chow-DSS). Among these cytokines, IL-1α, IL-6, IL-12p40, IL-12p70, G-CSF, MCP-1, and TNF-α concentrations was significantly decreased in mice fed the ASH diet (ASH-DSS) (Fig. 5A-G). Thereafter, we measured the degree of oxidation and antioxidant defense capacity. Decreased diacron-reactive oxygen metabolites (dROMs) and increased biological antioxidant potential (BAP) were observed in the sera of mice fed the ASH diet (ASS-DSS) (dROMs; Chow-DSS: 137.4±25.16 U.CARR, ASH-DSS: 95.43±6.97 U.CARR; P<0.05, BAP; Chow-DSS: 3,572±333 μmol/l, ASH-DSS: 4,175±550 μmol/l; P<0.01; Fig. 5H and I). These results suggest that reduced inflammatory cytokines and oxidative

Table II. Measurement of cytokines in mouse sera.

Cytokine	Chow-H_2O (pg/ml)	Chow-DSS (pg/ml)	ASH-DSS (pg/ml)
IL-1α	0.50±1.73[a]	7.81±2.34	4.58±0.46[a]
IL-1β	n.d.	3.16±1.16	2.53±0.37
IL-2	n.d.	n.d.	n.d.
IL-3	3.17±1.61	1.95±0.89	1.40±0.22
IL-4	0.82±1.00	2.03±1.34	2.04±0.75
IL-5	n.d.	6.00±4.43	3.63±0.57
IL-6	5.90±1.15[a]	54.58±20.66	20.20±5.88[a]
IL-9	n.d.	n.d.	n.d.
IL-10	0.46±1.00[a]	20.81±4.68	18.85±1.27
IL-12p40	972.45±2.93[a]	3,400.23±1,916.89	1,545.61±244.91[b]
IL-12p70	23.11±1.04[a]	39.49±15.59	26.70±2.31[b]
IL-13	0.50±15.62[a]	45.48±25.10	33.60±11.05
IL-17A	15.62±2.08[a]	36.30±12.70	25.01±6.59
Eotaxin	n.d.	n.d.	n.d.
G-CSF	84.22±1.15[a]	3,814.11±1,052.78	2,411.41±243.65[a]
GM-CSF	n.d.	n.d.	n.d.
IFN-γ	0.84±2.02[a]	5.95±4.28	4.21±0.90
KC	32.72±2.52[a]	339.14±235.79	186.66±49.26
MCP-1	85.13±1.00[a]	351.22±156.13	171.48±24.44[a]
MCP-1α	n.d.	n.d.	n.d.
MCP-1β	36.05±1.61[a]	60.97±21.78	44.32±8.60
RANTES	n.d.	65.97±10.65	78.51±11.98
TNF-α	29.20±1.73	43.42±18.61	28.03±4.93[b]

[a]P<0.01 vs. chow-DSS. [b]P<0.05 vs. chow-DSS. n.d., not detectable; DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normal T cell expressed and secreted.

stress resulting from an ASH diet contributes to the mitigation of DSS-induced colitis.

ASH restored antioxidant capacity by suppressing NOX2 expression in mice colon. We examined the expression of NOX2, which produced oxidants and contributed to the development of experimental models of IBD, in mouse colon (Fig. 6A-C). The results of immunostaining and RT-qPCR showed that NOX2 expression at the protein and mRNA levels were lower in mice fed the ASH diet (ASH-DSS) than in those fed the chow diet (Chow-DSS). In mice fed the ASH diet (ASH-DSS), the reduction in Nrf2 expression was mitigated (Fig. 6D), while the expression of Cu/Zn-SOD, an intracellular superoxide dismutase, was restored (Fig. 6E). The RT-qPCR results of NOX2, Nrf2, and Cu/Zn-SOD genes were verified using two housekeeping genes, GAPDH and 18S ribosome (Fig. S1). These results suggest that the restoration of antioxidant capacity by ASH intervention suppressed inflammation and inflammatory cell infiltration in the intestinal mucosa.

Discussion

The present study examined the effects of ASH on DSS-induced colitis in mouse models. Mice fed the ASH diet showed a tendency toward reduced severity of colitis compared with mice fed the chow diet. In addition, inflammatory cytokine production and oxidative stress tended to be lower in ASH-fed mice. Since ASH was administered prior to DSS exposure, our results support only its prophylactic effects and do not demonstrate its efficacy against colitis that has already developed. If ASH were administered after disease onset, its effects might be weaker than those observed with prophylactic administration, as epithelial colon damage and the inflammation would already begin. Nevertheless, the reductions in oxidative stress and inflammatory cytokines observed in this study suggest that a partial therapeutic effect is biologically plausible, and it is worthwhile to conduct direct evaluations in future post-onset treatment designs.

NOX2, also known as GP91phox, is a member of the NOX superfamily that catalyzes the formation of oxidants; it is mainly expressed in leukocytes such as neutrophils and macrophages (21). When activated, NOX2 forms a complex with p22phox and p67phox, followed by the production of a superoxide, which includes reactive oxygen species (ROS). NOX2 also contributes to the development of experimental animal models of IBD (22,23). Furthermore, Nrf2-deficient mice was reported to increase susceptibility to DSS-induced colitis (24). Our results suggest that the ASH diet inhibits oxidative stress by partially affecting Nrf2-NOX2 axis. The

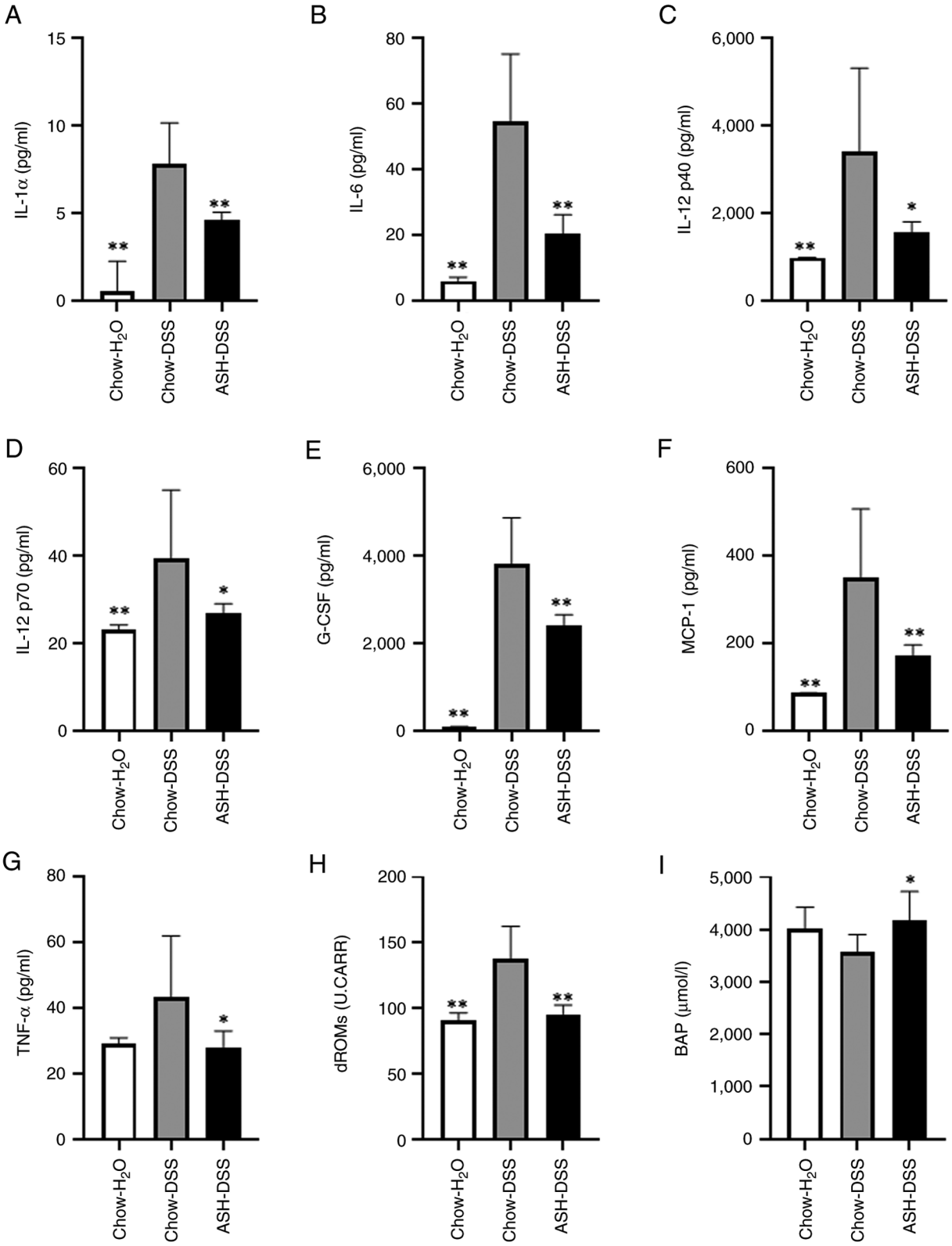

Figure 5. ASH diet decreases inflammatory cytokine production and oxidative stress. Measurement of cytokines in mouse sera: (A) IL-1α, (B) IL-6, (C) IL-12 p40, (D) IL-12 p70, (E) G-CSF, (F) MCP-1 and (G) TNF-α. The degree of oxidation and antioxidant defense capacity: (H) dROMs and (I) BAP (n=7). All units (y-axis), except for dROMs and BAP, are pg/ml. *P<0.05 vs. chow-DSS. **P<0.01 vs. chow-DSS. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms; G-CSF, granulocyte colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; dROMs, diacron-reactive oxygen metabolites; BAP, biological antioxidant potential.

other findings that Cu/Zn-SOD, the antioxidant enzyme, was restored in ASH-fed mice during DSS treatment, followed by the mitigation of oxidative stress by decreased degree of oxidation (dROMs) and increased antioxidant defense capacity (BAP). These findings suggest an association between ASH pretreatment and modulation of oxidative

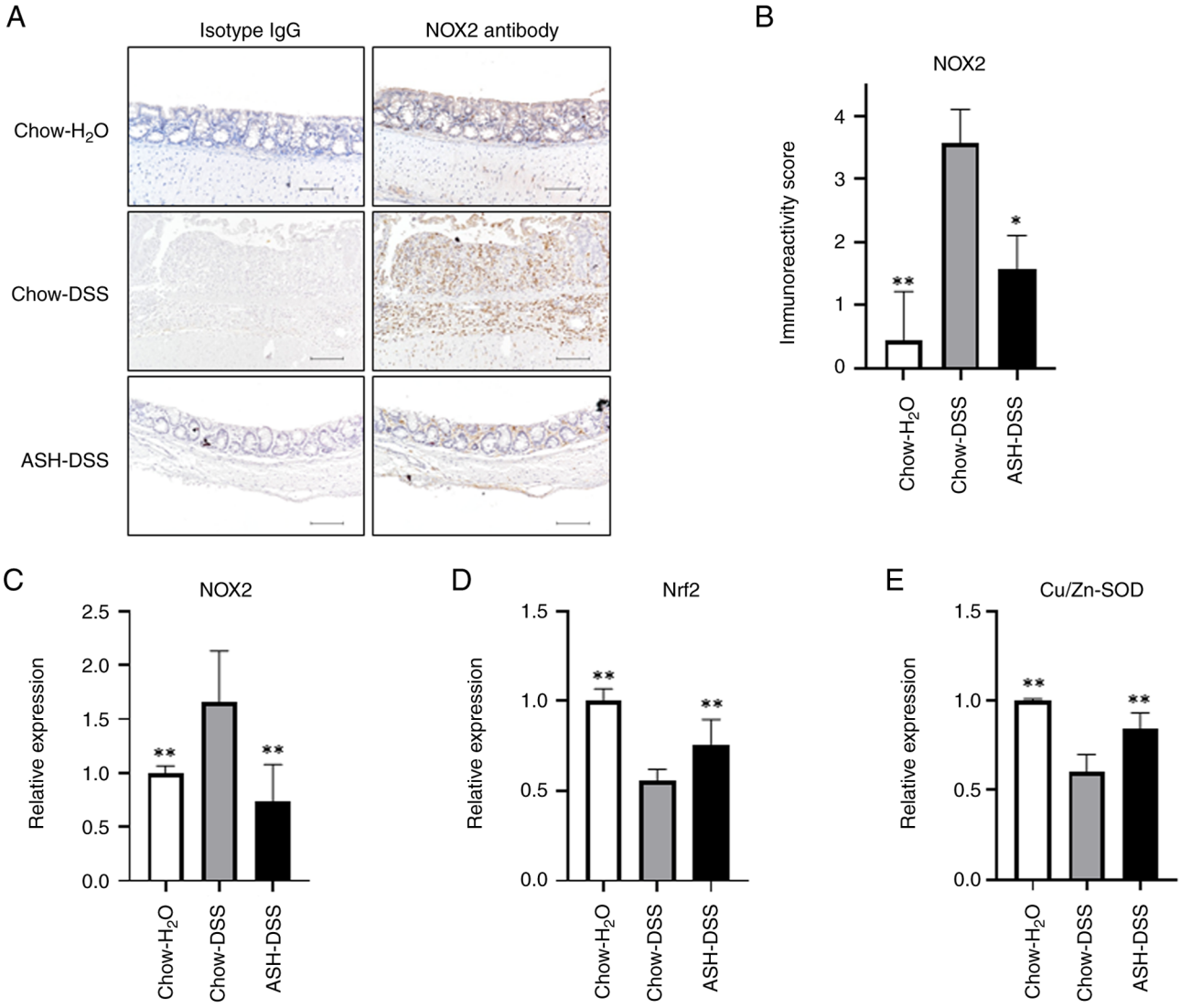

Figure 6. Effect of 5% ASH diet on oxidative stress-related factors in colonic tissue of mice. (A) Representative immunohistochemical staining for NOX2 protein in inflamed colorectal tissues of mice. Scale bar, 100 μm. (B) Immunoreactivity score of NOX2 protein expression. mRNA expression levels of (C) NOX2, (D) Nrf2 and (E) Cu/Zn-SOD in mouse colons. The GAPDH gene was used as an internal control. *P<0.05 vs. chow-DSS. **P<0.01 vs. chow-DSS. DSS, dextran sulfate sodium; ASH, *Acanthopanax senticosus* Harms; NOX2, NADPH oxidase 2; Nrf2, nuclear factor erythroid 2-related factor 2; Cu/Zn-SOD, Cu/Zn-superoxide dismutase.

stress-related pathways. However, direct activation of Nrf2 signaling at the protein level was not demonstrated in this study.

DSS binds to medium-chain fatty acids in the colon and induces inflammation, leading to colitis in mice and rats (25). These experimental animal models of IBD show increased production of numerous inflammatory cytokines, followed by exacerbation in ulcerative colitis. Previous studies on DSS-induced colitis suggest that targeting inflammatory cytokines such as IL-6 (26), IL-1α (27), IL-12p40/p70 (28), and TNF-α (29) is promising treatment with IBD. Furthermore, MCP-1 and G-CSF are potential biomarkers for patients with IBD (30). In the present study, ASH treatment significantly decreased the concentration of these cytokines in the serum, suggesting that the anti-inflammatory effect of ASH plays an important role in a DSS-induced colitis model, resulting in the prevention of colitis exacerbation.

ASH is a widely used component of Chinese traditional medicine, and it is prescribed for the treatment of several clinical diseases such as heart disease, hypertension, and allergies, which are related to inflammation and oxidative stress (31). Recent studies have elucidated the mechanisms underlying the clinical effects of ASH. Takahashi *et al* (16) investigated the effects of ASH on collagen-induced arthritis in mice and demonstrated that ASH delayed the onset of arthritis and reduced its severity. Moreover, ASH exhibited antioxidant properties, suppressing the production of ROS and inflammatory cytokines such as TNF-α and IL-6. Other studies have shown that the anti-inflammatory effects of ASH can be attributed to its inhibition of the expression of inducible nitric oxide synthase and cyclooxygenase-2 in activated macrophages (32). Regarding antioxidative stress, a study on the effect of ASH on patients with cancer experiencing cancer-related fatigue demonstrated an increased ratio of BAP to dROMs (33). Both the anti-inflammatory and antioxidant

effects of ASH were exerted in DSS-induced colitis models, showing improved clinical manifestations. Several bioactive constituents of ASH have been reported to exert colon-protective effects in DSS-induced colitis models. Isofraxidin attenuated DSS-induced colitis by reducing reactive oxidative species and promoting Nrf2 activation (34). Cotreatment with Syringin and DSS also mitigated colitis by suppressing IL-1β, IL-6, TNF-α, iNOS, and COX-2 expression, accompanied by inhibition of NF-κB and activation of Nrf2 signaling (35). Pretreatment with Chlorogenic acid before DSS exposure reduced disease activity, colon shortening, and TNF-α levels, and dysbiosis (36).

The limitations of this study were as follows. First, the present model represents acute DSS-induced experimental colitis rather than chronic IBD. For future studies, chronic models such as repeated-cycle DSS colitis, chronic TNBS colitis, oxazolone-based chronic colitis, and genetically engineered models including IL-10-deficient mice may provide complementary information regarding persistent inflammation, fibrosis, and long-term disease mechanisms (37). Second, the main bioactive compound in ASHE is not determined. There are several reports for the effect of ASHE compounds, isofraxidin (34), syringin (35), and chlorogenic acid (36), on the experimental colitis model. We are not sure whether these compounds could synergistically ameliorate colitis, but these reports support that ASHE has a prophylactic role as a dietary supplement. Another limitation is that we did not perform gain-of-function or loss-of-function experiments targeting NOX2. Therefore, although ASH pretreatment was associated with reduced NOX2 expression and improved colitis severity, a direct causal role of NOX2 suppression in mediating the protective effect of ASH cannot be concluded from the present data alone. Finally, female mice were used in the present proof-of-concept study to minimize biological variability associated with a mixed-sex design. This choice was also informed by previous reports showing that DSS-induced colitis severity differs by sex and is influenced by estrogen-related signaling (38,39). However, because only female mice were studied, the present findings should not be generalized across sexes, and future studies in male mice will be required.

In conclusion, ASH prophylaxis prevented the exacerbation of colitis in experimental mice models. Decreased inflammatory cytokine levels and oxidative stress caused by the inhibition of NOX2 expression would lead to improvements in the clinical and pathological features of IBD. As ASH is not only safe but also widely available for purchase as a commercial food product, regular intake may be considered for IBD prophylaxis.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YKa, DT, HF, STI and MT performed the experiments. TM and HTakek provided and prepared the *Acanthopanax senticosus* Harms extracts. YS, YT, KM, YKu, MS and HTaked participated in the design of the study. YKa and MT wrote the main manuscript, and YKa prepared Figs. 1-6 and Tables I and II. All authors reviewed the manuscript. MT and HF confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Animal Experiments Committee of Health Sciences University of Hokkaido (approval no. 21-016; Sapporo, Japan). The present study was performed in compliance with the guidelines of the Committee of Animal Care and Use of Health Sciences University of Hokkaido and Animal Research: Reporting of *In Vivo* Experiments guidelines.

Patient consent for publication

Not applicable.

Competing interests

YKa received research grants from Sun Chlorella Co., Ltd. MT received research grants from Sun Chlorella Co., Ltd. TM and HTakek are employees of Sun Chlorella Co., Ltd. This company had no role in interpreting, drafting or deciding whether to publish the manuscript, although the company Sun Chlorella Co., Ltd. provided funding. All other authors declare that they have no competing interests.

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