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Anti-Inflammatory Effects of Fermented Products with *Avena sativa* on RAW264.7 and HT-29 Cells via Inhibition of Inflammatory Mediators

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Abstract – This study investigated therapeutic candidates with anti-inflammatory potential among traditional dietary ingredients targeting inflammatory bowel disease (IBD). Both *Avena sativa* and traditional fermented products, such as Korean soy paste, are popular health foods. We investigated the anti-inflammatory effects of soy paste combined with *A. sativa* (KDA), compared with soy paste without *A. sativa* (KD) by evaluating the expression of pro-inflammatory cytokines in lipopolysaccharide-stimulated RAW264.7 mouse macrophages and HT-29 human colon epithelial cells. KDA significantly inhibited the production of nitric oxide (NO) and downregulated the pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in lipopolysaccharide (LPS)-induced RAW264.7 cells. In another in vitro experiment involving LPS-stimulated HT-29 cells, KDA suppressed the levels of IL-8, which is the chemokine elevated in IBD. In addition, KDA exhibited anti-oxidative properties, such as 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) radical scavenging activity. Our findings revealed that *A. sativa* combined with soy paste exhibits a synergistic anti-inflammatory and anti-oxidant effect following fermentation. These results suggest that KDA may be used as a potential anti-inflammatory therapy against IBD.
Keywords – *Avena sativa*, inflammatory mediators, anti-oxidant, macrophages, colon epithelial cells, inflammatory bowel diseases

Introduction

Inflammatory bowel disease (IBD) is a complex disease triggered by the interaction between environmental and genetic factors leading to immunological reactions and inflammation in the intestine.[1] IBD is characterized by an imbalance in the activation of proinflammatory and anti-inflammatory signaling pathways in the gut.[2] Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions of the gastrointestinal system.[3] CD can affect any portion of the gastrointestinal tract from the mouth to the perianal area. UC, distinguished by inflammation of the mucosal layer, is limited to the colon.[4] A change in gut microbial composition in genetically susceptible individuals, an altered immune system, and environmental factors are all assumed to play a role in the pathogenesis of IBD[3]. Complications include diarrhea, chronic inflammation, abdominal pain, cramping, fever, weight loss, wasting, internal bleeding, and ultimately cancer.[5] The prevalence of IBD has increased to almost 1% during the past decades, especially in Western countries, with poor prognosis and resistance to medications[5].

Inflammation is a crucial biological response to various stimuli and leads to the release of pro-inflammatory cytokines and mediators.[6] It is commonly accepted that acute inflammatory response is necessary for normal wound repair, while chronic or excessive inflammation may lead to pathological scarring and fibrosis.[7] IL-1β is a pro-inflammatory cytokine secreted as a biologically inert pro-peptide, which is cleaved by caspase-1 to an active state.[8] The pro-inflammatory cytokine interleukin (IL)-6 plays a pivotal role in IBD via uncontrolled intestinal inflammation.[9] TNF-α is a pro-inflammatory cytokine with antimicrobial and immunomodulatory features, and is mainly involved in the activation of endothelial and immune cells.[10] Nitric oxide (NO) and TNF-α are mainly released by activated mononuclear phagocytes, macrophages and monocytes.[11] NO is a short-lived gaseous molecule released by endothelial cells and influences the function

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of many cells.[12] The high levels of NO released in the mucosa of IBD patients, appear to be strongly associated with the maintenance of chronic inflammation.[13] Further, the increased levels of proinflammatory chemokine, IL-8 in the inflamed bowel probably play a key role in tissue fibrosis and injury.[14] In the present study, the anti-inflammatory response of IL-8 was assessed in HT-29 human colon carcinoma cells. Meanwhile, as the formation of reactive oxygen is related to chronic intestinal inflammation, oxidative stress is considered as one of the major factors in IBD, and thus antioxidants represent potential therapeutic agents for IBD.[15]

Avena sativa (Gramineae), known as the common oat, is cultivated worldwide. It is an important dietary staple for people in many countries. *A. sativa* has potent pharmacological effects such as antitumor, diuretic, neurotonic, and antispasmodic activities, and thus considered important to human health.[16] Recent reports suggested that *A. sativa* may protect against colorectal cancer and have benefits in inflammatory bowel disease and coeliac disease. Thies et al. suggested that long-term dietary intake of *A. sativa* has benefits for patients with IBS and UC, and also acts as an adjuvant in pharmaceutical treatment of UC patients.[17] Meanwhile, in Korea, various traditional fermentation products such as Korean soy paste are used as health foods. The bioactivities of Korean tradition fermented soy paste, known as 'Doen-jang', have been investigated in recent studies.[18] In the present study, we investigated the potential anti-inflammatory response of traditional fermentation products, such as Korean soy paste combined with *A. sativa,* by measuring the levels of inflammatory mediators in vitro using RAW 264.7 mouse macrophage cells and HT-29 human colon carcinoma cells. We sought to demonstrate the therapeutic potential against IBD.

Experimental

Materials – *A. sativa* (oat) which were grown in Gangjin, Korea, were powdered for preparation as additives and contained 2% weight of whole soy paste. Korean traditional soy paste and soy paste-containing powders of *Avena sativa* were fermented for 12 and 24 weeks at 4 ºC in porcelain pot to use as test samples. They were manufactured by the Jeonnam Food Research Center in Naju-si, Jeollanam-do, Korea.

Sample preparation and extraction – Samples were freeze-dried, powdered, and stored at -20 ºC. A 10 g portion of each dried sample was extracted in ethanol (100 mL) for 2 h. The ethanol solution was filtered with Whatman paper (No. 2), and the ethanol was evaporated using a rotary vacuum evaporator (EYELA, Tokyo. Japan). The extracts from Korean traditional soy paste (KD) and KD containing powders of *A. sativa* (KDA) were transferred to vials, and dissolved in ethanol and dimethylsulfoxide (DMSO) (Sigma) for anti-oxidant testing and anti-inflammation assay in RAW264.7 cells, respectively.

Cell culture – The mouse macrophage cell line, RAW 264.7, and a human colon epithelial cell line, HT-29, were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 μg/mL Streptomycin Solution and 100 U/mL Penicillin (HyClone, Logan, UT, USA) and maintained in an incubator, under humidified atmosphere with 4.5% CO_2 and a temperature of 37 ºC.

Cell viability – The mouse macrophage cell line, RAW 264.7 cells (1×10^5 cells/well) were seeded and incubated in 96-well plates with DMEM containing 10% FBS for 24 h. Cells were treated with different concentrations of KD and KDA for 1 h, followed by LPS (1 μg/mL) for 16 h. Cell viability was measured via MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide) assay. The cultured cells were incubated with MTT solution (0.05 mg/mL) at 37 ºC for 4 h. The supernatants were then removed and the formazan crystals dissolved using 100 μL of dimethyl sulfoxide. The absorbance was assessed at 570 nm using a microplate reader.

NO assay – The mouse macrophage cell line, RAW 264.7 cells were preincubated for 1 h with different concentrations of KD and KDA, followed by stimulation with 1 μg/mL of LPS for 16 h. The supernatant of cultured cells was harvested and treated with an equal amount of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylene]. The supernatant containing the mixed Griess reagent was incubated at room temperature for 10 min, and the absorbance was measured at 550 nm using a microplate reader.

Determination of PGE2 – The amount of PGE2 in culture medium after LPS-stimulation for 24 h was measured via PGE2 assay according to the manufacturer's instruction (KGE004B; R&D Systems Inc., Minneapolis, MN, USA). The average PGE2 concentration of the three wells per group was calculated. A standard curve was obtained by plotting the optical density of each standard on a linear y-axis against a logarithmic x-axis. B0 (the zero standard sample) was excluded in the standard curve.

The sample concentrations were obtained using the standard curve. The optical density of each well was determined at 450 nm by a Microplate Reader.

Measurement of cytokine production-The expression of IL-1β, IL-6, and TNF-α in the RAW 264.7 macrophages and IL-8 in the HT-29 cell cultures was measured with ELISA kits (BD OptEIA^{TM}, CA, USA) following the manufacturer's instructions.

DPPH radical scavenging assay – DPPH (2,2-diphenyl-β-picrylhydrazine, Sigma-Aldrich, Co.) was used to measured the anti-oxidant activiites of KD and KDA. Its radical scavenging activities were evaluated using a modified Blois method.[19] Equal volumes (100 μL) of 0.6 mM DPPH reagent and samples were added to the 96-well plates and mixed for 25 s, followed by incubation for 30 min in the dark. Absorbance was measured at 517 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA).

ABTS radical scavenging assay – The radical scavenging effects of ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, Sigma-Aldrich, Co.) were assessed using a modified Proestos method.[20] In a 96-well plate, the samples (100 μL each) were mixed for 25 s after adding 100 μL of 7.4 mM ABTS solution and reacted for 6 min. The absorbance was measured at 734 nm in a microplate reader (BioTek Instruments, Winooski, VT, USA).

Statistical analysis – All experiments were independently measured in at least triplicate and expressed as the means ± standard deviation (SD). The data were compared using

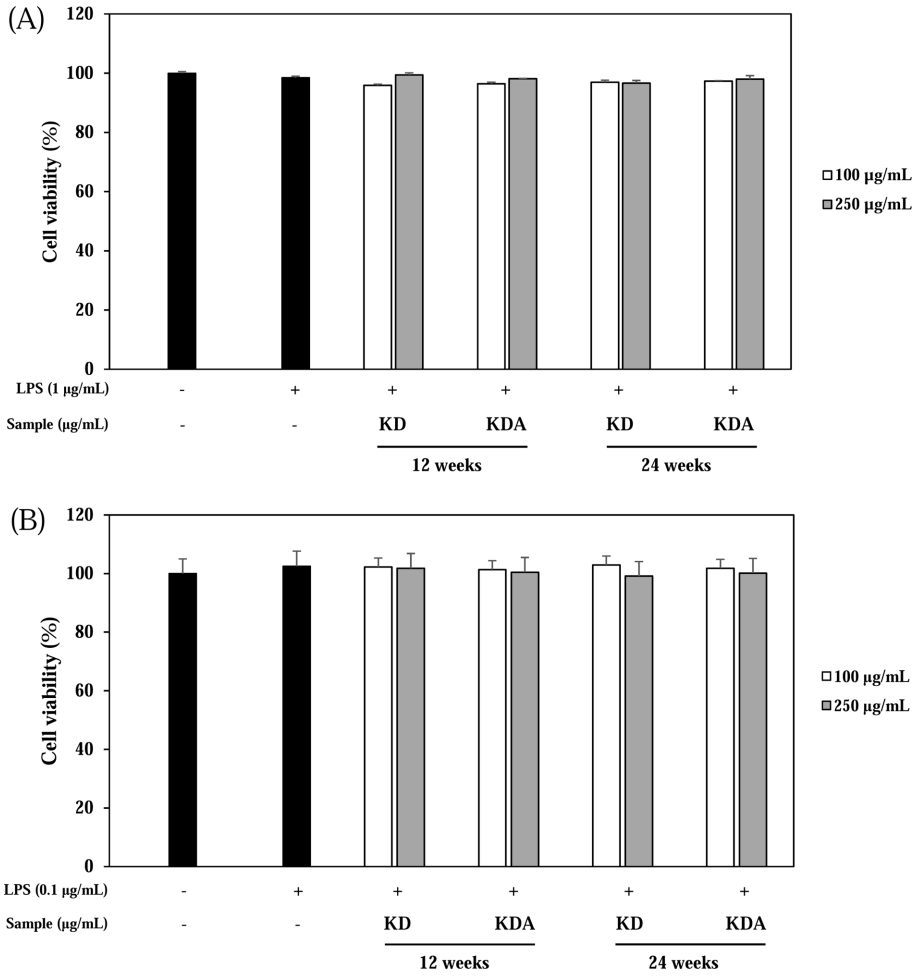

Fig. 1. Effects of KD and KDA on cell viability during fermentation. RAW 264.7 cells were cultured in the presence of ethanol extract (100 and 250 μg/mL) for 1 h and induced with LPS (1 μg/mL) for 16 h. HT-29 colon epithelial cells were pretreated with ethanol extract (100 and 250 μg/mL) for 2 h, followed by induction with LPS (100 ng/mL) for 12 h. Cell viability of RAW 264.7 cells (A) and HT-29 cells (B) in the culture media were measured by MTT assay.

one-way ANOVA and Duncan's multiple range tests using the software package SPSS statistics V20, and $p < 0.05$ was considered significant.

Results and Discussion

RAW 264.7 mouse macrophages were pretreated with KD and KDA at different concentrations (100 and 250 μg/mL) for 1 h before stimulation with LPS (1 μg/mL) for 18 h. We compared the cell viability of the three groups: the control group that was not treated with LPS or samples, the group treated with only LPS, and the sample group containing LPS. The viability of the cells treated with KD and KDA was measured via MTT assay. None of the samples, KD and KDA according to the incubation period (12 and 24 weeks) and concentration (100 and 250 μg/mL) showed cytotoxicity against LPS-stimulated RAW264.7 cells (Fig. 1A). We determined the effect of KD and KDA on the cell viability of HT-29 human colon epithelial cells via MTT assay. The KD and KDA had no cytotoxicity on HT-29 cells (Fig. 1B). Therefore, the KD and KDA were used in subsequent experiments to investigate their anti-inflammatory activity.

NO is a free radical gas messenger molecule with both intracellular and extracellular regulatory functions. NO is one of the proposed etiological factors in the inflammatory process underlying these diseases, and increased NO production in IBD was recently reported.[21] NO overexpression was alleviated in altered inflammatory mucosa of patients with IBD, which may contribute to deterioration and exacerbation of immune responses.[22]

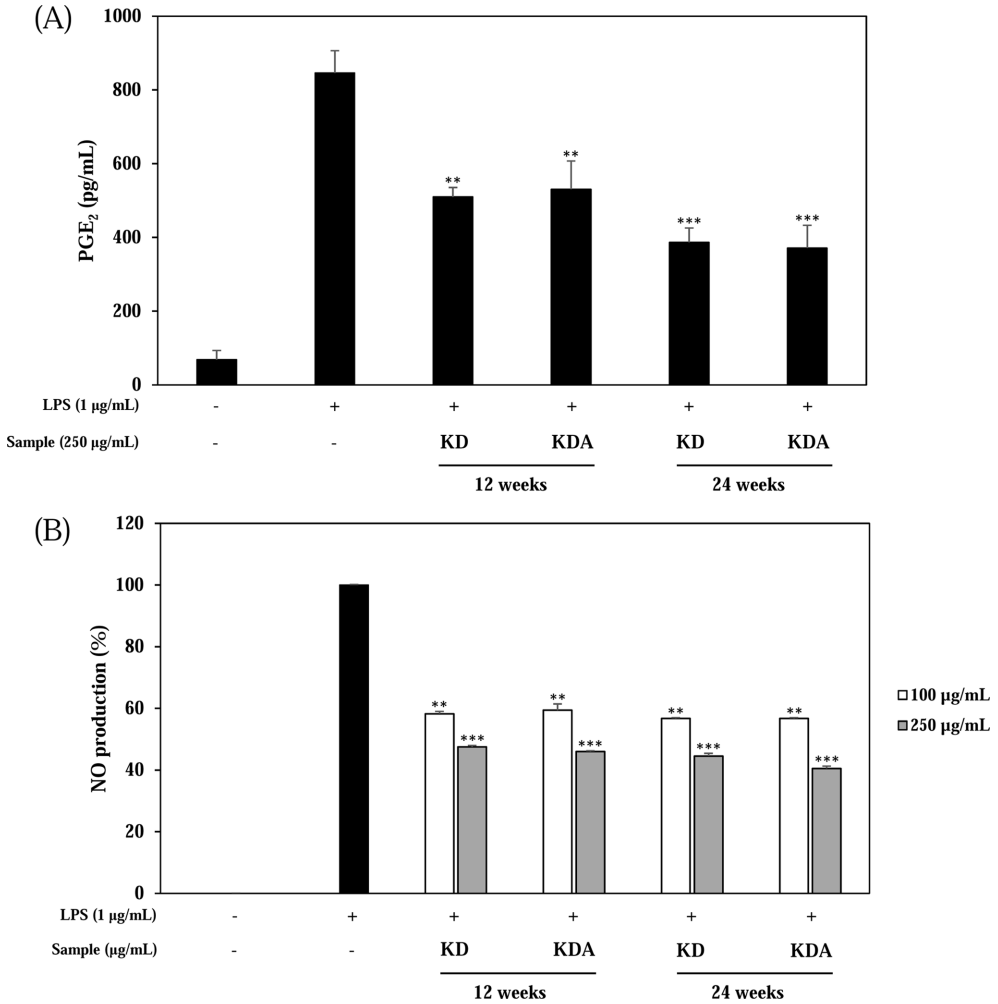

Fig. 2. Effect of KD and KDA on NO and PGE2 production during fermentation. RAW264.7 cells were incubated with ethanol extract (100 and 250 μg/mL) for 1 h and stimulated with LPS (1 μg/mL) for 16 h. NO (A) and PGE2 (B) production was measured using Griess reagent and PGE2 assay, respectively. Nitrite concentrations of non-treated and LPS-treated controls were 0.7 ± 0.1 μM and 21.2 ± 0.2 μM, respectively. Each experiment was performed in triplicate. The data are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated g roup.

Thus, the anti-inflammatory effects of KD and KDA were evaluated by analyzing the suppression of NO synthesis in LPS-induced RAW 264.7 cells. KD and KDA attenuated NO production at 100 and 250 μg/mL, in a concentration-dependent manner (Fig. 2A). In fermented period of 24 weeks, KDA exhibited the most potent inhibition by reducing NO synthesis by 59.5% at a concentration of 250 μg/mL ($p < 0.001$), compared to 55.5% of KD. The level of PGE2 concentration was increased in the intestinal mucosa of IBD patients and PGE2 attenuated fibroblast migration in intestinal wound healing.[23] To investigate the effect of KD and KDA treatment on PGE2 production, RAW264.7 cells were pretreated with KD and KDA for 1 h followed by stimulation with LPS (1 μg/mL) for 24 h. As shown in Fig. 2B, PGE2 production was induced by LPS. Pretreatment with both KD and KDA significantly decreased the levels of LPS-induced PGE2 synthesis at a concentration of 250 μg/mL ($p < 0.001$). Treatment with KD and KDA for 24 weeks (386.4±25.7 and 370.9±76.8 pg/mL) inhibited the level of PGE2 more than in 12 weeks (509.6±39.0 and 530.6±62.0 pg/mL). However, in terms of NO production, the effect of KDA on PGE2 synthesis, which is considered a potential biomarker in IBD, was not superior to that of KD. Rather, PGE2 synthesis following KDA treatment was slightly upregulated compared with KD in 12 weeks. These results showed that the addition of *A. sativa* to KD has a synergistic effect on NO production, but no obvious effect on PGE2 production.

The increased production of cytokines that are released by inflammatory cells such as macrophages and endothelial cells is associated with active colon inflammation and stimulation of NO production resulting in the formation of reactive products such as superoxide anions and hydrogen peroxide.[21] Especially, levels of inflammatory cytokines such as IL-1β, IL-6 and TNF-α have been reported to be elevated in patients with inflammatory bowel disease.[24] Therefore, the inhibitory effects of KD and KDA on pro-inflammatory mediators were measured using ELISA kits. Compared with the untreated control group, the levels of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α increased in LPS-stimulated macrophages. Pretreatment of macrophages with these compounds with KDA reduced the expression of IL-1β by 35.0% and 47.1% in 12 and 24 weeks, respectively, which was significantly higher than that of KD, which resulted in 28.2% and 39.3% reduction at the concentration of 250 μg/mL (Fig. 3A). The results with KDA and KD exposure showed a similar inhibitory pattern of IL-1β expression at low concentrations of 100 μg/mL. KD and KDA decreased the expression of IL-6 at 100 and 250 μg/mL in a concentration-dependent manner (Fig. 3B). Especially, KDA in 24 weeks dramatically suppressed the level of IL-6 expression up to 61.7%, compared with LPS-treated group, and 10%, compared with KD. At concentrations of 100 and 250 μg/mL, KD and KDA significantly inhibited the TNF-α expression in 12 and 24 weeks. As shown in Fig. 3C, anti-inflammatory activities of *A. sativa* range from 3.3% to 6.9%, according to the concentration and fermentation duration. The foregoing results indicated that KDA meaningfully suppressed the pro-inflammatory cytokines stronger than KD in LPS-stimulated RAW 264.7 cells. Indeed, KDA dramatically decreased the IL-6 expression compared with other cytokines and also had the most synergistic effect when combined with *A. sativa*.

IL-8 is genetically related to inflammation in IBD and increased in the intestinal mucosa of patients with UC and CD.[25] The increased IL-8 expression may initiate or maintain IBD via interactions with TNF-α and/or IL-1 in neutrophil activation.[26] The inhibitory effects of KD and KDA on IL-8 production were evaluated using ELISA kits. Compared with untreated cells, colon epithelial cells exposed to LPS stimulation (100 ng/mL) showed increased levels of IL-8. KD and KDA inhibited IL-8 production in a concentration-dependent manner; and the IL-8 level in these cells was lower than in LPS-stimulated cells (Fig. 4). In particular, treatment with 250 μg/mL (12, 24 weeks) of KDA resulted in potent anti-inflammatory activities (32.4% and 61.4% in 12 and 24 weeks) compared with KD (27.8% and 50.6%). Treatment with *A. sativa* reduced IL-8 level up to 10.8% in 24 weeks. Similar to the result of IL-6, the synergistic effect of KDA was clearly observed by the inhibition of IL-8, which is an important etiological factor in UC and CD. Thus, KDA is a potential anti-inflammatory agent against IBD and acts via down-regulation of IL-8.

To investigate the therapeutic role of IBD, we mainly focused on anti-inflammation. Recent studies reported the oxidative stress signaling in the gastrointestinal tract and pathologic mechanisms of oxidative stress in IBD. Tian et al., proposed the development of anti-oxidants into potential medications for IBD.[15] Consequently, a variety of studies identified the role of new therapeutic agents such as curcumin and polyphenol in IBD based on their anti-oxidant effect. Several assays are available for the measurement of antioxidant activity with varying sensitivities. The ABTS and DPPH assays, which are associated with free radical scavenging, have been developed to evaluate the anti-oxidant activity of plants and food.[27] ABTS is a relatively stable free radical and is

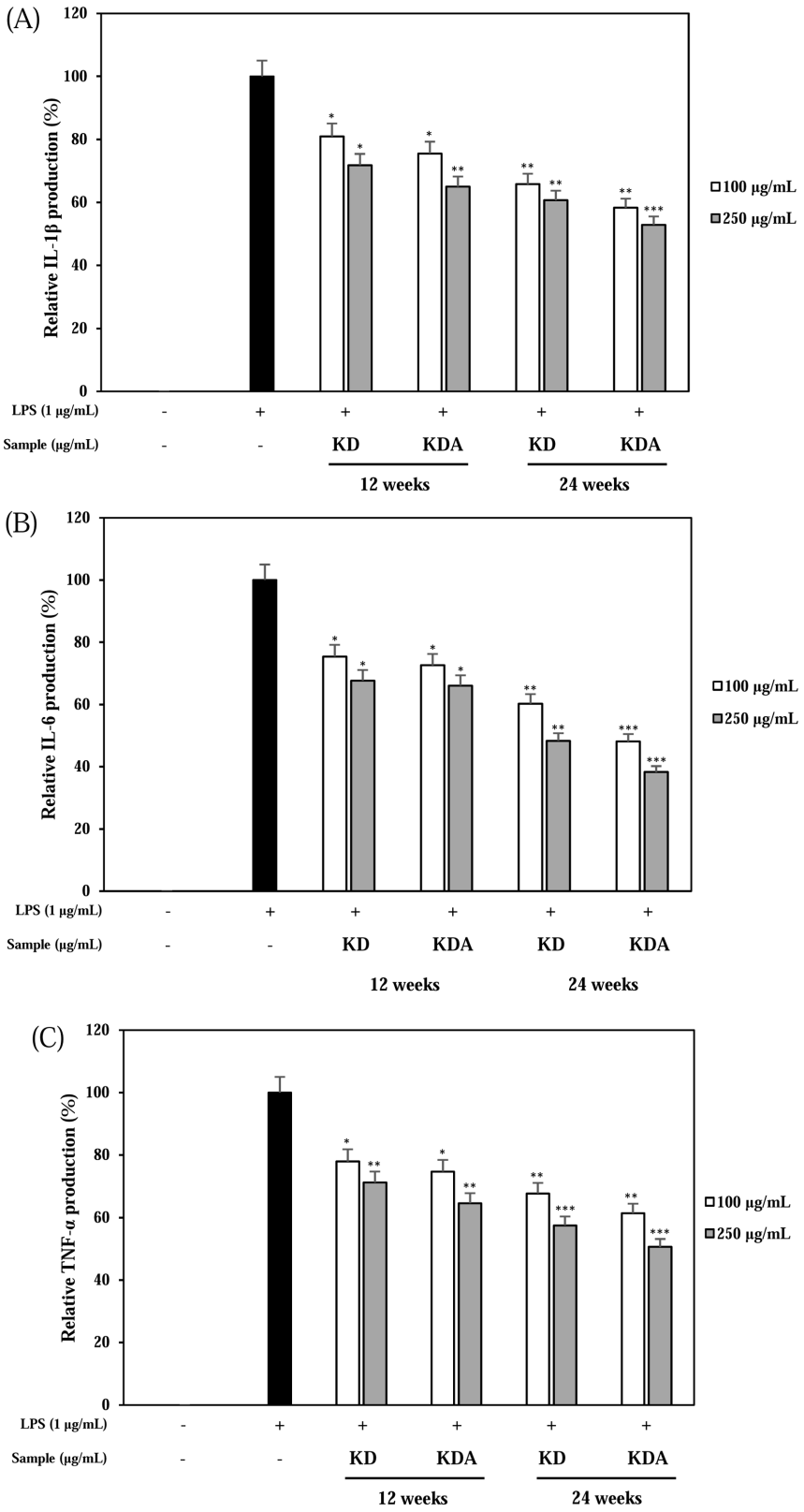

Fig. 3. Effect of KD and KDA on the expression of proinflammatory cytokines, IL-1β, IL-6 and TNFα. RAW264.7 cells were incubated with ethanol extract (100 and 250 μg/mL) for 1 h and stimulated with LPS (1 μg/mL) for 16 h. Levels of IL-1β (A), IL-6 (B) and TNF-α (C) in the culture media were measured by ELISA. Data are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

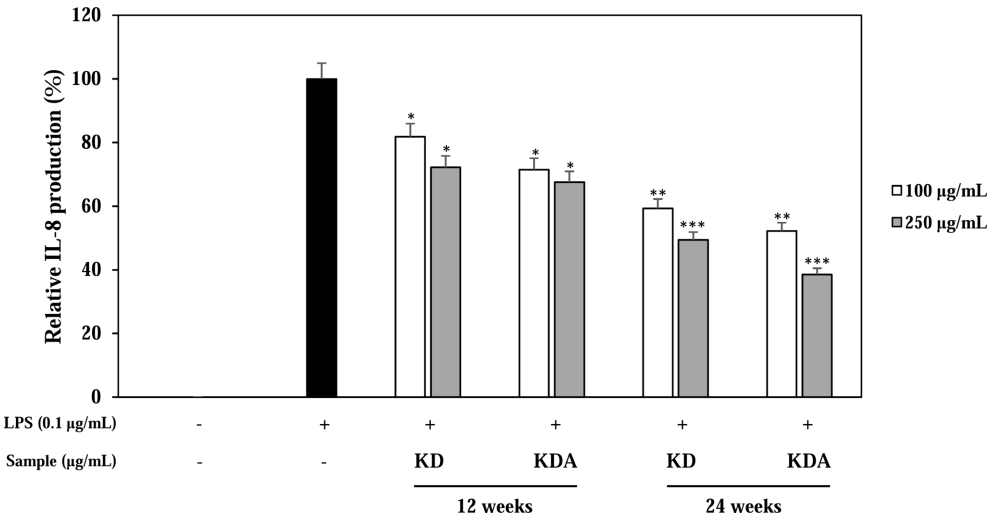

Fig. 4. Effect of KD and KDA on cell viability and IL-8 expression. HT-29 colon epithelial cells were pretreated with ethanol extract (100 and 250 μg/mL) and for 2 h, followed by induction with LPS (100 ng/mL) for 12 h. IL-8 levels in the culture media were measured by ELISA. Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

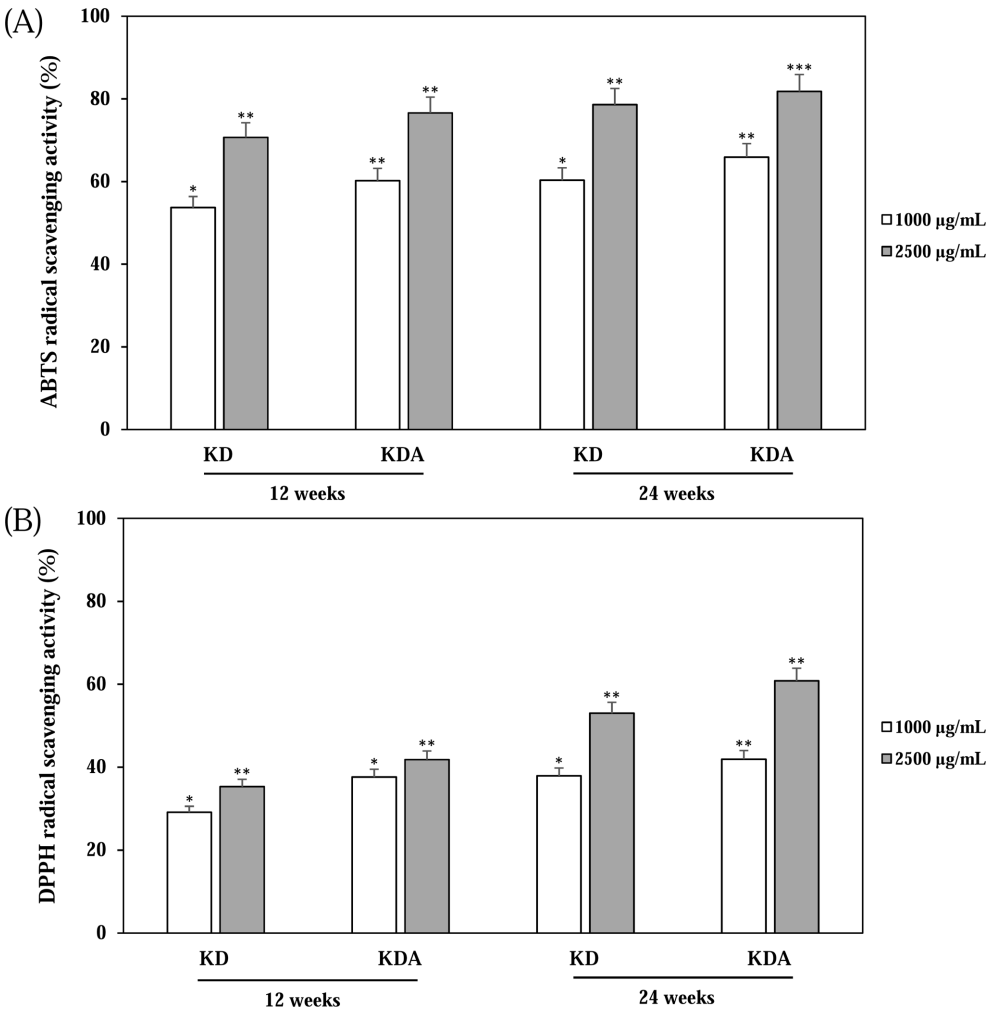

Fig. 5. Radical scavenging activities of KD and KDA using ABTS (A) and DPPH (B) assays. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group.

widely used to measure antioxidant activity in combination with DPPH, which can measure both lipophilic and hydrophilic substances.[28] In studies conducted to elucidate the anti-oxidant activities of KD and KDA, the ABTS scavenging activity of KDA was 76.6% and 81.8% in 12 and 24 weeks at a concentration of 2500 μg/mL, compared with that of KD, which was 70.7% and 78.6%, respectively (Fig. 5). The DPPH scavenging activities of KD and KDA at similar concentrations of ABTS were 23.5% and 30.0% in 12 weeks, 41.2% and 49.0% in 24 weeks, respectively. In the present study, the DPPH radical scavenging activity was relatively lower than the ABTS radical scavenging activity and was similar to the general result. These results revealed that similar to anti-inflammatory results, the anti-oxidant effect was elevated when *A. sativa* was added to KD, which was affected by the fermentation period.

In summary, we evaluated the anti-inflammatory effects of KD and KDA by measuring proinflammatory mediators in LPS-induced macrophage cells. Compared with KD, KDA showed stronger inhibitory effects against NO production and the expression of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α. In another in vitro system, LPS-stimulated HT-29 cells, KDA suppressed the levels of IL-8, more than KD. Based on these results, we identified the synergistic anti-inflammatory effects of KDA. In addition, our results showed that *A. sativa* may affect the anti-inflammatory and anti-oxidant activity depending on the fermented period. Therefore, KDA represents a potential candidate for treating IBD due to its anti-inflammatory effects. To confirm its potential therapeutic application in IBD, further studies are needed to investigate the therapeutic effects of KDA against IBD using animal models such as dextran sodium sulfate-induced colitis.

Acknowledgments

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