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Fermented Product Extract with *Lentinus edodes* Attenuate the Inflammatory Mediators Releases and Free Radical Production

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Abstract – *Lentinus edodes* contains functional metabolites such as polysaccharopeptides, lectins, and secondary metabolites. Fermented soybean paste is representative fermented materials in Korea, and is gradually increasing due to various biological activities. In the present study, ethanol extracts of fermented products with/without *L. edodes* were designated as SPL and SP, and prepared to develop safer and therapeutic functional foods with antioxidant and anti-inflammatory activities for treatment of inflammatory disorders. SP and SPL extracts exhibited antioxidant effects via inhibiting radical activities. Inflammatory mediators, nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS) production and nuclear factor-kappa B (NF-κB) activation were down-regulated by two extracts. SPL extract more strongly enhanced the antioxidant and anti-inflammatory activities than SP extract. Its' activities shown more longer fermentation period and more strong inhibitory effects. Taken together, our results suggested that fermented product with medicinal plant has synergic effect and SPL can be a potential candidate for treatment of inflammatory bowel diseases.
Keywords – *Lentinus edodes*, antioxidant, anti-inflammation, iNOS, NF-κB

Introduction

Oxidative stress which caused by continuously deteriorating environment with increasing pressures of work results in chronic diseases involving coronary and Alzheimer's diseases, diabetes and inflammatory bowel diseases (IBD).[1] Among the chronic diseases, IBD comprises the two disorders that are characterized by chronic inflammation of the gastrointestinal tract: ulcerative colitis (UC) and Crohn's disease (CD), and its prevalence has been increasing worldwide.[2] The mucosal population of immune cells such as macrophages, plasma cells, T and B cells as well as neutrophils is increased in IBD.[3] During the propagation and initiation of IBD, the mucosal epithelial barrier is compromised and macrophages secrete chemokines, inflammatory mediator [nitric oxide (NO)], and cytokines [interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-8], which lead to damage of the intestinal epithelial monolayers and subsequent mucosal inflammation.[2,4] Nuclear transcription factor kappa-B (NF-κB) regulates various inflammatory responses, is activated via the phosphorylation of various inflammation-related signaling factors, and induces the production of inducible nitric oxide synthase (iNOS). In macrophages activated during inflammation, iNOS is responsible for production of NO. Thus, the inhibition of iNOS could provide new targets for the prevention and treatment of inflammatory diseases.[5]

Fermented materials have been produced and consumed since the development of human civilization and are generally defined as those made through controlled microbial growth and enzymatic conversions of major and minor food components.[6,7] Fermented soybean paste (SP), is a popular Korean traditional product, and is used as an ingredient in stews, soups, sauces, and salad dressing. It has a long fermentation period that extends from two to 24 months, and contains a high level of bioactive compounds and a good source of essential amino acids, minerals, and vitamins.[8,9] It showed various physiological effects involving anti-obesity, anti-diabetic,

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anti-cancer, anti-inflammatory and hypotensive blood pressure effects.[10,11]

Lentinus edodes (the shiitake mushroom) is a basidiomycete that has been consumed for more than 2000 years, and is the second most popular cultivated edible mushroom in the world. This medicinal plant also contains vitamins and minerals in addition to a wide range of functional metabolites involving polysaccharides, polysaccharopeptides, lectins, and secondary metabolites such as lentinan, β-glucan with immunomodulatory activity.[12] It possesses anti-tumor, anti-oxidant, anti-viral, immunomodulating, anti-microbial activities.[12,13] Many studies have focused on the development of safer and therapeutic agents and functional foods using natural products or traditional Chinese medicine for various diseases. In our present study, the antioxidants and anti-inflammatory effects of fermented SP and SP supplemented with *L. edodes* (SPL) were investigated using various *in vitro* system.

Experimental

Preparation of fermented materials – Raw materials of soybean paste (RSP) were purchased from Jangheungfood Co. Ltd in 2019. Boiled soybeans were added to the raw materials to make SP with a salinity adjusted to 9%. *L. edodes* was grown as special resource in Jangheung, Korea, and the powders were purchased from market. *L. edodes* fermented product (LFP) was prepared by mixing a material with 2.0% weight of *L. edodes* powder and 18.0% weight of boiled soy beans, and then dispensing the *Bacillus subtilis*. SPL was made by mixing 75% of RSP, 20% of LFP, and 5% of boiled soy beans. SP and SPL were fermented for 12 and 24 weeks at 30°C, 75% humidity in porcelain pot for use as the test samples and provided by the Jeonnam Food Research Center (Naju-si, Jeollanam-do, Korea).

Sample preparation and extraction – Four samples of fermented materials were freeze-dried, powdered, and stored at -20 °C. A 10 g portion of each dried sample was extracted in ethanol (100 mL) for 2 h. The ethanol solution was filtered through Whatman filter paper of No. 2, and then evaporated for removal of ethanol using a rotary vacuum evaporator (EYELA, Tokyo. Japan). The extracts of SP and SPL were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO, USA) for the experiments.

***In vitro* antioxidant assay** – *In vitro* antioxidant activities of SP and SPL extracts were assessed by 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and 1,1-diphenyl-β-picrylhydrazine (DPPH) radical scavenging activities. The ABTS (Sigma) radical scavenging activity was measured using a previously reported method by Proestos with slight modification.[14] 2,2'-Azobis (2-aminopropane) dihydrochloride (7 mM) (Sigma) was mixed with 2.45 mM ABTS and allowed to react for 16 h at 4 °C. Typically, 50 μL of the sample and 100 μL of the ABTS solution were mixed and allowed to react at 23 °C for 20 min after addition to a 96 well plate. The absorbance was measured at 734 nm. Ascorbic acid (100 μg/mL) was used as a positive control. The radical scavenging effect of DPPH (Sigma) was measured using the method reported elsewhere with a slight modification.[15] A 100 μL of DPPH solution (0.2 mM) was added to 100 μL of the sample on a 96 well plate, mixed for five seconds, and allowed to react for 30 min under shade. The absorbance was measured at 517 nm using a microplate spectrophotometer (Epoch, Biotek Instruments, Inc., VT, USA); ascorbic acid (100 μg/mL) (Sigma) was used as the positive control.

Cell culture, treatment and stimulation – The mouse macrophage, RAW 264.7 cells and human colon epithelial, HT-29 cells were acquired from the Korean Cell Lines Bank (KCLB) (Seoul, Korea). These cells were grown as monolayers in a 37 °C humidified atmosphere with 5% CO_2. The cell lines were maintained in culture medium [Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL penicillin solution (HyClone, Logan, UT, USA)].

Cell viability assay – Cells were plated at the density of 1×10^5 cells/well in 96-well plates in culture medium for 24 h. Cells were treated with various concentrations of SP and SPL extracts for 1 h and then were stimulated with LPS (1 μg/mL) for 20 h. Cell viability was evaluated by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] (Sigma) assay. The cultured cells were incubated with MTT (5 mg/mL) at 37 °C for 4 h. The supernatants were removed and 100 μL dimethyl sulfoxide (DMSO) (Sigma) was added. After 5 minutes, the absorbance of the formazan crystals was measured at 570 nm in a microplate reader (Bio Tek Instruments, Winooski, VT, USA).

Measurement of NO and prostaglandin E2 (PGE_2) production – RAW 264.7 cells were treated with SP and SPL extracts extracts for 1 h and then stimulated 1 μg/mL of lipopolysaccharide (LPS) (Sigma) for 16 h (NO) and 24 h (PGE 2). The NO assay and PGE_2 determination were done as previously described experimental.[16] Curcumin (Sigma) was used as the positive control.

Enzyme linked immunosorbent assay (ELISA) – The protein expressions of IL-1β, IL-6, and TNF-α in the mouse macrophages and IL-8 in human colon epithelial cells were measured by ELISA kits (BD OptEIATM, CA, USA) following the manufacturer's instructions.

Western blot analysis – Cells were seeded at the density of 1×10^6 cells/6-well plates in culture medium for 24 h, treated with SP and SPL extracts 2 h, and then stimulated with LPS (1 μg/mL). After 16 h (iNOS and COX-2) or 1 h (NF-κB), the cells were washed twice with cold phosphate-buffered saline (PBS) and whole cell lysates were extracted with protein extraction solution (Pro-Prep, iNtRON, Biotechnology, Daejeon, Korea). The protein concentration was determined by the Bradford assay method. Equal amounts of control, LPS and SP and SPL extracts-treated cell lysates (30 μg) were separated by 10% SDS-PAGE gels and transferred on PVDF nitrocellulose membranes. The membranes were blocked with 5% skim milk powder in plain buffer [20 mM Tris-HCl (pH 7.4) and 4 M NaCl] for 1 h at RT. The membranes were incubated with primary antibodies (iNOS, COX-2, NF-κB, and β-actin) at 4 °C overnight and then washed three times with a wash buffer [1 M Tris-HCl (Ph 7.4), 4 M NaCl, Tween-20 in DW] for 10 min. They were incubated in specific secondary antibodies (1:2,000 dilution) conjugated with horseradish peroxidase (HRP) at RT for 2 h and washed. The protein signals were obtained using chemiluminescence detection reagents (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using a Bio imaging system (MicroChemi 4.2 Chemilumineszenz-System, Israel).

Statistical analysis – All data are expressed as the means ± standard deviation (SD) of at least three independent experiments. The data were compared using one-way ANOVA and Duncan's multiple range tests using the software package SPSS statistics V20. A p-value of < 0.05 was considered significant.

Results and discussion

Oxidative stress has been widely implicated in many inflammatory diseases such as IBD. Therefore, the attenuation of inflammation by functional foods and agents has been used to treat IBD and protect the gastrointestinal immune system. This research investigated the antioxidant and anti-inflammatory activities of Korean traditional fermented SP, the ethanol extract of SP and SPL. To examine antioxidant effects, ABTS and DPPH radical scavenging assays were conducted at 100 and 250 μg/mL on the 12th and 24th weeks. SP and SPL extracts exhibited ABTS (Fig. 1A) and DPPH (Fig. 1B) radical-scavenging activities, but SPL more strongly inhibited ABTS and DPPH radicals, with longer fermentation periods showing greater activity. Ascorbic acid, which used as positive control, shown 36.01% of ABTS and 62.27% of DPPH radical scavenging activities at 100 μg/mL. These results suggest that antioxidant activity was the bioactive functional components of *L. edodes* to the substances produced during the fermentation process of SP, resulting in stronger antioxidant capacity.

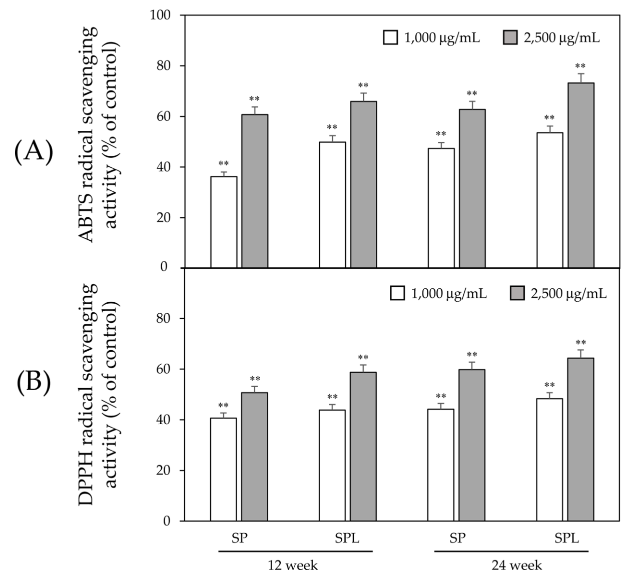

Fig. 1. Anti-oxidative effects of SP and SPL extracts on ABTS (A) and DPPH (B) radical scavenging activities. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group.

Inflammation is a non-specific immune response that occurs in reaction to any type of physical injury. Macrophages play an important roles in the inflammatory response, and are activated by the exposure to bacterial LPS and proinflammatory cytokines.[17] In this study, mouse macrophage RAW 264.7 cells were used to examine anti-inflammatory effects of SP and SPL extracts. To determine the non-toxic level of SP and SPL extracts had any cytotoxic effects on RAW 264.7 cells was investigated using an MTT assay. Each extracts had no cytotoxic effect on RAW 264.7 cells after treatment with 100 and 250 μg/mL for 24 h (Fig. 2A). The subsequent experiments were conducted with the SP and SPL extracts with no cytotoxicity. Macrophages produced inflammatory mediator such as NO in inflammation. Excessive NO levels are associated with inflammatory diseases.[18] RAW 264.7 cells were treated with SP and SPL extracts for 1 h and induced inflammatory response with LPS stimulation

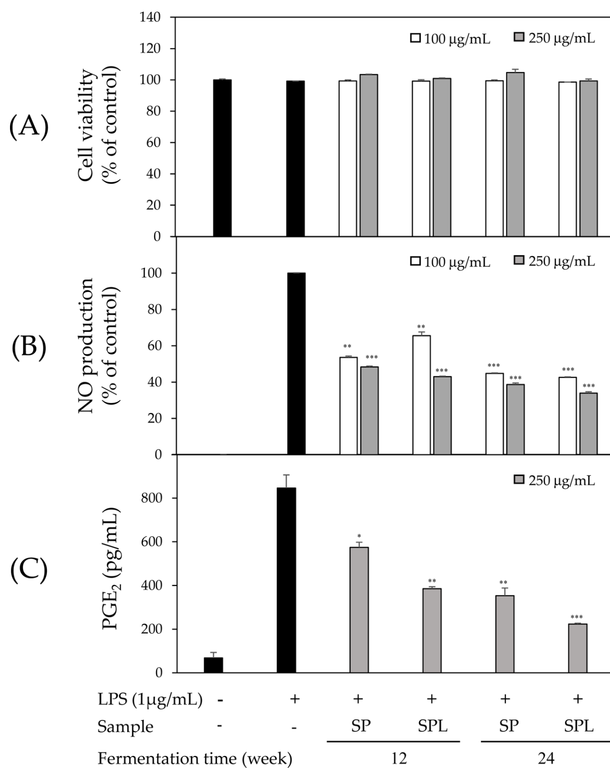

Fig. 2. Effects of SP and SPL extracts on cell viability (A), NO (B) and PGE_2 (C) production in LPS-stimulated RAW 264.7 cells. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

(1 μg/mL) for 16 h. The control group was not treated with either LPS or the extracts. To measure NO production, supernatant (100 μL) was harvested and NO was measured by the Griess assay. NO is generally a tightly controlled mediator that initiates and maintains inflammation.[19] SP and SPL extracts inhibited NO production in a dose-dependent manner at 100 and 250 μg/mL on the 12th and 24th weeks (Fig. 2B). Curcumin, as used positive control, inhibited 35.50% of LPS-induced NO production at 25 μM. Also, they decreased PGE_2, which negatively regulates induction of NO synthase by interleukins (Fig. 2C).[20] Among two extracts, SPL more strong inhibited NO and PGE_2 production, the longer the fermentation period and the greater the activities (Fig. 2).

Proinflammatory cytokines is released by LPS and inflammatory mediators-stimulated macrophages.[19,] ELISA assay was performed to examine the inhibitory effects of SP and SPL extracts on the production of proinflammatory cytokines. Compared to the untreated control group, the production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) increased in LPS-stimulated RAW 264.7 cells. Pretreatment with SP and SPL extracts at 100

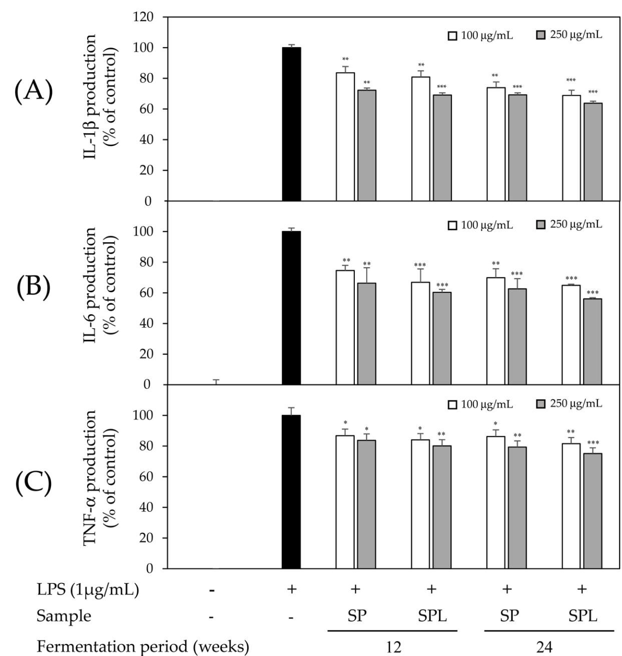

Fig. 3. Effects of SP and SPL extracts on IL-1β (A), IL-6 (B), and TNF-α (C) production in LPS-stimulated RAW 264.7 cells. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

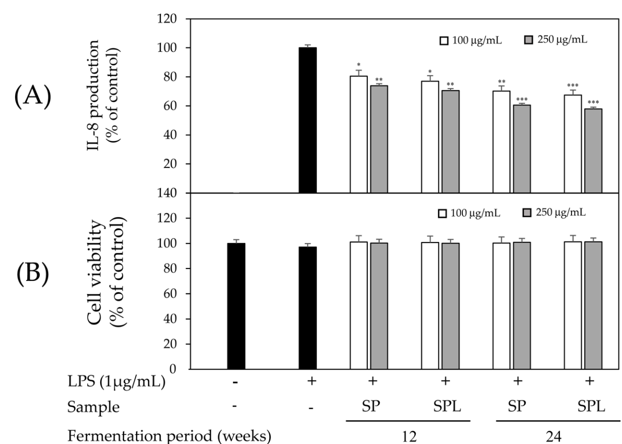

Fig. 4. Effects of SP and SPL extracts on IL-8 (A) production and cell viability (B) in LPS-stimulated HT-29 cells. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

and 250 μg/mL down-regulated IL-1β, IL-6 and TNF-α production at 100 and 250 μg/mL (Fig. 3A~3C). Additionally, the two extracts inhibited IL-8 production in LPS-stimulated HT-29 cells at 100 and 250 μg/mL (Fig. 4A) without cytotoxicity (Fig. 4B). SPL supplemented with *L. edodes* exhibited more strong inhibition of proinflammatory cytokines production, with longer fermentation

periods showing greater activity (Fig. 3 and 4A). Proinflammatory cytokines (IL-1β, IL-6 and TNF-α) play important roles in the inflammatory response in the pathogenesis of IBD.[4,21] Moreover, the synthesis of IL-8, a chemotactic cytokines, produced by inflammation stimulated-macrophages, neutrophils, keratinocytes, fibroblasts and neutrophils and is enhanced in the mucosa of patients with IBD.[22-24] Our results showed that the level of IL-1β, IL-6, TNF-α and IL-8 was decreased by SP and SPL extracts in LPS-induced mouse macrophages and human colon epithelial cells.

The inflammatory enzyme, iNOS is necessary for NO production.[18] To investigate iNOS protein level, RAW 264.7 cells were pretreated with SP and SPL extracts, and stimulated with LPS for 16 h. Western blot analysis was performed to examine the inhibitory effect of the two extracts on expresstion of iNOS protein. As shown in Fig. 5A, iNOS was not detected in unstimulated cells, but LPS considerably enhanced the protein expression. SP and SPL extracts inhibited the LPS-induced iNOS expression in RAW 264.7 cells at 250 μg/mL. Among the two extracts, the inhibition by SPL was stronger than that of SP. The inhibition by extracts fermented for 24 weeks was greater than that of the extracts fermented for 12 weeks . Thus, the inhibition of NO production after LPS stimulation mediated by the two extracts was likely attributable to the down-regulation of iNOS. Meanwhile, SP and SPL extracts did not distinctly show the effect on COX-2 expression, compared to iNOS expression and PGE_2 production (Fig. 5A).

NF-κB plays an important roles in the early stages of immune and inflammatory responses by regulating various inflammatory transcriptional factors.[5,25] To assess the inhibitory effects of SP and SPL extracts on NF-κB expression, western blot analysis was performed using specific antibodies. SP and SPL extracts down -regulated NF-κB act ivation and SPL suppl emented with *L. edodes* exhibite d more st ronger inhibition than SP (Fig. 5B). Our results indicated that SP and SPL extracts effectively inhibited the production of NO and various proinflammatory cytokines possibly through down-regulation of NF-κB. Activation of NF-κB by LPS, inflammatory mediators and cytokines induces the phos- phorylation of MAPK (ERK1/2, p38, JNK) and IκB-α in inflammatory signaling.[5] Further molecular mechanism studies on transcriptional factors regulation of SP and SPL in inflammatory response.

SP made by soybean with plant protein is representative traditional fermentation material in Korea and its function is being increased by the fermentation, a

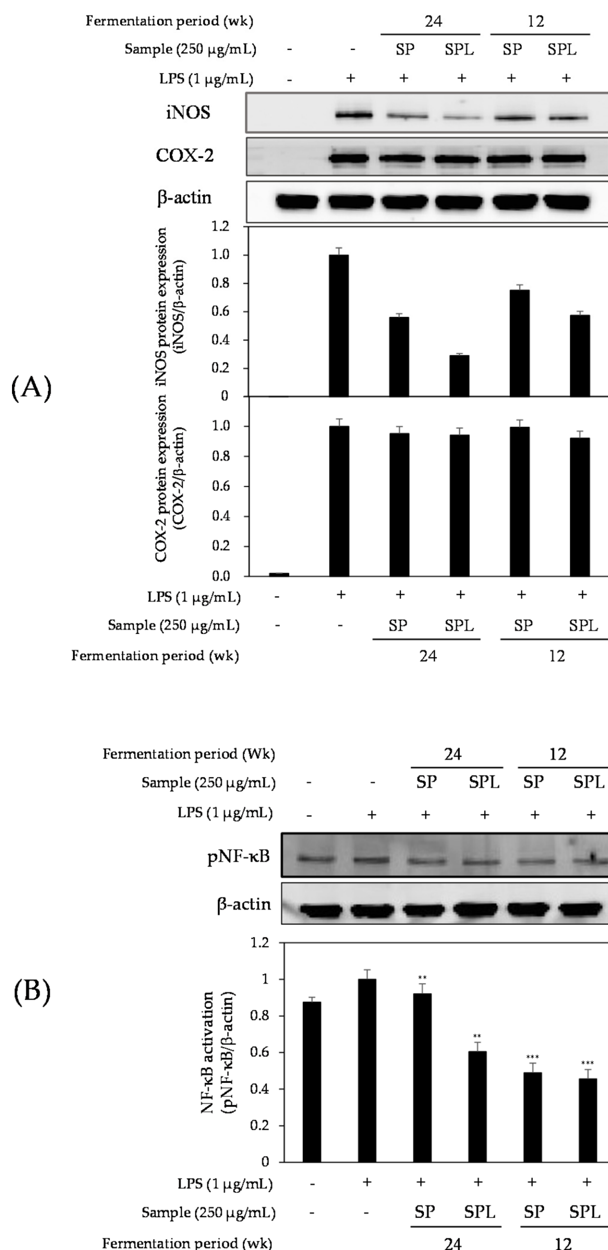

Fig. 5. Effects of SP and SPL extracts on iNOS and COX-2 expression (A) and NF-κB activation (B) in LPS-stimulated RAW 264.7 cells. The data are expressed as the mean ± SD (n = 3) of three individual experiments. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

processing method increasing biological and nutritional properties, and supplementation of various functional materials.[7,26,27] It has been reported that fermented production, especially soybean, has higher bioactivity such as antioxidant and anti-obesity activity than unfermented soybean.[26,27] We prepared SPL supplemented with *L. edodes* to develop therapeutic functional food for protection of oxidation and inflammation.

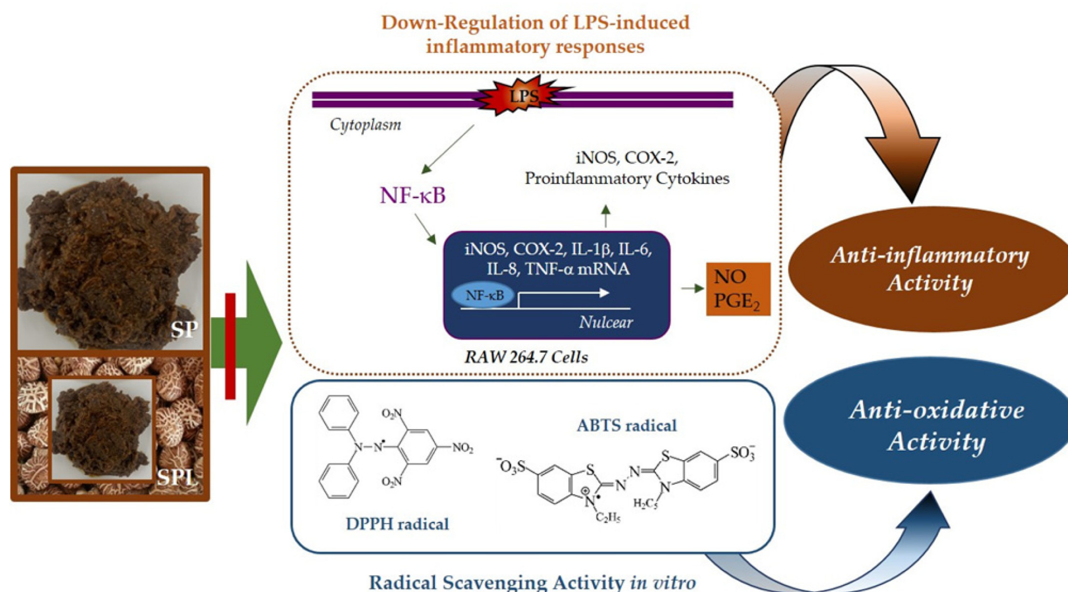

Fig. 6. Summary of anti-oxidative and anti-inflammatory effects of SP and SPL extracts.

Taken together, SP and SPL extracts showed antioxidant and antiinflammatory effects. SP and SPL extracts exhibited ABTS and DPPH radical scavenging activities. Two extracts suppressed production of inflammatory mediator, NO production and iNOS expression in LPS-stimulated RAW 264.7 cells. Moreover, SP and SPL extracts inhibited inflammatory cytokines (IL-1β, IL-6, TNF-α, and IL-8) production in LPS-induced mouse macrophage and human colon epithelial cells. The two extracts exhibited stronger inhibitory effects with longer fermentation periods. Moreover, transcriptional factor, NF-κB activation was down-regulated by SP and SPL extracts (Fig. 6). In addition, SPL supplemented with *L. edodes* showed stronger inhibition of oxidation and inflammation. Overall, SP and SPL extracts could be useful candidates for the protection of inflammatory diseases such as IBD.

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References

(1) He, Y.; Yue, Y.; Zheng, X.; Zhang, K.; Chen, S.; Du, Z. *Molecules* **2015**, *20*, 9183-9213.

(2) Kim, K. M.; Kim, Y. S.; Lim, J. Y.; Min, S. J.; Ko, H. C.; Kim, S. J.; Kim, Y. *Nutr. Res. Pract.* **2015**, *9*, 3-10.

(3) German, A. J.; Hall, E. J.; Day, M. J. *J. Vet. Intern. Med.* **2001**, *15*, 14-25.

(4) Bode, H.; Schmitz, H.; Fromm, M.; Scholz, P.; Riecken, E. O.; Schulzke, J. D. *Cytokine* **1998**, *10*, 457-465.

(5) Jeong, D. E.; Shim, S. Y.; Lee, M. *Int. Immunopharmacol*. **2020**, *86*, 106576.

(6) Kim, S. S.; Kwak, H. S.; Kim, M. J. *Food Chem.* **2020**, *328*, 127176.

(7) Marco, M. L.; Heeney, D.; Binda, S.; Cifelli, C. J.; Cotter, P. D.; Foligné, B.; Gänzle, M.; Kort, R.; Pasin, G.; Pihlanto, A.; Smid, E. J.; Hutkins, R. *Curr. Opin. Biotechnol.* **2017**, *44*, 94-102.

(8) Lee, D. H.; Kim, M. J.; Park, S. H.; Song, E. J.; Nam, Y. D.; Ahn, J.; Jang, Y. J.; Ha, T. Y.; Jung, C. H. *J. Food Sci.* **2018**, *83*, 2212-2221.

(9) Park, N. Y.; Rico, C. W.; Lee, S. C.; Kang, M. Y. *J. Clin. Biochem. Nutr.* **2012**, *51*, 235-240.

(10) Kwak, C. S.; Park, S. C.; Song, K. Y. *J. Med. Food* **2012**, *15*, 1-9.

(11) Jang, S. E.; Kim, K. A.; Han, M. J.; Kim, D. H. *J. Med. Food* **2014**, *17*, 67-75.

(12) Gaitán-Hernández, R.; López-Peña, D.; Esqueda, M.; Gutiérrez, A. *Int. J. Med. Mushrooms* **2019**, *21*, 841-850.

(13) Kang, M. Y.; Rico, C. W.; Lee, S. C. *Food Sci. Biotechnol.* **2012**, *21*, 167-173.

(14) Proestos, C.; Lytoudi, K.; Mavromelanidou, O. K.; Zoumpoulakis, P.; Sinanoglou, V. J. *Antioxidants* **2013**, *2*, 11-22.

(15) Blois, M. S. *Nature* **1958**, *181*, 1199-1200.

(16) Shin, J.; Lee, M. *Nat. Prod. Sci.* **2020**, *26*, 244-251.

(17) Ferrero-Miliani, L.; Nielsen, O. H.; Andersen, P. S.; Girardin, S. E. *Clin. Exp. Immunol.* **2007**, *147*, 227-235.

(18) Gao, Y.; Fang, L.; Cai, R.; Zong, C.; Chen, X.; Lu, J.; Qi, Y. *Phytomedicine* **2014**, *21*, 461-469.

(19) Choy, E. H.; Panayi, G. S. *N. Engl. J. Med.* **2001**, *344*, 907-916.

(20) Blázovics, A.; Hagymási, K.; Prónai, L. *Orv. Hetil.* **2004**, *145*, 2523-2529.

(21) Neurath, M. F. *Nat. Rev. Immunol.* **2014**, *14*, 329-342.

(22) Kolios, G.; Robertson, D. A.; Jordan, N. J.; Minty, A.; Caput, D.; Ferrara, P.; Westwick, J. *Br. J. Pharmacol.* **1996**, *119*, 351-359.
(23) Baggiolini, M.; Clark-Lewis, I. *FEBS Lett.* **1992**, *307*, 97-101.
(24) Grimm, M. C.; Elsbury, S. K.; Pavli, P.; Doe, W. F. *Gut* **1996**, *38*, 90-98.
(25) Marks-Konczalik, J.; Chu, S. C.; Moss, J. *J. Biol. Chem*. **1998**, *273*, 22201-22208.
(26) Cha, Y. S.; Park, Y.; Lee, M.; Chae, S.; Park, K.; Kim, Y.; Lee, H. *J. Med. Food* **2014**, *17*, 119-127.
(27) Chung, S. I.; Rico, C. W.; Kang, M. Y. *Nutrients* **2014**, *6*, 4610-4624.

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