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Effects of fruiting body extracts of *Lentinus edodes* cultivated using corn cob on biological activities

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Abstract−We investigated the antioxidant, nitrite scavenging, immune-enhancing and antimicrobial effects of the *Lentinus edodes* fruiting body extracts (LEFB extract) cultivated in the solid state using corn cob. The CAT, SOD and GSH-Px activities in the LEFB extract (400mg)-fed group were approximately 1.70, 1.64, and 2.2 times higher than those of the control group. The nitrite scavenging activity was highest (86.4%) when the LEFB extract concentration was 600 μg/mL. The immune-enhancing and antimicrobial effects of LEFB extract after an experimental *Bordetella bronchiseptica* infection were examined. White blood and neutrophil in the LEFB extract (400 mg)-fed group were approximately 2.1 and 2.2 times higher, respectively, than those of the control group. The total leukocyte was 30% higher than that of the control group. The CD4 : CD8 ratio in the LEFB extract (200 mg)-fed group was increased approximately 54.3% compared to that of the control group. The levels of TNF-α and IFN-γ mRNA expression in the LEFB extract (400 mg)-fed group were approximately 49 and 50% lower than those of the control group. The bacteria and inflammatory cell in the LEFB extract (400 mg)-fed group were approximately 48.7 and 29% lower, respectively, than that of the control group. These results suggest that the LEFB extract may be useful in immune function improvement.

Key words: *Lentinus edodes*, Corn Cob, Antioxidant Activity, Nitrite Scavenging Activity, and Immune-enhancing Activity, Antimicrobial Effect

INTRODUCTION

Lentinus edodes, a species of mushroom associated with the shii tree (*Castanopsis cuspidate* Schottky) and *take*, is one the five most commonly produced edible mushrooms in the world. It is becoming popular in nutritional and medicinal products throughout Asia, Europe, and North America [1]. Currently, the *L. edodes* fruiting body is being produced on an industrial scale from sawdust obtained from a range of wood sources. Alternative processes have been explored extensively because of the high production cost and environmental impact associated with the excessive utilization of natural wood sources. A variety of agricultural and industrial by-products or wastes have been used as inexpensive growth substrates for economical production from various mycelial species. Of these alternatives, mushroom production from various agricultural wastes [3-8] is quite attractive. Although there have been many reports on the cultivation of mushrooms using agriculture and industrial wastes, there are no reports on the biological activities, such as antioxidant, immune-enhancing and antimicrobial effects of extracts of *L. edodes* fruiting body cultivated on corn cob as an energy source.

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Among the agricultural waste materials, corn cob is a promising agricultural resource for mushroom cultivation due to the extensive cultivation of corn. Corn cob is a by-product generated during the processing of corn, which is rich in hemicellulose and one of the most widely planted crops in the world. Traditionally, the corn cob waste generated has been sent to landfill, but this causes serious environmental problems, primarily because of the waste volume and high organic material concentration. Recently, Zdena et al. [9] extracted xylan from corn cob, which is applicable as an additive in papermaking, textile printing and the pharmaceutical industry. Rivas et al. [10] prepared a fermentable hydrolysate from corn cob. Cao et al. [11] examined the pyrolysis behavior of corn cob as an energy source in a fixed bed. Perotti and Molina [12] used corn cob as a bacterial substrate for the production of forage protein. The preparations of ordinary activated carbon by a combination of chemical and physical activation have also been reported [13]. Ninawe and Kuhad [14] investigated xylanase production from *Streptomyces cyaneus* using corn cob as a carbon source. Tota et al. [15] showed xyltol production from corn cob by *Candida magnolia* in high yield. Recently, we attempted to use corn cob as an alternative substrate for mushroom cultivation [16]. We investigated the biochemical and enzymatic properties of a fibrinolytic agent purified from *Pleurotus eryngii* cultivated under solid-state conditions using corn cob as the energy source [17] and the antioxidant and the nitrite scav-

enging activities of the extracts from *Pleurotus ferulae* fruiting body grown on the solid state using mixture of corn cob and activated bleaching earth [18].

This study investigated the biological activities of extracts of *Lentinus edodes* fruiting body (LEFB extract) cultivated in the solid state using corn cob by analyzing the antioxidant, nitrite scavenging, immune-enhancing and antimicrobial effects after an experimental *Bordetella bronchiseptica* infection.

MATERIALS AND METHODS

1. Strain and Cultivation

Fruiting body cultures of *Lentinus edodes* were performed in propylene bags. The media were prepared as follows: 90% corn cob, 4.5% populous sawdust, 5% rice bran, 0.2% $NH_4H_2PO_4$, and 0.3% $CaCO_3$. Five hundred grams of the mixture containing 70% moisture was dispersed into the bag. After autoclave sterilization at 121 °C for 40 min followed by cooling, a 5% inoculation was used in each sample. The inoculated blocks were incubated at 25 °C in the dark. After 25 days, the substrates were completely colonized by the mycelium. The blocks were then shocked at 4-5 °C for 48 hrs to stimulate the production of fruiting bodies.

2. Preparation of Sample Extract

A sample (100 g) of fresh fruiting body was extracted with 500 mL of hot distilled water using a soxhlet apparatus at 90 °C for 3 hrs, centrifuged at 5,000 ×g for 15 min, and filtered through filter paper (0.2 μm, MN sterilizer PES; Macherey-Nagel GmbH & Co., KG, Duren, Germany). After standing overnight at 4 °C, the mixture was centrifuged and the supernatant was then lyophilized. The solid mass was used as a sample (designated the LEFB extract). The dried extract was used directly to analyze the antioxidant components and stored at 4 °C for further use.

3. Test of Animals for Antioxidant Enzyme Assay

White male rats (Sprague-Dawley), weighing approximately 200-230 g, were raised for eight weeks under constant conditions for acclimatization to the laboratory environment. The temperature and relative humidity were maintained at 20 °C and 50%, respectively. Over the course of the experiment, the rats were provided solid feed from Sam Yang Ltd. (Korea) and water. After the rats were predomesticated for two weeks, oxidative stress was induced by administering 10 mL (5.0 mL at 10:00, and 5.0 mL at 18:00) of ethanol (30%) to the control group, silymarin-fed group, and LEFB extract-fed group, respectively, for 2 weeks. To obtain the enzyme solution from the liver, 10 mL of ethanol (30%) to the control group, 10 mg of silymarin/kg/body weight/day to the silymarin-fed group, and 200 and 400 mg of LEFB extract/kg/body weight/day to the LEFB extract-fed groups were administered daily over a period of 8 weeks.

4. Antioxidant Enzyme Activity

Catalase activity (CAT): A 0.1 mL aliquot of surfactant was added to potassium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (10.5 mM). The reaction took place for 30 sec at 25 °C. The amount of enzyme activity required to decompose 1 μmole H_2O_2/sec via this reaction was defined as one unit of activity. Superoxide dismutase activity (SOD): The SOD activity was determined by recording the inhibition of ferricytochrome C reduction with EDTA. In each sample, the amount of enzyme sufficient to inhibit the rate of cytochrome C reduction by 50% was determined. Glutathione peroxidase activity (GSH-Px): A 500 μL mixture of potassium phosphate buffer (0.1 M, pH 7.0) containing 1×10^{-3} M sodium azide, 1 mM EDTA, 10 μL of enzyme solution, 100 μL of glutathione reductase (2.768 U/mL), and 100 μL of glutathione (1×10^{-2} M) was mixed and precultured for 10 min at 37 °C. Subsequently, an aliquot of $NaHCO_3$ (0.1%) containing 100 μL of NADPH (1.5×10^{-3} M) and 100 μL of H_2O_2 (1.5×10^{-3} M) was added to the reaction mixture. The absorbance was measured for 1 min at 340 nm.

5. Nitrite Scavenging Activity

One milliliter of $NaNO_2$ (1 mM) was added to 1 mL of the sample, and the pH of the resulting mixtures was adjusted to 1.2. The final sample volume was adjusted to 10 mL. The sample was allowed to react at 30 °C for 1 hrs, after which 1 mL of sample was obtained, mixed thoroughly with 5 mL of acetic acid (2%) and 0.4 ml of Griess reagent, and kept at room temperature for 15 min. A blank was prepared by adding 0.4 mL of distilled water instead of the Griess reagent. The nitrite scavenging activity was determined at 520 nm.

6. Test of Animals for Immune Enhancing and Antimicrobial Assay

Specific pathogen-free female 6-week-old ICR mice (DBL, Daejeon, Korea) were divided randomly into three groups consisting of five mice each. The control group was given a commercial, nutritionally complete, extruded dry rodent feed (Feed lab, Gyeonggi province, Korea). The experiment groups received the same extruded dry rodent feed supplemented with 200 mg of LEFB extract/kg/body weight/day (LEFB extract (200 mg)-fed group) and 400 mg of LEFB extract/kg/body weight/day (LEFB extract (400 mg)-fed group). All mice were housed in an air-conditioned room, kept in polypropylene cages and allowed free access to their particular diet and tap water from drinking bottles with stainless steel sipper tubes.

7. *Bordetella bronchiseptica* Infection

All mice were acclimatized to their particular diet for 25 days before the experimental bacterial infection. The original stock of *B. bronchiseptica* was cultured overnight on MacConkey agar (BD Biosciences, Franklin Lakes, NJ, USA). A single colony was selected and inoculated on Brain Heart Infusion broth (BD Biosciences) in a universal bottle and incubated at 250 ×g for 16 hrs at 37 °C. Viable counts of the broth culture were prepared using the pour plate method. The inoculated broth was diluted with phosphate-buffered saline (PBS) to obtain the infective dose of colony forming units (cfu/mL). Ten microliters of the *B. bronchiseptica* culture was inoculated intranasally in each nostril of each mouse. All mice were sacrificed for collection of blood, bronchial lymph node (BLN), Bronchoalveolar lavage (BAL) at five days post-infection (DPI).

8. Differential Ratios of Leukocytes in Peripheral Blood

Blood was collected in tubes coated with ethylene diaminetetraacetic acid from the retro-orbital plexus of the mice. The blood leukocytes and differential counts were determined using an automated veterinary hematology analyzer (Oxford Science, Oxford, CT, U.S.A.) configured for mouse blood. Each sample was counted independently three times.

9. Determination of Lymphocyte Subpopulation in BLN

The BLN and single cell suspensions were prepared by pushing the tissue through a 40 μm nylon mesh (BD Biosciences). The isolated cells were analyzed to determine the $CD3^-CD19^+$ B lymphocyte, $CD3^+CD19^-$ T lymphocyte, $CD4^+CD8^-$ T lymphocyte, and $CD4^-CD8^+$ T lymphocyte component ratio. The cells were stained

with both phycoerythrin (PE)-conjugated anti-mouse CD3 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD19 (BD Biosciences) to determine the ratio of the $CD3^-CD19^+$ B lymphocyte and $CD3^+CD19^-$ T lymphocyte component. To determine the ratios of the $CD4^+CD8^-$ T lymphocyte and $CD4^-CD8^+$ T lymphocyte components, the cells were stained with FITC-conjugated anti-mouse CD4 (BD Biosciences) and PE-conjugated anti-mouse CD8 (BD Biosciences). After incubation at 4 ℃ for 30 min in the dark, the cells were washed twice with PBS and the lymphocyte subpopulation was analyzed using a FACSort flow cytometer (BD Biosciences). The viable lymphocytes were gated by their forward and side-scatter characteristics (FSC/SSC), and 10,000 events were analyzed for positive staining with FITC or PE. The results for each lymphocyte subpopulation are expressed as the percentage of events in the FSC/SSC lymphocyte gate.

10. Nitric Oxide Inhibitory Activity

RAW264.7 cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO, USA) in a CO_2 incubator (5% CO_2 in air) at 37 ℃ for 24 hrs. The cells were cultured in a 24-well plate (5×10^5) containing DMEM supplemented with 10% FBS for one day to become almost confluent. The cells were then cultured with various extracts of LEFB in the presence of 10 μg/mL of LPS for 24 hrs. The nitrite concentration was measured by using the supernatant from the RAW264.7 cells with the Griess reagent. This mixture was then incubated at room temperature for 10 min and the absorbance was read at 540 nm on an ELISA reader (Ceres UV, Bio-Tek instrument, Basel, Switzerland).

11. Evaluation of mRNA Expression Levels of TNF-α and IFN-γ in BLN

The total RNA extraction from BLN was performed with an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and the target RNA was reverse transcribed using a QuantiTect® reverse transcription kit (Qiagen) according to the manufacturer's instructions. To minimize the variations in reverse transcriptase efficiency, all samples were transcribed simultaneously. The primers and probes for murine tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-α) were designed. The probes were dual labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and the quencher dye 6-carboxytetramethyrhodamine (TAMRA) at the 3′ end. The TNF-α and IFN-γ mRNA levels were determined by a real-time polymerase chain reaction (PCR) assay using MyiQ™2 (Bio-Rad Laboratories, Hercules, CA, USA) with 0.5 μg of cDNA. The threshold cycle (Ct; the cycle number at which the amount of amplified gene of interest reaches a fixed threshold) was then determined. The relative quantitation of TNF-α and IFN-γ mRNA expression was calculated using a comparative Ct method. The relative quantitation value of the target (TNF-α or IFN-γ) was normalized to an endogenous control β-actin gene and relative to a calibrator.

12. Viable Cell Count in BAL

The viable cell count was conducted in samples. Briefly, the samples were collected by inserting a suitable cannula towards the lungs into the exposed trachea. A 2 mL syringe was attached to the cannula and the lungs were washed with 1 mL of PBS by flushing the solution in and out of the lungs several times. The wash solutions were collected into a suitable tube. One hundred microliters of the wash solutions were serially diluted 10-fold in PBS and 100 μL of each dilution was spread onto a MacConkey agar plate (BD Biosciences) and incubated at 37 ℃ for 48 hrs. The colonies that were characteristically small, pale with a pinkish hue were counted and expressed as log cfu/mL but only for those colonies between 30 and 300 per plate. In addition, the representative colonies were subjected to gram staining and biochemical tests for identification purposes. Each sample was tested in duplicate.

13. Statistical Analysis

Results are expressed as the mean±standard deviation.

RESULTS

1. Effect of LEFB Extract on Antioxidant Enzyme Activity

Vegetables and fruits are rich sources of antioxidants that prevent free radical damage and reduce the risk of chronic diseases. Therefore, the consumption of dietary antioxidants from these sources is beneficial for preventing cardiovascular diseases, such as atherosclerosis. The concentrations of total polyphenol, total flavonoid, polysaccharide, and beta-glucan were 18.63, 1.58, 5.92 and 1.23 mg/g of the dried extract, respectively (data not shown). Fig. 1 shows the effect of LEFB extract on CAT, SOD, and GSHPx activities in livers. The CAT activity in the normal group fed without ethanol and LEFB extract was 14.9 U/mg protein but was 7.5 U/mg protein in the control group. The activity was increased from 9.1 to 12.7 U/mg protein when the administered concentration of the LEFB extract was increased from 200 to 400 mg/kg/ body weight/day. In the case of the Silymarin group, it was 13.6 U/mg protein. The SOD activity in the normal group was 21.6 U/mg protein. On the other hand, it was 10.6 U/mg protein in the control group. When the concentration of LEFB extract administered was increased from 200 to 400 mg// kg/body weight/day, the SOD activity was increased from 12.9 to 17.4 U/mg protein. In the case of the silymarin group, it was 18.3 U/mg protein. The GSH-Px activity in the normal group was 7.9 U/mg protein, but was 3.8 U/mg protein in the control group. When the concentration of LEFB extract administered was increased from

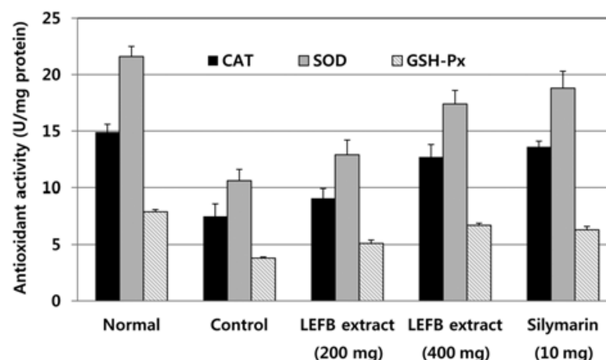

Fig. 1. Effect of LEFB extract on the CAT, SOD, and GSH-Px activities in the liver of rats. Normal group: without ethanol and LEFB extract. Control group: 10 mg of ethanol/kg/body weight/day. LEFB extract (200 mg): 10 mg of ethanol and 200 mg of LEFB extract/kg/body weight/day. LEFB extract (400 mg): 10 mg of ethanol and 400 mg of LEFB extract/ kg/body weight/day. Silymarin group: 10 mg of ethanol and 10 mg of silymarin/kg/body weight/day.

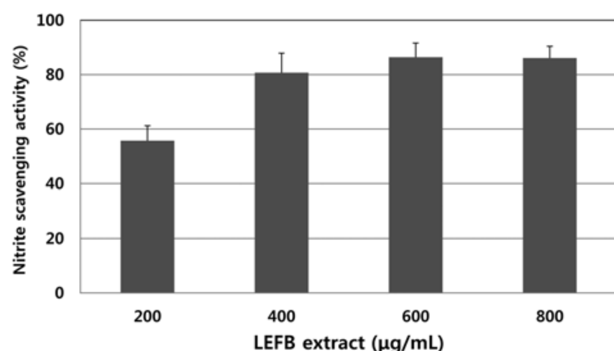

Fig. 2. Effect of LEFB extract on the nitrite scavenging activity.

200 to 400 mg//kg/body weight/day, the GSH-Px activity was increased from 5.1 to 6.7 U/mg protein, which was similar to that of the silymarin-fed group. In the case of GSH production, it was increased 82.7 to 101.3 nmol/mg protein, which was similar to that of the silymarin-fed group (data not shown). This suggests that the LEFB extract might be useful as a material to prevent hepatoxicity in humans.

2. Effect of LEFB Extract on the Nitrite Scavenging Activity

To examine the effect of the LEFB extract concentration on the nitrite scavenging activity, 200, 400, 600, and 800 μg/mL of LEFB extract were used. Fig. 2 shows the effect of LEFB extract on the nitrite scavenging activity. The nitrite scavenging activity increased with increasing LEPC extract concentration by 600 μg/mL. The nitrite scavenging activity was increased from 55.7 to 86.4% when the extract concentration of LEFB was increased from 200 to 600 μg/mL. On the other hand, it was not increased in the cases over 800 μg/mL (data not shown). These present results suggest that the LEFB extract can be useful for preventing nitrosamine formation in foods.

3. Effect of LEFB Extract on the Differential Ratio of Leukocyte, the Total Leukocyte Number and Lymphocyte Subpopulation in the Bronchial Lymph Node

The differential count of leukocytes and erythrocytes was measured to examine the effect of the LEFB extract on the biochemical parameters of the peripheral blood of mice after an experimental *B. bronchiseptica* infection. Table 1 shows the effect of LEFB extract on the differential ratio of leukocyte in the peripheral blood of *B. bronchiseptica* infected mice. When the concentration of LEFB extract administered was increased from 200 to 400 mg/kg/body weight/day, the number of white blood cells and neutrophils increased significantly from 4.308 to 4.964×10^3/μL and from 1.567 to 1.592×10^3/μL, respectively. The lymphocyte, eosinophil, and basophil in the LEFB extract (400 mg/kg/body weight/day)-fed group were 1.93, 0.04, and 0.008×10^3/μL, respectively. Table 2 shows effect of LEFB extract on the total leukocyte number and Lymphocyte subpopulation in the bronchial lymph node of experimentally *B. bronchiseptica* infected mice. When the concentration of LEFB extract administered was increased from 200 to 400 mg/kg/body weight/day, the total leukocyte number in the bronchial lymph node did not increase. In the case of the lymphocyte subpopulation, when the concentration of LEFB extract administered was increased from 200 to 400 mg/kg/body weight/day, the CD3, CD4 and CD8 levels increased from 54.06 to 58.53%, 46.35 to 48.44%, and 8.52 to 12.07%, respectively. On the other hand, the CD19 level decreased from 41.57 to 36.41%. The lymphocyte subpopulations increased approximately 20-30% compared to that of the control group.

4. Effects of LEFB Extract on the Nitric Oxide Inhibitory Activity and the TNF-α and IFN-γ Expression

The effects of the LEFB extract concentrations on the nitric oxide inhibitory activity were examined by using 100, 200, 300, or 400 μg/mL of the LEFB extract. The results are listed in Fig. 3. The nitric oxide inhibitory activity was dependent on the LEFB extract concentration. In particular, when the LEFB extract concentration was increased from 100 to 300 μg/mL, the nitric oxide inhibitory activity increased from 15.6 to 44.2%. On the other hand, no increase was observed when the LEFB concentration was more than 400 μg/mL of LEFB extract. Fig. 4 shows the effects of the LEFB extract on the relative TNF-α and IFN-γ mRNA expression levels in the bronchial lymph node of the experimentally *B. bronchiseptica* infected mice. The relative TNF-α and IFN-γ mRNA expression levels in the BLN was decreased in a dose-dependent manner during the entire experimental infected period. In particular, when the concentration of LEFB extract administered was increased from 200 to 400 mg/kg body weight/day, the relative TNF-α and IFN-γ mRNA expression levels in the BLN were decreased from 0.68 to

Table 1. Effect of LEFB extract on the differential ratio of leukocyte in the peripheral blood of *B. bronchiseptica* infected mice

Group	Number (×10^3/μL)					
	White blood cell	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Control	2.40±0.12	0.69±0.025	1.92±0.11	0.19±0.022	0.03±0.001	0.007±0.0001
LEFB extract (200 mg)	4.30±0.28	1.56±0.02	2.10±0.12	0.16±0.003	0.02±0.001	0.005±0.0001
LEFB extract (400 mg)	4.96±0.51	1.59±0.152	1.93±0.13	0.16±0.004	0.04±0.001	0.008±0.0001

Table 2. Effect of LEFB extract on the total leukocyte number and Lymphocyte subpopulation in the bronchial lymph node of *B. bronchiseptica* infected mice

Group	Total leukocyte (× 10^6 cell)	Lymphocyte subpopulation (%)			
		CD3	CD19	CD4	CD8
Control	3.35±1.61	44.75±2.35	50.93±4.78	38.27±2.78	10.57±1.02
LEFB extract (200 mg)	4.35±1.84	54.06±4.52	41.57±3.89	46.35±4.20	8.52±0.45
LEFB extract (400 mg)	4.35±1.23	58.53±5.01	36.41±3.10	48.44±4.17	12.07±1.07

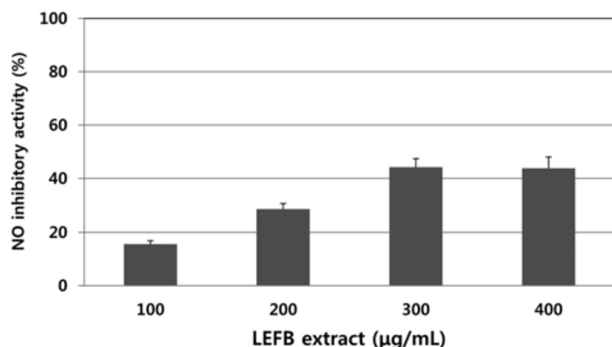

Fig. 3. Effect of LEFB extract on the nitric oxide inhibitory activity.

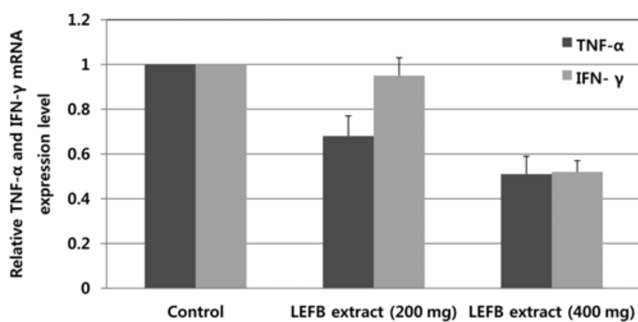

Fig. 4. Effect of LEFB extract on the relative TNF-α and IFN-γ mRNA expression levels in the bronchial lymph node of *B. bronchiseptica* infected mice.

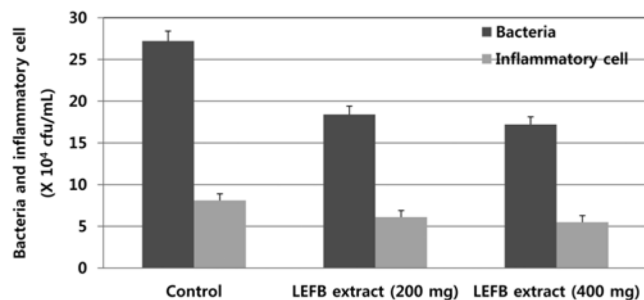

Fig. 5. Effect of LEFB extract on the number of bacteria and inflammatory cell in the broncho-alveolar lavage of *B. bronchiseptica* infected mice.

0.51 and 1.05 to 0.52, respectively. This suggests that the LEFB extract can enhance the systemic immune responses in mice.

5. Effect of LEFB Extract on the Antimicrobial Effect

The bacteria clearance and inflammatory cell number were measured to determine the effect of the LEFB extract concentration on the antimicrobial effect in broncho-alveolar lavage of experimentally *B. bronchiseptica* infected mice. Fig. 5 shows the number of bacteria and inflammatory cells. When the concentration of LEFB extract administered was increased from 200 to 400 mg/kg/body weight/day, the number of bacteria and inflammatory cells decreased from 18.4 to 17.2×10^4 cfu/mL and from 6.1 to 5.5×10^4 cells/mL, respectively.

DISCUSSION

L. edodes has been reported to exhibit biological activity, such as anticarcinogenic action, anti-tumor effects, antimicrobial properties, improved liver function and a reduction of viremia in patients with chronic hepatitis B, inhibition of human immunodeficiency virus infection *in vitro*, antiatherogenesis, anti-HIV effect, and hepatoprotective effect etc. [19]. Although there are many reports on the biological activities of the *L. edodes* extract, there are none on the antioxidant, nitrite scavenging effect, immune-enhancing and antimicrobial effects of extracts of the *L. edodes* fruiting body cultivated on corn cob as an energy source. To determine the biological effects of LEFB extract, we researched the CAT, SOD and GSH-Px activities, nitrite scavenging activity, leukocytes and erythrocyte, total leukocyte and lymphocyte subpopulation, nitric oxide inhibitory activity, TNF-α and IFN-γ expression levels, and number of bacteria and inflammatory cells.

Currently, synthetic antioxidants are used widely in the food industry [20], but restrictions on synthetic antioxidants are being imposed because of their liver toxicity and carcinogenicity. Therefore, the development and utilization of more effective antioxidants of natural origin is desirable. Previously we examined the antioxidant properties of natural origins, such as mushroom extracts (e.g., *Pleurotus ferulae*, *Pleurotus nebrodensis* Inzenga, *Fomitopsis pinicola*, and *Antrodia camphorata*) [19,21-23], extracts of a fish, seaweed, and mushroom mixture [24], bamboo oil extracted from *Phyllostachys nigra* var. *henonis* [25], *Achyranthis Radix* extract [26], and *Astragalus sinicus* L. seed extract [27]. Shahidi and Wanasundara [28] reported that the antioxidant activity of plant materials correlates well with the concentration of antioxidant components. Therefore, it is important to consider the effects of antioxidant components, such as polyphenol, polysaccharide, flavonoid, and beta-glucan, of extracts from *L. edodes* grown on corn cob. The polyphenol, polysaccharides, flavonoid, and beta-glucan concentrations in the extracts were dependent on the extractants used for extraction. In particular, when hot water was used to extract the fruiting body, they were approximately 2-3 times higher than those extracted with ethanol or methanol (data not shown). The total polyphenol, total flavonoid, polysaccharide, and beta-glucan concentrations of LEFP extract cultivated using corn cob were also about 20-30% higher than those of the commercial LEFB extract (data not shown). In general, living tissues are endowed with innate antioxidant defense mechanisms. The damaging effects of oxidative stress appear to be related to the inability of the cellular defense enzymes, such as CAT, SOD, and GSH-Px, to reduce the reactive oxygen species and oxygen-centered free radicals to levels that prevent their destructive effects. A decrease in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, which have deleterious effects, such as loss of integrity and function of cell membranes [27]. The CAT activity in the LEFB extract (400 mg)-fed group was approximately 1.7 times higher than that of the control group and similar to that of the silymarin-fed group. This suggests that the high CAT activity induced by the LEFB extract can protect cells from the damaging effects of hydrogen peroxide. The SOD activity in the LEFB extract (400 mg)-fed group was 1.64 times higher than that of the control group and similar to that of silymarin-fed group. This indicates that the LEFB extract induces significant SOD activity in the liver, which can reduce the potential damage caused by free radicals. The GSH-Px activity in the LEFB extract (400 mg)-fed group was 2.2 times higher than that of the control group and similar to that of the sily-

marin-fed group. The increased liver GSH-peroxidase activity with the LEFB extract suggests that such a treatment can protect the cells or tissues against the cytotoxic or genotoxic effects of peroxide and hydroxyl radicals. The thiobarbituric reactive substance was also decreased (data not shown), which suggests that the LEFB extract might be a valuable antioxidant from a natural source that would be applicable in the medicine, cosmetics and the food industry.

Nitrite reacts with amines in protein-rich foods, medicines and residual pesticides to form nitrosamines, and is present in large quantities in meat color and both leaf and root vegetables. Nitrosamine is converted to diazoalkane, proteins and intracellular components, which can increase the risk of cancer [29]. Among the various extracts of the *L. edodes* fruiting body using different solvents, the nitrite scavenging activity using polar solvents was higher than when non-polar solvents were used (data not shown). The nitrite scavenging activities of the LEFB extracts were affected by pH. The maximum nitrite scavenging activity was observed at pH 1.2, irrespective of the extracts (data not shown). The nitrite scavenging activity increased with increasing LEFB extract concentration. In particular, the activity was highest (86.4%) when the extract concentration of LEFB was 600 μg/mL. Overall, the antioxidant and nitrite scavenging activities of the LEFB extract indicate it to be a potential source for the development of a range of health supplements and pharmaceutical and nutraceutical applications.

B. bronchiseptica is a piliated coccobacillus that is important as a cause of disease in the respiratory tract of dogs, pigs, laboratory rodents and a variety of wild mammalian species. In particular, it is a primary etiological agent or a predisposing factor that results in atrophic rhinitis, pneumonia and porcine reproductive and respiratory disease complex in animal [30]. This study evaluated the immune enhancing effects of the LEFB extract concentration in mice against *B. bronchiseptica*, as an initial step towards promoting the immune activity and preventing disease. The white blood cells and neutrophils in the LEFB extract (400 mg)-fed group were approximately 2.1 and 2.2 times higher than of the control group, respectively. On the other hand, the lymphocyte, eosinophil and basophil counts were similar to those of the control group. In the case of monocytes, however, the counts were slightly lower than that of the control group. The total red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were not affected by the LEFB extract concentration. However, the total red blood cell, hemoglobin, and hematocrit were slightly higher than those of the control group. The mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were similar to those of the control group (data not shown). This suggests that leukocyte was strongly affected by the LEFB extract concentration. The total leukocyte number was not affected by the LEFB extract concentration. On the other hand, the total leukocyte in the LEFB extract group was approximately 30% higher than that of the control group. In the case of the lymphocyte subpopulation, the CD3, CD4 and CD8 but not CD19 showed a slight increase when the concentration of LEFB extract administered was increased from 200 to 400 mg/kg/body weight/day. In the LEFB extract (200 mg)-fed group, the CD4 : CD8 ratio was significantly higher (~54.3%) than that of the control group. The lysozyme activity in serum was slightly higher that of the control group when the concentration of LEFB extract administered was 400 mg/kg body weight/day (data not shown). This indicates that the lymphocyte subpopulation was strongly affected by the LEPC extract.

Nitric oxide (NO) is a short-lived biomolecule that mediates many biological functions, including the host defense, vasoregulation, platelet aggregation and neurotransmission. The level of NO production by activated macrophages mediates the immune functions including the antiviral, antimicrobial and antitumor activities [31]. On the other hand, excess NO production is associated with several diseases, such as arthritis and other chronic inflammatory diseases, autoimmune diseases and septic shock by increasing the vascular permeability and extravasations of fluid and proteins at the inflammatory sites [32]. Therefore, inhibiting high-output nitric oxide production has been a therapeutic strategy for the treatment of a range of inflammatory diseases. The NO inhibitory activity is dependent on the LEFB extract concentration. In particular, the NO inhibitory activity was highest (44.2%) when LEFB extract concentration was 300 μg/mL. This indicates that the NO inhibitory activity is strongly affected by the LEFB extract concentration. The dysregulation of cytokines plays an essential role in many inflammatory conditions, such as septic shock, hemorrhagic shock, rheumatoid arthritis, and atherosclerosis. Inhibition of inflammatory cytokines and mediator production or function serves as a key mechanism in the control of inflammation, and agents that suppress the expression of these inflammation-associated genes have therapeutic potential in the treatment of inflammatory diseases [33]. In particular, TNF-α and IFN-γ are produced mainly by activated macrophages and stimulate T cells. These cytokines play important roles in the host defense against infection by viral and microbial pathogens, and induce a range of physiologically significant responses that contribute to immunity [34]. The levels of TNF-α and IFN-α mRNA expression in mice were measured to evaluate the effects of the LEFB extract concentrations on the cytokines released in the bronchial lymph node of experimentally *B. bronchiseptica* infected mice. The levels of TNF-α and IFN-γ mRNA expression in the BLN in the LEFB extract (400 mg)-fed group were approximately 49 and 50% lower than those of the control group. These results suggest that the LEFB extract inhibits the production of several inflammatory mediators and could be used in the treatment of a range of inflammatory diseases.

The antimicrobial effects after an experimental *B. bronchiseptica* infection increased with increasing LEFB extract concentration. The bacteria count in the bronchio-alveolar lavage of the experimentally *B. bronchiseptica* infected mice in the LEFB extract (400 mg)-fed group was approximately 48.7% lower than that of the control group. In the case of inflammatory cells, the number of bacteria was 29% lower than that of the control group. This suggests that bacteria clearance and inflammatory cells in the bronchoalveolar lavage were strongly affected by the LEFB extract.

In conclusion, the antioxidant, nitrite scavenging, immune-enhancing and antimicrobial effects are strongly affected by the LEFB extract concentration, and the LEFB extract also has high immune-enhancing and antimicrobial effects against *B. bronchiseptica* in mice. Corn cob media can be a suitable substrate for fruiting body production and can be recycled for the culture of *L. edodes* fruiting bodies. Nevertheless, further studies will be needed to determine the relationship between biological properties and the pharmacological activity of the *L. edodes* fruiting body and mycelium extract in

pre-clinical animal studies. We are investigating the antihypercholesterolemic effect of the LEFB extract, as well as the biochemical and enzymatic properties of a fibrinolytic enzyme purified from the *L. edodes* fruiting body cultivated in the solid state using corn cob. We are also researching the large-scale production of *L. edodes* mycelium in an air-lift bioreactor based on the reported design [35].

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