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A Polysaccharide Isolated from the Liquid Culture of *Lentinus edodes* (Shiitake) Mushroom Mycelia Containing Black Rice Bran Protects Mice against a *Salmonella* Lipopolysaccharide-Induced Endotoxemia

Sung Phil Kim,[†] Sun Ok Park,[§] Sang Jong Lee,[§] Seok Hyun Nam,*[,†] and Mendel Friedman*[,#]

[†]Department of Biological Science, Ajou University, Suwon 443-749, Republic of Korea

[§]STR Biotech Company, Ltd., Chuncheon 200-160, Republic of Korea

[#]Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710, United States

ABSTRACT: Endotoxemia (sepsis, septic shock) is an inflammatory, virulent disease that results mainly from bacterial infection. The present study investigates the inhibitory effect of a bioprocessed polysaccharide (BPP) isolated from the edible *Lentinus edodes* liquid mycelial mushroom culture supplemented with black rice bran against murine endotoxemia induced by the *Salmonella* lipopolysaccharide and D-galactosamine (LPS/GalN). BPP was obtained after dialysis against water using a cellulose tube with a molecular weight cutoff of 10000. BPP eluted as a single peak on an HPLC chromatogram. Acid hydrolysis of BPP showed the presence of the following sugars: fucose, galactose, galactosamine, glucose, glucosamine, mannose, rhamnose, and xylose. Treatment of BPP with β-glucanase reduced its immunostimulating activity, suggesting that the polysaccharide has a β-glucan structure. Pretreatment of mice with BPP via oral or intraperitoneal (ip) administration for 2 weeks resulted in the suppression of LPS/GalN-induced catalase, superoxide dismutase (SOD), and transaminase (GOT/GPT) liver enzymes, amelioration of necrotic liver lesions, and reduction of tumor necrosis factor α (TNF-α) and nitrite serum levels as well as myeloperoxidase (MPO) activity, an index of necrotic injury. Immunostimulating macrophage activity was up to 5.4-fold greater than that observed with the culture without the rice bran. BPP also extended the lifespan of the toxemic mice. These positive results with inflammation biomarkers and lifespan studies suggest that the BPP can protect mice against LPS/GalN-induced liver, lung, and kidney injuries and inflammation by blocking oxidative stress and TNF-α production, thus increasing the survival of the toxic shock-induced mice. The polysaccharide has the potential to serve as a new functional food.

KEYWORDS: *Lentinus edodes mushroom mycelia culture, black rice bran, biopolymer, HPLC, β-glucan, oxidative enzymes, myeloperoxidase, cytokines, immunostimulation, mice, histopathology, kidney/liver/lung injury prevention, endotoxemia prevention*

INTRODUCTION

Endotoxemia is a systemic infection caused by release into the bloodstream of endogenous mediators of inflammation and the response of the immune system to infections by bacteria, fungi, viruses, and parasites in the blood, liver, kidney, lungs, and skin. The often fatal disease, to which pregnant women and immunocompromised individuals are especially susceptible, is accompanied by failure of multiple organs, including liver, lungs, and kidneys.[1−3] In the United States, about 750000 individuals are afflicted with sepsis annually, with a 28−50% mortality rate.[4] In England, about 7.7% of all deaths during 2010 are estimated to be sepsis-related.[5]

Lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria, is a major virulence factor for the pathogenesis of toxemias (endotoxin shock).[6] Reported biomarkers associated with sepsis, septic shock, and endotoxemia in humans, reviewed by Samraj et al.,[7] include changes in mean cell volume of neutrophils and monocytes,[8] acute kidney injury,[9] increases in pro-inflammatory cytokine levels (TNF-α, IL-6, IL-10, and IL-1RA),[10] immunopathologic markers,[11] and the production of numerous amino acid and lipid metabolites.[12,13] Studies with mice showed that endothelial activation and oxidant injury are key events in the pathogenesis of sepsis-induced lung injury, that MAPK kinase-deficient mice were protected against injury,[14] and that a high-fat diet increased LPS levels, causing metabolic endotoxemia, cardiac fibrosis, and mortality.[15]

Because treatment has proven unsuccessful, there is a need for new approaches to protect against endotoxemias. In previous studies we reported that black rice brans, rich in bioactive bran components such as γ-oryzanol and anthocyanins, and rice hulls exhibited in vitro and in vivo antioxidative, anti-inflammatory, antiallergic, antibiotic, and anticarcinogenic effects.[16−26] In related studies, we also reported that mushroom[27−30] and herbal[31] extracts exhibited similar beneficial effects. Because oral administration of extracts of the mushroom *Lentinus edodes* mycelia protected rats against induced liver injury and infectious disorders,[32] the objective of the present study is to determine whether a polysaccharide isolated from cultures of *L. edodes* mycelia supplemented with black rice bran can protect mice against adverse effects caused by induced endotoxemia more effectively than the mycelia culture alone. The results suggest that this is indeed the case.

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MATERIALS AND METHODS

Materials. *Salmonella enterica* LPS and GalN were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and used without further purification. The RPMI 1640 medium, Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), and other cell culture reagents were obtained from Hyclone Laboratories (Logan, UT, USA). The enzyme kit for glutamic-oxaloacetic transaminase/glutamic-pyruvic transaminase (GOT/GPT) was the product of Asan Pharmaceutical Co. (Seoul, Korea). The ELISA kit for the quantification of tumor necrosis factor-α (TNF-α) was obtained from Biosource USA (Camarillo, CA, USA). Black rice bran was prepared from grain of *Oryza sativa* cv. Heugjinjubyeo as described in our previous paper.[17]

Cultivation of Mushroom Mycelia and Preparation of a Bioprocessed Polysaccharide (BPP). *L. edodes* fungal mycelia were isolated from the mushroom fruitbody and cultured on potato dextrose agar medium (PDA, Difco Laboratory, Detroit, MI, USA). The genetic identity of the fungus was confirmed by the Korean Center of Microorganisms (Seoul, Korea). The mycelia cultured on PDA media were inoculated in 50 mL of the liquid medium (Table 1).

Table 1. Composition of Liquid Media Used for *L. edodes* Mycelia Culture

ingredient	amount (%)
glucose	2
yeast extract	0.5
soy peptone	0.5
KH_2PO_4	0.2
$MgSO_4$	0.05
$FeSO_4$	0.002

The culture experiments were conducted in 250 mL Erlenmeyer flasks at 28 °C for 5 days in a rotary shaker (120 rpm) to be used to seed the main liquid culture. The main liquid medium contained black rice bran (20 g/L) and dried soybean powder (2 g/L). The medium was then treated with amylase and protease at 60 °C for 60 min for enzymatic digestion of particulate materials containing carbohydrate and protein. Subsequently, the culture mass was adjusted to pH 6.0 with HCl, followed by sterilization in the autoclave. The experiment with the main liquid culture was started using a 5 L fermentor (working volume of 3 L) at 28 °C and 150 rpm by inoculating with the inoculum (10%) of the preliquid cultured mycelia. After 7 days, the culture mass was ground in a colloid mill (model PUC60 Hankook Power Technology System, Seoul, Korea). The powder was treated with 0.1 M lactic acid for 60 min, followed by treatment with an enzyme mixture for cell wall lysis containing cellulose, hemicellulase, pectinase, glucanase, mannose, and arabinase at 50 °C for 60 min. The acid- and enzyme-treated culture mass was then extracted with hot water at 90 °C for 1 h and freeze-dried to a solid material.

Chemical Analysis of BPP. The purity of the resultant BPP fraction was confirmed by HPLC analysis with the HPLC instrument (model SPD-20A, Shimazu Corp., Kyoto, Japan) equipped with an ELSD-LTH detector (Shimazu Corp.). The chromatographic column WAT11535 packed with hydroxylated polymethacrylated-based gel, 7.8 × 300 mm (Waters, Milford, MA, USA), was set at 45 °C with water as the carrier at a flow rate of 0.6 mL/min.

Component sugars of BPP were analyzed following the method of Kim et al.[30] with some modification. Briefly, each of the polysaccharide fractions (0.1 mg) was subjected to different acid hydrolysis conditions. For analysis of neutral and amino sugars, the polysaccharide was hydrolyzed by treatment with 2 M trifluoroacetic acid or 6 M HCl at 100 °C for 4 h, respectively. The hydrolysate was evaporated to dryness using a SpeedVac model 3100 (EYELA, Tokyo, Japan), resuspended in distilled water, and injected into a Bio-LC model ICS-5000 chromatogram with pulsed amperometric detection (Dionex Corp., Sunnyvale, CA, USA). A CarboPac PA1 column (Dionex, 4 × 50 mm) was used to separate monosaccharides by elution with 16 mM NaOH or 100 mM NaOH/150 mM NaOAc at a flow rate of 1 mL/min for neutral and amino sugars, respectively.

Animals and Treatments. The protocol for the mice studies was approved by the Ethics Committee for Animal Care and Use, Ajou University, Republic of Korea. All experiments were performed in compliance with the relevant laws and institutional guidelines. Five-week-old female BALB/c mice were purchased from Orient Bio Inc. (Seongnam, Korea) and were hosted under a 12 h light/dark cycle at 20−22 °C and relative humidity of 50 ± 10%. The mice were fed freely a pelletized commercial chow diet obtained from Orient Bio Inc. (catalog no. 5L79) and sterile tap water during the entire period. After acclimation for 1 week, the mice were then divided into four groups (n = 10), avoiding any intergroup difference in body weight. Two groups were given BPP, and two were given water only. BPP was dissolved in water and given to mice via dietary (oral) or the intraperitoneal (ip) route, respectively. The dose was adjusted to 10 mg/mL for both routes. After 14 consecutive days, the mice were intravenously injected with LPS and GalN (5 μg/kg and 700 mg/kg, respectively). For determination of the survival rate, lethal doses of LPS and GalN (20 μg/kg and 700 mg/kg, respectively) were employed. The mice were observed for 60 h after the LPS and GalN challenge. Except for determining the survival rate, the other experiments were carried out 4 h after challenge of a sublethal dose of the LPS and GalN (5 μg/kg and 700 mg/kg, respectively). The mice were sacrificed by CO_2 asphyxia, and livers and kidneys from each group of mice were excised and weighed. The blood from the four groups of mice was collected by cardiac puncture and allowed to clot at room temperature for 30 min. Subsequently, the blood was subjected to centrifugation at 2000g for 30 min. The separated serum was stored at −20 °C until use.

Purification of Murine Peritoneal Macrophages. Isolation and purification of peritoneal macrophage cells from BALB/c mice were performed according to the method of Narumi et al.[33] Mice were injected with 4% thioglycollate (1 mL). Four days later, they were sacrificed by CO_2 inhalation, followed by lavaging of the peritoneal cavity with 10 mL of HBSS. Repeated washings were pooled and centrifuged at 200g for 10 min at 4 °C. The exudated cell pellet was then resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). After the number of viable cells had been counted by trypan blue staining, the exudated cells were plated onto 60 mm tissue culture dishes [(1−5) × 10^6 cells/dish)] and incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO_2 to produce macrophage cells firmly adhered onto the dishes. The culture dishes were thoroughly washed with HBSS to remove nonadherent cells, and the adhered cells were collected by detaching with 0.25% trypsin digest and equilibrated with RPMI 1640 medium containing 10% FBS.

Macrophage Stimulating Activity. The cellular lysosomal enzyme activity was measured according to the method of Suzuki et al.[34] to evaluate the peritoneal macrophage stimulation level. Briefly, the samples (20 μL) were added to a macrophage suspension (20 μL; 1 × 10^6 cells/mL) and RPMI 1640 medium (40 μL) in a flat-bottom 96-well plate at 37 °C in humidified atmosphere with 5% CO_2 for 24 h. The medium was then discarded, and the adherent macrophage monolayer in each well was solubilized with Triton X-100 (0.1%; 20 μL). To each well were added 10 mM p-nitrophenyl phosphate (p-NPP) solution (100 mL) and citrate buffer (0.1 M; 50 μL; pH 9.8) to terminate the reaction. The absorbance (OD) at 405 nm was measured using a microplate reader (model 550, Bio-Rad, Hercules, CA, USA). The percentage of lysosomal enzyme activity was calculated by using the following formula:

$$\text{lysosomal enzyme activity (\%)} = (OD_{sample} - OD_{control}/OD_{control}) \times 100$$

Serum Nitrite and Nitrate Levels. Serum nitrite and nitrate levels were measured primarily following the method of Misko et al.[35] with some modification. Briefly, serum was filtered through an Ultrafree-MC microcentrifuge filter unit (Millipore, Bedford, MA, USA) for 1 h at 14000 rpm to remove the hemoglobin released by cell

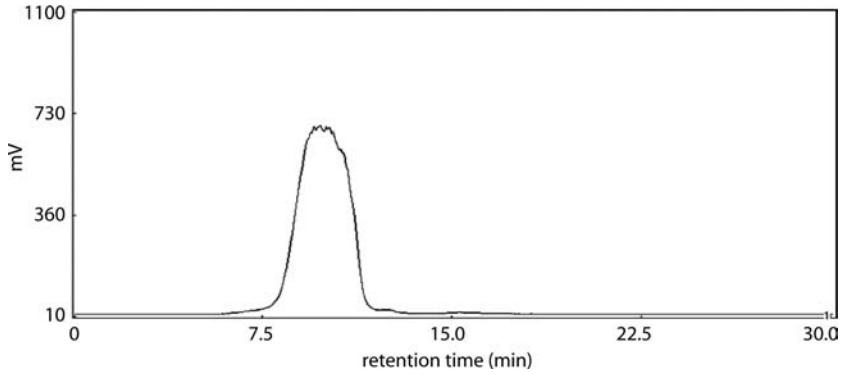

Figure 1. HPLC-ELSD chromatogram of the polysaccharide produced by mycelia of *Lentinus edodes* mushroom cultured in black rice bran supplemented medium.

lysis. The serum (50 μL) was incubated in a reaction mixture (40 mM Tris Cl, 40 μM reduced β-nicotinamide adenine dinucleotide phosphate, 40 μM flavine adenine dinucleotide, and nitrate reductase (0.05 U/mL) at pH 7.9 at 37 °C for 15 min. Reduced samples were incubated with an equal volume of Greiss reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid] at room temperature for 15 min. The absorbance was read at 570 nm using a microplate reader (model 550, Bio-Rad). The total nitrate/nitrite concentration was determined by comparison with a reduced $NaNO_3$ standard curve.

TNF-α and Serum Transaminases. TNF-α level was determined using an enzyme-linked immunosorbent assay (ELISA) kit specific for murine TNF-α according to the manufacturer's instruction. After termination of all reactions, the absorbance of the chromophore at 420 nm was measured using a microplate reader. The activities of serum enzymes glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were estimated using a commercial colorimetric kit. Briefly, appropriately diluted serum (200 μL) was added to the reaction solution (0.1 mL; L-asparaginic acid and α-ketoglutaric acid mixture). The resultant mixture was incubated at 37 °C for 30 min (GPT) or 60 min (GOT), and 2,4-dinitrophenylhydrazine (1 mL) was added. After incubation at room temperature for 20 min, 0.4 N sodium hydroxide (10 mL) was added, and the absorbance of the solution was measured at 505 nm using a microplate reader.

Antioxidant Enzyme Activities in Mouse Liver. The liver tissue was homogenized in 10 volumes of phosphate-buffered saline (PBS) on ice for 30 s using a power-driven Polytron homogenizer (Pro Scientific, Monroe, CT, USA). The homogenate was centrifuged at 9000*g* at 4 °C for 20 min. The supernatant was recovered and used to measure the following two antioxidant enzyme activities: for catalase activity assay at time zero, each supernatant (1.8 mL) was mixed with PBS (0.2 mL) containing hydrogen peroxide (10 mM). The reaction mixture (1 mL) was immediately added to a cuvette and placed into a UV−vis spectrophotometer (model V-550, Jasco, Tokyo, Japan). The enzyme activity was observed via degradation of hydrogen peroxide as determined by a decrease in UV light absorbance at 240 nm over time. Measurement of absorbance was taken at 15 s intervals after addition of the homogenate to the hydrogen peroxide buffer. Units of catalase activity per milligram of protein were calculated following the method of Nandi et al.[36] For superoxide dismutase (SOD)-like activity assay, the supernatant (100 μL) was mixed with a homogenizing buffer (1.5 mL; 50 mM Tris, 10 mM EDTA, pH 8.5), then pyrogallol (100 μL; 7.2 mM) was added, and the reaction mixture was incubated at 25 °C for 10 min. The reaction was terminated by addition of 1 M HCl (50 μL), and the absorbance was then measured at 420 nm. One unit was defined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units per milligram of protein.[37]

Myeloperoxidase (MPO) Activity. MPO activity was assayed according to the method of Bralley et al.[38] Briefly, the supernatants recovered from lung and kidney tissue homogenates (30 μL) were added to the reaction mixture (200 μL) consisting of 100 μL of PBS (80 mM phosphate, 140 mM NaCl, pH 5.4), 85 μL of PBS2 (220 mM phosphate, 140 mM NaCl, pH 5.4), and 0.017% H_2O_2 (15 μL). The reaction was started by adding tetramethybenzidine (18.4 μM) in aqueous dimethylformamide (8%; 20 μL) and was left standing for 3 min at 37 °C. The reaction was stopped by the addition of sodium acetate buffer (30 μL; 1.46 M; pH 3.0). The absorbance (OD) of the supernatant was then read in a microplate reader at 655 nm for 30 min. The reaction rate (change in OD/time) was derived from an initial slope of the curve. A calibration curve was used to show the rate of reaction plotted against the concentration of a standard human MPO preparation (Sigma-Aldrich).

Serum Urea Nitrogen and Creatinine. Concentrations of urea nitrogen and creatinine were determined using the commercial QuantiChrom creatinine assay kit and QuantiChrom urea assay kit, respectively (Bioassay System, Hayward, CA, USA).

Histology of Liver Tissue. For histological analysis, the liver tissue of the mice was fixed with paraformaldehyde (4%) in phosphate buffer (0.5 M; pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted onto glass slides. The sections were then dewaxed using xylene and ethanol and stained with hematoxylin and eosin Y (H&E) to reveal the hemorrhagic necrosis in the liver. Histological changes were observed under a light microscope at 100× magnification.

Statistical Analysis. Results are expressed as the mean ± SD of three independent experiments. Differences between means were determined by ANOVA test using the Statistical Analysis Software package SAS (Cary, NC, USA). $p < 0.05$ is regarded as significant.

RESULTS AND DISCUSSION

Isolation and Characterization of BPP from the Mushroom−Black Rice Bran Culture. The analytical data described under Materials and Methods indicate that BPP is a pure compound with a molecular weight >10000 that eluted as a single peak on the chromatogram shown in Figure 1. Small molecular weight compounds would be lost during dialysis.

Component sugar analysis following acid hydrolysis shown in Table 2 indicates that BPP contained mainly the neutral sugars glucose, galactose, rhamnose, fucose, mannose, and xylose (33.8 g/100 g proteins) and basic amino sugars glucosamine and galactosamine (0.9 g/100 g BPP). It is likely that additional sugar compounds might have been degraded during the acid hydrolysis procedure used to analyze the liberated sugars with the Bio LC system. Because the BPP treatment with β-glucanase reduced the macrophage-stimulating activity (results not shown), it seems that BPP might have a β-glucan structure.

Although we do not know the mechanisms of BPP biosynthesis, one possibility is that enzymes associated with

Table 2. Sugar Composition of Macrophage Stimulating Polysaccharide (BPP) Isolated from *L. edodes* Mushroom Mycelia Cultured in Liquid Medium Supplemented with Black Rice Bran

component sugar	g/100 g BPP
fucose	0.8
rhamnose	2.7
galactosamine	0.1
glucosamine	0.8
galactose	9.1
glucose	18.2
mannose + xylose	3.0

the mushroom mycelia plus exogenously added enzymes in the presence of the black rice bran catalyzed the conversion of cell wall and bran compounds to the bioactive polysaccharide.

Bioactivity of Black Rice Bran. Because black rice bran facilitates production of BPP, it is relevant to mention our previous study on the vivo bioactivity of the bran.[23] We reported that tumor inhibition in mice by black rice bran was associated with increases in cytolytic activity of splenic natural killer (NK) cells; partial restoration of NO production and phagocytosis in peritoneal macrophages; increases in released TNF-α, IL-1β, and IL-6 cytokines from macrophages; increases in infiltration of leukocyte into the tumor; and reduction in angiogenesis inside the tumor. Pro-angiogenic biomarkers vascular endothelial growth factor, cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX) were also reduced in mRNA and protein expression. ELISA of tumor cells confirmed reduced expression of COX-2 and 5-LOX. Reduced COX-2 and 5-LOX expression down-regulated vascular endothelial growth factor and inhibited neo-angiogenesis inside the tumors. The bran component γ-oryzanol exhibited similar beneficial antitumor effects in mice.[22] Whether BPP would exhibit similar antitumor effects merits study.

Macrophage-Stimulating Activity of BPP. To find new uses for black rice bran as a source of biopolymers with immune system macrophage-stimulating activity, *L. edodes* mycelia were cultured in the black rice bran liquid medium for 7 days. As an internal control, *L. edodes* mycelia were also cultured in the liquid medium without black rice bran. BPP was tested for the ability to enhance macrophage activity using murine peritoneal macrophage cells. As shown in Table 3, macrophage-stimulating activity in the presence of black rice bran was increased from 15 to 81% at 10 μg/mL treatment (~5.4-fold change) and, in the absence of black rice bran, from 49 to 120% at 100 and 10 μg/mL treatment (~2.5-fold change). This study revealed that BPP can elicit a similar (at 10 μg/mL) or much greater level (at 100 μg/mL) macrophage activation than that observed with the 100 ng/mL LPS-treated positive control.

These results support the use of black rice bran-supplemented *L. edodes* liquid mycelia culture as a biomaterial for the production of biopolymers with much greater macrophage-stimulating activity than those produced in cultures without rice bran supplementation.

Table 3. Effect of Black Rice Bran Supplementation on Mouse Peritoneal Macrophage-Stimulating Activity of BPP Isolated from *L. edodes* Mushroom Mycelia Culture[a]

sample	lysosomal enzyme activity OD at 405 nm (% of activity)
vehicle	0.088 ± 0.005 d (4.2)
LPS (100 ng/mL)	1.44 ± 0.11 b (83)
BPP from culture without black rice bran (10 μg/mL)	0.273 ± 0.010 d (15)
BPP from culture without black rice bran (100 μg/mL)	0.845 ± 0.053 c (49)
BPP from culture with black rice bran (10 μg/mL)	1.39 ± 0.11 b (81)
BPP from culture with black rice bran (100 μg/mL)	2.08 ± 0.16 a (121)

[a]Data are expressed as the mean ± SD (n = 3). Values with the same letter are not significantly different between groups at $p < 0.05$.

Effects of BPP on Antioxidative Enzyme Activities in Mouse Liver. To find out whether reactive oxygen species (ROS) level is a crucial factor for the incidence of liver injury associated with LPS/GalN-induced endotoxemia, the activity of hepatic antioxidant enzymes including catalase and SOD-like enzymes was evaluated in the control and experimental mice groups. Table 4 shows that in the liver of LPS/GalN-treated and untreated mice, the oral treatment with 10 mg/kg body wt/day BPP induced significant changes in the catalase and SOD-like enzyme activities from 22.6 to 12.19 U/mg protein (~56% change) and from 16.9 to 11.20 U/mg protein (~49% change), respectively.

Table 4. Effects of BPP on Antioxidant Catalase and Superoxide Dismutase (SOD)-like Enzyme Activities in LPS/GalN-Induced Endotoxemic Mouse Liver[a]

sample	catalase activity (U/mg protein)	SOD-like activity (U/mg protein)
normal	3.92 ± 0.30 d	5.28 ± 0.33 d
LPS/GalN (5 μg/kg and 700 mg/kg)	22.6 ± 1.6 a	16.9 ± 1.1 a
BPP (10 mg/kg) oral administration	12.19 ± 0.86 b	11.20 ± 0.54 b
BPP (10 mg/kg) ip injection	9.53 ± 0.45 c	9.67 ± 0.72 c

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

Table 4 also shows that ip administration of BPP was more effective in suppressing the LPS/GalN-induced activation of hepatic catalase and SOD-like enzymes. The decrease ranged from 22.6 to 9.53 U/mg protein (~70% change) and from 16.9 to 9.67 U/mg protein (~62% change), respectively.

These results show that BPP administration reduced oxidative damage in the liver induced by the LPS/GalN challenge.

Effects of BPP on Weights and Histopathology of Mouse Livers. Figure 2 shows that the induction of endotoxemia by LPS/GlN resulted in significant increased liver weights as well as in the formation hemorrhagic lesions in the liver tissues. By contrast, no apparent hepatic injury was observed in the vehicle-treated control group. As expected, BPP treatment via both the oral and ip routes (10 mg/kg body weight each) markedly blocked the hemorrhagic liver injury as well as hepatomegaly induced by a sublethal dose of the LPS/GalN challenge.

These results demonstrate strikingly that BPP protected the mice against injury of a major organ associated with endotoxemia.

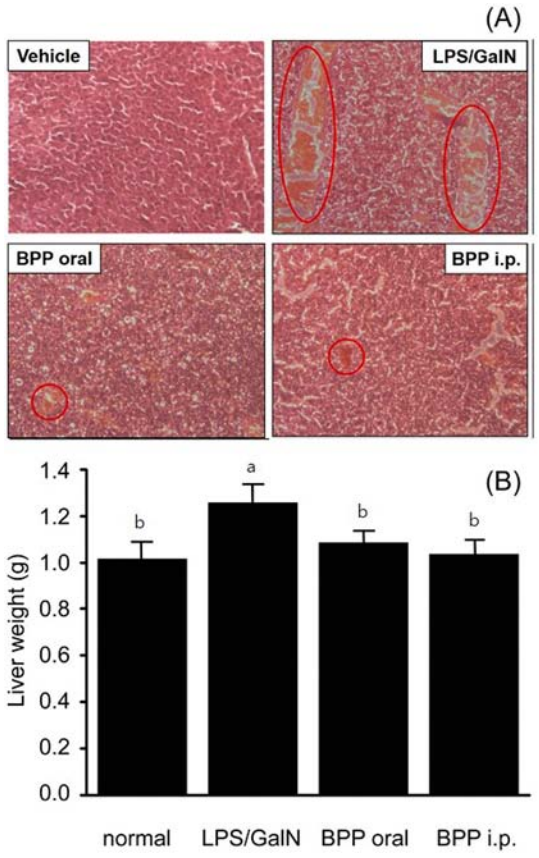

Figure 2. Modulation of liver injury in LPS/GalN-induced endotoxemic mice by BPP (10 mg/kg). (A) Liver sections from LPS/GalN-administered mice show disorganized hepatic architecture, intense cellular necrosis, and marked hemorrhagic lesions compared with the liver section of vehicle-treated mice. Oral or ip administration of BPP markedly ameliorated LPS/GalN-induced liver damage. (B) Liver weight was decreased by administration of BPP through both oral and ip routes. Mice were sacrificed 4 h after LPS/GalN treatment, and the livers were weighed. Each liver specimen was fixed with 4% paraformaldehyde, and sections were stained with hematoxylin and eosin (H&E). Magnification, ×100. Circular areas indicate local hemorrhage and necrosis regions. Bars not sharing a common letter are significantly different between groups at $p < 0.05$.

Effects of BPP on Liver Transaminase Enzyme Activities in Mouse Serum. Figure 3 shows that as compared to the normal control group, the liver enzyme levels of the transaminases GOT and GPT, primary enzymes that serve as an index of hepatic injury, increased significantly in the serum of only LPS/GalN-treated mice. Thus, in the mice treated orally with 10 mg/kg body wt/day BPP, GOT and GPT activities decreased substantially from 256.3 to 155.9 U/mL (~58% change) and from 189.6 to 60.4 U/mL (~84% change), respectively. It was also found that ip delivery of BPP was more effective in suppressing the LPS/GalN-induced activation of GOT and GPT, from 256.3 to 121.3 U/mL (~78% change) and from 189.6 to 48.7 U/mL (~91% change), respectively.

These results show that BPP protected the liver against necrotic injury associated with endotoxemia.

Effects of BPP on TNF-α and Nitrite Concentrations in Mouse Serum. Table 5 shows that TNF-α and nitrite/nitrate levels in the serum of LPS/GalN-induced endotoxemic mice were markedly increased compared with those observed in the normal control mice group (~9- and 21-fold increases,

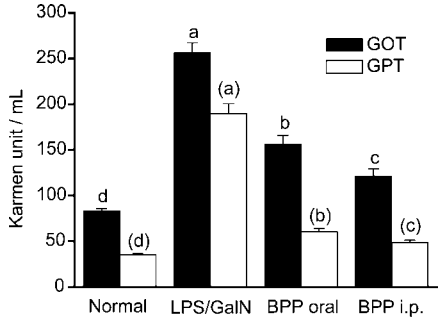

Figure 3. Effect of BPP (10 mg/kg) on serum glutamic-oxaloacetic/glutamic-pyruvic transaminases (GOT/GPT) in LPS/GalN-induced endotoxemic mice. The mice were pretreated with BPP for 2 weeks through oral or ip administration before the LPS/GalN treatment. Mice were sacrificed 4 h after LPS/GalN treatment, and blood was collected to produce serum. Bars not sharing a common letter are significantly different between groups at $p < 0.05$.

Table 5. Effects of BPP on Tumor Necrosis Factor-α (TNF-α) and Nitrite Concentrations in Mouse Serum[a]

sample	TNF-α (pg/mL)	nitrite (μM)
normal	40.6 ± 2.0 d	0.893 ± 0.016 c
LPS/GalN (5 μg/kg and 700 mg/kg)	357 ± 15 a	18.9 ± 1.0 a
BPP (10 mg/kg) oral administration	176 ± 12 b	6.19 ± 0.44 b
BPP (10 mg/kg) ip injection	138.0 ± 8.9 c	5.59 ± 0.28 b

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

respectively). The administration of BPP via the oral route significantly decreased the TNF-α and nitrite/nitrate concentrations from 357 to 176 pg/mL (~57% change) and from 18.9 to 6.19 mM (~71% change), respectively. Table 5 also shows that ip injection of BPP was more effective in suppressing LPS/GalN-induced activation of TNF-α and nitrite/nitrate concentrations, from 357 to 138.0 pg/mL (~69% change) and from 18.9 to 5.59 M (~74% change), respectively.

These results show that BPP protected the treated mice against increased production of nitrite/nitrate, a source of bioactive nitric oxide (NO), and TNF-α, a critical factor associated with the cause of the endotoxemia syndrome.

Effects of BPP on Lung and Kidney Injuries in Endotoxemic Mouse. The evidence of necrotic cell injuries in the lung and kidney in LPS/GalN challenged endotoxemia was further confirmed by the observed decreases in the MPO enzyme activity induced by BPP (Table 6). The table shows that BPP treatment via the oral route significantly decreased the MPO activities from 11.94 to 5.63 U/g protein (~72% change) in the lung and from 6.00 to 3.59 U/g protein (~61% change) in the kidney, respectively. Intrapeitoneal delivery of BPP was more effective in suppressing LPS/GalN-induced activation of MPO activities, from 11.94 to 5.15 U/g protein (~78% change) in the lung and from 6.00 to 3.11 U/g protein (~73% change) in the kidney, respectively.

The protective effect of BPP treatment against LPS/GalN-induced renal injury was also evaluated by measuring creatinine and urea nitrogen concentrations in the serum. Table 6 also shows that creatinine and urea nitrogen levels in the serum of the endotoxemic mice were significantly greater than those in the normal mice group (~2- and ~4-fold increases, respectively), indicating the association of endotoxemia with

Table 6. Effects of BPP on Lung and Kidney Injuries in LPS/GalN-Induced Endotoxemic Mice Assessed by Changes in Myeloperoxidase, Creatine, and Blood Urea Nitrogen (BUN) Concentrations[a]

sample	myeloperoxidase (U/g protein)		creatinine (mg/dL)	BUN (mg/dL)
	lung	kidney		
normal	3.22 ± 0.15 c	2.022 ± 0.077 d	0.318 ± 0.024 d	12.73 ± 0.82 c
LPS/GalN (5 μg/kg and 700 mg/kg)	11.94 ± 0.86 a	6.00 ± 0.21 a	0.551 ± 0.027 a	52.6 ± 2.3 a
BPP (10 mg/kg) oral administration	5.63 ± 0.14 b	3.59 ± 0.16 b	0.433 ± 0.020 b	40.4 ± 2.1 b
BPP (10 mg/kg) ip injection	5.15 ± 0.27 b	3.11 ± 0.13 c	0.414 ± 0.023 c	38.7 ± 1.8 b

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

the release of these amino acid metabolites from the damaged kidney. By contrast, BPP administration via both the oral and ip routes significantly lowered LPS/GalN-triggered increases in creatinine and urea nitrogen levels by up to 59 and 35%, respectively.

These results imply that despite the observed general immunostimulating activity in macrophages, BPP suppresses the signaling pathway for the production of TNF-α and ROS, possibly analogous to the reported inhibition of the innate immune receptor Toll-like receptor 4 (TRL-4) synthetic lipodisaccharide endotoxin antagonist.[39] This receptor is also essential for the expression of chronic inflammatory allergic airway diseases such as asthma.[40]

The present study shows that BPP neutralizes the toxic effect of the LPS-induced endotoxin by reducing TNF-α expression and production of ROS. Whether BPP might also interrupt TLR4 signaling and protect against allergic inflammation merits study.

Effects of BPP on Mortality of Endotoxemic Mice. To determine the therapeutic effects of BPP on life expectancy, a lethal dose of LPS/GalN was used to induce sepsis and to determine the protection by BPP against mortality. Each group of 10 mice was treated with BPP (10 mg/kg body wt/day) via both the oral and ip routes during the entire experimental period. Figure 4 shows that the mice treated with a lethal dose of LPS/GalN all died after 45 h. By contrast, the groups treated with BPP survived >60 h.

These observations demonstrate strikingly the potential of BPP to protect mice against sepsis-induced lethality, presumably via down-regulating the production of the pro-inflammatory cytotoxic factors such as reactive oxygen radicals, cytokines, and nitric oxide.

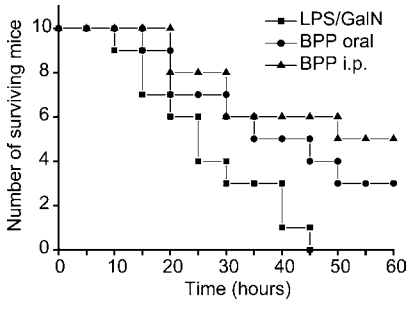

Figure 4. Histogram showing the effect of BPP (10 mg/kg) on LPS/GalN-induced endotoxemia-induced lethality. BALB/c mice were preadministered BPP through an oral or ip route every 24 h for 2 weeks and then challenged ip with a lethal dose of LPS/GalN (20 μg and 700 mg per kg body weight). BPP was then again administered through the same route during the entire experimental period. Plotted values are mean values of triplicate determinations.

The reason why ip administration of BPP was more effective against endotoxemia than oral administration might be because the high-molecular-weight compound is less bioavailable from the oral route. Some of the BPP might also be partially hydrolyzed or degraded by the acid conditions of the digestive tract and/or by digestive glycosidases.

Related Studies. To place the findings of the present study in proper perspective, we will mention reported efforts to overcome the symptoms of sepsis in rodents with other plant-derived compounds.

- Ailanthoidol, a neolignan isolated from *Zanthoxylum ailanthoides* and *Salvia miltiorrhiza* Bunge, inhibited inflammatory reactions by macrophages and protected mice against LPS-induced endotoxin shock, suggesting that this compound is a functional candidate for the prevention of inflammatory diseases.[41]
- Cathelicidin, a peptide antibiotic, neutralized the biological activity of LPS and protected mice from lethal endotoxin shock.[42]
- Ethyl acetate, a flavor compound present in fruits and wines, protected mice against LPS-GalN-induced endotoxic shock, suggesting that the compound possesses anti-inflammatory properties.[43]
- Honokiol, a phenolic compound isolated from the medicinal herb *Magnolia officinalis*,[44] suppressed the lethal response and acute lung injury in mice associated with LPS-induced septic shock, suggesting that the compound could be used as a therapeutic agent to treat conditions associated with endotoxemia.[45]
- Nicotinamide, a group B3 vitamin, provided therapeutic benefits, attenuated inflammatory injury, and improved the survival rate of mice suffering from LPS/GalN-induced sepsis.[46]
- Paeonol (2′-hydroxy-4′-methoxyacetophenone), the main phenolic compound of the radix peony *Paeonia suffruticosa* used as a traditional Chinese medicine, improved the survival of mice with LPS-induced endotoxemia.[47] The compound also exhibited neuroprotective properties, suggesting that it can also protect against inflammation and oxidative stress-induced neuronal damage.[48]
- Phosvitin, a hen egg yolk protein, exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* bacteria under thermal stress and increased the survival rate of mice with induced endotoxemia.[49]
- Resolvin D1, a polyunsaturated fatty acid (trihydroxydocosahexaenoic acid), protected mice against induced sepsis/endotoxin shock by suppressing the release and production of the septic mediators high-mobility group box-1 (HMGB1) protein and cytokines.[50]
- The traditional Chinese medicinal plant *Rhizoma coptidis* protected mice against LPS-induced acute liver injury,[51]

suggesting its potential value against endotoxemia-related liver damage.

These observations suggest the possibility that combinations of BPP with some of the above-mentioned bioactive formulations could be useful in the prevention or treatment of endotoxemias. Such combinations might exhibit additive or synergistic beneficial effects at lower concentrations than individual antiendotoxic compounds.

Summary and Research Needs. We evaluated the potential of the natural biopolymer BPP produced in cultures of *L. edodes* mushroom mycelia with supplemented black rice bran to protect mice against LPS/GalN-induced endotoxemia. BPP contained both sugars and amino sugars and eluted as a single peak on an HPLC chromatogram. BPP administered by ip injection was more effective than that delivered by oral feeding in protecting the mice against the induced sepsis. The protective effect was accompanied by stimulation of the innate immune system without activation of TNF-α expression and ROS production associated with LPS-induced endotoxemia.

Collectively, the results show that BPP protected mice against the following biomarkers associated with induced endotoxemia by: (a) stimulation of the innate immune system and (b) amelioration of pathological effects in the liver, lung, and kidney tissues as determined by changes in the myeloperoxidase activity and serum creatinine and urea nitrogen levels that serve as an index of sepsis-associated oxidative tissue damage.

We did not determine whether BPP also exerts its beneficial effect by inhibiting the extracellular release of HMGB1, a DNA-binding signaling protein that is released from injured cells during inflammation.[52] This protein also seems to play an important role as a late-phase mediator of endotoxic/septic shock.[50] The detailed mechanism of the protective effect of BPP and the potential for food-derived BPP to protect against liver failure and endotoxemia in humans both merit further study. We agree with a journal reviewer that the observed beneficial properties suggest that BPP has the potential to serve as a functional food.

AUTHOR INFORMATION

Corresponding Authors

*(S.H.N.) Phone: 82-31-219-2619. Fax: 82-31-219-1615. E-mail: shnam@ajou.ac.kr.

*(M.F.) E-mail: Mendel.Friedman@ars.usda.gov.

Notes

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ABBREVIATONS USED

BPP, bioprocessed polymer; BUN, blood urea nitrogen; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; GalN, D-galactosamine; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; HBSS, Hanks balanced salt solution; HMGB1, high-mobility group box-1 protein; HPAEC-PAD, high-performance liquid chromatography with pulsed amperometric detection; LPS, lipopolysaccharide; MPO, myeloperoxidase; *p*-NPP, *p*-nitrophenyl phosphate; PBS, phosphate saline buffer; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

REFERENCES

(1) Beers, M. H. *The Merck Manual of Diagnosis and Therapy*, 18th ed.; Merck Research Laboratories: Whitehouse Station, NJ, 2006.

(2) Dogné, J.-M.; Hanson, J.; de Leval, X.; Pratico, D.; Pace-Asciak, C. R.; Drion, P.; Pirotte, B.; Ruan, K.-H. From the design to the clinical application of thromboxane modulators. *Curr. Pharm. Des.* **2006**, *12*, 903−923.

(3) Nakayama, M.; Niki, Y.; Kawasaki, T.; Takeda, Y.; Horiuchi, K.; Sasaki, A.; Okada, Y.; Umezawa, K.; Ikegami, H.; Toyama, Y.; Miyamoto, T. Enhanced susceptibility to lipopolysaccharide-induced arthritis and endotoxin shock in interleukin-32α transgenic mice through induction of tumor necrosis factor α. *Arthritis Res. Ther.* **2012**, *14*, Article number R120. DOI: 10.1186/ar3850.

(4) National Institute of Health; National Institute of General Medical Sciences. Sepsis Fact Sheet; http://www.nigms.nih.gov/Publications/factsheet_sepsis.htm (accessed Oct 17, 2013).

(5) McPherson, D.; Griffiths, C.; Williams, M.; Baker, A.; Klodawski, E.; Jacobson, B.; Donaldson, L. Sepsis-associated mortality in England: an analysis of multiple cause of death data from 2001 to 2010. *BMJ Open* **2013**, *3*, article e002586, DOI: 002510.001136/bmjopen-002013-002586.

(6) Sepsis Alliance. http://www.sepsisalliance.org/.

(7) Samraj, R. S.; Zingarelli, B.; Wong, H. R. Role of biomarkers in sepsis care. *Shock* **2013**, *40*, 358−365.

(8) Lee, A. J.; Kim, S. G. Mean cell volumes of neutrophils and monocytes are promising markers of sepsis in elderly patients. *Blood Res.* **2013**, *48*, 193−197.

(9) White, L. E.; Hassoun, H. T.; Bihorac, A.; Moore, L. J.; Sailors, R. M.; McKinley, B. A.; Valdivia, A.; Moore, F. A. Acute kidney injury is surprisingly common and a powerful predictor of mortality in surgical sepsis. *J. Trauma Acute Care Surg.* **2013**, *75*, 432−438.

(10) King, E. G.; Bauza, G. J.; Mella, J. R.; Remick, D. G. Pathophysiologic mechanisms in septic shock. *Lab. Invest.* **2013**, available online Sept 23, DOI: 10.1038/labinvest.2013.1110.

(11) van Eijk, L. T.; van der Pluijm, R. W.; Ramakers, B. P.; Dorresteijn, M. J.; van der Hoeven, J. G.; Kox, M.; Pickkers, P. Body mass index is not associated with cytokine induction during experimental human endotoxemia. *Innate Immun.* **2013**, available online April 19, DOI: 10.1177/1753425913481821.

(12) Kamisoglu, K.; Sleight, K. E.; Calvano, S. E.; Coyle, S. M.; Corbett, S. A.; Androulakis, I. P. Temporal metabolic profiling of plasma during endotoxemia in humans. *Shock* **2013**, available online Oct 1, DOI: 10.1097/SHK.0000000000000063.

(13) Luche, E.; Cousin, B.; Garidou, L.; Serino, M.; Waget, A.; Barreau, C.; André, M.; Valet, P.; Courtney, M.; Casteilla, L.; Burcelin, R. Metabolic endotoxemia directly increases the proliferation of adipocyte precursors at the onset of metabolic diseases through a CD14-dependent mechanism. *Mol. Metab.* **2013**, *2*, 281−291.

(14) Mannam, P.; Zhang, X.; Shan, P.; Zhang, Y.; Shinn, A. S.; Lee, P. J. Endothelial MKK3 is a critical mediator of lethal murine endotoxemia and acute lung injury. *J. Immunol.* **2013**, *190*, 1264−1275.

(15) Lew, W. Y.; Bayna, E.; Molle, E. D.; Dalton, N. D.; Lai, N. C.; Bhargava, V.; Mendiola, V.; Clopton, P.; Tang, T. Recurrent exposure to subclinical lipopolysaccharide increases mortality and induces cardiac fibrosis in mice. *PLoS One* **2013**, *8*, article e61057, DOI: 61010.61371/journal.pone.0061057.

(16) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Koh, H. J.; Kozukue, N.; Friedman, M. Bran extracts from pigmented rice seeds inhibit tumor promotion in lymphoblastoid B cells by phorbol ester. *Food Chem. Toxicol.* **2005**, *43*, 741−745.

(17) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Kozukue, N.; Friedman, M. Antioxidative, antimutagenic, and anticarcinogenic activities of rice bran extracts in chemical and cell assays. *J. Agric. Food Chem.* **2005**, *53*, 816−822.

(18) Nam, S. H.; Choi, S. P.; Kang, M. Y.; Koh, H. J.; Kozukue, N.; Friedman, M. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem.* **2006**, *94*, 613−620.
(19) Choi, S. P.; Kang, M. Y.; Koh, H. J.; Nam, S. H.; Friedman, M. Antiallergic activities of pigmented rice bran extracts in cell assays. *J. Food Sci.* **2007**, *72*, S719−S726.
(20) Choi, S. P.; Kim, S. P.; Kang, M. Y.; Nam, S. H.; Friedman, M. Protective effects of black rice bran against chemically-induced inflammation of mouse skin. *J. Agric. Food Chem.* **2010**, *58*, 10007−10015.
(21) Chen, M.-H.; Choi, S. H.; Kozukue, N.; Kim, H.-J.; Friedman, M. Growth-inhibitory effects of pigmented rice bran extracts and three red bran fractions against human cancer cells: relationships to composition and antioxidative activities. *J. Agric. Food Chem.* **2012**, *60*, 9151−9161.
(22) Kim, S. P.; Kang, M. Y.; Nam, S. H.; Friedman, M. Dietary rice bran component γ-oryzanol inhibits tumor growth in tumor-bearing mice. *Mol. Nutr. Food Res.* **2012**, *56*, 935−944.
(23) Choi, S. P.; Kim, S. P.; Nam, S. H.; Friedman, M. Antitumor effects of dietary black and brown rice brans in tumor-bearing mice: relationship to composition. *Mol. Nutr. Food Res.* **2013**, *57*, 390−400.
(24) Kim, S. P.; Yang, J. Y.; Kang, M. Y.; Park, J. C.; Nam, S. H.; Friedman, M. Composition of liquid rice hull smoke and anti-inflammatory effects in mice. *J. Agric. Food Chem.* **2011**, *59*, 4570−4581.
(25) Yang, J.-Y.; Moon, E.; Nam, S. H.; Friedman, M. Antidiabetic effects of rice hull smoke extract on glucose-regulating mechanism in type 2 diabetic mice. *J. Agric. Food Chem.* **2012**, *60*, 7442−7449.
(26) Kim, S. P.; Kang, M. Y.; Park, J. C.; Nam, S. H.; Friedman, M. Rice hull smoke extract inactivates *Salmonella* Typhimurium in laboratory media and protects infected mice against mortality. *J. Food Sci.* **2012**, *77*, M80−M85.
(27) Kim, S. P.; Kang, M. Y.; Choi, Y. H.; Kim, J. H.; Nam, S. H.; Friedman, M. Mechanism of *Hericium erinaceus* (Yamabushitake) mushroom-induced apoptosis of U937 human monocytic leukemia cells. *Food Funct.* **2011**, *2*, 348−356.
(28) Kim, S. P.; Kang, M. Y.; Kim, J. H.; Nam, S. H.; Friedman, M. Composition and mechanism of antitumor effects of *Hericium erinaceus* mushroom extracts in tumor-bearing mice. *J. Agric. Food Chem.* **2011**, *59*, 9861−9869.
(29) Kim, S. P.; Moon, E.; Nam, S. H.; Friedman, M. *Hericium erinaceus* mushroom extracts protect infected mice against *Salmonella* Typhimurium-induced liver damage and mortality by stimulation of innate immune cells. *J. Agric. Food Chem.* **2012**, *60*, 5590−5596.
(30) Kim, S. P.; Nam, S. H.; Friedman, M. *Hericium erinaceus* (lion's mane) mushroom extracts inhibit metastasis of cancer cells to the lung in CT-26 colon cancer-transplanted mice. *J. Agric. Food Chem.* **2013**, *61*, 4898−4904.
(31) Kim, S. P.; Moon, E.; Nam, S. H.; Friedman, M. Composition of Herba Pogostemonis water extract and protection of infected mice against *Salmonella* Typhimurium-induced liver damage and mortality by stimulation of innate immune cells. *J. Agric. Food Chem.* **2012**, *60*, 12122−12130.
(32) Chen, M. F.; Chung, H. H.; Lu, H. L. Protection of the extracts of *Lentinus edodes* mycelia against carbon-tetrachloride-induced hepatic injury in rats. *Sci. World J.* **2012**, *2012*, article 231586, DOI: 231510.231100/232012/231586.
(33) Narumi, S.; Finke, J. H.; Hamilton, T. A. Interferon-γ and interleukin 2 synergize to induce selective monokine expression in murine peritoneal macrophages. *J. Biol. Chem.* **1990**, *265*, 7036−7041.
(34) Suzuki, I.; Tanake, H.; Kinoshita, A.; Oikawas, S.; M., O.; Yadomae, T. Effect of orally administered glucan on macrophage function in mice. *Immunopharmacol. Immunotoxicol.* **1990**, *12*, 675−684.
(35) Misko, T. P.; Schilling, R. J.; Salvemini, D.; Moore, W. M.; Currie, M. G. A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* **1993**, *214*, 11−16.
(36) Nandi, D.; Mishra, M. K.; Basu, A.; Bishayi, B. Protective effects of interleukin-6 in lipopolysaccharide (LPS)-induced experimental endotoxemia are linked to alteration in hepatic anti-oxidant enzymes and endogenous cytokines. *Immunobiology* **2010**, *215*, 443−451.
(37) Aebi, H. Catalase in vitro. *Methods Enzymol.* **1974**, *105*, 121−126.
(38) Bralley, E. E.; Greenspan, P.; Hargrove, J. L.; Wicker, L.; Hartle, D. K. Topical anti-inflammatory activity of *Polygonum cuspidatum* extract in the TPA model of mouse ear inflammation. *J. Inflamm.* **2008**, *5*, 1−7.
(39) Mullarkey, M.; Rose, J. R.; Bristol, J.; Kawata, T.; Kimura, A.; Kobayashi, S.; Przetak, M.; Chow, J.; Gusovsky, S.; Christ, W. J.; Rossignol, D. P. Inhibition of endotoxin response by e5564: a novel Toll-like receptor 4-directed endotoxin antagonist. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1093−1102.
(40) Millien, V. O.; Lu, W.; Shaw, J.; Yuan, X.; Mak, G.; Roberts, L.; Song, L. Z.; Knight, J. M.; Creighton, C. J.; Luong, A.; Kheradmand, F.; Corry, D. B. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* **2013**, *341*, 792−796.
(41) Kim, H. J.; Jun, J.-G.; Kim, J.-K. 2-(4-Hydroxyphenyl)-5-(3-hydroxypropenyl)-7-methoxybenzofuran, a novel ailanthoidol derivative, exerts anti-inflammatory effect through downregulation of mitogen-activated protein kinase in lipopolysaccharide-treated RAW 264.7 cells. *Korean J. Physiol. Pharmacol.* **2013**, *17*, 217−222.
(42) Suzuki, K.; Murakami, T.; Kuwahara-Arai, K.; Tamura, H.; Hiramatsu, K.; Nagaoka, I. Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells. *Int. Immunol.* **2011**, *23*, 185−193.
(43) Liu, T.; Xiao, N.; Liu, Y.; Wilson, B.; Li, J.; Wang, Y.; Cao, X.; Liu, Z. The anti-inflammatory effects of ethyl acetate on Lipopolysaccharide/D-galactosamine challenged mice and Lipopolysaccharide activated RAW264.7 cells. *Biochem. Biophys. Res. Commun.* **2012**, *427*, 518−524.
(44) Woodbury, A.; Yu, S. P.; Wei, L.; García, P. Neuro-modulating effects of honokiol: a review. *Front. Neurol.* **2013**, *4*, article 130, DOI: 110.3389/fneur.2013.00130.
(45) Weng, T. I.; Wu, H. Y.; Kuo, C. W.; Liu, S. H. Honokiol rescues sepsis-associated acute lung injury and lethality via the inhibition of oxidative stress and inflammation. *Intensive Care Med.* **2011**, *37*, 533−541.
(46) Yuan, H.; Wan, J.; Li, L.; Ge, P.; Li, H.; Zhang, L. Therapeutic benefits of the group B3 vitamin nicotinamide in mice with lethal endotoxemia and polymicrobial sepsis. *Pharmacol. Res.* **2012**, *65*, 328−337.
(47) Chen, N.; Liu, D.; Soromou, L. W.; Sun, J.; Zhong, W.; Guo, W.; Huo, M.; Li, H.; Guan, S.; Chen, Z.; Feng, H. Paeonol suppresses lipopolysaccharide-induced inflammatory cytokines in macrophage cells and protects mice from lethal endotoxin shock. *Fundam. Clin. Pharmacol.* **2013**, available online Feb 17, DOI: 10.1111/fcp.12019.
(48) Tseng, Y. T.; Hsu, Y. Y.; Shih, Y. T.; Lo, Y. C. Paeonol attenuates microglia-mediated inflammation and oxidative stress-induced neurotoxicity in rat primary microglia and cortical neurons. *Shock* **2012**, *37*, 312−318.
(49) Ma, J.; Wang, H.; Wang, Y.; Zhang, S. Endotoxin-neutralizing activity of hen egg phosvitin. *Mol. Immunol.* **2013**, *53*, 355−362.
(50) Murakami, T.; Suzuki, K.; Tamura, H.; Nagaoka, I. Suppressive action of resolvin D1 on the production and release of septic mediators in D-galactosamine-sensitized endotoxin shock mice. *Exp. Ther. Med.* **2011**, *2*, 57−61.
(51) Choi, Y. Y.; Kim, M. H.; Cho, I.-H.; Kim, J. H.; Hong, J.; Lee, T. H.; Yang, W. M. Inhibitory effect of *Coptis chinensis* on inflammation in LPS-induced endotoxemia. *J. Ethnopharmacol.* **2013**, *149*, 506−512.
(52) Vitali, R.; Palone, F.; Cucchiara, S.; Negroni, A.; Cavone, L.; Costanzo, M.; Aloi, M.; Dilillo, A.; Stronati, L. Dipotassium glycyrrhizate inhibits HMGB1-dependent inflammation and ameliorates colitis in mice. *PLoS One* **2013**, *8*, article 66572, DOI: 66510.61371/journal.pone.0066527.