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# *Hericium erinaceus* Mushroom Extracts Protect Infected Mice against *Salmonella* Typhimurium-Induced Liver Damage and Mortality by Stimulation of Innate Immune Cells

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**ABSTRACT:** The present study investigated the antibacterial effect of four extracts from the fruitbody of the edible medicinal mushroom *Hericium erinaceus* (hot water extract, HWE; microwave/50% ethanol extract, MWE; acid extract, ACE; and alkaline extract, AKE) against murine salmonellosis. The extracts had no effect on *Salmonella* ser. Typhimurium growth in culture. Nor were the extracts toxic to murine macrophage cells, RAW 264.7. HWE and MWE stimulated uptake of the bacteria into the macrophage cells as indicated by increased colony-forming unit (CFU) counts of the contents of the lysed macrophages infected with *Salmonella* Typhimurium for 30 and 60 min. Two hours postinfection, the bacterial counts increased in the macrophages, but 4 and 8 h postinfection the HWE- and MWE-treated cells showed greater activity against the bacteria than the control. HWE- and MWE-treated noninfected macrophages had altered morphology and elevated inducible nitric oxide (NO) synthase (iNOS) mRNA expression. In the presence of *S.* Typhimurium, iNOS mRNA expression was further increased, accompanied by an increase in NO production. Histology assays of the livers of mice infected with a sublethal dose ($1 \times 10^4$ CFU) of *S.* Typhimurium showed that HWE and MWE, administered by daily intraperitoneal injection, protected against necrosis of the liver, a biomarker of in vivo salmonellosis. The lifespans of mice similarly infected with a lethal dose of *S.* Typhimurium ($1 \times 10^5$ CFU) were significantly extended by HWE and MWE. $\beta$-Glucan, known to stimulate the immune system, was previously found to be present in high amounts in the active extracts. These results suggest that the mushroom extract activities against bacterial infection in mice occur through the activation of innate immune cells.

**KEYWORDS:** *Hericium erinaceus mushroom, macrophage, β-glucan, Salmonella Typhimurium, liver necrosis, phagocytosis, mice, functional food*

## INTRODUCTION

In previous studies, we described the preparation, chemical characterization, and bioactivities of four extracts from the edible medicinal mushroom *Hericium erinaceus*.[1,2] We discovered that two of the extracts induced apoptosis (cell death) in human leukemia cells and inhibited tumor growth in tumor-bearing mice via the induction of natural killer cells, the activation of macrophages, and the inhibition of angiogenesis (restriction of blood flow to tumor cells). Several studies found that extracts from a variety of mushrooms and isolated pure mushroom compounds exhibited antimicrobial effects against foodborne pathogens such as *Escherichia coli* and *Salmonella* in laboratory media[3−11] and stimulated the immune system in vitro.[12−16]

To place the findings of the present study in proper perspective, we will briefly outline recent findings on the immune-stimulating effects of mushroom extracts and isolated pure compounds, reviewed in Lima et al.[17] and Li et al.,[18] that are relevant to the present study. Maity et al.[19] found that a water-soluble glucan isolated from an alkaline extract of fruitbodies of somatic hybrid mushroom of *Pleurotus florida* and *Calocybe indica* varieties activated innate immune cells of macrophages, splenocytes, and thymocytes. Li et al.[20] isolated an immunostimulant protein from the medicinal mushroom *Trametes versicolor* that enhanced the immune response of splenocytes. Bobovcák et al.[21] found that supplementation of the diet with an insoluble $\beta$-glucan from *Pleurotus ostreatus* mushrooms seems to modulate exercise-induced changes in natural killer (NK) cell activity (NCKA) in intensively training athletes. Gaullier et al.[22] reported that oral feeding of a soluble $\beta$-glucan derived from the shiitake medicinal mushroom *Lentinus edodes* to healthy elderly people was safe and induced an increase in the number of circulating B-cells, which play a role in immune response.

The primary objective in this study was to evaluate the potential of the four *H. erinaceus* extracts to inhibit growth of the virulent pathogen *S.* Typhimurium in laboratory media. Surprisingly, the results turned out to be negative. We then determined whether the mushroom extracts could exhibit antisalmonellosis effects by activation or stimulation of the innate immune potential of macrophage cells, rather than via direct bactericidal activity, by evaluating the effects of the mushroom extracts on the murine macrophage RAW 264.7 cell

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line and in mice infected with *Salmonella*. The results show that two bioactive mushroom extracts both stimulated the activity of macrophage cells in vitro and protected infected mice against mortality and liver damage, presumably via stimulation of the immune system. To our knowledge, this is the first report on the inhibition of adverse in vivo effects of *Salmonella* in mice via stimulation of the immune system by mushroom extracts.

## MATERIALS AND METHODS

**Materials.** RPMI 1640 medium, Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), and other miscellaneous cell culture reagents were purchased from Hyclone Laboratories (Logan, UT, USA). The AMV reverse transcriptase and dNTP mixture were obtained from Takara Bio (Kyoto, Japan). PCR primers were custom-synthesized and purified by Bioneer (Daejon, Korea). All reagents of analytical grade were purchased from Sigma Chemicals (St. Louis, MO, USA) and used without further purification.

**Preparation of *H. erinaceus* Extracts.** The dried fruitbody of *H. erinaceus* was obtained from Forest Environment Science Laboratory, College of Agriculture and Life Science, Kyungpook National University (Daegu, Korea). The production of *H. erinaceus* extracts [hot water extract (HWE); microwave/50% ethanol extract (MWE); acid extract (ACE); and alkaline extract (AKE)] and their chemical characterization by gas chromatography and mass spectrometry is described in our previous publication.[1]

**Bacterial Strain and Culture Condition.** *Salmonella enterica* subsp. *enterica* ser. Typhimurium (*S.* Typhimurium) ATCC 140 was obtained from the American Type Culture Collection (Manassas, VA, USA) and kept as frozen glycerol stock. Cells in frozen stock were streaked onto agar medium to produce cell colonies, from which a single colony was transferred to liquid medium. For preparation of inocula, cells were grown for 20 h at 37 °C in nutrient broth (NB). For infection, cultured bacterial cells were recovered by centrifugation at 13000 rpm for 30 s, then washed with phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS. The turbidity of the cell suspensions was measured. The cell suspensions were diluted with PBS to the desired concentration of bacteria using a standard curve of optical density versus bacterial number determined as colony-forming units (CFU).

**Determination of Antibacterial Activity.** The 18 h-cultured *Salmonella* cells were diluted with PBS to a density of $2 \times 10^8$ CFU/mL and inoculated to rich medium NB to a final cell density of $2 \times 10^4$ CFU/mL. After inoculation, *H. erinaceus* extract was added to make a final concentration of 100 μg/mL, the highest concentration of extract readily soluble in PBS, and incubated at 37 °C for 0, 2, 4, and 8 h. PBS was used as a vehicle for negative control. Aliquots were then plated onto NB agar to assess CFU.

**Mammalian Cell Culture.** Murine macrophage cell line RAW 264.7 from the American Type Culture Collection were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS containing 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37 °C in humidified air containing 5% $CO_2$. Cell density was adjusted to optimize each of the following macrophage tests.

**Macrophage Cytotoxicity Assay.** Cell viability was assessed by MTT staining as previously described.[23] Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of $1 \times 10^4$ cells/well and cultured for 16 h at 37 °C in humidified air containing 5% $CO_2$. The cells were then subjected to treatment with each *H. erinaceus* extract in PBS (1, 10, and 100 μg/mL and a saturated solution) for 48 h. Whereas 100 μg/mL is the highest concentration readily soluble in PBS, we prepared a saturated solution by suspending 500 μg/mL in PBS, agitating for 1 h at room temperature, and subsequently centrifuging to remove the insoluble precipitate. PBS was used as a vehicle for negative control indicating cell viability in this medium. After treatment, cells were stained by the addition of MTT. The resultant intracellular chromogen formazan product was solubilized by adding DMSO. Absorbance of the chromogen was determined using a microplate reader (model 550, Bio-Rad, Hercules, CA, USA) at 570 nm and a reference wavelength of 655 nm. Cell viability was expressed as a percentage of live cells relative to that of normal control group treated with PBS as a vehicle alone.

**Morphologic Changes in Macrophages.** RAW 264.7 cells were grown in the presence of each *H. erinaceus* extract (100 μg/mL, the highest concentration of extract readily soluble in PBS while showing no cytotoxicity) in 24-well cell culture plates with coverslips for 8 h. The coverslips were examined under a microscope (100×) to observe morphologic changes in macrophages. Cells were selected in six blindly chosen random fields, and the morphologically changed cells were then counted and recorded. This assay was performed for at least three individual experiments.

**Determination of Bacteria in Macrophages.** To measure internalization and intracellular survival of bacteria in macrophages, RAW 264.7 cells were infected with *S.* Typhimurium following the method of Lu et al.[24] To analyze the efficiency of bacterial uptake by macrophages, inoculum (10 μL) containing $1 \times 10^4$ CFU was added to macrophage cells ($1 \times 10^4$ cells) pretreated with each extract (100 μg/mL) and incubated for 30 or 60 min in a 5% $CO_2$ atmosphere. Cells were washed once with RPMI 1640 medium after incubation at 37 °C and then treated with the same medium containing 10% FBS and gentamicin (30 μg/mL) for 30 min to kill extracellular bacteria, an optimal time determined in a preliminary study (results not shown). For viable cell counting, the infected macrophage cells were washed three times and then lysed with distilled water. Aliquots of lysates were plated onto NB agar medium to measure bacterial CFUs.

To measure intracellular survival of the bacteria after uptake by the macrophages, *S.* Typhimurium ($1 \times 10^4$ CFU) was added to each extract-treated (100 μg/mL for 4 h) macrophage sample. The samples were incubated at 37 °C for 1 h, followed by washing once with medium and further incubation in the presence of RPMI 1640 medium containing 10% FBS with gentamicin (30 μg/mL) for 2, 4, and 8 h. Cell washing, lysis, and plating procedures onto NB agar medium were carried out following the same protocol as for the analysis of bacterial uptake efficiency.

**Nitric Oxide (NO) Assay.** RAW 264.7 cells ($1 \times 10^5$ cells/well) in a 96-well plate were incubated in the presence of each extract (100 μg/mL) for 4 h. Then, cells were washed once with PBS and infected with *S.* Typhimurium ($1 \times 10^4$ CFU/well) for 2, 4, and 8 h. NO was measured by determining the concentration of its stable oxidative metabolite nitrite using a microplate assay by a described method.[25] After bacterial incubation, cell-free culture medium (100 μL) was collected and mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid] at room temperature for 15 min. The absorbance was then read at 570 nm using a microplate reader. Sodium nitrite was used as the standard.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR) of Cellular RNA.** Total cellular RNA was prepared following acid phenol guanidium thiocyanate−chloroform extraction.[26] For reverse transcription, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT18) as primer (100 ng). DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), *Taq* polymerase (2.5 U), and primer sets (20 μM each) representing target genes as follows: inducible nitric oxide synthase (iNOS) sense primer, 5′-ATGTCCGAAGCAAACATCAC-3′; iNOS antisense primer, 5′-TAATGTCCAGGAAGTAGGTG-3′; β-actin sense primer, GTGGGGCGCCCCAGGCACCA-3′; β-actin antisense primer, 5′-GTCCTTAATGTCACGCACGATTTC-3′. PCR was conducted using a thermocycler (model PTC-200, MJ Research Inc., Reno, NV, USA) with 1 cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, and finally 1 cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with a UV illuminator. The intensity of the separated bands of DNA was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

**Mice.** Pathogen-free female Balb/c mice, aged from 6 to 8 weeks, were obtained from Orient Bio Inc. (Seongnam, Republic of Korea). After acclimation for 1 week, the mice were hosted under a 12 h light/

dark cycle with a temperature range of 20−22 °C and a relative humidity of 50 ± 10%. The mice were fed freely a pelletized commercial chow diet obtained from Orient Bio Inc. (catalog no. SL79) and sterile tap water during the entire experimental period. Food was withheld for a period of 12−15 h before the experiments. 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.

**Histology of Liver Tissue.** Because salmonellosis elicits liver necrosis, the level of liver injury was used as the marker for incidence and progression of salmonellosis. Mice were intraperitoneally treated with each of the extracts every 24 h for 2 days after intraperitoneal infection of a sublethal dose ($1 \times 10^4$ CFU) of *S.* Typhimurium. For histological analysis, the liver tissue of the mice was fixed with 4% paraformaldehyde in 0.5 M phosphate buffer (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.

**Mice Salmonellosis Study.** The salmonellosis assay was carried out following the method of Kim et al.[27] with some modification. Five groups of 10 mice (a group for each of the four extracts and a PBS-vehicle positive control) were used for bacterial infection. Mice were infected intraperitoneally with a lethal dose of *Salmonella* ($1 \times 10^5$ CFU). After bacterial infection, each of four groups of mice was treated with an extract (10 mg/kg) and the fifth group with vehicle via the intraperitoneal route every 24 h during the entire experimental period. The dose of the extract (10 mg/kg) for intraperitoneal delivery was adopted from data from previously reported relevant studies on natural product extracts. We confirmed this dose with additional preliminary experiments (results not shown). To determine the survival rate, mice were observed for an additional 25 days after bacterial infection.

**Statistical Analysis.** Results are expressed as the mean ± SD of three independent experiments. Significant 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

**Effect of *H. erinaceus* Extracts on Growth of *Salmonella*.** To find out whether *H. erinaceus* extracts have antibacterial activities against *S.* Typhimurium, bacteria were incubated with 100 μg/mL of each extract (HWE, MWE, ACE, and AKE) dissolved in PBS at 37 °C for 0, 2, 4, and 8 h. Figure 1 shows that bacteria treated with the extracts for different incubation times showed the same proliferating patterns as observed in the control PBS used as a vehicle. The data show that *H. erinaceus* extracts did not kill bacteria directly, as was the case in previous studies with rice hull smoke extract.[28]

**Cytotoxicity of *H. erinaceus* Extracts.** To evaluate the cytotoxic effects of HWE, MWE, ACE, and AKE, RAW 264.7 cells were treated in the presence of the extracts (1, 10, and 100 μg/mL and a saturated solution) for 48 h. The MTT assay was used to assess the cytotoxicity of each extract in RAW 264.7 cells. Table 1 shows that there were no detectable cytotoxic effects (100% RAW 264.7 cell survival) of the extracts under these assay conditions at any of the concentrations tested. *H. erinaceus* extract-treated cells showed statistically the same survival rates as vehicle-treated cells.

**Induction of Morphologic Changes in Macrophages.** To determine whether *H. erinaceus* extracts can induce changes in the morphology of the macrophage cells, RAW 264.7 cells were cultivated in the presence of each extract (100 μg/mL) for 8 h. Microscopic observation of cell morphology (Figure 2A) revealed that the cells treated with HWE and MWE changed to dendrite-like cells, reaching up to 65% and 68% levels, respectively (Figure 2B). Dendritic morphologic changes were not observed with ACE or AKE.

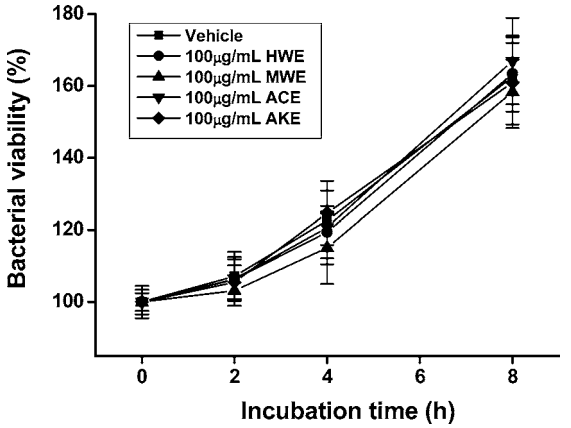


**Figure 1.** Effects of HWE, MWE, ACE, and ACE on growth of *S.* Typhimurium. Each extract (100 μg/mL) was incubated with *S.* Typhimurium ($2 \times 10^4$ CFU) for 2, 4, and 8 h. Bacterial viability was measured based on CFU counts on NB agar medium, and the rate of viability was compared with the control treated with PBS as vehicle. Plotted values are the mean ± SD of triplicate experiments.

**Table 1. Cell Cytotoxicity of the Extracts from *Hericium erinaceus* in RAW 264.7 Cells**

| extract | dose (mg/mL) | cell survival[a] (%) |
|---|---|---|
| HWE | 1 | 101.7 ± 5.3 |
| | 10 | 99.7 ± 2.1 |
| | 100 | 103.1 ± 4.7 |
| | saturated | 102.1 ± 4.6 |
| MWE | 1 | 98.1 ± 2.3 |
| | 10 | 100.5 ± 4.9 |
| | 100 | 103.6 ± 6.6 |
| | saturated | 102.2 ± 8.2 |
| ACE | 1 | 102.6 ± 3.6 |
| | 10 | 99.4 ± 3.6 |
| | 100 | 101.1 ± 4.8 |
| | saturated | 101.0 ± 5.5 |
| AKE | 1 | 100.6 ± 3.1 |
| | 10 | 102.0 ± 5.7 |
| | 100 | 100.0 ± 3.3 |
| | saturated | 102.7 ± 6.6 |

[a]Data are expressed as the mean ± SD of triplicate experiments. The treatments were not significantly different at $p < 0.05$.

**Phagocytotic Stimulatory Effects of *H. erinaceus* Extracts.** To examine whether *H. erinaceus* extracts affect phagocytotic activity, RAW 264.7 cells were cultivated in the presence of each extract (100 μg/mL) for 4 h before infection with *S.* Typhimurium. Thereafter, the cells were incubated for 30 and 60 min before they were lysed and their contents enumerated for bacteria. The internalization of bacteria into cells was increased for HWE- and MWE-treated macrophages. This increase in internalization was not observed, however, with ACE and AKE treatments. Figure 3 shows that the bacterial

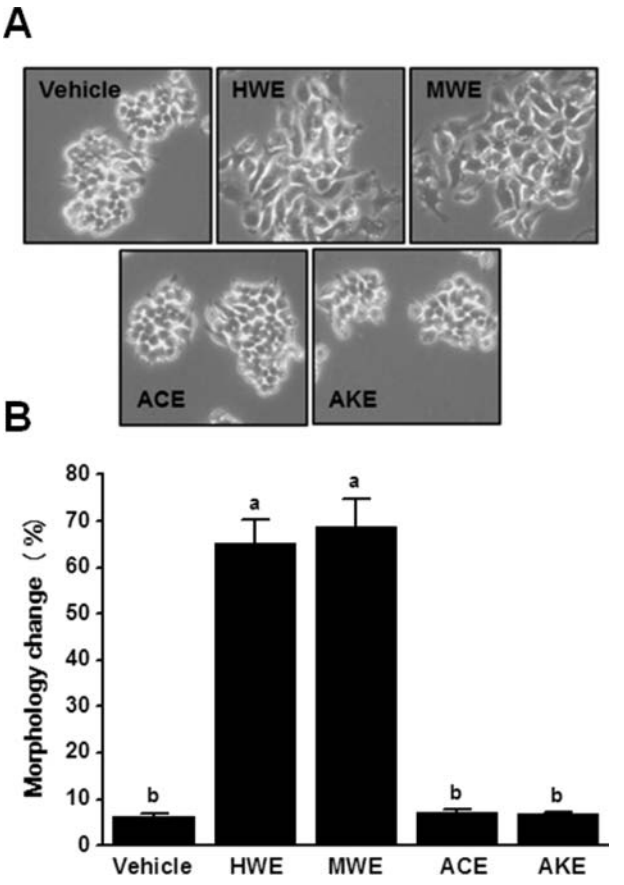


**Figure 2.** Effects of HWE, MWE, ACE, and AKE on morphologic changes in macrophages. Murine macrophage cell line RAW 264.7 cells were cultured in the presence of each extract (100 $\mu$g/mL) for 8 h. (A) After 8 h of incubation, the morphologic changes were photographed at 100× magnification. (B) One hundred cells in five blindly chosen random fields were examined per coverslip, and the rates of morphologically changed cells were scored. Data are expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different within groups at $p < 0.05$.

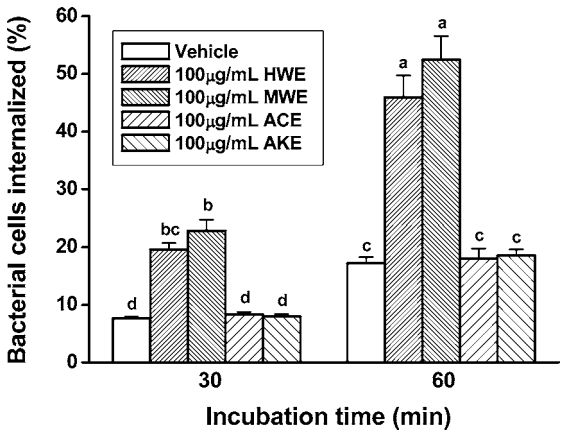


**Figure 3.** Changes in phagocytotic stimulatory effects of HWE, MWE, ACE, and AKE on *S.* Typhimurium-infected macrophages. Murine macrophage cell line RAW 264.7 cells ($1 \times 10^4$) were incubated with each extract (100 $\mu$g/mL) for 4 h and then infected with *S.* Typhimurium ($1 \times 10^4$ CFU). Incubation continued at 37 °C for the indicated periods. After incubation, bacteria-infected macrophages were then cultured in the presence of gentamicin (30 $\mu$g/mL) for 30 min. Bacterial internalization efficiency by macrophages was determined by evaluating the protection of internalized bacteria from bacteriocidal action of the antibiotic gentamicin. Data are expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

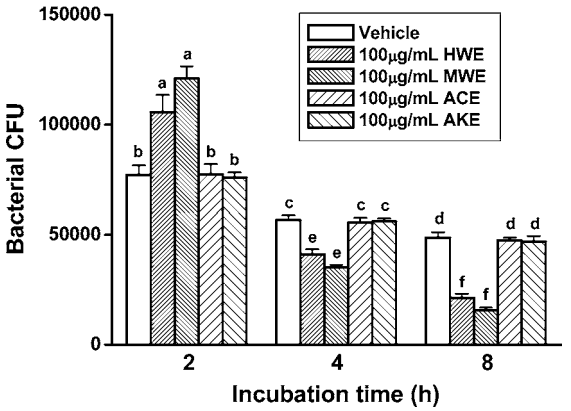


**Figure 4.** Survival and/or proliferation of *S.* Typhimurium within mushroom-extract-treated macrophages. Murine macrophage cell line RAW 264.7 cells ($1 \times 10^4$) were incubated with each extract (100 $\mu$g/mL) for 4 h and then infected with *S.* Typhimurium ($1 \times 10^4$ CFU) at 37 °C for 1 h. The cells were washed and then further incubated in the presence of gentamicin (30 $\mu$g/mL) for the indicated periods. At each time point, the cells were lysed and the numbers of viable intracellular bacteria counted. Data are expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

uptake rates of HWE- and MWE-treated macrophages were about 2-fold greater after 30 min of incubation and about 3-fold greater after 60 min of incubation than that of macrophages treated with PBS alone.

**Effects of *H. erinaceus* Extracts on Intracellular Bacterial Survival.** To examine whether *H. erinaceus* extracts affect *S.* Typhimurium survival within macrophage cells, RAW 264.7 cells were treated with each extract for 4 h, incubated in the presence of bacteria for 1 h, and then incubated for another 2, 4, or 8 h postinfection in the presence of gentamicin. Figure 4 shows that 2 h after infection, the intracellular growth of *S.* Typhimurium within the cells treated with HWE and MWE was about 37 and 57% greater, respectively, than in the PBS-treated control. However, 8 h after infection, marked decreases in intracellular bacteria were observed in the cells treated with HWE and MWE (56 and 68%, respectively) compared with the control. In contrast, ACE and AKE treatments were not significantly different than the control.

**Effects of *H. erinaceus* Extracts on NO Production.** NO is generated by macrophages as part of the immune response. It is a signaling molecule to host cells for altering physiological status, including modulation of immune response,[29] but toxic to bacteria through the action of peroxynitrite, a reaction product of NO and $O_2^-$.[30] Inducible NO synthase (iNOS) activity is stimulated by lipopolysaccharide, the endotoxin found in the outer cell membrane of Gram-negative bacteria such as *Salmonella*.[31]

NO production was assessed to determine whether infected RAW 264.7 cells could be stimulated by *H. erinaceus* extracts at a dose of 100 $\mu$g/mL. Aliquots of culture supernatant were collected after 2, 4, and 8 h of incubation with *S.* Typhimurium, and the NO concentration was then measured. Even without *S.* Typhimurium infection, HWE- or MWE-treated macrophages showed marked increases in NO production at all experimental


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times up to 8 h (data not shown). When infected with *S.* Typhimurium after a 4 h treatment with the extracts (100 μg/mL), NO production in HWE- and MWE-treated RAW 264.7 cells increased by about 60 and 70%, respectively, at 8 h postinfection compared with vehicle-treated or ACE- and AKE-treated macrophages (Figure 5).

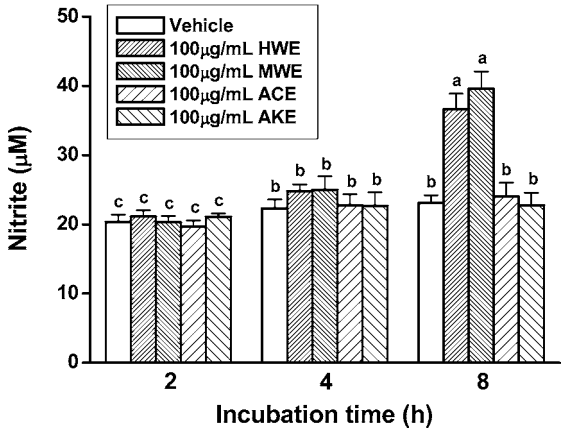


**Figure 5.** Effects of HWE, MWE, ACE, and AKE on the production of NO from *S.* Typhimurium-infected macrophages. Murine macrophage RAW 264.7 cells ($1 \times 10^5$) cells were incubated in the presence of each extract (100 μg/mL) for 4 h. Then, *S.* Typhimurium ($1 \times 10^4$ CFU) was added to RAW 264.7 cells and incubated for the indicated time periods. After incubation, cell-free medium was collected and nitrite concentration was determined. Data are expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

RT-PCR was performed to examine whether the increase in NO production by HWE and MWE was mediated through changes in iNOS mRNA expression.[31] Test samples included untreated macrophages (negative control), *S.* Typhimurium infected macrophages (positive control), and macrophages treated with each of the extracts, with and without *S.* Typhimurium infection. Figure 6 shows that untreated and

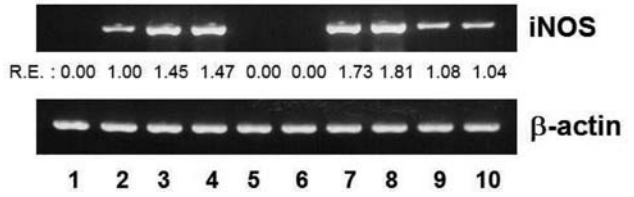


**Figure 6.** Effects of HWE, MWE, ACE, and AKE on iNOS mRNA expression in *S.* Typhimurium-infected macrophages. Murine macrophage RAW 264.7 cells ($1 \times 10^6$) were cultured with or without each extract (100 μg/mL) for 4 h. Then, *S.* Typhimurium ($1 \times 10^4$ CFU) was added to the cells, which were then incubated for 8 h. After incubation, total RNA was purified and iNOS mRNA expression was determined using RT-PCR analysis. Lanes: 1, vehicle (PBS)-treated without bacterial infection; 2, bacterial infection alone; 3, HWE-treated without bacterial infection; 4, MWE-treated without bacterial infection; 5, ACE-treated without bacterial infection; 6, AKE-treated without bacterial infection; 7, HWE-treated with bacterial infection; 8, MWE-treated with bacterial infection; 9, ACE-treated with bacterial infection; 10, AKE-treated with bacterial infection. The relative proportion of iNOS gene mRNA is expressed as an RE (relative expression) value calculated from iNOS gene/β-actin gene expression. Figures represent results from at least three individual experiments.

ACE- and AKE-treated macrophages had no measurable iNOS mRNA expression. iNOS mRNA was strongly expressed in uninfected cells treated with HWE or MWE, about 46% greater than in *Salmonella* alone infected cells. All of the *Salmonella*-infected cells expressed iNOS, but expression in HWE- and MWE-treated infected cells was greater. For the HWE- and MWE-treated cells, expression was 9 and 24% greater, respectively, in the *Salmonella*-infected cells than in the noninfected cells, showing that the effects are possibly additive.

**Effects of *H. erinaceus* Extracts on Histopathology of Mouse Livers.** To examine whether *H. erinaceus* extracts can ameliorate liver injury induced by salmonellosis, mice were intraperitoneally treated with each of the extracts every 24 h for 2 days after intraperitoneal infection of a sublethal dose ($1 \times 10^4$ CFU) of *S.* Typhimurium. Figure 7 shows that extensive

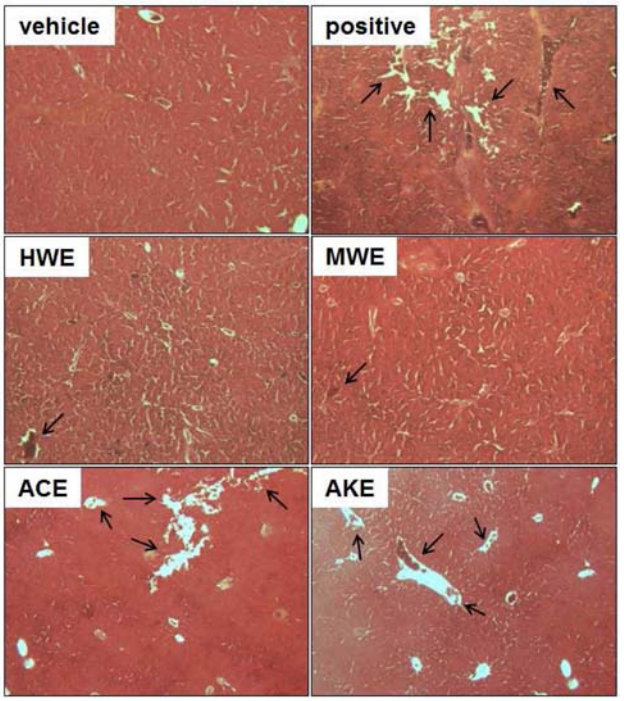


**Figure 7.** Effects of HWE, MWE, ACE, and AKE on the formation of pathological lesions in livers of *S.* Typhimurium-infected mice. Liver specimens from extract-treated mice infected with *S.* Typhimurium ($1 \times 10^4$ CFU) were fixed with 4% paraformaldehyde. The sections were then stained with hematoxylin and eosin (H & E). Magnification 100×. Arrows indicate representative hemorrhagic necrosis. Figures represent results from at least three individual experiments.

liver injuries such as necrosis and hemorrhage were present in the liver tissues from bacteria-infected control mice. However, liver tissues from bacteria-infected mice treated with HWE and MWE showed minimal liver damages. ACE and AKE treatments failed to ameliorate liver injury caused by infection of *S.* Typhimurium. These results demonstrate that the two extracts that were most effective in inducing macrophage activity also protected the livers of mice against *Salmonella*-induced necrosis.

**Effects of *H. erinaceus* Extracts on Mortality of Infected Mice.** To determine the therapeutic effects of the extracts on murine salmonellosis, mice were infected with a lethal dose ($1 \times 10^5$ CFU) of *S.* Typhimurium intraperitoneally and assessed for mortality. Each group of 10 mice was treated with each extract (10 mg/kg) or PBS-vehicle control every 24 h

during the entire experimental period. Figure 8 shows that the mortality rate in the vehicle-treated control group was 100% on

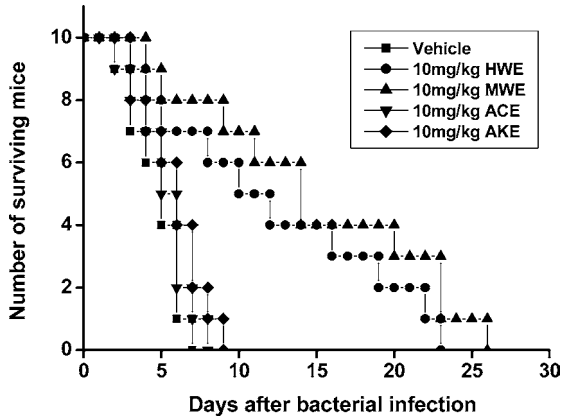


**Figure 8.** Histogram showing effects of HWE, MWE, ACE, and AKE on *S.* Typhimurium infection-induced lethality. Balb/c mice (10 mice per group) were infected with lethal dose of *S.* Typhimurium ($1 \times 10^5$ CFU) through the intraperitoneal route. Then, each extract (10 mg/kg) was intraperitoneally injected every 24 h during the entire experimental period. PBS was used as the vehicle in the control group. Plotted values are the mean values of triplicate determinations.

day 7. However, the groups treated with HWE and MWE survived until days 23 and 26, respectively. ACE and AKE treatments failed to suppress the mortality caused by infection of *S.* Typhimurium. These observations strikingly demonstrate the potential of HWE and MWE to protect mice against the lethal effects of the bacteria, presumably via stimulation of the immune system.

**Relationship of Composition of *H. erinaceus* Extracts to Bioactivities.** The relative bioactivities of the four extracts in the macrophage cells are consistent with the previously reported bioactivities against cancer cells in vitro and in mice.[1,2] The two extracts with a higher content of compounds as characterized by mass spectrometry,[1] as well as a high content of $\beta$-glucans determined using a commercial kit,[1] exhibited immunomodulatory effects in the macrophage cells, whereas the other two were not active. $\beta$-Glucans are known to participate in the adaptive immune response.[32] The $\beta$-glucan contents of the bioactive HWE and MWE of 0.51 and 0.47% on a dry weight basis, respectively, were about 3−4-fold higher than those of of the inactive ACE and AKE extracts, with contents of 0.17 and 0.13%, respectively.[1] The active extracts also contained a greater number of characterized compounds than did the inactive extracts. Active HWE contained 40 compounds, and MWE contained 27; inactive ACE contained 16, and AKE contained 13.[1] We do not know which individual compound or mixtures of compounds may contribute to the beneficial effects.

**Health and Dietary Aspects.** We demonstrated the protective effect of two mushroom extracts of *H. erinaceus* against the adverse effects of *Salmonella* in mice. Liver morphology and life expectancy were improved in *Salmonella*-infected mice by intraperitoneal administration of these extracts. The beneficial effects were paralleled by changes in several parameters measured in cultured murine macrophage cells associated with the immune system.

Because systemic salmonellosis induces liver necrosis, liver injury was used as the biomarker for the incidence of in vivo salmonellosis in the mice. We do not know whether the extracts offer any direct protection of the liver. It seems that primarily the protection of the liver by the mushroom extracts is due to stimulation of the innate immune cells, which then kill the *Salmonella* bacteria that induced the liver injury, analogous to that observed with the cultured macrophage cells. It would be of interest to find out whether the bioactive mushroom extracts would also protect against adverse effects caused by other pathogenic microorganisms such as *E. coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*, as well as against Vibrio infections in seafood,[33] and HIV infections in humans.[34]

The results of the present and of the previous[1,2] studies suggest that functional foods (nutraceuticals) containing *H. erinaceus* mushrooms or mushroom extracts could be used to improve microbial food safety and protect against cancer. The underlying mechanisms of the immunomodulatory effects of the extracts also merit further study.

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### Notes

The authors declare no competing financial interest.

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