JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

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

Sung Phil Kim,[†] Eunpyo Moon,[‡] Seok Hyun Nam,*[,‡] and Mendel Friedman*[,§]

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

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

ABSTRACT: GC-MS analysis of a hot water extract of Herba Pogostemonis (HP) revealed the presence of 131 compounds. HP slightly inhibited *Salmonella* Typhimurium bacteria in culture and stimulated uptake of the bacteria into RAW 264.7 murine macrophage cells as indicated by both increased fluorescence from internalized FITC-dextran and increased colony-forming unit (CFU) counts of the lysed macrophages. Postinfection, the HP-treated cells showed lower bacterial counts than the control. HP elicited altered morphology, elevated inducible NO synthase (iNOS) mRNA, and reduced pro-inflammatory cytokine expression in macrophage cells. *Salmonella* induced increased expression of iNOS mRNA, cognate polypeptides, and NO. Histology of mice infected with a sublethal dose (1×10^4 CFU) of *Salmonella* showed that intraperitoneally administered HP protected against necrosis of the liver, a biomarker of in vivo salmonellosis. The lifespan of mice infected with a lethal dose (1×10^5 CFU) was significantly extended. These results suggest that the activity of HP against bacterial infection in mice occurs through the activation of innate immune macrophage cells. The relationship of composition of HP to bioactivity is discussed.

KEYWORDS: *Herba Pogostemonis, composition, Salmonella typhimurium, antimicrobial, mice, innate immune cells, liver damage prevention, immunostimulant*

INTRODUCTION

The immune response to pathogenic microorganisms occurs in two stages. Cells of the innate immune system respond rapidly to an infection, blocking the invading pathogen and signaling to lymphocytes. These cells and the adaptive immune system then neutralize the infection. In previous studies, we reported that rice hull smoke[1] and mushroom extracts[2] protected mice against *Salmonella*-induced liver necrosis and mortality. We are interested in finding other natural substances that may act similarly. Herba Pogostemonis (HP) from the dried leaves of *Pogostemon cablin* Bentham is widely used in traditional Chinese medicine to treat inflammation and other disorders.[3]

Previous studies have identified patchouli alcohol as a biologically active component of HP. Patchouli alcohol isolated from HP exhibited anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, in part by down-regulation of inflammatory mediators TNF-α, IL-1β, IL-6, iNOS, and COX-2.[3] Another study[4] found that the patchouli alcohol also protected mice against influenza viral infection. However, other components of HP may also be bioactive. A water-soluble extract of Herba Pogostemonis, which would not contain patchouli alcohol, protected mice against induced gastrointestinal pain and diarrhea.[5] A water extract also inhibited the activity of *Escherichia coli*, *Salmonella*, *Shigella flexneri*, and *Staphylococcus aureus* bacteria.[6]

The main objective of this study was to prepare a hot water extract of Herba Pogostemonis from freeze-dried leaves, determine the composition of the powder by GC-MS, and evaluate the antimicrobial activities of the extract in laboratory media and in mice infected with the virulent *Salmonella* foodborne pathogens.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, phosphate-buffered saline (PBS), 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, Republic of Korea). All reagents of analytical grade were purchased from Sigma (St. Louis, MO, USA) and used without further purification.

Preparation of Herba Pogostemonis Extract. HP (*P. cablin* Bentham) was kindly provided from a Korean traditional clinic in Yongin City, Gyeonggi-do. Briefly, dried HP (12 g) was extracted with 10-fold deionized water (120 mL) by autoclaving for 30 min, followed by filtration using Whatman filter paper no. 2 (Whatman International, Maidstone, UK). The filtrate was concentrated and lyophilized by sequential use of a rotary evaporator and lyophilizer and dissolved in phosphate-buffered saline (PBS, pH 7.4) to yield ~19.5%. The extract was stored at −20 °C until use.

Component Analysis by GC-MS. Lyophilized HP was derivatized in two steps to protect carbonyl function following the method of Kim et al.[7] Dried samples were dissolved in methoxyamine hydrochloride (100 μL; 20 mg/mL) in pyridine and reacted at 60 °C for 1 h. The acidic protons were exchanged against the trimethylsilyl group to increase the volatilities of the polar compounds using 100 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 70 °C for 1 h.

Received: July 17, 2012
Accepted: November 15, 2012
Published: November 27, 2012

The HP was analyzed by GC-MS using a gas chromatograph, model 6890N (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a mass spectrometer detector 5973 and DB-1 column (Agilent Technologies, stationary phase; polyethylene glycol, 30 m × 0.25 mm; i.d. = 0.25 μm). The temperature was programmed at 70 °C (4 min) with an increase of 10 °C/min until 300 °C (6 min) was reached. Helium gas was used as the carrier with a flow rate of 1 mL/min. Both injector and detector temperatures were set at 250 °C. The injection was a split ratio of 25:1 in all cases. The injection volume was 1 μL. Mass spectra were recorded in electron ionization mode with an ionization energy of 70 eV. Components were identified by retention times in the mass spectra and by comparing the mass spectra with the aid of ADMIS software (National Institute of Technology and Standards, USA) with those in a public mass spectral and retention index library.[8]

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 Nutrient agar (NA) medium to produce cell colonies, from which a single colony was transferred to Nutrient broth (NB). For preparation of inocula, cells were grown for 20 h at 37 °C in NB. For infection, cultured bacterial cells were recovered by centrifugation at 13000 rpm for 30 s, then washed with 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. *Salmonella* cells were diluted with PBS to a density of 2×10^4 CFU/mL, treated with various concentrations of HP (1, 10, and 100 μg/mL), and incubated at 37 °C for 0, 2, 4, and 8 h. After incubation, the culture (100 μL) diluted to appropriate concentration was plated onto NA medium to assess bacterial CFUs.

Mammalian Cell Culture. Murine macrophage cell line RAW 264.7 from the American Type Culture Collection was 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 Viability Assay. Cell viability was assessed by MTT staining as previously described.[9] Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of 1×10^4 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 three concentrations of extract (1, 10, and 100 μg/mL and 1 mg/mL) for 48 h. 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 (vehicle).

Morphologic Changes in Macrophages. RAW 264.7 cells were grown in the presence of various concentrations of the HP (100 μg/mL) 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.

Phagocytotic Uptake Assay. The phagocytotic activity of the HP-treated RAW 264.7 cells was measured following the method of Duperrier et al.[10] with some modification. Briefly, RAW 264.7 cells were cultured in a 60 mm culture dish (1×10^5 cells) with three concentrations of the HP extract (1, 10, and 100 μg/mL) for 48 h. LPS (100 ng/mL)-treated cells were used as positive control. After stimulation, cells (1×10^4 cells) were resuspended in 1 mL of PBS containing 5% FBS and left at 37 °C for 15 min. They were then incubated with Dextran-FITC (1 mg/mL) at 37 °C for 1 h. The reaction was stopped with cold PBS containing 5% FBS and 1% sodium azide. The cells were washed three times with cold PBS and analyzed on a FACSvantage Instrument (Becton-Dickinson, Franklin Lakes, NJ, USA).

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.[11] For analysis of macrophage bacterial uptake efficiency, inoculum (10 μL) containing 1×10^4 CFU was added to macrophage cells (1×10^4 cells) pretreated with three concentrations of the HP (1, 10, and 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 gentamycin (30 μg/mL) for 30 min to kill extracellular bacteria. For viable cell counting, the infected macrophage cells were washed three times and then lysed with distilled water. Aliquots of lysates were plated onto NA medium to measure bacterial CFUs.

To measure intracellular survival, *S.* Typhimurium (1×10^4 CFU) was added to serially diluted extract-treated macrophage sample for 4 h. The samples were incubated at 37 °C for 1 h, followed by washing once with medium and subsequent treatment with RPMI 1640 medium containing 10% FBS with gentamycin (30 μg/mL) for 2, 4, and 8 h. Cell washing, lysis, and plating onto NA medium were carried out following the same protocol as for the analysis of bacterial uptake efficiency.

Nitric Oxide (NO) Generation Assay. RAW 264.7 cells (1×10^5 cells/well) in a 96-well plate were incubated in the presence of three concentrations of the HP (1, 10, and 100 μg/mL) for 4 h. Then, cells were washed once with PBS and infected with *S.* Typhimurium (1×10^4 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 according to a described method.[12] 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 read at 570 nm using a microplate reader. $NaNO_2$ was used as the standard.

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 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. Food was withheld for a period of 12−15 h before the experiments.

Mice Salmonellosis Study. The salmonellosis assay was carried out following the method of Kim et al.[13] with some modification. Briefly, mice were acclimated for 1 week before the experiments were begun. Two groups of 10 mice each (PBS-treated control and HP-treated experimental group) were used for bacterial infection. Mice were infected intraperitoneally with a lethal dose of *Salmonella* (1×10^5 CFU). After bacterial infection, the experimental group of mice was treated with the HP (10 mg/kg) via the intraperitoneal route every 24 h during the entire experimental period. To determine the survival rate, mice were observed for an additional 20 days after bacterial infection. All experiments were performed in compliance with the relevant laws and institutional guidelines.

Histology of Liver Tissue. 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, embedded in paraffin, sectioned into 4 μm, mounted onto glass slides, 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.

ELISA of Cytokines. After RAW 264.7 cells (1×10^6 cells) were stimulated with the HP (100 μg/mL), the culture medium was recovered and stored at −20 °C until use. The pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) released into the culture medium were measured with corresponding assay kits (Biosource International, Camarillo, CA, USA) according to the manufacturer's instruction. The absorbance of the final reaction mixture at 420 nm was measured in a microplate reader (model 550, Bio-Rad).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) of Cellular RNA. Total cellular RNA was prepared following acid phenol guanidium thiocyanate−chloroform extraction.[14] For reverse transcription, total RNA (1 μg) was incubated with AMV reverse

Table 1. Retention Times (t_R) of Identified Compounds in HP and Relative Proportions in Percent Peak Area

peak	t_R (min)	compound (based on mass spectral data)	peak area (%)
1	5.114	1-methylhydantoin	0.044
2	5.390	2-amino-2-methyl-1,3-propanediol	0.183
3	5.822	*N*-ethylglycine	0.049
4	6.746	2-hydroxypyridine	0.006
5	7.185	L-(+)-lactic acid	0.632
6	7.517	glycolic acid	0.993
7	10.814	malonic acid	0.105
8	10.924	L-valine	0.061
9	12.134	2-amino-1-phenylethanol	0.005
10	12.225	L-norleucine	0.050
11	12.363	L-leucine	0.033
12	12.488	glycerol	3.495
13	12.532	phosphoric acid	2.678
14	12.905	DL-isoleucine	0.021
15	12.944	L-proline	0.061
16	13.179	glycine	0.034
17	13.325	methylmalonic acid	0.002
18	13.328	maleic acid	0.004
19	13.412	succinic acid	1.254
20	13.970	glyceric acid	3.021
21	14.242	uracil	0.006
22	14.277	fumaric acid	0.254
23	14.309	tartronic acid	0.116
24	14.499	L-alanine	0.081
25	14.622	pipecolic acid	0.003
26	14.718	L-serine	0.018
27	15.388	L-threonine	0.023
28	16.051	3-hydroxypropanoic acid	0.044
29	16.238	β-alanine	0.002
30	17.479	iminodiacetic acid	0.097
31	17.663	mandelic acid	0.004
32	17.939	D-malic acid	6.140
33	17.939	oxalic acid	5.198
34	18.472	synephrine	0.003
35	18.508	D-threitol	2.500
36	18.667	aspartic acid	0.011
37	18.727	4-guanidinobutyric acid	0.349
38	18.727	L-pyroglutamic acid	0.338
39	18.750	L-glutamic acid 3 (dehydrated)	0.131
40	19.506	acetol	0.193
41	19.915	2-isopropylmalic acid	0.008
42	20.427	maleamic acid	0.001
43	20.508	3-hydroxy-3-methylglutaric acid (dicrotalic acid)	1.681
44	21.097	4-hydroxybenzoic acid	0.018
45	21.297	D-sphingosine	0.014
46	22.040	ribose	0.050
47	22.168	D-lyxose	0.265
48	23.347	6-deoxy-D-glucose	3.713
49	23.457	xylitol	10.729
50	23.639	*N*-methylglutamic acid	0.005
51	23.731	L-(−)-fucose	0.013
52	24.141	4-hydroxy-3-methoxybenzoic acid	0.040
53	24.716	tartaric acid	0.592
54	25.158	shikimic acid	0.072
55	25.178	3,4-dihydroxybenzoic acid	0.119
56	25.388	citric acid	0.713
57	26.188	tagatose	0.068
58	26.348	quinic acid	2.107
59	26.694	fructose	13.024
60	26.796	gluconic acid lactone	0.564
61	26.895	DL-glyceraldehyde	4.903
62	26.940	D-allose	0.194
63	27.126	2-keto-L-gulonic acid	0.055
64	27.210	D-glucose	15.064
65	27.764	D-mannitol	2.891
66	27.989	talose	0.044
67	28.047	galacturonic acid	0.051
68	28.483	gluconic acid	0.236
69	29.217	DL-4-hydroxy-3-methoxymandelic acid	0.010
70	29.356	D-saccharic acid	0.032
71	29.407	palmitic acid	0.226
72	29.940	mucic acid	0.027
73	30.680	*allo*-inositol	2.823
74	30.826	5-aminoimidazole-4-carboxamide	0.023
75	31.237	caffeic acid	0.034
76	31.686	D-(+)-altrose	0.085
77	32.015	putrescine	0.004
78	32.584	oleic acid	0.017
79	32.883	stearic acid	0.231
80	33.007	D-sorbitol	0.062
81	35.726	glucoheptonic acid	0.260
82	37.881	methyl β-D-galactopyranoside	0.041
83	38.786	neohesperidin	0.184
84	39.057	1-hydroxy-2-naphthoic acid	0.002
85	39.459	adenosine	0.015
86	40.002	sucrose	7.663
87	40.337	ribonic acid-γ-lactone	0.070
88	40.983	lactose	0.052
89	41.076	isopropyl β-D-1-thiogalactopyranoside	0.006
90	41.202	cellobiose	0.055
91	41.420	D-(+)-trehalose	0.813
92	41.423	melezitose	0.257
93	41.599	guanosine	0.034
94	41.842	maltose	0.022
95	42.070	sophorose	0.027
96	42.197	palatinitol	0.011
97	42.594	D-lyxosylamine	0.030
98	42.606	arabitol	0.039
99	42.831	turanose	0.047
100	42.928	*O*-acetylsalicylic acid	0.003
101	43.049	nonane	0.008
102	43.122	palatinose	0.066
103	43.127	leucrose	0.041
104	43.282	melibiose	0.412
105	43.289	isomaltose	0.130
106	44.086	galactinol	0.085
107	45.361	phenyl-β-glucopyranoside	0.380
108	46.176	chlorogenic acid	0.028
109	46.696	lactobionic acid	0.035
110	48.753	octadecane	0.007
111	48.764	nonadecane	0.001
112	49.965	raffinose	0.131
113	50.288	DL-3,4-dihydroxyphenylglycol	0.001

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′-ATGCCGAAGCAAACATCAC-3′; iNOS antisense primer, 5′-TAATGTCCAGGAAGTAGGTG-3′; β-actin

dx.doi.org/10.1021/jf304466t | J. Agric. Food Chem. 2012, 60, 12122−12130

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 one 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 one 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).

Western Blot Analysis of Cell Proteins. The HP-treated RAW 264.7 cells were lysed and extracted using RIPA buffer (50 mM Tris Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Kit. Bovine serum albumin (BSA) was used as standard. The cell extracts containing proteins (30 μg) were separated on 10% SDS−polyacrylamide gels and electrophoretically transferred onto nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membrane was incubated with rabbit anti-mouse iNOS polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-mouse β-actin monoclonal antibody (Millipore), followed by HRP-conjugated anti-IgG antibodies. Blots were developed using the ECL detection kit (Pierce, Rockford, IL, USA). The intensity of separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co.). At least three separate replicates were determined for each experiment.

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

Component Analysis of HP Extract. The analysis by GC-MS revealed that HP contained 113 characterized compounds (Table 1). The compounds listed in Table 1 can be subdivided into the following structural categories (number in parentheses: (a) alcohols and derivatives (5); (b) free amino acids and amino acid metabolites (12); (b) monosaccharides, disaccharides, and metabolites (28); (c) organic acids, derivatives, and metabolites (42); and (d) miscellaneous compounds such as acetol, D-sphignosin, 5-aminoimidazole-1-carboxyamie, caffeic acid, chlorogenic acid, neohesperedin, *O*-acetylsalicylic acid, quinic acid, 3,4-dihydroxybenzoic acid, and DL-hydroxyphenylglycol.

The structures of many of the listed compounds, which include rare carboxylic acids, monosaccharides such as D-allose, arabitol, talose, and xylitol, and compounds with multiple OH groups (polyols), appear to be quite unusual. The secondary metabolites listed above in (d) are expected to contribute to the bioactivity of the extract. Because the chemical nature of the identified compounds varied widely, we do not know whether individual or combinations of compounds are responsible for the bioactivity.

Here, we will also briefly mention some reported bioactivities of the rare sugars and other compounds listed in Table 1, which may help to provide a chemical basis for the observed beneficial effects in the present study. D-Allose possesses antimicrobial,[15] antioxidant,[16] and immunostimulant[17] properties. Talose affects the permeability of cell membranes.[18] Replacement of galactose with talose in galectin-1, a protein produced by tumor cells, provides an important approach against cancer.[19] Xylitol-containing chewing gum was effective in controlling caries (oral bacteria) in children.[20] Caffeic, chlorogenic, and quinic acids are known antioxidants.[21,22] 3,4-Dihydroxybenzoic acid inhibited the growth of foodborne bacteria.[23] *N*,*N*-Dimethylglycine causes membrane damage to *Escherichia coli* bacteria.[24] Inositol, widely distributed in vegetables,[25] possesses antimicrobial[26] and insulin-like activities.[27]

This study covers in vivo bioactivity of a water extract, which is not expected to contain the bioactive water-insoluble tricyclic sesquiterpenes such as patchouli alcohol also present in HP.[28] Several studies reported that this compound (a) exhibited anti-inflammatory activities in mice via enhancement of host immune responses[3] and (b) protected against influenza viral infection of the lungs in mice by down-regulating the mRNA expression of inflammatory mediators TNF-α, IL-1β, IL-6iNOS, and COX-2.[4,29] The results of the present study on the in vitro and in vivo antimicrobial effects by compounds present in the water extract show that water-soluble constituents of HP also possess beneficial health-promoting activities.

Effect of HP on in Vitro Growth of *Salmonella*. To find out whether the HP has antibacterial activity against *S.* Typhimurium, bacteria were incubated with serially diluted extracts in PBS (1, 10, and 100 μg/mL) at 37 °C for 0, 2, 4, and 8 h. Figure 1 shows that bacteria treated with the extracts at

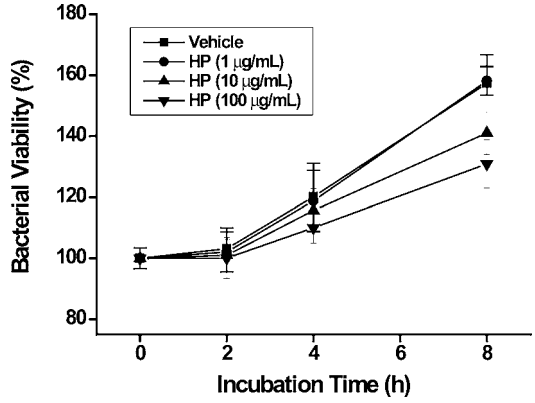

Figure 1. Effects of the HP on growth of *S.* Typhimurium. Serially diluted extracts (1, 10, and 100 μg/mL) were incubated with *S.* Typhimurium (2×10^4 CFU) for 2, 4, and 8 h. Plotted values are the mean ± SD of triplicate experiments.

each concentration for different incubation times showed slight but significant bactericidal activities at 10 or 100 μg/mL doses during the 8 h incubation. Our findings are consistent with the observation that water extracts and the volatile oil of HP inhibited *E. coli*, *Shigella*, and *Staphylococcus* bacteria.[6] We then examined whether HP can kill *Salmonella* in infected mice through activation of innate immune macrophage cells.

Cytotoxicity of HP and Induction of Morphologic Changes in Macrophages. To evaluate possible cytotoxicity, RAW 264.7 macrophage cells (1, 10, and 100 μg/mL) were treated with HP for 48 h. The MTT assay was used to assess the cytotoxicity. Table 2 shows that at concentrations of up to

Table 2. Cell Cytotoxicity of HP in RAW 264.7 Cells

sample, HP extract	cell survival[a] (%)
vehicle (PBS)	100.000 ± 2.437 a
1 μg/mL	97.945 ± 2.968 a
10 μg/mL	98.756 ± 1.765 a
100 μg/mL	97.977 ± 2.589 a
500 μg/mL	90.135 ± 1.609 b
1 mg/mL	85.112 ± 2.931 b

[a]Values, expressed as the mean ± SD of triplicate experiments, with the same letters are not significantly different at $p < 0.05$.

100 μg/mL, the survival of the HP-treated cells was statistically the same as those of (PBS)-treated cells. To determine whether the HP can induce changes in the morphology in the macrophage cells, the cells were cultured in the presence of HP (100 μg/mL), the highest concentration with no cytotoxicity. The cytotoxicity at this concentration is the same as that of the vehicle-treated control.

The microscopic observation of cell morphology revealed that the cells treated with HP changed to dendrite-like cells (Figure 2), reaching up to 2.9-, 5.2-, and 8.9-fold increases

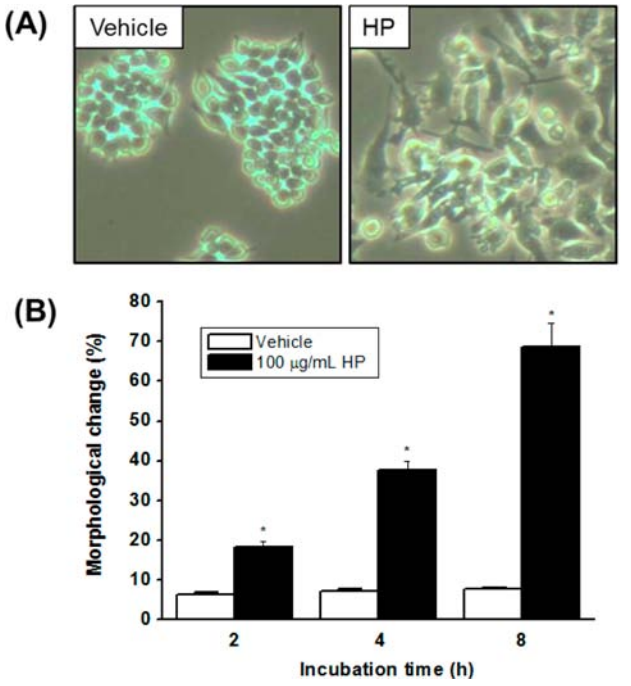

Figure 2. Effects of HP on morphologic changes in macrophages. Murine macrophage cell line RAW 264.7 cells were cultured in the presence of the extract (100 μg/mL) for 2, 4, and 8 h. (A) After 8 h of incubation, the morphologic changes were photographed at 100× magnification. (B) One hundred cells in 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. Asterisks indicate statistical difference at $p < 0.05$, compared to each vehicle (PBS)-treated control.

compared to those of vehicle-treated control. Dendritic morphologic changes were not observed with PBS-treated control cells. These results indicate that HP is not cytotoxic to the macrophage cells, altered the morphology (appearance) of the cells, and activated the macrophages by immunostimulation.[30]

Phagocytotic Stimulatory Effects of HP. To examine whether the HP affect phagocytotic activity, RAW 264.7 cells were cultivated in the presence of three concentrations (1, 10, and 100 μg/mL) for 48 h to stimulate the cells. This was followed by determining the uptake of Dextran-FITC, used as a parameter for phagocytotic activity. Table 3 shows that the HP treatment stimulated phagocytosis in a dose-dependent manner; treatment with 100 μg/mL extract elicited the same level of phagocytotic activity as that of LPS-treated positive control. Similar experiments were performed using *S.* Typhimurium instead of Dextran-FITC. RAW 264.7 cells were cultivated in the presence of three concentrations of HP

Table 3. Effect of HP on Phagocytotic Activity in RAW 264.7 Murine Macrophage Cells

sample, HP extract	phagocytotic activity[a] (%)
negative control (vehicle)	45.302 ± 2.098 cc
positive control (100 ng/mL LPS)	78.632 ± 3.371 a
1 μg/mL	47.304 ± 2.303 c
10 μg/mL	52.973 ± 4.004 b
100 μg/mL	80.752 ± 3.129a

[a]Values, expressed as the mean ± SD of triplicate experiments, with the same letters are not significantly different at $p < 0.05$.

(1, 10, and 100 μg/mL) for 4 h before infection. Thereafter, the cells were incubated for 30 and 60 min before they were lysed and their contents enumerated for bacteria. Figure 3 shows that

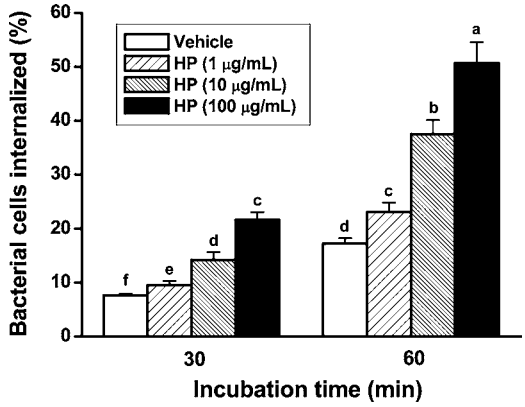

Figure 3. Changes in phagocytotic stimulatory effects of HP on *S.* Typhimurium-infected macrophages. Murine macrophage cell line RAW 264.7 cells (1×10^4) were incubated with three concentrations of the extract (1, 10, and 100 μg/mL) for 4 h and then infected with *S.* Typhimurium (1×10^4 CFU). Incubation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

after 60 min of incubation, the internalization of bacteria into the macrophage cells increased in a dose-dependent manner. The bacterial uptake rates of macrophages treated with 1, 10, and 100 μg/mL HP were about 1.3-, 2.1-, and 2.9-fold greater than that of macrophages treated with the PBS control. These data indicate that the macrophage cells associated with the immune system seem to have a strong affinity for the *Salmonella* bacteria and that the HP treatment induced internalization of the bacteria in the cells.

Effects of HP on Pro-Inflammatory Cytokine Release. To examine whether HP stimulates pro-inflammatory release of cytokine macrophage from RAW 264.7 cells, the cells were treated with 100 μg/mL HP. Table 4 shows that HP-treated macrophage cells released the inflammatory TNF-α, IL-1β, and IL-6 to about 89, 80, and 78% levels, respectively, observed in LPS-treated positive control cells. These in vitro results suggest the reduction (down-regulation) in the release of pro-inflammatory cytokines by HP may contribute to its beneficial effects in vivo.

Related studies found that *S.* Typhimurium caused liver injury in mice through involvement of TNF-α[31] and that inhibition of

Table 4. Effect of HP on the Pro-inflammatory Cytokine Productions (TNF-α, IL-1β, and IL-6) from RAW 264.7 Murine Macrophage Cells

	cytokines[a] (pg/mL)		
sample, HP extract	TNF-α	IL-1β	IL-6
vehicle (PBS)	20.085 ± 1.047 c	8.040 ± 0.413 c	17.740 ± 1.115 c
100 ng/mL LPS	703.781 ± 17.458 a	284.900 ± 15.60 b a	317.891 ± 21.505 a
100 μg/mL HA	625.046 ± 22.604 b	226.784 ± 10.564	247.003 ± 15.601 b

[a]Values, expressed as the mean ± SD of triplicate experiments, in each column with the same letters are not significantly different at $p < 0.05$.

lipid peroxidation by probiotics protected mice against *Salmonella*-induced liver damage.[31,32] We do not know whether HP acts by a similar mechanism.

Effects of HP on Intracellular Bacterial Survival. To examine whether the HP affects *S.* Typhimurium survival within macrophage cells, RAW 264.7 cells were treated with the extract for 4 h, followed by bacterial infection for 1 h. After removal of bacteria from the culture medium, infected macrophages were incubated for another 2, 4, or 8 h in the presence of gentamycin. Figure 4 shows that 2 h after infection, the

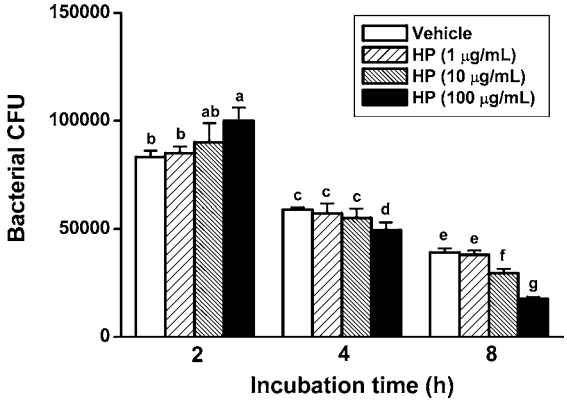

Figure 4. Survival and/or proliferation of *S.* Typhimurium within the HP-treated macrophages. Murine macrophage cell line RAW 264.7 cells (1×10^4) were incubated with three concentrations of the extract (1, 10, and 100 μg/mL) for 4 h and then infected with *S.* Typhimurium (1×10^4 CFU) at 37 °C for 1 h. The cells were washed and then further incubated in the presence of gentamycin (30 μg/mL) for the indicated periods. At each time point, the cells were lysed and the number 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 $p < 0.05$.

intracellular growth of *S.* Typhimurium within the cells treated with 100 μg/mL HP was about 22% greater than in the PBS-treated control. However, 8 h after infection, marked decreases in intracellular bacteria were observed in the cells treated with 100 μg/mL HP. The decrease induced by HP was about 72% greater than that by the PBS-treated control. It seems that HP can induce destruction of *Salmonella* in infected macrophage cells associated with the immune system.

Effects of HP on NO Production. NO is generated by macrophages as part of the immune response. It is both a signaling molecule and is toxic to bacteria. Inducible NO synthase (iNOS) activity is stimulated by lipopolysaccharide from Gram-negative bacteria such as *Salmonella*.[33]

NO production was assessed to determine whether RAW 264.7 cells could be stimulated by HP in a dose-dependent manner (Figure 5). Aliquots of culture supernatant were recovered after 2, 4, and 8 h of incubation with *S.* Typhimurium

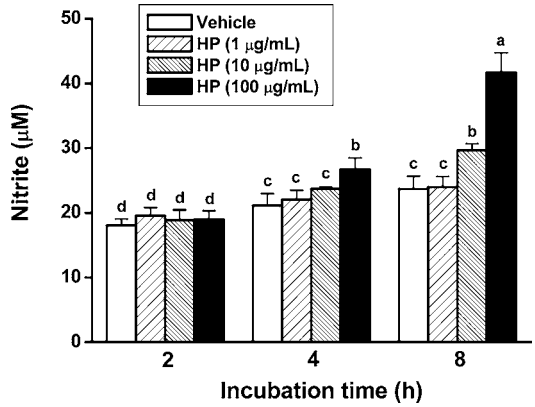

Figure 5. Effects of HP on the production of NO from macrophages. Murine macrophage RAW 264.7 cells (1×10^5) cells were incubated in the presence of three concentrations of the extract (1, 10, and 100 μg/mL) for 4 h. Then, *S.* Typhimurium (1×10^4 CFU) was added to RAW 264.7 cells and incubated for the 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 $p < 0.05$.

followed by measuring the NO concentration. Without infection, HP elicited NO production in the cells at all experimental times up to 8 h (data not shown). Compared to PBS-treated macrophages infected with *S.* Typhimurium, after a 4 h treatment of infected cells with HP (100 μg/mL), NO production increased by 76% at 8 h postinfection.

RT-PCR was performed to examine whether the increase in NO production by HP was mediated through changes in iNOS mRNA expression.[33] Figure 6A shows that PBS-treated macrophages did not express iNOS mRNA. By contrast, iNOS mRNA was strongly expressed in *Salmonella*-infected control cells and in the HP-treated cells. All of the *Salmonella*-infected cells expressed iNOS, but expression in the HP-treated cells was greater. For 4 or 8 h HP-treated cells, expression was about 13 or 14% greater, respectively, than in the cells infected with *Salmonella* without HP. These results show a possible additive effect. Induction of iNOS gene expression by HP was confirmed by the level of protein expression using Western blotting analysis (Figure 6B). The expression profile was similar to that of the mRNA expression. These in vitro data suggest that induction of toxic NO and iNOS by the bacteria may contribute to their death in vivo.

Effects of HP on Histopathology of Mouse Livers. To examine whether HP can ameliorate liver injury induced by salmonellosis, mice were intraperitoneally treated with HP (10 mg/kg) every 24 h for 2 days after intraperitoneal infection of a sublethal dose (1×10^4 CFU) of *S.* Typhimurium. Figure 7 shows that liver necrosis and hemorrhage were present in the tissues from bacteria-infected mice. However, liver tissues from

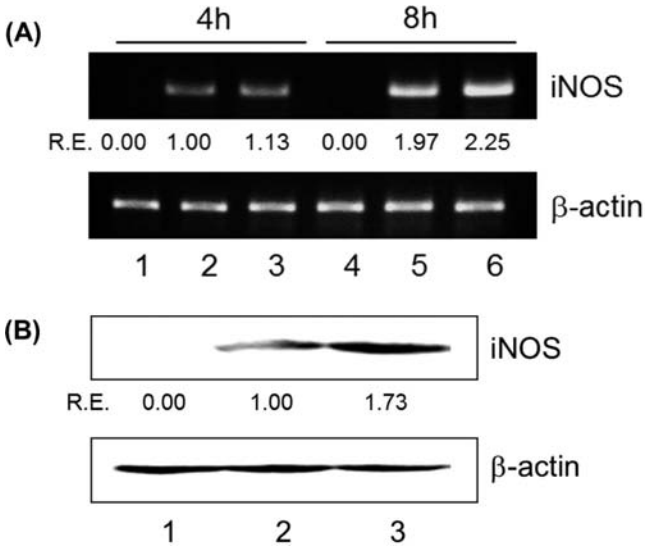

Figure 6. Effects of HP on iNOS gene expression in *S.* Typhimurium-infected macrophages. (A) iNOS mRNA expression profiles assessed by RT-PCR. Murine macrophage cell line RAW 264.7 cells (1×10^6) were cultured with or without the extract (100 μg/mL) for 4 or 8 h. Then, *S.* Typhimurium (1×10^4 CFU) was added to RAW 264.7 cells for 8 h. After incubation, total RNA was purified and iNOS mRNA expression was determined using RT-PCR analysis. Lanes: 1 and 4, PBS-treated without bacterial infection; 2 and 5, bacterial infection alone; 3 and 6, HP-treated with bacterial infection. (B) iNOS polypeptide expression profiles assessed by Western blot. RAW 264.7 cells (1×10^6) treated with the extract (100 μg/mL) for 24 h and subsequent infection of *S.* Typhimurium were lysed, and iNOS polypeptide in the cell supernatant was identified by Western blot using anti-mouse iNOS polyclonal antibody. Lanes: 1, PBS-treated without bacterial infection; 2, bacterial infection alone; 3, HP-treated with bacterial infection. The relative proportions of iNOS mRNA and polypeptide are expressed as the R.E. (relative expression) values calculated from iNOS gene/β-actin gene expression. Figures represent results from at least three individual experiments.

bacteria-infected mice treated with HP showed minimal liver damage. These results show that the HP protected the mouse livers against *Salmonella*-induced hepatic necrosis, a known biomarker of *Salmonella* toxicity.

Effects of HP on Mortality of Infected Mice. To determine the therapeutic effects of HP on life expectancy, mice were infected with a lethal dose (1×10^5 CFU) of *S.* Typhimurium intraperitoneally and assessed for mortality. Each group of 10 mice was treated with HP (10 mg/kg) every 24 h during the entire experimental period. Figure 8 shows that the

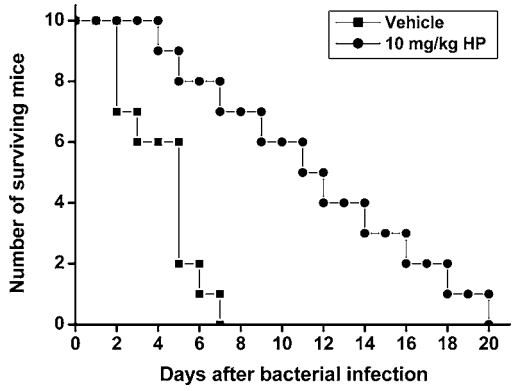

Figure 8. Histogram showing the effect of HP on *S.* Typhimurium infection-induced lethality. Balb/c mice (10 mice per group) were infected with a lethal dose of *S.* Typhimurium (1×10^5 CFU) through intraperitoneal route. Then, the extract (10 mg/kg) was intraperitoneally injected every 24 h during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

mortality rate in the PBS-treated control group was 100% on day 7. By contrast, the groups treated with HP survived until day 20. These observations strikingly demonstrate the potential of HP to protect mice against the lethal effects of the bacteria, presumably via stimulation of the immune system.

In summary, we used a series of in vitro and in vivo bacterial and other bioassays to demonstrate the protective effect of a water extract of the medicinal herb Herba Pogostemonis containing 113 characterized compounds against adverse effects of the virulent foodborne pathogen *Salmonella* Typhimurium in macrophage cells and against liver necrosis and mortality in infected mice. Liver morphology and life expectancy were improved in *Salmonella*-infected mice by peritoneal administration of the extract. The beneficial effects were accompanied by changes in several biomarkers in cultured murine macrophage cells associated with the immune system. Because the bioactive extracts protected mice administered a lethal dose of

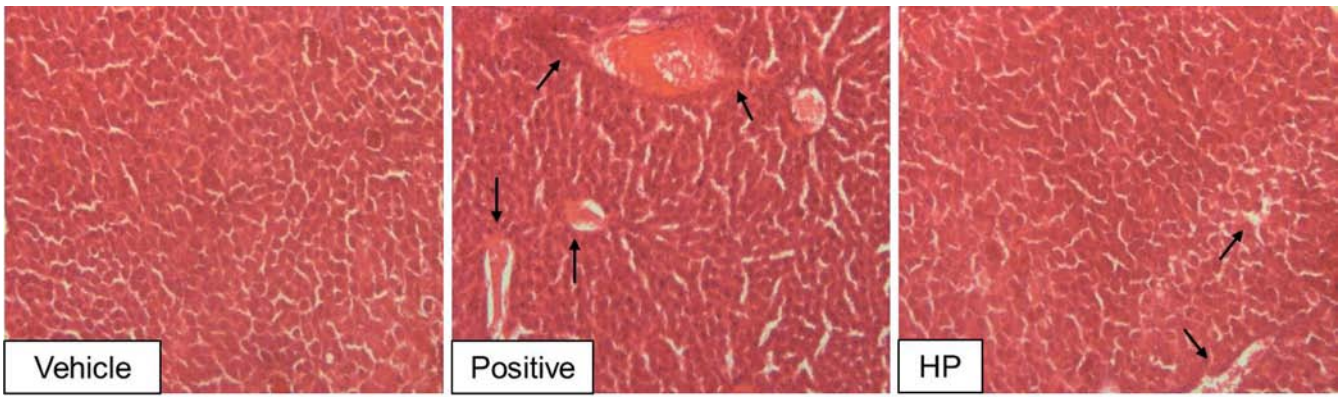

Figure 7. Effect of HP on the formation of pathological lesions in liver of *S.* Typhimurium-infected mice. Liver specimens from extract-treated mice (10 mg/kg) infected with *S.* Typhimurium (1×10^4 CFU) were fixed with 4% paraformaldehyde. The sections were then stained with hematoxylin and eosin (H&E).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

the bacteria against liver damage and mortality, it seems that the protective effect is associated with a potentiation of the immune system. These beneficial results suggest that the bioactive extract merits further study for its potential to improve microbial food safety and to protect animals and humans against adverse effects associated with infectious salmonellosis.

AUTHOR INFORMATION

Corresponding Author

*(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

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Carol E. Levin for assistance with the preparation of the manuscript and Han Soon Kwak of the Molecular and Aquatic Ecology Laboratory of Ajou University for assisting with GC-MS analysis.

REFERENCES

(1) 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.

(2) 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.

(3) Li, Y.-C.; Xian, Y.-F.; Ip, S.-P.; Su, Z.-R.; Su, J.-Y.; He, J.-J.; Xie, Q.-F.; Lai, X.-P.; Lin, Z.-X. Anti-inflammatory activity of patchouli alcohol isolated from Pogostemonis Herba in animal models. *Fitoterapia* **2011**, *82*, 1295−1301.

(4) Li, Y.-C.; Peng, S.-Z.; Chen, H.-M.; Zhang, F.-X.; Xu, P.-P.; Xie, J.-H.; He, J.-J.; Chen, J.-N.; Lai, X.-P.; Su, Z.-R. Oral administration of patchouli alcohol isolated from Pogostemonis Herba augments protection against influenza viral infection in mice. *Int. Immunopharmacol.* **2012**, *12*, 294−301.

(5) Chen, X.; He, B.; Li, X.; Luo, J. Effects of Herba Pogostemonis on gastrointestinal tract. *Zhongyaocai* **1998**, *21*, 462−466.

(6) Liu, H.; Luo, J.; Lai, P. Studies on the anti-enteropathogenic bacteria action of Herba Pogostemonis extracts. *Zhongyaocai* **1999**, *22*, 408−411.

(7) 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.

(8) UC Davis Genome Center. FiehnLib − a mass spectral and retention index library for comprehensive metabolic profiling; http://fiehnlab.ucdavis.edu/projects/FiehnLib (accessed Oct 10, 2012).

(9) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.

(10) Duperrier, K.; Eljaafari, A.; Dezutter-Dambuyant, C.; Bardin, C.; Jacquet, C.; Yoneda, K.; Schmitt, D.; Gebuhrer, L.; Rigal, D. Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplements. *J. Immunol. Methods* **2000**, *238*, 119−131.

(11) Lu, S.; Manges, A. R.; Xu, Y.; Fang, F. C.; Riley, L. W. Analysis of virulence of clinical isolates of *Salmonella enteritidis* in vivo and in vitro. *Infect. Immun.* **1999**, *67*, 5651−5657.

(12) Murakawa, M.; Yamaoka, K.; Tanaka, Y.; Fukuda, Y. Involvement of tumor necrosis factor (TNF)-α in phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem. Pharmacol.* **2006**, *71*, 1331−1336.

(13) Kim, G. S.; Kim, D. H.; Lim, J. J.; Lee, J. J.; Han, D. Y.; Lee, W. M.; Jung, W. C.; Min, W. G.; Won, C. G.; Rhee, M. H.; Lee, H. J.; Kim, S. Biological and antibacterial activities of the natural herb *Houttuynia cordata* water extract against the intracellular bacterial pathogen *Salmonella* within the RAW 264.7 macrophage. *Biol. Pharm. Bull.* **2008**, *31*, 2012−2017.

(14) Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **1987**, *162*, 156−159.

(15) Al-Baarri, A. N.; Hayashi, M.; Ogawa, M.; Hayakawa, S. Effects of mono- and disaccharides on the antimicrobial activity of bovine lactoperoxidase system. *J. Food Prot.* **2011**, *74*, 134−139.

(16) Ishihara, Y.; Katayama, K.; Sakabe, M.; Kitamura, M.; Aizawa, M.; Takara, M.; Itoh, K. Antioxidant properties of rare sugar D-allose: effects on mitochondrial reactive oxygen species production in Neuro2A cells. *J. Biosci. Bioeng.* **2011**, *112*, 638−642.

(17) Tanaka, S.; Sakamoto, H. Effects of D-allose on the endocytic activity of dendritic cells and the subsequent stimulation of T cells. *Cell. Immunol.* **2011**, *271*, 141−146.

(18) Denda, M. Effects of topical application of aqueous solutions of hexoses on epidermal permeability barrier recovery rate after barrier disruption. *Exp. Dermatol.* **2011**, *20*, 943−944.

(19) Collins, P. M.; Öberg, C. T.; Leffler, H.; Nilsson, U. J.; Blanchard, H. Taloside inhibitors of galectin-1 and galectin-3. *Chem. Biol. Drug Des.* **2012**, *79*, 339−346.

(20) Campus, G.; Cagetti, M. G.; Sale, S.; Petruzzi, M.; Solinas, G.; Strohmenger, L.; Lingström, P. Six months of high-dose xylitol in high-risk caries subjects − a 2-year randomised, clinical trial. *Clin. Oral Invest.* **2012**, published online July 13, DOI: 10.1007/s00784-00012-00774-00785.

(21) Li, X.; Zhang, J.-Y.; Gao, W.-Y.; Wang, Y.; Wang, H.-Y.; Cao, J.-G.; Huang, L.-Q. Chemical composition and anti-inflammatory and antioxidant activities of eight pear cultivars. *J. Agric. Food Chem.* **2012**, *60*, 8738−8744.

(22) Jurgonski, A.; Juskiewicz, J.; Zdunczyk, Z.; Król, B. Caffeoylquinic acid-rich extract from chicory seeds improves glycemia, atherogenic index, and antioxidant status in rats. *Nutrition* **2012**, *28*, 300−306.

(23) Friedman, M.; Henika, P. R.; Mandrell, R. E. Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* **2003**, *66*, 1811−1821.

(24) Vanhauteghem, D.; Janssens, G.; Lauwaerts, A.; Sys, S.; Boyen, F.; Kalmar, I.; Meyer, E. Glycine and its *N*-methylated analogues cause pH-dependent membrane damage to enterotoxigenic *Escherichia coli*. *Amino Acids* **2012**, *43*, 245−253.

(25) Hernández-Hernández, O.; Ruiz-Aceituno, L.; Sanz, M. L.; Martinez-Castro, I. Determination of free inositols and other low molecular weight carbohydrates in vegetables. *J. Agric. Food Chem.* **2011**, *59*, 2451−2455.

(26) Fortuna, A. M.; Juárez, Z. N.; Bach, H.; Nematallah, A.; Av-Gay, Y.; Sánchez-Arreola, E.; Catalán, C. A.; Turbay, S.; Hernández, L. R. Antimicrobial activities of sesquiterpene lactones and inositol derivatives from *Hymenoxys robusta*. *Phytochemistry* **2011**, *72*, 2413−2418.

(27) Yao, Y.; Shan, F.; Bian, J.; Chen, F.; Wang, M.; Ren, G. D-Chiro-inositol-enriched tartary buckwheat bran extract lowers the blood glucose level in KK-Ay mice. *J. Agric. Food Chem.* **2008**, *56*, 10027−10031.

(28) Zhao, Z.; Lu, J.; Leung, K.; Chan, C. L.; Jiang, Z.-H. Determination of patchoulic alcohol in Herba Pogostemonis by GC-MS-MS. *Chem. Pharm. Bull.* **2005**, *53*, 856−860.

(29) Xian, Y.-F.; Li, Y.-C.; Ip, S.-P.; Lin, Z.-X.; Lai, X.-P.; Su, Z.-R. Anti-inflammatory effect of patchouli alcohol isolated from Pogostemonis Herba in LPS-stimulated RAW264.7 macrophages. *Exp. Ther. Med.* **2011**, *2*, 545−550.

(30) Berghaus, L. J.; Moore, J. N.; Hurley, D. J.; Vandenplas, M. L.; Fortes, B. P.; Wolfert, M. A.; Boons, G.-J. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to

ligands of Toll-like receptors 2, 3, and 4. *Comp. Immun. Microbiol. Infect. Dis.* **2010**, *33*, 443−454.
(31) Lincopan, N.; Mamizuka, E. M.; Carmona-Ribeiro, A. M. Low nephrotoxicity of an effective amphotericin B formulation with cationic bilayer fragments. *J. Antimicrob. Chemother.* **2005**, *55*, 727−734.
(32) Rishi, P.; Kaur, P.; Virdi, J. S.; Shukla, G.; Koul, A. Amelioratory effects of zinc supplementation on *Salmonella*-induced hepatic damage in the murine model. *Dig. Dis. Sci.* **2008**, *53*, 1063−1070.
(33) Chakravortty, D.; Hensel, M. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect.* **2003**, *5*, 621−627.