Biological and Antibacterial Activities of the Natural Herb *Houttuynia cordata* Water Extract against the Intracellular Bacterial Pathogen *Salmonella* within the RAW 264.7 Macrophage

Gon Sup Kim,[a,#] Dong Hyeok Kim,[a,#] Jeong Ju Lim,[a] Jin Ju Lee,[a] Dae Yong Han,[a] Whi Min Lee,[b] Won Chul Jung,[a] Won Gi Min,[a] Chung Gil Won,[a] Man Hee Rhee,[b] Hu Jang Lee,[a] and Suk Kim*,[a,c]

[a] Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University; [c] Institute of Agriculture and Life Science, Gyeongsang National University; 900 Gazwa, Jinju 660–701, Republic of Korea: and [b] Department of Veterinary Medicine, College of Veterinary Medicine, Kyungpook National University; Daegu 702–701, Republic of Korea.

Salmonellosis is a major bacterial zoonosis that causes a variety of disease syndromes, from self-limiting enteritis to fatal infection in animals and food-borne infection and typhoid fever in humans. Recently, the emergence of multidrug-resistant strains of *Salmonella* sp. has caused more serious problems in public health. The present study investigated the antibacterial effects of *Houttuynia cordata* water extract (HCWE) against murine salmonellosis. In RAW 264.7 cells, there was no detectable cytotoxic effect of HCWE at any concentration between 25 and 100 μg/ml after 8-h incubation. The antibacterial activity of HCWE was then examined in a *Salmonella enterica* serovar (*Salmonella typhimurium*), and was found to increase in a dose-dependent manner at concentrations from 25 to 100 μg/ml during 8-h incubation. HCWE also affected RAW 264.7 cells including morphologic change and bacterial uptake, but there was no significant difference in bacterial replication in RAW 264.7 cells. With HCWE alone, nitric oxide (NO) production by RAW 264.7 cells did not increase, but when RAW 264.7 cells were infected by *S. typhimurium*, with or without HCWE, NO production with HCWE was 2-fold higher than that without HCWE. Treatment with HCWE did not affect inducible NO synthase (iNOS) mRNA expression by RAW 264.7 cells, but when RAW 264.7 cells with HCWE were infected by *S. typhimurium*, iNOS mRNA expression was increased during 8-h incubation. Furthermore, HCWE showed virulence reduction effects in *S. typhimurium*-infected BALB/c mice. After a lethal dose of *S. typhimurium*, the mortality rate in the HCWE untreated group was 100% at 7 d, but the HCWE 25, 50, and 100 μg/ml groups survived until 11, 17, and 23 d, respectively. These data suggest that HCWE is stable and beneficial in the treatment of bacterial infection including intracellularly replicating pathogens and may solve antimicrobial misuse and overuse.

Key words	salmonellosis; *Houttuynia cordata*; antibacterial activity; macrophage; *Salmonella typhimurium*; nitric oxide

Houttuynia cordata has a long history of use in indigenous systems of medicine as an antiseptic, febrifuge, diuretic, and deobstruant. It is frequently used in many traditional medicines for its antimicrobial, antiviral, and antiinflammatory properties.[1—4] *H. cordata* contains of methyl nonyl ketone, caryophyllene, bornyl acetate, α-pinene, β-pinene, limonene, and other components. These components have antibacterial properties against the Gram-positive bacteria *Staphylococcus aureus* and *Sarcina ureae*.[5] In experimental studies, *H. cordata* exhibited antibacterial activity, antileukemic activity, antiinflammatory activity, anaphylactic reaction and mast cell activation, and modulated nitric oxide (NO) production.[6—10] However, to date, studies to understand the other antibacterial effects, including the intracellularly replicating pathogens of these plants, are lacking.

The mammalian immune response is divided broadly into the innate and adaptive responses. Innate immune responses of mammals involve the first line of defense against invading microorganisms, including the release of antimicrobial peptides at epithelial surfaces, phagocytosis, and intracellular killing of microorganisms by phagocytes and the activation of the complement cascade. In contrast, the adaptive immune response is antigen specific and modified by previous antigen exposure.[11] In the innate immune response, the reactive oxygen intermediates (ROIs) such as superoxide and reactive nitrogen intermediates (RNIs) such as NO have a key role in intracellular killing of microorganisms within phagocytes.[12] Several studies reported that RNIs are important for control-

ling *Salmonella* multiplication within macrophages.[13,14]

Salmonellosis is a major bacterial zoonosis that causes a variety of disease syndromes, from self-limiting enteritis to fatal infection in animals and food-borne infection and typhoid fever in humans.[15] The etiologic agents of salmonellosis are *Salmonella* sp. characterized by motile, Gram-negative, rod-shaped bacteria and facultative intracellular pathogens that can multiply within professional and nonprofessional phagocytes. Recently, the emergence of multidrug-resistant strains of *Salmonella* sp. has caused more serious problems in public health.[16] Multidrug resistance is a worldwide problem that does not recognize international borders and can indiscriminately affect the treatment and prevention of various diseases.

To kill intracellular bacteria including *Salmonella* sp., *Brucella* sp., *Listeria* sp., *Shigella* sp., *etc.*, much effort is needed to develop antibiotics, determine treatment duration, and resolve other issues.

In this study, we examined the antibacterial effects, and macrophage activation of *H. cordata* water extract (HCWE) such as morphologic changes, NO production, phagocytic activity, and effects against the *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo*.

MATERIALS AND METHODS

HCWE Preparation	*H. cordata* powder was obtained from the Korea National Animal Bio Resource Bank

(Gyeongnam, Korea). Five grams of powder was extracted with 100 ml of distilled water by stirring at room temperature for 8 h. The supernatant from the plant extraction was centrifuged at 5000 rpm for 10 min, then filtered through Advantec filter paper number 2 (Japan). The filtrates were evaporated to dryness at 54 °C. The residue was dissolved in distilled water, adjusted to 5 mg/ml final concentration, and sterilized by passage through a Corning syringe filter (0.20 μm, Japan).[9]

Bacterial Culture and Media *S. enterica* serovar Typhimurium (*Salmonella typhimurium*) ATCC 14028 cells were maintained as frozen glycerol stock and cultured in Luria-Bertani (LB) broth or LB broth containing 1.5% agar. Bacteria were grown at 37 °C with vigorous shaking to a stationary phase in LB broth.

Determination of Antibacterial Activity Bacteria were diluted with phosphate-buffered saline (PBS) solution, pH 7.4, to 2×10^4/ml, added to different concentrations (0, 25, 50, 100 μg/ml) of HCWE, and incubated at 37 °C for 0, 2, 4, and 8 h. After incubation and proper dilution, 100 μl of each solution was plated onto LB agar to assess bacterial colony-forming units (CFUs).

Cell Culture The murine macrophage cell line RAW 264.7 cells were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine 2 mM, penicillin 100 U/ml, and streptomycin 100 μg/ml (Invitrogen). RAW 264.7 cells were seeded (1×10^4 per well) in 96-well cell culture plates 1 d before infection for all assays and incubated at 37 °C in 5% CO_2 atmosphere. RAW 264.7 cells were incubated with HCWE (0, 25, 50, 100 μg/ml) for 4 h before infection with *S. typhimurium*.

Morphologic Changes in Macrophages RAW 264.7 cells were grown in the presence of 100 μg/ml of HCWE in 24-well cell culture plates with coverslips for 0, 2, 4, and 8 h. The coverslips were examined with a microscope ($\times$200) to assay morphologic changes in macrophages. One hundred RAW 264.7 cells were selected randomly, and the morphologically changed cells were counted. This assay was performed at least three times.

Nitrite Assay For the nitrite assay, RAW 264.7 cells were cultured in 12-well cell culture plates (1×10^5 cells/well) and incubated in the presence of different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 2, 4, and 8 h. After incubation, 100 μl of cell-free supernatant was collected, and the nitrite concentration was determined based on the Griess reaction. Briefly, 100 μl of cell-free supernatant was transferred to a 96-well plate and incubated with equal volumes of Griess reagent (Sigma) in the dark for 10 min, and absorbance (450 nm) was determined in a Biotrak II Reader. The nitrite concentration was determined using sodium nitrite as a standard.

Cytotoxic Assay To determine whether HCWE exhibited cytotoxicity, RAW 264.7 cells were grown in the presence of different concentrations of HCWE (0, 25, 50, 100 μg/ml) in 96-well cell culture plates for 8 h. Cell viability was examined in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cleavage assay.[6] In brief, cells were incubated MTT with solution (5 mg/ml in PBS) and incubated at 37 °C in 5% CO_2 for 4 h. After incubation, the plates were centrifuged at $450 \times \boldsymbol{g}$ and super-

natants were removed. Acid/isopropanol was added to the wells and mixed to dissolve completely the crystalline material. The absorbance at 540 nm was measured using a spectrophotometer.

Determination of Bacterial Uptake and Intracellular Growth To determine internalization and intracellular growth of bacteria, macrophages were infected with *S. typhimurium* as described previously.[17,18] For analysis of bacterial uptake efficiency, cells were incubated with 10 μl of *S. typhimurium* (1×10^4) for 30 and 60 min at 37 °C in a 5% CO_2 atmosphere. Cells were washed once with medium after 30 and 60 min of incubation at 37 °C and then incubated with Dulbecco's modified Eagle's medium containing 10% FBS with genatamicin (30 μg/ml) for 30 min to kill extracellular bacteria. For viable cell counting at different time points (30, 60 min), the infected macrophage monolayers were washed 3 times with PBS and then lysed with distilled water. Aliquots of lysates were plated onto LB agar to assess bacterial CFUs. For intracellular growth efficiency, bacteria-infected macrophages were incubated at 37 °C for 1 h, washed once with medium, incubated with Dulbecco's modified Eagle's medium containing 10% FBS with gentamicin (30 μg/ml), and then incubated for 2, 4, and 8 h. Cell washing, lysis, and plating procedures were the same as for the analysis of the efficiency of bacterial uptake. All assays were conducted in triplicate and repeated at least thee times on different days. The results are presented as the mean$\pm$standard deviation (S.D.).

RNA Preparation and iNOS mRNA Analysis Using RT-PCR RAW 264.7 cells were cultured in 12-well cell culture plates (1×10^5 cells/well) and incubated with or without HCWE (100 μg/ml) for 4 h before bacterial infection. Bacterial infection and gentamicin treatment were performed as described above. After 4 and 8 h of incubation, total RNA was isolated using an RNA Purification Kit (Qiagen). Purified RNA samples were stored at -75 °C until use. RNA was quantified by absorption at 260 nm using an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech). RT-PCR was carried out using a Superscript II kit (Invitrogen). Expression of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. The RT reactions were performed using primers for iNOS, 5′-GCC TTC AAC ACC AAG GTT GTC TGC A-3′ (sense) and 5′-TCA TTG TAC TCT GAG GGC TGA CAC A-3′ (anti-sense), and for GAPDH, 5′-CAA TGC CAA GTA TGA TGA CAT-3′ (sense) and 5′-CCT GTT ATT ATG GGG GTC TG-3′ (anti-sense), respectively.[19] Prior to reverse transcription, total RNA was treated with DNase (Takara) to eliminate residual DNA contamination. Total RNA was quantified, and RT reactions were carried out for equal amounts of RNA (5 μg) from each sample. For preparation of the cDNA, each RNA was incubated at 45 °C for 60 min using a first-strand cDNA synthesis kit (Invitrogen). The cDNAs were denatured at 94 °C for 5 min and then they were amplified by 27 cycles of denaturation at 94 °C for 2.5 min, annealing at 59 °C for 2 min, extension at 72 °C for 2 min for iNOS, and denaturation at 94 °C for 2.5 min, annealing at 49 °C for 2 min, extension at 72 °C for 2 min for GAPDH.[19]

HCWE Treatment for Murine Salmonellosis Pathogen-free female BALB/c mice aged 6—8 weeks, weighing 25$\pm$3 g each, were used in this study. All mice were kept at 23$\pm$1 °C

with a 12-h light/dark cycle. They had free access to water and diet and were acclimatized for at least 2 weeks before starting the experiments. Four groups of 10 mice each were used for bacterial infection. Mice were infected intraperitoneally with 2×10^4 CFUs of *S. typhimurium*, which is a lethal dose for mice *via* the intraperitoneal route. After bacterial infection, four groups of mice were orally treated with 0.1 ml of sterile PBS with/without HCWE (25, 50, 100 mg/ml) every 24 h during all experiments. Infected mice were examined for the viability every 24 h. All procedures described were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University.

Statistical Analysis　The results obtained are expressed as mean±S.D. for the number of experiments. Student's *t*-test was used to make statistical comparisons between groups. Results of $p < 0.05$ were considered to represent a statistically significant difference.

RESULTS

Antibacterial Activity of HCWE　To determine the antibacterial effects of HCWE against *S. typhimurium*, bacteria were incubated with different concentrations (0, 25, 50, 100 μg/ml) of HCWE in PBS at 37 °C for 0, 2, 4, and 8 h. Bacteria incubated with all concentrations of HCWE showed slightly increased antibacterial effects in a dose-dependent manner up to 8 h. In contrast, bacteria without HCWE showed slightly proliferating patterns up to 8 h (Fig. 1).

Cytotoxic Effects and Morphologic Changes in Macrophages　To evaluate the cytotoxic effects of HCWE, RAW 264.7 cells were grown in the presence of different concentrations of HCWE (0, 25, 50, 100 μg/ml) in culture plates for 8 h. The MTT assay was used to assess the cytotoxicity of HCWE in RAW 264.7 cells. There were no detectable cytotoxic effects of HCWE at any concentration between 0 and 100 μg/ml. The survival rates were 99±1% in the 25 μg/ml, 98±2% in the 50 μg/ml, and 98±1% in the 100 μg/ml of HCWE-treated macrophages compared with untreated macrophages, which was set at 100%. To determine whether HCWE affects morphologic changes in macrophages, RAW 264.7 cells were grown in the presence of HCWE (100 μg/ml) and incubated for 2, 4, and 8 h. Microscopic observations of cell morphology showed that all the cells cultured with HCWE showed dendritic changes compared with untreated cells (Fig. 2A). As shown in Fig. 2B, HCWE affected morphologic changes in RAW 264.7 cells time dependently, and the morphologically changed cells reached up to 50% after 8-h incubation.

Phagocytic Stimulation Effects of HCWE　To determine whether HCWE affects phagocytic activity, RAW 264.7 cells were incubated with different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 4 h before bacterial infection and then infected with *S. typhimurium* as described in Materials and Methods. The results showed that bacterial internalization was increased in HCWE-treated macrophages in a dose-dependent manner, and the bacterial uptake rate of HCWE-treated macrophages was 2- to 2.5-fold higher than that in untreated macrophages after 60-min incubation (Fig. 3).

Effects of HCWE on Bacterial Survival within Macrophages　To investigate whether HCWE affects *S. typhimurium* survival within macrophages, RAW 264.7 cells
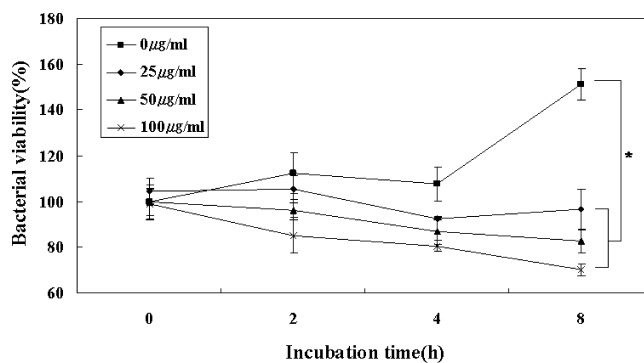

Fig. 1.　Antibacterial Activity of HCWE against *S. typhimurium*

　Various concentrations of HCWE diluted with PBS were incubated with *S. typhimurium* for 2, 4, and 8 h. Bacterial viability was measured based on CFUs on culture plates, and the rate of bacterial viability was compared with HCWE-untreated PBS as a control. The data represent the mean±S.D. of triplicate experiments. Statistically significant differences between bacterial viability of HCWE-treated PBS and that of HCWE-untreated PBS are indicated by an asterisk ($* p < 0.01$).
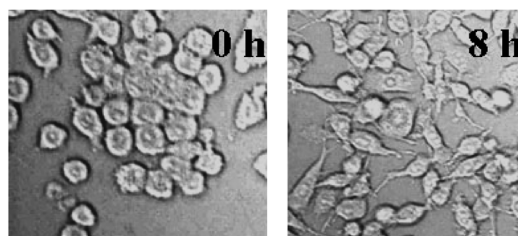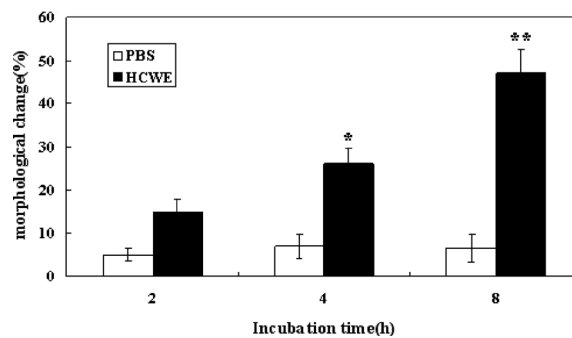

Fig. 2.　Effects of HCWE on the Morphologic Changes in Macrophages

　Murine macrophage cell line RAW 264.7 cells were cultured in the presence of 100 μg/ml of HCWE for 0, 2, 4, and 8 h. (A) After 0 and 8 h of incubation, the morphologic changes were photographed at ×200 magnification. (B) One hundred RAW 264.7 cells were examined per coverslip, and the rates of morphologically changed cells such as dendritic forms were measured. Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences between morphologically changed cell number of HCWE-untreated macrophages and that of HCWE-treated macrophages are indicated by asterisks ($* p < 0.01$; $** p < 0.001$).

were treated as for the phagocytic activity assay and incubated for 2, 4, and 8 h after bacterial infection. The results showed that the intracellular replication of *S. typhimurium* within all RAW 264.7 cells showed a similar pattern, but was slightly decreased in HCWE-treated RAW 264.7 cells in a dose-dependent manner at 8 h (Fig. 4).

Nitrite Assay and iNOS mRNA Expression　We determined whether RAW 264.7 cells could be stimulated by
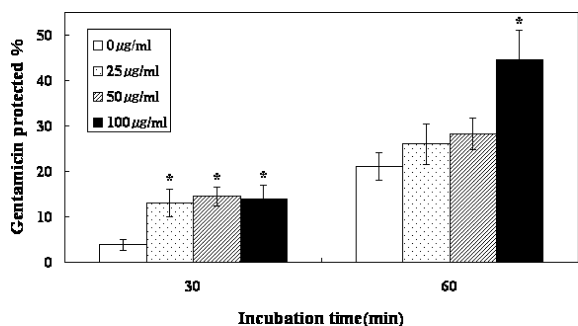
Fig. 3.   Phagocytic Stimulatory Effects of HCWE on Bacterially Infected Macrophages

Murine macrophage cell line RAW 264.7 cells were incubated with different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 4 h before bacterial infection, and then *S. typhimurium* were deposited onto RAW 264.7 cells and incubated at 37 °C for the indicated periods. Bacterial internalization efficiency by macrophages was determined by evaluating the protection of internalized bacterial from gentamicin killing and quantitated as described previously (see Materials and Methods). Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences between bacterial internalization of HCWE-untreated macrophages and that of HCWE-treated macrophages are indicated by asterisks (∗ *p* < 0.01).
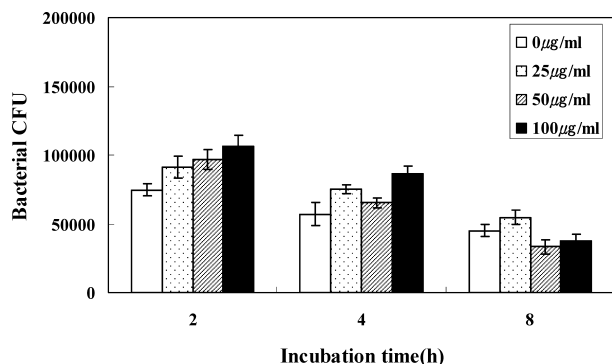

Fig. 4.   Intracellular Replication of *S. typhimurium* within HCWE-Treated Macrophages

Murine macrophage cell line RAW 264.7 cells were incubated with different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 4 h before bacterial infection, and then *S. typhimurium* were deposited onto RAW 264.7 cells and incubated at 37 °C for the indicated periods. At different times of incubation, the cells were lysed, and the numbers of viable intracellular bacteria were determined. Datum points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least three times) and their standard deviations.

HCWE, either alone or in combination with *S. typhimurium*, to alter NO production. RAW 264.7 cells were incubated with different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 8 h. With HCWE alone, there was no significant change in NO production at all experimental times up to 8 h (data not shown). Furthermore, to determine whether HCWE affects NO production from bacteria-infected macrophages, RAW 264.7 cells were incubated with different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 4 h before bacterial infection, and then *S. typhimurium* was infected into RAW 264.7 cells as described in Materials and Methods. After bacterial infection, aliquots of medium were collected after 2, 4, and 8 h of incubation and the NO concentration was measured. The NO production by HCWE-treated RAW 264.7 cells was similar to or a slightly increased compared with that by HCWE untreated cells after 4-h incubation. NO production from HCWE 100 μg/ml-treated macrophages was 2-fold higher than that by HCWE-untreated macrophages at 8 h of incubation (Fig. 5). Infection with *Salmonella* sp. induces
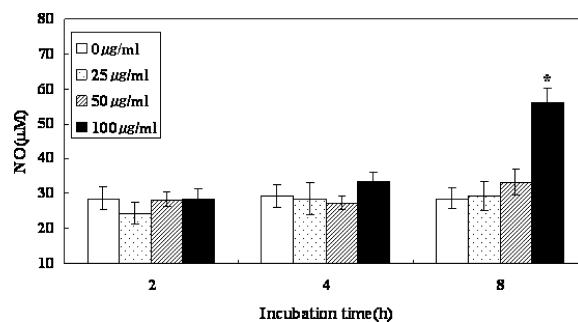

Fig. 5.   Effects of HCWE on the Production of Nitric Oxide (NO) from Macrophages

RAW 264.7 cells were preincubated in the presence of different concentrations of HCWE (0, 25, 50, 100 μg/ml) for 4 h. After incubation, the medium was changed, and *S. typhimurium* (MOI 20) was added to RAW 264.7 cells. After the indicated times, cell-free supernatant was collected and nitrite concentration was determined. Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences between NO production by HCWE-untreated macrophages with bacterial infection and that of HCWE-treated macrophages are indicated by an asterisk (∗ *p* < 0.01).
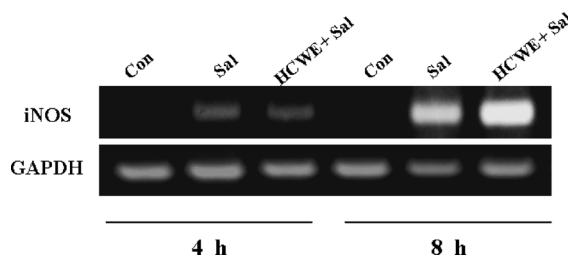

Fig. 6.   Effects of HCWE on iNOS mRNA Expression in *S. typhimurium*-Infected RAW 264.7 Macrophages

RAW 264.7 cells were cultured in 12-well cell culture plate (1×10^5 cells/well) and incubated with (HCWE+Sal) or without (Sal) HCWE (100 μg/ml) for 1 h before bacterial infection. After bacterial infection and incubation for 4 and 8 h, total RNA was purified and iNOS mRNA expression was determined using RT-PCR analysis. Untreated RAW 264.7 cells served as negative controls (Con) without HCWE or bacterial infection.

a strong inflammatory response and leads to the induction of iNOS from macrophages.[20] RT-PCR was performed to examine whether the increase in NO production by HCWE was mediated through changes in iNOS mRNA expression. In the RAW 264.7 cells without *S. typhimurium* infection and HCWE treatment, iNOS mRNA was not expressed during any experiment. However, in the *S. typhimurium*-infected RAW 264.7 cells without HCWE treatment, iNOS mRNA was expressed in a time-dependent manner up to 8 h. The iNOS mRNA expression of *S. typhimurium*-infected RAW 264.7 cells with HCWE treatment showed a similar pattern at 4 h and was greater than that by *S. typhimurium*-infected RAW 264.7 cells without HCWE at 8 h (Fig. 6).

Virulence Reduction Effects of HCWE against Murine Salmonellosis   To determine the therapeutic effects of HCWE against murine salmonellosis, we experimentally infected mice with approximately 2×10^4 of *S. typhimurium* cells intraperitoneally and the mortality was scored. Each group of 10 mice was orally treated with 0.1 ml of sterile PBS with/without HCWE (25, 50, 100 μg/ml) every 24 h during all experiments. The mortality rate in the untreated group was 100% at 7 d. However, the groups treated with 25, 50, and 100 μg/ml of HCWE survived until 11, 17, and 23 d, respectively (Fig. 7).
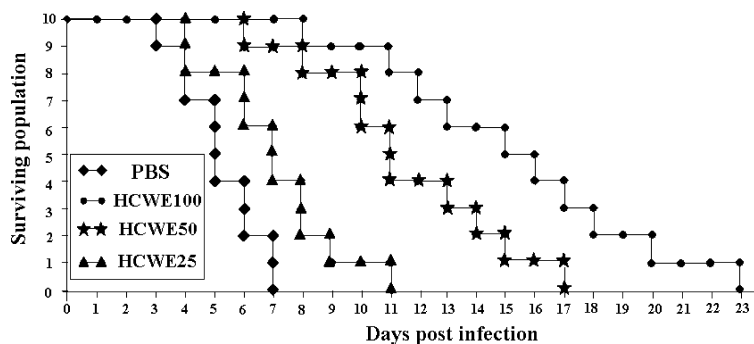
Fig. 7. Mortality Rate of HCWE-Treated Mice with Bacterial Infection

Mice ($n=10$ per group) treated or untreated with HCWE were infected with *S. typhimurium*. Each group was orally treated with 0.1 ml of sterile PBS with/without HCWE (0, 25, 50, 100 µg/ml) every 24 h. Mortality was observed daily for 23 d post-infection.

DISCUSSION AND CONCLUSION

Medicinal plants have been used to treat a variety of disorders including inflammatory conditions, infections with microorganisms, cancer, allergy, and other diseases. Recently, the consumption of antibiotics has increased worldwide, causing serious problems such as multidrug-resistant bacteria, antibiotic misuse/overuse, antibiotic residues in food, *etc*. For these reasons, many researchers have attempted to find natural materials to replace antibiotics to treat bacterial infections. *H. cordata* Thunb. (Saururaceae) is a perennial herb native to Southeast Asia with a thin stalk and heart-shaped leaf. It is called e-sung-cho in Korean and known to be effective in treating allergic inflammation, anaphylaxis, cancer, and viral infection.[21—24] A previous study reported that essential oils from *H. cordata* have antibacterial activities against *S. aureus* and *S. ureae*.[5] The chemical composition of *H. cordata* extract was investigated and found to be methyl nonyl ketone, bornyl acetate, β-myrcene, α-pinene, β-pinene, acetic acid geraniol ester, camphene, sabinene, *n*-decanal contents, caryophyllene, limonene, 4-terpineol, a-terpineol, acetic acid geraniol, tetradecanoyl-phorbol-acetate, tetradecanoyl ester, and others.[5] Many researches reported that these components have antibacterial activities. For example, methyl nonyl ketone exhibits the growth of *Escherichia coli*,[25] β-myrcene enhances the antibacterial activity of other components of *H. cordata*,[26] and caryophyllene, bornyl acetate, α-pinene, β-pinene, and limonene exhibit bacteriocidal and fungicidal activity.[5,27—30] Tetradecanoyl-phorbol-acetate and tetradecanoyl ester potently induce H_2O_2 and NO in phagocytes.[31,32] In this study, we investigated whether HCWE had antibacterial effects including direct antibacterial effects, phagocytic activity, bacterial clearance within macrophages, and clinical efficacy against murine salmonellosis. Some medical plant extracts such as *Salicornia herbacea* induce enlargement or dendritic morphologic changes in macrophage in a dose-dependent manner after coincubation for 48 h.[33] Similar to *S. herbacea*, the 100 µg/ml concentration of HCWE also caused morphologic changes in dendritic forms compared with untreated murine-derived RAW 264.7 cells, and the rates of morphologically changed cells reached to 50% in a time-dependent manner after 8-h incubation. The phagocytic activity of RAW 264.7 cells treated with HCWE increased dose dependently. The components of HCWE causing morphologic changes in RAW 264.7 cells are not clear, but the change from the round to dendritic form of macrophages appears to activate bacterial uptake.

Salmonellae can prevent the induction or neutralize the action of antimicrobial effector mechanisms within macrophages and can therefore survive and multiply within phagosomes.[34] *S. typhimurium* is the most common cause of food poisoning, an acute, self-limiting gastroenteritis that can be debilitating in the very young, the very old, and the immunocompromised. Moreover, it also causes economically important health problems in livestock populations. From these reasons, many researchers have attempted to develop preventive and clinical agents against salmonellosis in humans and animals, but there is no safe vaccine or effective drug without resistance problems to treat salmonellosis. NO and H_2O_2 have been identified as major effector molecules produced by activated macrophages and are involved in the host defense against microorganisms and tumor cells.[35—38] In mammalian organisms, NO is generated by the enzyme NOS. Three types of NOS, *i.e.*, NOS1, NOS2, and NOS3, are classified, and NOS1, also known as neuronal NOS (nNOS), and NOS3, known as endothelial NOS (eNOS), both depend on increased intracellular Ca^{2+} levels for their activity. NOS2, known as iNOS, however, is independent of increased intracellular Ca^{2+} levels.[39] iNOS is induced by various cytokines, bacterial components like lipopolysaccharide (LPS), lipoteiconic acid, or bacterial infection such as salmonellosis, leishmaniasis, and human tuberculosis.[20] iNOS has an important role in bacterial infection in macrophages. HCWE did not affect NO production or iNOS expression in the present study. However, *S. typhimurium* infection combined with HCWE pretreatment activated NO production and iNOS expression by RAW 264.7 cells. Although NO production and iNOS expression were increased by HCWE combined with *S. typhimurium* infection, the bacterial replication of *S. typhimurium* was not affected in RAW 264.7 cells until 8 h of incubation. Utaisincharoen *et al.*[40] reported that the NO production by RAW 264.7 macrophages was dependent on the *Salmonella* LPS concentration. NO and tumor necrosis factor (TNF) are essential host mediators in bacterial infection. *Salmonella* sp. and their LPS stimulate TNF and NO production *via* expression of iNOS in macrophages.[41,42]

This study showed that HCWE activates bacterial uptake and simultaneously induces NO production and iNOS expression in RAW 264.7 cells. It appears that the phagocytic activation of macrophages is directly affected by HCWE

and results in increased internalization of *Salmonella* by macrophages. Like bacterial LPS, the increased bacterial internalization into macrophages may increase NO production and iNOS expression within macrophages. In conclusion, there is no direct relationship between HCWE treatment and NO production or iNOS expression, but the increased phagocytic activation by HCWE induces increased NO production and iNOS expression in *Salmonella*-infected RAW 264.7 cells.

In *Salmonella* infections, the role of iNOS is controlling multiplication at later stages of infection without direct bacterial killing within macrophages.[11] Moreover, NO or its metabolites and H_2O_2 inhibit the growth of *Salmonella*, indicating that RNI would help to limit salmonella infection *in vivo*.[43,44] The increased and induced NO limits bacterial replication and helps in the clearance of intracellular bacteria by other bacteriocidal mechanisms including cytokine analysis and phagolysosome fusion within macrophages, although further study is required to clarify the mechanisms.

In conclusion, HCWE has antibacterial activity and activates macrophages, including in induction of morphologic change, bacterial uptake, and NO production, and shows virulence reduction effects against murine salmonellosis. Our findings suggest that HCWE can be used as important treatment agent for bacterial pathogens including intracellular bacteria such *Salmonella*, *Brucella*, *Listeria*, *Bordetella*, and *Helicobacter*, instead of misusing or overusing antibiotics to treat bacterial infections.

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