Immunostimulatory Action of High-Content Active Arabinoxylan in Rice Bran

Min Gun Ji, Yeong Ro Lee, Youn Hee Nam, Rodrigo Castañeda, Bin Na Hong, and Tong Ho Kang*

ABSTRACT: Immunostimulatory activity comprises specific and nonspecific immune responses stimulated by internal and external factors. Arabinoxylan is well known for its immunostimulatory activity in vivo and in vitro, although the biological activities of arabinoxylan oligosaccharides depend on their structural features. In this study, we aimed to evaluate in vitro and in vivo the immunostimulatory activity of high-content active arabinoxylan (HCAA) obtained from rice bran through bioconversion by microorganisms and acid hydrolysis. Three microorganisms, Penicillium rocheforti, Aspergillus oryzae, and Pleurotus ostreatus, and three different acid concentrations of hydrochloric acid (5, 10, and 20%) and acetic acid (25, 50, and 75%) were used for producing HCAA. HPLC analysis of arabinose and xylose content revealed that fermentation with P. rocheforti followed by hydrolysis with 5% hydrochloric acid was the most efficient to produce HCAA. GPC analysis of HCAA indicates that HCAA is a complex of various forms of saccharides and shows an average molecular weight of 62.5. Further, in vitro evaluation disclosed that exposure to HCAA (10–200 μg/mL) increased cell viability in mice splenic cells and RAW 264.7 cells. Additionally, exposure of mice to oral administration of HCAA (100 mg/kg) for 4–7 days increased lymphokine-activated killer (LAK)- and macrophage-mediated cytotoxic activity in cancer cells (YAC-1). Furthermore, in vitro exposure to HCAA and oral administrations in mice revealed increased interferon-γ (IFN-γ) and interleukin-10 (IL-10) protein expression through western blot analysis in RAW 264.7 cells and isolated splenic cells. Our results suggest that HCAA developed by bioconversion and acid hydrolysis may enhance immune responses in vivo and in vitro.

1. INTRODUCTION

Arabinoxylans are a major component of polysaccharides, which are found in the cell walls of oat, sorghum, rice, wheat, corn, and rye, that consist of a β-1,4-D-xylopyranose linked with α-L-arabinofuranosyl. The ratio of arabinose/xylose varies depending on the grain species. A previous report showed that the ratio for wheat (Triticum aestivum Var DWR 19S) is 0.6, maize (Zea mays Var NAC 6002) is 1.3, and rice (Oryza sativa Var Jaya) is 4.79. The spatial arrangement and proportions of xylose and arabinose in arabinoxylans vary depending on the source and method of their isolation, which also determines their functional properties. A previous study reported that arabinoxylan, also known as hemicellulose, has anticancer, anti-diabetes, immunomodulation, and gut microbiota regulation effects. The immune system can be divided into two main types, innate and adaptive immunity, and comprises various organs, such as thymus, bone marrow, and spleen. When invading organisms cause infection, the innate immune system is activated immediately. The activated innate immune system protects against pathogenic antigen by producing various immune cells, such as natural killer (NK) cells and macrophages, cytokines, and chemokines. Natural plants and their components were evaluated for potential immunostimulatory activity for functional ingredients. Particularly, it has been reported that processed arabinoxylan is a highly available biological response regulator for improved immunostimulatory activity.

The use of different methods to reduce the size of various polysaccharides demonstrates that a lower molecular weight increases bioavailability and bioactivity. Standardization of the factors affecting the degree of debranching required to produce arabinoxylan is fundamental to developing functional products with desired activity levels. Among the methods described to produce arabinoxylan, chemical hydrolysis by dilute acids is one of the simplest methods. This approach, which is often used for hydrolyzing polysaccharides, is time-consuming and can involve high temperatures, which have been associated with higher concentrations of degradation products and decreasing yield of oligosaccharides. Biocconversion combined with desired activity levels. Among the methods described to produce arabinoxylan, chemical hydrolysis by dilute acids is one of the simplest methods. This approach, which is often used for hydrolyzing polysaccharides, is time-consuming and can involve high temperatures, which have been associated with higher concentrations of degradation products and decreasing yield of oligosaccharides.

Received: May 26, 2020
Accepted: August 24, 2020
Published: September 22, 2020
with acid hydrolysis has been an alternative method used recently to reduce the size of polysaccharides more efficiently. Microorganisms that engage in cellulolytic activities have also been utilized to produce arabinoxylan. However, the optimal process to reduce the size of the macromolecule and maximize arabinoxylan content during bioconversion of rice bran has not been identified.

For the present study, we developed a high-content active arabinoxylan (HCAA) obtained through bioconversion of rice bran combined with acid hydrolysis to increase immunostimulatory activity. This involved assessing different hydrolysis conditions and bioconversion strategies in fungal species that break down cellulose to produce arabinoxylan. In addition, HCAA boosts the viability of splenocyte and RAW 264.7 cells and increases cytokine expression and lymphokine-activated killer (LAK) and macrophage cytotoxic-mediated activity in cancer cells (YAC-1).

2. RESULTS AND DISCUSSION

2.1. Results. 2.1.1. Establishment of the HCAA Production Process. To establish the optimized processing method of HCAA, a combination of acid hydrolysis and bioconversion was employed. Acid hydrolysis was processed in 25, 50, and 75% acetic acid and 5, 10, and 20% hydrochloric acid at 100 °C during 6 h. According to arabinose and xylose contents, acid hydrolysis using 5% hydrochloric acid revealed the highest concentrations (Table 1, Figure 1).

### Table 1. Concentration of Arabinose and Xylose in Acid Hydrolysis Samples

| Hydrolysis Solution | Arabinose (%) | Xylose (%) |
|---------------------|---------------|-----------|
| 25% acetic acid     | 0.035         | 0.004     |
| 50% acetic acid     | 0.014         | 0.002     |
| 75% acetic acid     | 0.015         | 0.003     |
| 5% hydrochloric acid| 0.753         | 0.942     |
| 10% hydrochloric acid| 0.708       | 0.930     |
| 20% hydrochloric acid| 0.271        | 0.260     |

Additionally, three types of microorganisms were used for bioconversion, and the highest content of arabinose and xylose was confirmed in *Penicillium roqueforti* (Table 2). To produce high-content active arabinoxylan (HCAA), a combination of bioconversion using *P. roqueforti* and acid hydrolysis with 5% hydrochloric acid was established (Figure 2).

2.1.2. Standardization of HCAA. The standardization of HCAA was carried out based on the loss on drying, residue on ash, appearance, and a pH test. The HCAA powder was shown to be a dark greenish-brown powder of irregular size through a microscope and was insoluble. The average loss on drying and ash residue were 17.51 ± 0.05%, respectively. The average pH was 6.19 ± 0.03 (Table 3).

2.1.3. Molecular Weight Analysis of HCAA. The molecular weight of HCAA was determined by gel permeation chromatography (GPC). HCAA showed molecular weights ranging from 169 to 1915 (Figure 3). Most HCAA consists of four forms, such as single arabinose or xylose (peak #4, 28%), disaccharide of arabinose and xylose (peak #3, 43%), combined with four of arabinose and/or xylose (peak #2, 11%), and approximately 16 of arabinose and/or xylose combination (peak #1, 18%). The mean value of HCAA molecular weight is 625 approximately.

2.1.4. HCAA Enhances the Viability of Primary Splenocyte and RAW 264.7 Cells. Spleens were isolated from 10-week-old ICR mice to evaluate the HCAA effect on viability of adherent primary splenocytes (Figure 4). After 24 h of exposure to 10 μg/mL HCAA, viability of splenocytes was significantly increased (p < 0.001). Similarly, viability of RAW 264.7 cells was significantly increased (p < 0.001) at 50, 100, and 200 μg/mL HCAA during 24 h of exposure (Figure 5A). Similarly, 50 and 100 μg/mL HCAA-treated groups showed a significant increase in viability after 48 h of exposure (Figure 5B, p < 0.001).

2.1.5. HCAA Increases Macrophage- and LAK-Mediated Cytotoxic Activity. Peritoneal macrophages and nonadherent primary splenocytes were isolated from 7-week-old ICR mice treated orally for 4 and 7 days with 100 mg/kg HCAA to evaluate cytotoxic activity mediated by macrophages and LAK (Figure 6). YAC-1 cell viability was significantly reduced (p < 0.01) after exposure to macrophages isolated from mice treated with HCAA for 4 and 7 days compared to untreated mice (Figure 6A). Similarly, LAK-mediated cell cytotoxicity in YAC-1 was significantly higher in the splenocytes isolated from mice treated with HCAA for 4 (p < 0.05) and 7 (p < 0.001) days than those isolated from untreated mice (Figure 6B).

2.1.6. HCAA Increases Cytokine Expression in Macrophage. Cytokine expression was analyzed by western blotting using RAW 264.7 cells exposed to HCAA and LPS (Figure 7). RAW 264.7 cells stimulated by 1 μg/mL LPS were used as positive controls of cytokine induction. The expression level of IFN-γ was significantly elevated by HCAA at 24 h (p < 0.01) and 48 h (p < 0.001) compared with cells treated with only media. After 24 h, IFN-γ expression was similar between HCAA- and LPS-treated groups. However, the IFN-γ expression level was 2.4-fold higher in the 48 h HCAA exposure group (Figure 7B).

IL-10 expression was also revealed to be significantly elevated in the 100 μg/mL HCAA-treated group at 24 and 48 h (p < 0.001). IL-10 expression was increased 1.96-fold at 24 h and 2.5-fold at 48 h in the HCAA exposure group (Figure 7C).

2.1.7. HCAA Increases Cytokine Expression in Primary Splenocytes. IL-10 and IFN-γ expression levels in primary adhesion splenocytes were evaluated. IL-10 and IFN-γ expression levels were significantly elevated in HCAA-treated mice (Figure 8). IFN-γ and IL-10 expression levels in the HCAA group were significantly increased 3.7- and 4-fold higher than the LPS-injected group, respectively (Figure 8B,C).

2.2. Discussion. Rice bran is a byproduct made by rice milling and it has been proposed as a rich source of hemicellulose. Hemicellulose has antitumor, anti-inflammatory, and gut microbiota regulation effects. Especially, arabinoxylan has an immunostimulatory activity, and the lower molecular weight has enhanced bioavailability and increased bioactivity. Our study tried to validate how to increase the content of arabinoxylan and evaluate the effect of immunostimulatory activity.

In this study, the established process of HCAA is microbial fermentation, acid hydrolysis, neutralization, filtration, and drying. Hydrochloric and acetic acids were used for acid hydrolysis, and three microorganisms known to produce plantase, cellulase, and hemicellulase, *Penicillium roqueforti*, *Aspergillus oryzae*, and *Pleurotus ostreatus*, were used for fermentation. A combination of acid hydrolysis and
microbial fermentation was employed to increase the effectiveness of the amount of arabinoxylan. As a result, *P. roqueforti*-fermented rice bran with acid hydrolysis using 5% hydrochloric acid was the most efficient way to obtain HCAA (Tables 1 and 2). In addition, after microbial fermentation, acid hydrolysis was performed to sterilize microorganisms through acid. This result indicates that the acid solvent type and concentration in the acid hydrolysis process, boiling time, and temperature, and, crucially, the type of microorganism were important factors (Figure 2). This approach is an alternative and efficient method used to reduce the size of polysaccharides.

Arabinose and xylose are water-soluble monosaccharides and have a molecular weight of 150. HCAA is filtered through the filtration process, and the water-soluble component remains in the final product. Therefore, it was expected that HCAA has a small molecular weight. Indeed, HCAA components represent 169–1915 of molecular weight range and 169, 375, 661, 1915 molecular weight peak approximately. Therefore, HCAA is a complex with monosaccharide, disaccharide, and hemicellulose (Figure 3).

Lower molecular weight of polysaccharides increases bioavailability and bioactivity. In addition, previous studies report that arabinoxylan isolated from rice bran has immunomodulatory properties by enhancing NK cells and

Table 2. Arabinose and Xylose Contents in the Microorganisms Used for Bioconversion

| microorganism for bioconversion | arabinose (%) | xylose (%) |
|--------------------------------|---------------|------------|
| *Penicillium roqueforti*       | 1.510         | 1.810      |
| *Aspergillus oryzae*           | 1.130         | 1.050      |
| *Pleurotus ostreatus*          | 0.980         | 1.030      |

Figure 1. Chromatogram of 100 μg/mL arabinose, 100 μg/mL xylose, and 100 mg/mL rice bran extract with acid hydrolysis by 25% acetic acid (25% AA), 100 mg/mL rice bran extract with acid hydrolysis by 50% acetic acid (50% AA), 100 mg/mL rice bran extract with acid hydrolysis by 75% acetic acid (75% AA), 100 mg/mL rice bran extract with acid hydrolysis by 5% hydrochloric acid (5% HA), 100 mg/mL rice bran extract with acid hydrolysis by 10% hydrochloric acid (10% HA), and 100 mg/mL rice bran extract with acid hydrolysis by 20% hydrochloric acid (20% HA).

Figure 2. Chromatogram of 100 mg/mL HCAA.
macrophage activity in aged mice. In addition, arabinoxylan increases NK cell activity that mediated antitumor activity on Ehrlich carcinoma-bearing mice. Antimicrobial activity of HCAA was demonstrated by enhanced phagocytosis of macrophages, neutrophils, and monocytes in vitro and in vivo. Moreover, a randomized clinical trial demonstrated that rice bran increased NK activity and augmented concentrations of T helper cell type 1-related cytokines in patients with multiple myeloma. In this study, the immunostimulatory activity effect of HCAA was determined by increased viability on splenocytes and RAW 264.7 cells. Splenocytes and RAW 264.7 cells were shown to have significantly increased viability in 24 and 48 h by HCAA (Figures 4 and 5). In addition, HCAA increases LAK and macrophage cytotoxic-mediated activity in YAC-1 cells (Figure 6). These results indicate that HCAA reinforces immune function through increasing immune-related cell viability and cytotoxic activity.

The expression level of immunostimulatory activity cytokines, IL-10 and IFN-γ, was evaluated using a cell model administrated with HCAA. RAW 264.7 and splenocyte cells showed significant increases in cytokine expression when treated with HCAA (Figures 7 and 8). This means that HCAA

| sample | loss on drying (%) ± SD | residue on ash (%) ± SD | pH ± SD |
|--------|-------------------------|-------------------------|---------|
| HCAA   | 17.51 ± 0.01            | 75.26 ± 0.05            | 6.19 ± 0.03 |

Figure 3. Gel permeation chromatograph of HCAA.

Figure 4. HCAA increases adherent splenocyte cell viability. Adherent splenocytes were exposed to 10 μg/mL HCAA for 24 h. Cell viability was evaluated using MTT assay. ***p < 0.001 compared to untreated cells.

Figure 5. HCAA increases macrophage viability. Cell viability was evaluated using MTT assay. RAW 264.7 cells were exposed to 50, 100, or 200 μg/mL HCAA for (A) 24 and (B) 48 h. ***p < 0.001 compared to untreated cells.
can affect cells related to cytokine expression and synthesis. It is also known that the increase in cytokines activates various immune systems. The inflammatory processes were regulated by proinflammatory and anti-inflammatory cytokines, which have a specific role in immunostimulatory activity. The anti-inflammatory cytokine, IL-10, is produced by monocytes, T cells, B cells, macrophages, natural killer cells, and dendritic cells. IL-10 plays a central role in limiting host immune response to pathogens, thereby preventing autoimmune damage and maintaining normal tissue homeostasis. IFN-γ was secreted from cells that have been invaded by external viruses, which stimulates cells in vivo to resist viruses and to protect from the antigen. In addition, IFN-γ combines the surface protein of cells that have not been invaded to protect from viruses and is a critical effector of immune surveillance against tumor cells. A different clinical trial demonstrated that supplementation of HCAA over 8 weeks in healthy patients produced increased IFN-γ secretion without producing immunomodulatory adverse effects. In this study, LPS was used as a positive control. LPS is a non-protein component of the outer membrane of Gram-negative bacteria and is devoid of direct antibacterial activity. However, it is widely known to enhance antibacterial functions of monocytic phagocytes (macrophages) via immunopotentiation. These results suggest that HCAA may increase the activity of NK and macrophages and prevent autoimmune damage by increasing IFN-γ and IL-10 expressions.

Therefore, we suggest that HCAA has immunostimulatory activity through increasing immune cell viability, cytotoxicity of LAK and macrophages, and cytokine expression in vivo and in vitro. However, since this study proved HCAA in part of the mechanism, further work is needed to investigate the action point on the immune system and to explore the causal relationships between these biological factors. Moreover, optimization of HCAA production processes is validated through changing microbial types.

3. CONCLUSIONS
We developed a product derived from rice bran by fermentation with P. rocheforti combined with 5% hydrochloric acid hydrolysis. HCAA demonstrated increased viability on splenocytes and RAW 264.7 cells. In addition, macrophage and LAK cell anticancer activity is increased by HCAA in YAC-1 cells. Furthermore, HCAA increased cytokine expression levels on splenocytes and RAW 264.7 cells. Our results suggest that HCAA may enhance immune responses in vitro and in vivo.

4. EXPERIMENTAL SECTION
4.1. Arabinoxylan Extraction Process Validation for Acid Hydrolysis. To ensure optimal arabinoxylan extraction, an isolation process was validated. The process of arabinoxylan
Figure 8. HCAA increased cytokine expression in spleen. Mice were orally administered with 100 mg/kg HCAA for 7 days, and then adherent splenocytes were isolated for IFN-γ and IL-10 expression. (A) Western blot results of normal, LPS-, and HCAA-treated groups. The relative expression level of (B) IFN-γ and (C) IL-10. **p < 0.01 and ***p < 0.001 compared to untreated cells.

extraction involved acid hydrolysis of rice bran, pH adjustment, filtration, bioconversion using microorganisms, concentration, and drying. To validate the arabinoxylan acid hydrolysis conditions, the initial weight of rice bran, types of hydrolysis solvent, solvent concentration, boiling temperature, and time were taken into account.

Typically, 5 or 10 g of rice bran was used for each test. Acid solvents were used with acetic acid (AA) and hydrochloric acid (HA). The acetic acid was used at concentrations of 25, 50, and 75%, while hydrochloric acid was used at concentrations of 5, 10, and 20%. The boiling temperatures were 90 °C (AA) and 100 °C (HA). Finally, the acid hydrolysis process was established by adding acid solvents at each concentration and boiling for 6 h without stirring at a fixed temperature each time.

4.2. Arabinoxylan Extraction Process Validation for Microorganisms. Rice bran bioconversion was initially assessed to maximize arabinoxylan extract, using three different cellulolytic agents: P. roqueforti KCCM 11269, A. oryzae KCCM 11886, and P. ostreatus KCCM 16855 (obtained from Korean Collection for Type Cultures). These microorganisms were first cultured in potato dextrose agar (PDA, Becton, Dickinson and Company, Sparks) at 25 °C for 4 days, then inoculated individually in potato dextrose broth (PDB, Becton, Dickinson and Company, Sparks), and incubated under shaking at 120 rpm for 5 days at 24 °C. Further extraction parameters were evaluated after the pretreatment process of bioconversion.

4.3. Preparation of High-Content Active Arabinoxylan (HCAA). After validation, P. roqueforti KCCM 11269 was cultured in PDB at 25 °C in a rotary shaker for 5 days as previously described. After the incubation period, parallel Erlenmeyer flasks containing rice bran and PDB were sterilized by autoclaving. The flasks containing rice bran were further inoculated with P. roqueforti and incubated in a rotary shaker (120 rpm for 4 days). After the incubation period, 50 mL of 5% hydrochloric acid was added and heated at 100 °C for 6 h. After cooling, pH was adjusted with NaOH to 6.5 and dried at 70 °C for 4 days.

4.4. HPLC Analysis. To quantify the completed and isolated arabinoxylan samples, amounts of arabinose and xylose were measured. Because it was difficult to measure the amount of arabinoxylan directly, we tried to measure the amount of arabinose and xylose, which are processed products, and to examine the processed result indirectly.

High-performance liquid chromatography (HPLC) analysis of arabinose and xylose was performed using a Shimadzu column (VP-ODS, 250 L × 4.6, 5 μm). The mobile phase consisted of a mixture of sodium phosphate buffer (40 mM)—acetonitrile 79:21 at 0.7 mL/min. The column temperature was set at 30 °C, and the sample injection volume was 10 μL. A standard solution of arabinose and xylose with conjugated 1-phenyl-3-methyl-5-pyrazolone (PMP) was prepared at a concentration of 100 mg/mL. Ultraviolet detection was performed at 245 nm.

4.5. Standardization of HCAA. HCAA characteristics were evaluated by an ash content test, loss on drying, appearance, and a pH test. The ash content test was performed by exposing samples to temperatures of 550–600 °C. After 3 h of burning, the samples were cooled and weights were measured. This was repeated for one more hour until constant weights were achieved. Loss on drying was evaluated at 105 °C for 4 h, and the sample was further dried until constant weights were achieved. HCAA appearance was analyzed by a microscope in a mixture with 50% glycerol. Finally, pH was measured by a pH meter in three different concentrations of HCAA.

4.6. Gel Permeation Chromatography. To measure the molecular weight of HCAA, gel permeation chromatography (GPC) was performed. HCAA was dissolved in water and filtered using a 0.45 μm nylon filter. Polysaccharide was used as a standard substance for GPC analysis.

GPC analysis of HCAA was performed using a Tskgel guard PWxl + 2x TSKgel GMPWxl + TSKgel G2500PWxl column (7.8 × 300 mm3). The mobile phase was 0.1 M NaOH solution at 1 mL/min. The column temperature was set at 40 °C, and the sample injection volume of 3 mg/mL HCAA was 100 μL. An RI-detector determined the molecular weight, and EcoSEC software was used for data analysis.

4.7. Animals. All of the experimental procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication, #80-23, revised 1996) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. Adult male Institute for Cancer Research (ICR) mice
CO2 and then were washed and transferred to new plates. Macrophages were washed twice with cold HBSS and adjusted to 4 × 10^6 cells/mL. The plates were treated with 1000 U/mL of interleukin-2 (IL-2) and incubated for 3 days to obtain LAKs. For macrophage and LAK cytotoxic activity, 5 × 10^4 and 1 × 10^5 cells were added to each well, respectively.

4.7. Detection of Macrophage and LAK Cell Cytotoxic Activity. Macrophages isolated from the peritoneal cavity of 7-week-old ICR mice were used as effector cells. Macrophages were washed twice with cold HBSS and centrifuged at 300g for 3 min, and 1 × 10^5 cells were added to each well. From the same mice, nonadherent splenocytes isolated from spleens were used as LAK effectors. To separate nonadherent from adherent cells, cells were incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO2 and then were washed and transferred to new plates adjusted to 4 × 10^6 cells/mL. The plates were treated with 1000 U/mL of interleukin-2 (IL-2) and incubated for 3 days to obtain LAKs. For macrophage and LAK cytotoxic activity, 5 × 10^4 and 1 × 10^5 cells were added to each well, respectively.

4.8. HCAA Administration. Animals assigned for the evaluation of the cytokine expression were divided into three groups (n = S/group). Nontreated ICR mice (the normal group) were treated orally with distilled water, and HCAA-treated ICR mice (HCAA group) were treated orally with 100 mg/kg HCAA for 7 days. LPS-treated ICR mice (LPS group) were injected intraperitoneally with 1 mg/kg LPS for 24 h.

4.9. Splenocyte Isolation from Mouse Spleen. For splenocyte isolation, each spleen was removed aseptically from an ICR mouse and kept in cooled Hank’s balanced salt solution (HBSS). A single-cell suspension was prepared by gently homogenizing a spleen with the distal end of a syringe in a cell strainer and washed with HBSS. Cell suspensions were centrifuged at 400g and further incubated with red blood cell lysis buffer for 15 min. The cells were then centrifuged at 400g, washed, and stabilized in RPMI 1640 with 10% FBS.

4.10. Cell Culture. YAC-1 (murine T-cell lymphoma cell line) and RAW 264.7 (murine macrophage cell line) were obtained from Korean Cell Line Bank (Seoul). YAC-1, RAW 264.7, and primary splenocyte cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (FBS; Welgene, Seoul, Korea) at 37 °C in a humidified atmosphere of 5% CO2 atmosphere incubator.

RAW 264.7 cells and primary splenocytes were employed for the cell viability test, and YAC-1 cells were used for evaluation of macrophage- and LAK-mediated cytotoxic activity. Cells were used for tests at a passage number of less than 5 and were washed before the start of the assay.

4.11. Cell Viability Analysis. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), purchased from Dutchefa Biochemies B.V (Haarlem, The Netherlands). After cells were incubated for 24 h in 96-well plates at 37 °C in a humidified atmosphere of 5% CO2 in an incubator, 20 μL of 5 mg/mL sterile MTT solution was added to each well. Additional incubation was carried out under the same conditions for 4 h. The supernatant was removed, and the formazan crystals were resolubilized in 100 μL of dimethyl sulfoxide under agitation. Microtiter plates were measured in a microtiter plate reader (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT) at 570 and 630 nm. Cell viability was represented as an index compared with that of the control.

4.12. Western Blot Analysis. The HCAA- or 1 μg/mL lipopolysaccharide (LPS)-treated RAW 264.7 and adherent splenocytes were washed twice in phosphate-buffered saline (PBS) and lysed in Pierce Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Thermo Fisher Scientific, Inc., Waltham) for 30 min on ice. The lysates were centrifuged at 14 000g for 20 min at 4 °C. The supernatant was collected, and the protein content in the supernatant was measured using a bichinolinic acid protein kit assay kit (Thermo Fisher Scientific, Inc., Waltham) prior to analysis. The total protein samples (10 μL) were loaded and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat powdered milk in 1× Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h and were then incubated with interleukin-10 (IL-10, Peprotech, California), interferon-γ (IFN-γ, Santa Cruz, Dallas), and β-actin (Santa Cruz, Dallas) primary antibodies at 4 °C overnight. Finally, the membranes were treated with a horseradish peroxidase-conjugated secondary antibody (Bethyl, Montgomery) for 1 h. The membranes were washed with Tris-buffered saline, 0.1% Tween 20 (TBS-T), after each antibody binding reaction. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc, Waltham). Band intensities were determined using Image-Quant LAS 4000 (GE Healthcare, Chicago) and Quantity One software (Bio-Rad, Hercules). β-actin was used as a constitutive control for normalization.

4.13. Statistical Analysis. Data were analyzed using the Prism 5 Statistical Software package (Graph-Pad, Sandi ego, CA). All data are expressed as the mean ± standard error mean (SEM). Statistical comparisons between the groups were performed using one-way repeated measures analysis of variance with Tukey’s post hoc test. Values of p < 0.05, 0.01, and 0.001 were considered statistically significant.

AUTHOR INFORMATION

Corresponding Author
Tong Ho Kang — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea; Phone: +82-31-201-3862; Email: panjae@khu.ac.kr; Fax: +82-330-0300-0030

Authors
Min Gun Ji — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea
Yeong Ro Lee — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea
You Hee Nam — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea
Rodrigo Castañeda — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea
Bin Na Hong — Department of Oriental Medicine Biotechnology, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do 17104, Republic of Korea

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02472
**Funding**
This work (Grant No. C0268459) was supported by Business for Academic–industrial Cooperative establishments funded by Korea Small and Medium Business Administration in 2015. This research was supported by the Basic Science Research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2015R1D1A1A09060469).

**Notes**
The authors declare no competing financial interest. Access to these data will be considered by the author upon request, with permission of Tong Ho Kang (panjae@khu.ac.kr).

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