

Evaluation of the Immunological Activity of *Gryllus bimaculatus* Water Extract

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ABSTRACT: Edible insects are commonly consumed across the world because of their size, availability, and nutritional benefits. They have also been recommended as a potential solution to food shortage because of their high nutritional value. In this study, we demonstrated the immunological effects of *Gryllus bimaculatus* on RAW 264.7 cells and splenocytes obtained from mouse. This is the first study to evaluate the immunological effects of *G. bimaculatus* water extract. Innate and adaptive immunity were evaluated and measured in RAW 264.7 cells and/or mouse splenocytes using a cell viability assay; changes in cytokine abundance, nitric oxide production, and cell surface molecule abundance were determined using flow cytometry; and western blotting analysis was performed for various immune signaling pathways. *G. bimaculatus* water extract showed no cytotoxicity in cells, and the results suggest that treatment with *G. bimaculatus* water extract can induce macrophage activation through mitogen-activated protein kinase and nuclear factor- κ B signaling, induction of proinflammatory cytokines [interleukin (IL)-6, IL-1 β , and tumor necrosis factor- α] and activation of the expression of cell surface molecules [cluster of differentiation (CD)80, CD86, major histocompatibility complex (MHC) class I, and MHC class II]. Treatment with *G. bimaculatus* water extract increased the production of cytokines (IL-2, IL-4, and interferon- γ) in splenocytes. The results indicate that *G. bimaculatus* water extract can regulate innate and adaptive immunity via modulation macrophages and splenocytes activation and can serve as an immunological agent. We inferred that *G. bimaculatus* is a safe and efficient natural material that enhances immunological activity.

Keywords: adaptive immunity, edible insect, *Gryllus bimaculatus*, immunological activity, innate immunity

INTRODUCTION

Immunity is a balanced state of biological defenses to fight infections, diseases, or other unwanted invasions, i.e., it is the capability of a multicellular organism to resist harmful pathogenic microorganisms from entering the body. The environment contains a wide range of pathogenic microorganisms and toxic or allergenic proteins, which are removed by the immune system (Chaplin, 2010). Because pathogens can evolve and adapt rapidly, immune system disorders caused by pathogens can result in autoimmune diseases, inflammatory diseases, and cancer (O'Byrne and Dalgleish, 2001). In many species, there are two subsystems of the immune system: the innate and adaptive immune systems. The innate immune system, also called native immunity, consists of primitive bone marrow cells that are programmed to recognize for-

eign materials and react. This system is ready to act rapidly after encountering an invading pathogen or toxin. The innate immune system includes physical barriers (epithelial cell layers, secreted mucus layer, and epithelial cilia), soluble proteins, and bioactive small molecules that are constitutively present in biological fluids, membrane bound receptors, and cytoplasmic proteins. The adaptive immune system consists of lymphatic cells that are programmed to recognize self-substances and not react. It is composed of a small numbers of cells with specificity for individual pathogens. Therefore, the adaptive immune system manifests specificity for its target antigens. The innate and adaptive immune systems usually act together, with the innate response being the first line of host defense and the adaptive response becoming prominent after several days, after antigen-specific T and B cells have undergone clonal expansion.

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Globally, edible insects are commonly consumed because of their size, availability, and nutritional benefit. They have also been recommended as a potential solution to the shortage of food sources (Kim and Jung, 2013). Humans have consumed many insects as a food source for many years because they are naturally rich in proteins and micronutrients (Dobermann et al., 2017). In particular, the general nutritional content of *Gryllus bimaculatus* includes fats, proteins, polyunsaturated fatty acids, minerals, and fiber. *G. bimaculatus* is also rich in essential amino acids, such as lysine, leucine, valine, and isoleucine (Belluco et al., 2013). *G. bimaculatus* contains high concentrations of fatty acids, especially unsaturated fatty acids (68.6%) such as linoleic and oleic acid. Ahn et al. (2014; 2016) demonstrated that glycosaminoglycans isolated from crickets showed anti-inflammatory effects in a chronic arthritis rat model and antioxidant activity in liver tissues.

Insects are popularly consumed because of their size and availability (Bukkens, 1997). In addition, the United Nations has recommended the practice of consuming insects as a potential solution to the shortage of food sources, and *G. bimaculatus* has recently been listed as a food in Korea (Cho et al., 2019). Many insect species are consumed by humans as food (Paoletti et al., 2007) at various life stages and are consumed raw, fried, boiled, and roasted. *G. bimaculatus* has a long history of traditional use in Asian medicine. *G. bimaculatus* showed cytoprotective effects in the human epithelial cell line Caco-2 as well as increased cell viability and decreased the levels of inflammatory cytokines in inflammatory bowel disease (Kim et al., 2021). In addition, attempts have been made to investigate functional of *G. bimaculatus* and pharmacological potential, including immunomodulatory effect (Seo et al., 2004; Park et al., 2020), hepatoprotective properties (Im et al., 2018; Hwang et al., 2019), anti-inflammatory effect (Ahn et al., 2014; Hwang et al., 2019; Kim et al., 2021), antioxidant activity and neuroprotective effect (Boonsin et al., 2018), hyperglycemic control activity (Park et al., 2019), and skin protective effect (Jeong et al., 2020).

Another edible insect, the larva of *Allomyrina dichotoma*, which is listed in the Korean Food Standards Codex (Ministry of Food and Drug Safety, 2021) as a food resource, has anti-inflammatory effects in human endothelial cells due to stabilization of vascular barrier integrity, which suppresses lipopolysaccharide (LPS)-induced vascular inflammation by inhibiting the nuclear factor (NF)- κ B pathway (Park et al., 2020). Cell adhesion molecules (CAMs), such as vascular CAM-1 and intercellular adhesion molecule-1, play a crucial role in the inflammatory pathway (Lee et al., 2018), and blocking of CAM activity is regarded as a promising therapeutic method for the treatment of vascular inflammatory diseases. A compound

from *A. dichotoma* larva was shown to downregulate the level of CAMs.

In this study, we examined the immunological activity of *G. bimaculatus* water extract, as well as its possible mechanisms of action. This is the first study of macrophage activation using *G. bimaculatus* extract.

MATERIALS AND METHODS

Sample preparation of experimental extract for cell treatment

G. bimaculatus water extract was prepared, and a concentration of 10 mg/mL was used in the experiments.

Cell line and culture condition

RAW 264.7, a murine macrophage cell line, was purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) under relative humidity at 37°C and 5% CO₂ in an incubator.

Cell viability assay

RAW 264.7 cells were cultured in 96-well plates at a density of 1×10^4 cells per well. The cells were allowed to attach for 24 h and were then treated with *G. bimaculatus* water extract. At the end of the treatment period, 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) reagent (5 mg/mL) was added to each well. After 3 h of incubation at 37°C, the supernatant was aspirated, and formazan crystals were dissolved in 100 μ L of dimethyl sulfoxide at 37°C for 10 min with gentle agitation. The absorbance per well was measured at 540 nm using a microplate reader.

Measurement of cytokine production

Supernatants from experimental RAW 264.7 cell cultures were collected and stored at -70°C until use. The levels of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β in the supernatants were determined using a cytokine detection enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions, with detection at 450 nm using a microplate reader. The production of cytokines was also measured after treatment of splenocytes with *G. bimaculatus* water extract. Supernatants from experimental splenocyte cultures were collected and stored at -70°C until use. The levels of interferon (IFN)- γ , IL-2, and IL-4 in the supernatants of splenocyte cultures was determined using a cytokine detection ELISA kit according to the manufacturer's instructions, with detection at 450 nm using a microplate reader.

Measurement of nitric oxide (NO) production

The NO concentration in the culture supernatants was determined by measuring its oxidation product, nitrite, using the Griess method. Briefly, the culture supernatants were mixed with Griess reagent (1:1) and incubated at room temperature for 15 min. Then, the absorbance 517 nm was measured using a microplate reader. NaNO₂ freshly prepared in deionized water was used to generate a standard curve (0~100 µM) to calculate the nitrite concentration in the cell culture supernatants.

Western blotting analysis

The cells were harvested and washed twice in 1× phosphate-buffered saline (PBS). For western blot analysis, total proteins were prepared using PRO-PREP™ Protein Extraction Solution and quantified using protein assay reagent (iNtRON Biotechnology, Seongnam, Korea). The extracted proteins were denatured by boiling in sample buffer at 100°C for 5 min and then electrophoresed using 8% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The gels were transferred to polyvinylidene fluoride membranes at 100 V for 60 min in transfer buffer, and the membranes were incubated with 5% non-fat dry milk in 1× Tris-buffered saline (TBS) buffer at room temperature for 1 h to block nonspecific antibody response. Next, the membranes were incubated with specific primary antibodies at 4°C overnight. After washing for 1 h with 1× TBS buffer, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody or anti-rabbit antibody for 30 min at room temperature and then washed for 1 h with 1× TBS buffer. The proteins were detected using enhanced chemiluminescence western blotting detection reagents.

Nuclear protein extraction

The cells were placed in 2 mL of hypotonic buffer A [10 mM hydroxyethyl piperazine ethane sulfonic acid (HEPES, pH 7.8), 2 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)] and mixing sample in an ice bath. 10% NP-40 solution was added to the sample, and the mixture was centrifuged at 12,000 rpm for 4 min at 4°C. The nuclei pellets were washed twice with 500 µL of buffer A+62.5 µL of 10% NP-40, centrifuged, and resuspended in 300 µL of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol]. After incubation with vortexing every 5 min at 4°C for 30 min, the samples were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant containing nuclear proteins was collected and stored at -80°C in aliquots until use. Nuclear proteins were collected in duplicate using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of cell surface molecules using flow cytometry

After experimental treatment, the RAW 264.7 cells were washed with 1× PBS and resuspended in washing buffer (2% FBS and 0.1% sodium azide in PBS) for fluorescence-activated cell sorting (FACS) on the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were preincubated with 0.5% bovine serum albumin in 1× PBS for 30 min and washed with 1× PBS. The cells were then stained with PE-conjugated anti-mouse major histocompatibility complex (MHC) I, II, fluorescein-5-isothiocyanate (FITC) hamster anti-mouse cluster of differentiation (CD)80, or FITC rat anti-mouse CD86 for 45 min at 4°C. All antibodies were diluted 100-fold before use. The cells were washed three times and resuspended in a final volume of 500 µL of 1× PBS.

Preparation of murine splenocytes

Seven-week-old male C57BL/6 mice were placed on a clean dissection board and rinsed with 70% alcohol. An incision was made into the abdominal cavity, and the spleen, which is located to the left side of the abdomen, inferior to the stomach, was removed. The excised spleen was spliced into small pieces and then pressed through a strainer using the plunger end of a syringe. The cell suspension was centrifuged at 1,500 rpm for 5 min. Then, the supernatant was aspirated and the cell pellet was resuspended in 2 mL of lysing solution. 1× PBS was added, and the cells were centrifuged at 1,500 rpm for 5 min. Finally, the supernatant was discarded and the cells resuspended in 1× PBS. All animal experiments were assessed according to the established guidelines of Korea Atomic Energy Research Institute (KAERI, Jeongeup, Korea) and approved by the Institutional Animal Care and Use Committee (IACUC) of KAERI (permit number: KAERI-IACUC-2020-002).

Statistical analysis

Means and standard deviations were calculated using Graphpad Prism (version 5, GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance followed by Tukey's multiple comparison test was performed for comparisons of differences among multiple groups. $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of *G. bimaculatus* water extract on cell viability

Macrophage cells were treated with *G. bimaculatus* water extract at concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL. Cells treated with 0.2 µg/mL LPS were used as a positive control. After 24 h, cell proliferation

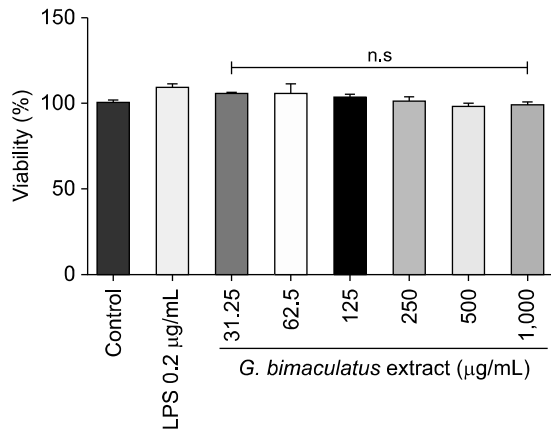


Fig. 1. Cytotoxicity of *Gryllus bimaculatus* water extract in the macrophage cell line RAW 264.7. Cells were treated with the indicated concentrations (31.25, 62.5, 125, 250, 500, and 1,000 µg/mL) of *G. bimaculatus* water extract for 24 h. After 24 h, cell cytotoxicity was measured using the MTT assay. Values are presented as mean±SD (n=3). LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide; n.s., not significant.

was measured using the MTT assay. *G. bimaculatus* water extract did not exert cytotoxicity at concentrations between 31.25 and 1,000 µg/mL (Fig. 1).

Effect of *G. bimaculatus* water extract on proinflammatory cytokine levels

The production of cytokines (TNF- α , IL-6, and IL-1 β) was highly increased in the groups treated with 250 and 500 µg/mL *G. bimaculatus* water extract compared with that in the control group. The production of TNF- α , IL-6, and IL-1 β significantly increased in a dose-dependent manner with the *G. bimaculatus* water extract concentration in the RAW 264.7 cells (Fig. 2). Therefore, *G. bimaculatus* water extract can induce macrophage activation through cytokine production and can show immunological activity.

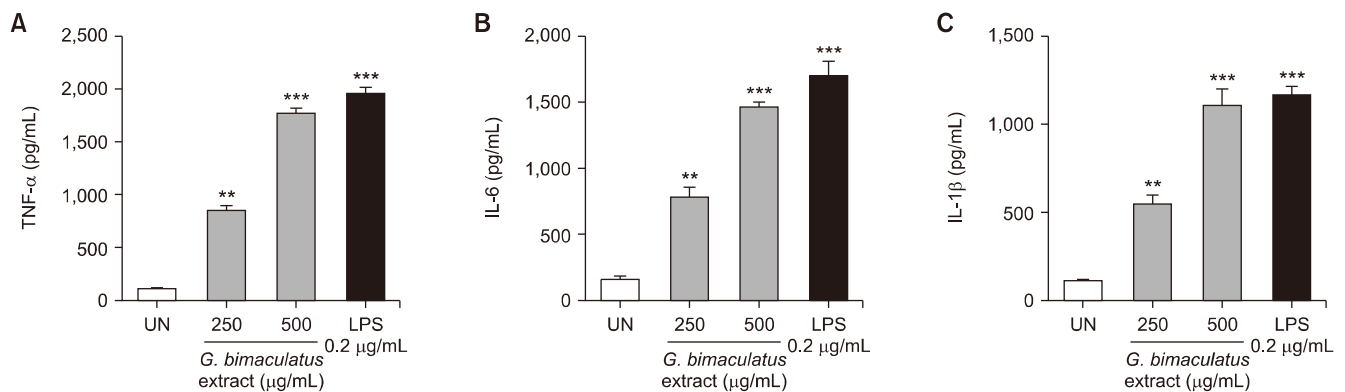


Fig. 2. Cytokine (TNF- α , IL-6, and IL-1 β) production in RAW 264.7 cells treated with *Gryllus bimaculatus* water extract. The levels of TNF- α (A), IL-6 (B), and IL-1 β (C) were quantified using enzyme-linked immunosorbent assay (ELISA). *G. bimaculatus* water extract was applied at concentrations of 250 and 500 µg/mL. After 24 h, cytokine production in the culture supernatant was measured using ELISA. Values are presented as mean±SD (n=3). One-way analysis of variance followed by Tukey's multiple comparison test was performed to compare differences among multiple groups. ** P <0.01 and *** P <0.001 were considered statistically significant. TNF, tumor necrosis factor; UN, untreated group; LPS, lipopolysaccharide; IL, interleukin.

Effect of *G. bimaculatus* water extract on NO, cyclooxygenase (COX)-2, and inducible NO synthase (iNOS) levels

There are two types of COXs: COX-1 and COX-2. COX-2 is an inducible isoform that shares features with iNOS. The product of iNOS catalysis, NO, is an important regulator of COX-2 expression and can also influence iNOS expression (Pérez-Sala and Lamas, 2001). RAW 264.7 cells were treated with *G. bimaculatus* water extract at concentrations of 250 and 500 µg/mL. LPS was also treated at a concentration of 0.2 µg/mL as a specific mitogen for macrophage cells. After 12 h, NO production in the culture supernatant was analyzed using the Griess assay. The COX-2 and iNOS levels in cell lysate were determined using western blot (Fig. 3). NO production (13.26 µM) was highly increased in the group treated with 500 µg/mL of *G. bimaculatus* water extract compared with that in the control group (5.05 µM) (Fig. 4). NO production and iNOS and COX-2 protein levels increased in a dose-dependent manner in RAW 264.7 cells after treatment with *G. bimaculatus* water extract.

Effect of *G. bimaculatus* water extract on the mitogen-activated protein kinases (MAPKs) pathway and NF- κ B signaling

MAPKs are serine and threonine protein kinases that can modulate cellular processes, such as apoptosis and immune defense. The proliferation of MAPKs is related to immune responses from the initiation phase of innate immunity (Dong et al., 2002). The immune response is one of several critical functions regulated by MAPKs, with the production of immunomodulatory cytokines, such as TNF- α and interleukin, induced by the activation of the p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) pathways (Arthur and Ley, 2013). *G. bimaculatus* water extract was applied at a concentration of 250 and 500 µg/mL for 30 min. The

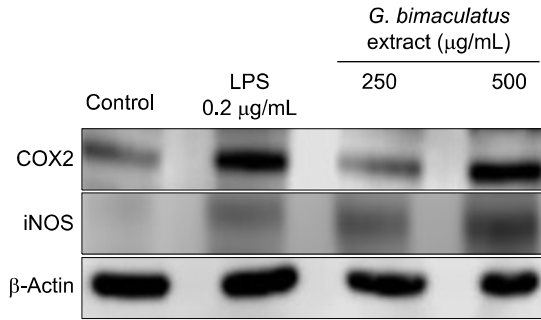


Fig. 3. Effect of *Gryllus bimaculatus* water extract on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 protein levels in RAW 264.7 cells. RAW 264.7 cells were treated with lipopolysaccharide (LPS, 0.2 µg/mL) only and with different concentrations (250 and 500 µg/mL) of *G. bimaculatus* water extract for 12 h and lysed for western blot analysis.

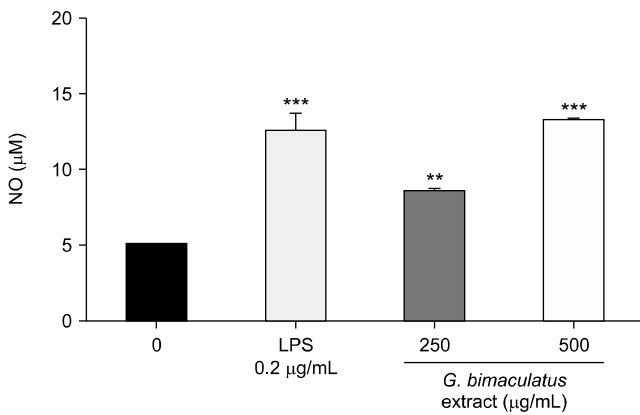


Fig. 4. Nitric oxide (NO) production in RAW 264.7 cells. Cells were treated with *Gryllus bimaculatus* water extract at concentrations of 250 and 500 µg/mL. Lipopolysaccharide (LPS) was used as a specific mitogen to RAW 264.7 cells at a concentration of 0.2 µg/mL. NO production in culture supernatant was estimated using the Griess assay. Values are presented as mean ± SD (n=3). One-way analysis of variance followed by Tukey's multiple comparison test was performed to compare differences among multiple groups. ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant.

phosphorylation level of phospho-JNK, phospho-ERK, and phospho-p38 increased following treatment. Therefore, *G. bimaculatus* water extract can induce macrophage activation through induction of MAPKs signaling (Fig. 5A).

NF-κB is a eukaryotic transcription factor that is activated in response to stimulations that induce cell growth, differentiation, apoptosis, and other events. NF-κB is involved in the regulation of immune responses and plays a key regulatory function in innate and adaptive immune responses (Dorrington and Fraser, 2019). The activated NF-κB signaling pathway is based on the degradation of IκB inhibitors. IκB is phosphorylated and degraded, which leads to the nuclear translocation of NF-κB and initiation of target gene transcription. Degradation of the IκB transcript exposes the nuclear localization signal of the NF-κB protein, leading to its nuclear translocation and binding to enhancers or promoters of target genes (Silverman and Maniatis, 2001). *G. bimaculatus* water extract was applied at a concentration of 250 and 500 µg/mL for 3 h. Treatment with LPS (0.2 µg/mL) served as a positive control. The level of NF-κB in nuclear fractions was determined using western blot analysis. Lamin B1 was used as a loading control for the nuclear fractions. The phosphorylation of NF-κB increased following treatment with *G. bimaculatus* water extract. The level of phospho-IκBα increased with treatment, whereas that of IκBα decreased in a dose-dependent manner. Thus, *G. bimaculatus* water extract can induce macrophage activation through induction of the phosphorylation of NF-κB signaling (Fig. 5B and 5C). Eventually, the innate immune system mounts a response, and the host produces cytokines and other mediators to defend itself. It has been observed that cellular signaling mechanisms in innate immunity provide an immediate reaction to utilize the NF-κB system at the heart of this first-step defense response (Hatada et al., 2000).

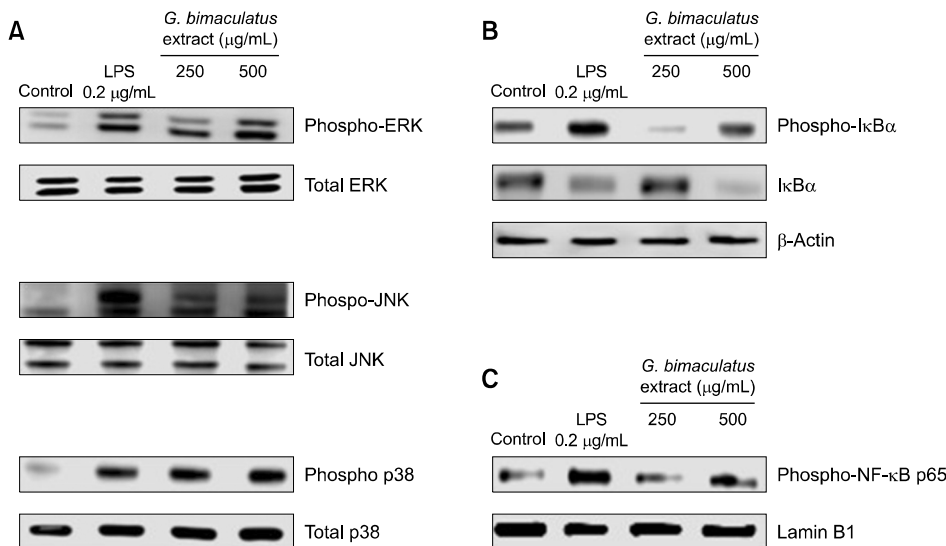


Fig. 5. Effect of *Gryllus bimaculatus* water extract on mitogen-activated protein kinase (MAPK) phosphorylation (A), phospho-IκBα, IκBα (B), and phospho nuclear factor (NF)-κB p65 (C) levels in RAW 264.7 cells. Cells were treated with lipopolysaccharide (LPS, 0.2 µg/mL) only and with *G. bimaculatus* extract at concentrations of 250 and 500 µg/mL for 30 min (A) and 3 h (B and C). Cell lysates were subjected to western blot analysis using specific MAPK and NF-κB signaling pathway antibodies. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

Effect of *G. bimaculatus* water extract on phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway expression

The PI3K family controls several cellular responses, including cell growth, proliferation, differentiation, survival, and cytoskeletal remodeling, and the transport of intracellular organelles in several types of cells. PI3K has important functions in the immune system, including immune cell development, differentiation, and function (Koyasu, 2003; Okkenhaug, 2013). Protein kinase B, also known as AKT, is a serine/threonine-specific protein kinase which plays an important role in multiple cellular processes, such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. PI3K activation leads to the phosphorylation and activation of AKT, localizing it in the plasma membrane. *G. bimaculatus* water extract was applied at a concentration of 250 and 500 $\mu\text{g}/\text{mL}$ for 24 h. LPS treatment (0.2 $\mu\text{g}/\text{mL}$) served as a positive control. The levels of phospho-PI3K and phospho-AKT in cell lysate were determined using western blot analysis. The levels of phospho-PI3K and phospho-AKT in macrophages treated with various concentrations

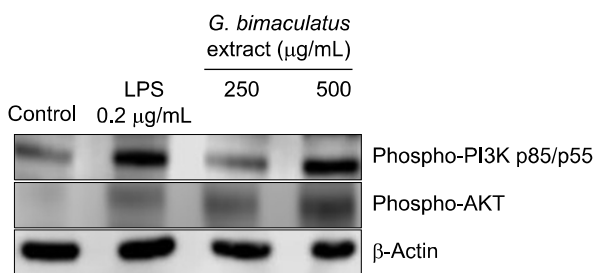


Fig. 6. Effect of *Gryllus bimaculatus* water extract on the levels of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway proteins in RAW 264.7 cells. RAW 264.7 cells were treated with lipopolysaccharide (LPS, 0.2 $\mu\text{g}/\text{mL}$) only and with a concentrations of 250 and 500 $\mu\text{g}/\text{mL}$ *G. bimaculatus* water extract for 24 h and lysed for western blot analysis. After 24 h, the cell lysates were subjected to western blot analysis using PI3K/AKT pathway antibodies (phospho-PI3K p85/p55, phospho-AKT).

of extract differed from that in the control group. *G. bimaculatus* water extract can induce phospho-PI3K and phospho-AKT activation (Fig. 6). The level of phospho-PI3K and phospho-AKT was increased by water extract treatment in the high-dose group (500 $\mu\text{g}/\text{mL}$).

Effect of *G. bimaculatus* water extract on CD80 and CD86

CD was used for the identification and investigation of cell surface molecules, providing targets for the immunophenotyping of cells (Chan et al., 1988). CD molecules serve as important cell receptors or ligands. CD80, which belongs to the immunoglobulin superfamily, possesses an extracellular immunoglobulin constant-like domain and a variable-like domain required for receptor binding. It is closely related to CD86, binding to the same receptors on primed T cells (Ho et al., 2009). CD80 is found on the surface of various immune cells, such as dendritic cells, B cells, monocytes, and antigen-presenting cells (APCs). CD86 is a protein expressed on APCs that provides costimulatory signals necessary for T cell activation and survival. The cells were treated with *G. bimaculatus* water extract at a concentration of 250 and 500 $\mu\text{g}/\text{mL}$. RAW 264.7 cells were treated with LPS at a concentration of 0.2 $\mu\text{g}/\text{mL}$. *G. bimaculatus* water extract was found to induce macrophage activation by activating the expression of CD80 and CD86 (Fig. 7).

Effect of *G. bimaculatus* water extract on MHC I and MHC II

MHC class I and MHC class II are a set of genes that encode cell surface proteins that are essential for the acquired immune system to recognize foreign molecules. MHC molecules bind to antigens derived from pathogens and present them on the cell surface for recognition by T cells. The function of MHC I molecules is to display peptide fragments of proteins from within the cell to cytotoxic T cells. MHC II molecules are a class of major histocompatibility complex molecules that are normally pres-

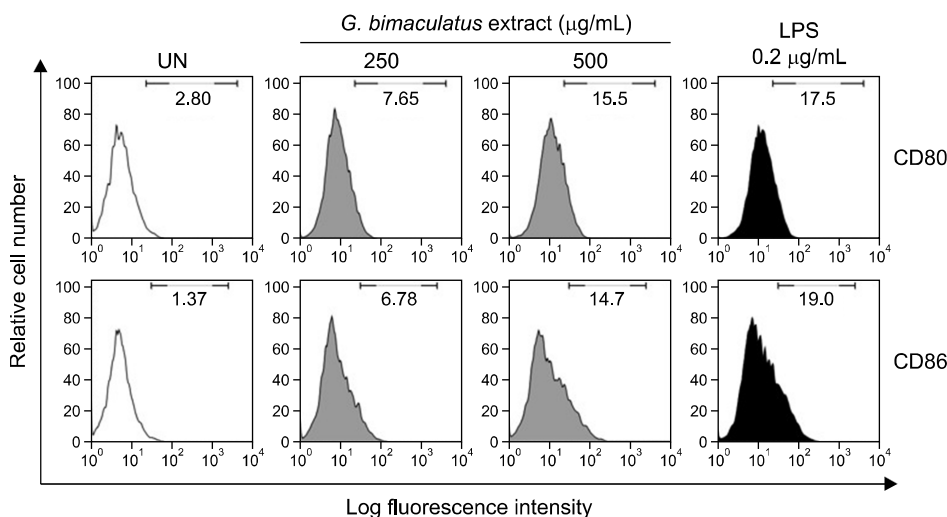


Fig. 7. Levels of cell surface molecules cluster of differentiation (CD) 80 and CD86 in RAW 264.7 cells. RAW 264.7 cells were treated with *Gryllus bimaculatus* water extract at concentrations of 250 and 500 $\mu\text{g}/\text{mL}$ and lipopolysaccharide (LPS) at a concentration of 0.2 $\mu\text{g}/\text{mL}$. After 24 h, cell surface marker levels were measured using flow cytometry using specific antibodies (n=3). UN, untreated group.

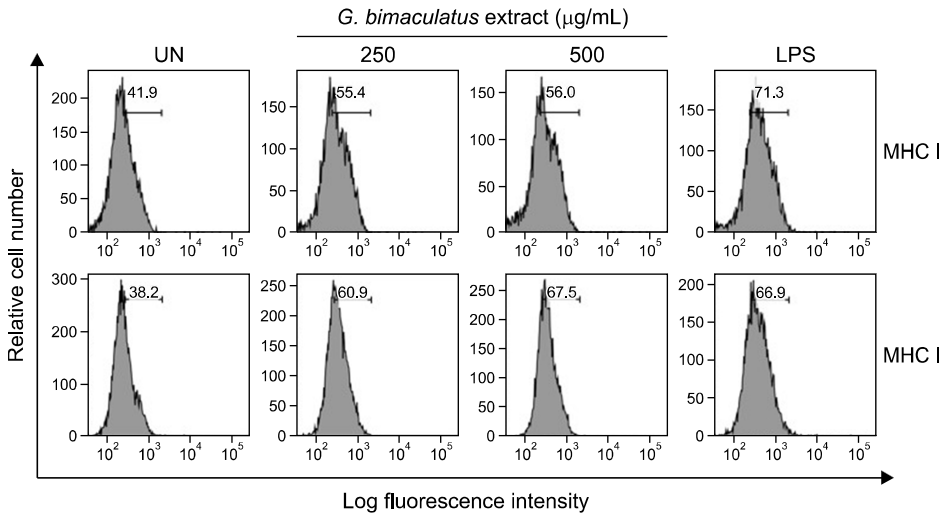


Fig. 8. Levels of cell surface molecules major histocompatibility complex (MHC) class I and II in RAW 264.7 cells. RAW 264.7 cells were treated with *Gryllus bimaculatus* water extract at concentrations of 250 and 500 µg/mL and lipopolysaccharide (LPS) at a concentration of 0.2 µg/mL. After 24 h, cell surface marker levels were measured using flow cytometry using specific antibodies (n=3).

ent only on professional APCs, including dendritic cells, mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells. These cells are important in initiating immune responses. The cells were treated with *G. bimaculatus* water extract at a concentration of 250 and 500 µg/mL. RAW 264.7 cells were treated with LPS at a concentration of 0.2 µg/mL. *G. bimaculatus* water extract was found to induce macrophage activation by activating the expression of MHC I and II molecules (Fig. 8).

Effect of *G. bimaculatus* water extract on cell viability in splenocytes

The word spleen is derived from the Ancient Greek word 'splén'. The spleen has important functions in red blood cells and the immune system. It removes old red blood cells and stores reserve blood. It synthesizes antibodies in its white pulp and removes antibody-coated bacteria and antibody-coated blood cells through the blood and lymph node circulation. Splenocytes are a splenic cell population that can be purified from splenic tissue. Splenocytes consist of many cell types, including T and B cells, dendritic cells, and macrophages, which have immune functions. *G. bimaculatus* water extract was applied to splenocytes at concentrations ranging from 62.5 to 1,000 µg/mL. *G. bimaculatus* water extract did not exert cytotoxicity at these concentrations (Fig. 9).

Effect of *G. bimaculatus* water extract on IFN-γ and IL-2 production

IL-2 is a cytokine that has an essential role in the immune system, primarily via its direct effects on T cells. The main sources of IL-2 are activated CD4+ T cells and activated CD8+ T cells (Liao et al., 2011). IL-2 promotes the differentiation of T cells into effector T cells and memory T cells. IL-4 regulates humoral and adaptive immunity and induces differentiation of naive helper T cells to Th2 cells. It also decreases the production of Th1 cells,

macrophages, IFN-γ, and dendritic cell IL-12. IFN-γ, a type II interferon, is a cytokine that is important for innate and adaptive immunity. IFN-γ is an important activator of macrophages and an inducer of MHC II expression. Its importance in the immune system is derived from its ability to inhibit viral replication directly as well as its immunostimulatory and immunomodulatory effects. IFN-γ is secreted by Th1 cells and has immunoregulatory properties (Schroder et al., 2004). In this study, *G. bimaculatus* water extract was applied at a concentration of 250 and 500 µg/mL, and Concanavalin A was used as a positive control at a concentration of 1 µg/mL. The levels of IL-2 and IFN-γ were increased compared with those in the control group at 250 and 500 µg/mL of water extract in a dose-dependent manner (Fig. 10). Treatment with *G. bimaculatus* water extract increased the production of IL-2 and IFN-γ but did not affect the production of IL-4 in splenocytes.

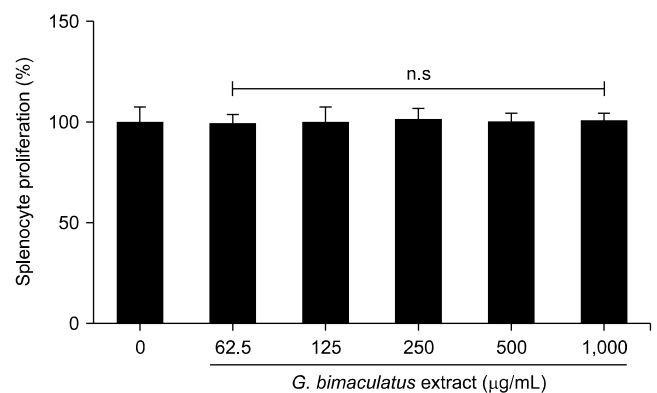


Fig. 9. Cell viability of splenocytes separated from mouse spleens using the WST-1 assay. Cells were treated with the indicated concentrations (62.5, 125, 250, 500, and 1,000 µg/mL) of *Gryllus bimaculatus* water extract for 24 h. After 24 h, cell viability was measured using the MTT assay. Values are presented as mean ± SD (n=3). MTT, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide; n.s., not significant.

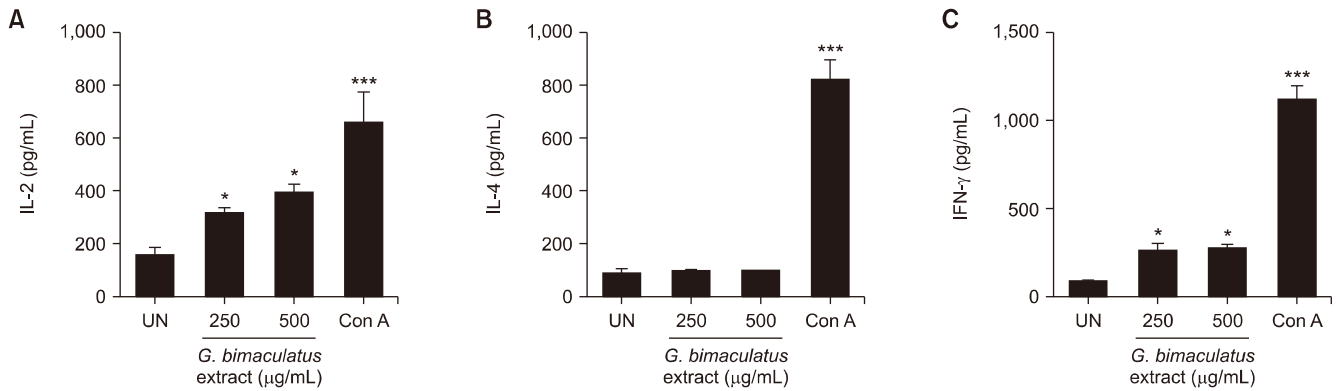


Fig. 10. Levels of cytokines (IL-2, IL-4, and IFN- γ) in splenocytes separated from mouse spleens after treatment with *Gryllus bimaculatus* water extract. The levels of IL-2 (A), IL-4 (B), and IFN- γ (C) were quantified using enzyme-linked immunosorbent assay. *G. bimaculatus* water extract was applied at concentrations of 250 and 500 $\mu\text{g/mL}$. Concanavalin A (Con A) was used as a specific mitogen to splenic T cells. One-way analysis of variance followed by Tukey's multiple comparison test was performed to compare differences among multiple groups. * $P < 0.05$ and *** $P < 0.001$ were considered statistically significant. UN, untreated group; IL, interleukin; IFN, interferon.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: EHB. Analysis and interpretation: HJL. Data collection: HJL and JMH. Writing the article: HJL. Critical revision of the article: HJL. Final approval of the article: all authors. Statistical analysis: HJL and JMH. Obtained funding: EHB. Overall responsibility: EHB.

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