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Evaluation of Anti-Cancer Activity of *Gryllus bimaculatus* Water Extract on Non-Small Cancer Lung Cell via Apoptosis

Hyun Jung Lim* **and Eui-Hong Byun**

Department of Food Science and Technology, Kongju National University, Chungnam 32439, Korea

ABSTRACT: *Gryllus bimaculatus*, traditionally used in oriental medicine, demonstrates functional and pharmacological potential through demonstrating immunomodulatory, hepato-protective properties, anti-inflammatory, antioxidant, and neuroprotective effects. In this study, we examined the effect of *G. bimaculatus* on cell proliferation and apoptosis in lung cancer cells. This is the first study to examine the anti-cancer effects of *G. bimaculatus* extracts on non-small cell lung cancer. Frozen *G. bimaculatus* was obtained, homogenized, and dissolved in distilled water. Using a freeze dryer, samples were concentrated until almost all the water was removed, and extracts were diluted in solutions of phosphate buffered saline. Anti-cancer effects of extracts on human non-small cancer lung cells were estimated based on cell cytotoxicity, western blot, and flow cytometry, using lipopolysaccharides as a positive control. H460 and A549 human non-small cell cancer lung cells were treated with *G. bimaculatus* water extracts of various concentrations, with lipopolysaccharide used as a positive control. The results showed that treatment with the extract for 24 or 48 h inhibited H460 proliferation, demonstrated by reduced cell numbers with morphological changes. Furthermore, flow cytometry analysis demonstrated that the extracts induced cell death on H460. However, extracts did not show cytotoxic effects on A549 cells. In conclusion, the extract induced apoptosis of lung cancer cells, possibly via caspase, Bcl-2 family signaling pathways. Therefore, *G. bimaculatus* water extracts are safe and efficient natural materials that may have great potential in the treatment of lung cancer.

Keywords: anti-cancer effect, apoptosis, edible insect, *Gryllus bimaculatus*, lung cancer

INTRODUCTION

Edible insects are commonly consumed worldwide due to their size, availability, and nutritional benefit. As an alternative source of animal proteins, edible insects can help solve food shortage problems (Rumpold and Schlüter, 2015; van Huis et al., 2015). Edible crickets have been approved as a food ingredient by the Korea Ministry of Food and Drug Safety because of their high contents of chitin, amino acids, and unsaturated fatty acids (Lee et al., 2016).

Gryllus bimaculatus is a species of cricket in the subfamily Gryllinae. It is also known as the African and Mediterranean field cricket or as the two-spotted cricket, and is one of the most abundant cricket species, inhabiting the tropical and subtropical regions of Asia, Africa, and Europe (Lee and Kwon, 2013). *G. bimaculatus* can be discriminated from other *Gryllus* species by two dot-like marks on the base of its wings. *G. bimaculatus* can be easily bread in laboratories where it is widely used in insect research, and is mass produced as feed for insectivorous animals in confined facilities such as zoos (Mito and Noji, 2008). *G. bimaculatus* are easy to breed and raise since they do not require prolonged exposure to cold to complete their life cycle. *G. bimaculatus* has a good nutritional content of fat, protein, polyunsaturated fatty acid, mineral, and fiber, and is high in essential amino acids including lysine, leucine, valine, and isoleucine (Belluco et al., 2013). *G. bimaculatus* also contains high concentrations of fatty acids especially unsaturated fatty acids (68.6%), such as linoleic and oleic acid.

G. bimaculatus has traditionally been used in oriental medicine. Efforts have been made to investigate the functional and pharmacological potential of *G. bimaculatus*, including its immunomodulatory (Seo et al., 2004), hepato-protective (Im et al., 2018), anti-diabetic (Cho et al., 2019), anti-inflammatory (Ahn et al., 2014; Hwang et al., 2019), antioxidant (Jang and Kim, 2021), and neuropro-

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Correspondence to Eui-Hong Byun , Tel: +82-41-330-1481, E-mail: ehbyun80@kongju.ac.kr
*Current Address: Institute of Fisheries Sciences, Pukyong National University, Busan 46041, Department of Liberal Arts, Gyeongbuk Provincial College, Gyeongbuk 36830, Korea
Author information: Hyun Jung Lim (Instructor), Eui-Hong Byun (Professor)

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tective effects (Boonsin et al., 2018), and its protective effect on the skin (Jeong et al., 2020). It is commonly recognized that the pathogenesis of human cancer is correlated with accumulation of multiple abnormalities over a long period of time.

Lung cancer is one of the serious types of cancer and is leading cause of cancer-related motility in both men and women. With an increasing number of smokers and environmental pollutants, the incidence of lung cancer has increased in recent years. Approximately 1.6 million people died from lung cancer in 2012, accounting for around 19% of all cancer deaths globally (Liu et al., 2017). Lung cancer motility rates exceed the sum of colon, breast, and prostate mortality rates (Siegel et al., 2016). Symptoms of lung cancer in the early stages are not always evident, and most patients with lung cancer are diagnosed in advanced stages, which often prevents effective treatment. Because lung cancer has a poor prognosis, over 85% of patients with lung cancer die from disease during the first 5 years (Panov, 2005). At present, the clinical treatment for lung cancer is mainly radiochemotherapy and surgery. However, effective treatment options are lacking. Therefore, it is important to develop novel treatments for lung cancer (Gu et al., 2018). However, research on the cytotoxic effect of lung cancer is limited, and there is limited research of *G. bimaculatus* in cancer. A foremost theory 'the inflammatory hypothesis' hypothesizes that inflammation is associated with the development and progression of cancer (Singh et al., 2019). Inflammation can cause immunosuppression, thereby providing tumors with growth factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), which are mediators of angiogenesis and up-regulate oncogene expression (Carmeliet, 2005). Glycosaminoglycan (GAG), a component of *G. bimaculatus*, has anti-inflammatory effects (Hwang et al., 2019), such as the ability to reduce levels of inflammatory cytokines (Park and Han, 2021) including VEGF (Ahn et al., 2014). Therefore, GAG from *G. bimaculatus* may play a central role in modulating cell signaling through FGF and VEGF, thereby inhibiting anti-cancer effects. In this study, we examined the effect of *G. bimaculatus* water extracts on proliferation and apoptosis of lung cancer cells, and explored the possible mechanism of action. This is a first study to examine the anti-cancer effects of *G. bimaculatus* extract on non-small cell lung cancer cells.

MATERIALS AND METHODS

Extraction of *G. bimaculatus*

Frozen *G. bimaculatus* was obtained from SWorm (Cheonan, Korea), homogenized in a kitchen blender and dissolved in distilled water. Homogenized samples were sonicated 3 times using a sonicator for 60 min. The juice was filtered with Whatman paper, and the filtrate was evaporated at 40°C under reduced pressure until the solvent was removed. Using a freeze dryer, samples were concentrated until most of the water was removed, and samples were collected in empty tubes and stored at −80°C in a defreezer. All extracts and substances were diluted in phosphate buffered saline solution before use.

Cell lines and culture condition

H460 and A549 cells, both human non-small cell lung carcinoma (NSCLC) cell lines, were purchased from the Korean cell line bank. H460 and A549 cells were maintained in Roswell Park Memorial Institute 1640 containing 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) under humidified conditions of 37°C and 5% CO_2 in an incubator.

Cell viability assays

H460 and A549 cells were grown in 96-well plates at a density of 1×10^4 cells per well. Cells were allowed to attach for 24 h, then were exposed to *G. bimaculatus* water extracts. At the end of the treatment period, 10 μL of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/mL) was added to each well. After 3 h incubation at 37°C, supernatants were aspirated and formazan crystals were dissolved in 100 μL 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.

Flow cytometry analysis

H460 cells were plated in complete medium in 24-well plates at a density of 1.5×10^5 cells/well and treated with *G. bimaculatus* water extracts for 24 or 48 h. Cells were harvested with trypsin, washed with phosphate buffered salines (PBS), and resuspended in binding buffer. Single-cell suspensions were incubated with annexin V-fluorescein isothiocyanate (FITC) for 30 min in the dark at room temperature. Propidium iodide (PI) was then added and cells were analyzed by flow cytometry (FACSort; Becton Dickinson, Rutherford, NJ, USA).

Western blot analysis

Cells were harvested and washed twice in PBS. For Western blot analysis, total proteins were prepared using PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Inc., Seongnam, Korea), and quantified using protein assay reagents. Extracted proteins were denatured by boiling in sample buffer at 100°C for 5 min, and electrophoresed using 8% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Gels were transferred onto polyvinylidene fluoride membranes at 100 V for 60 min in transfer buffer and membranes were incu-

bated with 5% non-fat dry milk in Tris-buffered saline (TBS) buffer at room temperature for 1 h to block non-specific responses. Next, membranes were incubated with specific primary antibodies at 4°C overnight, washed for 1 h with TBS buffer, incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 30 min at room temperature, and washed again with TBS buffer for 1 h. Proteins were detected using enhanced chemiluminescence western blotting detection reagents.

Statistical analysis

Means and standard deviations were calculated using Graphpad Prism version 5 program (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Turkey's multiple comparison tests were used to compare differences between multiple groups. $P<0.05$, $P<0.01$, and $P<0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of *G. bimaculatus* water extracts on cell viability of H460 cells

The H460 cell line was derived by Gazdar and associates in 1982 from the pleural fluid of a patient with large-cell lung cancer (Carney et al., 1985). H460 is a human large-cell lung carcinoma line containing mutant K-Ras and wild-type p53 (Mitsudomi et al., 1991). Cells were treated with *G. bimaculatus* water extracts concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL or lipopolysaccharide (LPS) at 0.2 μg/mL as a positive control (Fig. 1). Recently, gold nanoparticles and LPS were shown to be cytotoxic and to induce apoptosis MCF-7 breast cancer cells (Sameen et al., 2020). Therefore, LPS is a potential anti-cancer agent with the ability to induce apoptosis (Morishima and Inagawa, 2016), and can decrease viability of adenocarcinoma and squamous esophageal carcinoma cells (Gonçalves et al., 2016), contributing to tumor death.

After treatment with *G. bimaculatus* extracts or LPS for 24 or 48 h, cell proliferation was measured using MTT assays. Treatment with *G. bimaculatus* water extracts for 24 or 48 h inhibited H460 proliferation in a dose-dependent manner; inhibition was greater at 48 h than 24 h. Moreover, *G. bimaculatus* water extracts induced H460 cell apoptosis. Various concentrations of the extract reduced numbers of live H460 cell and induced morphology changes at 24 h. Morphology changes included rounding, detachment and cell shrinking, which are distinct morphological characteristics related with apoptotic cells (data are not shown). Thus, *G. bimaculatus* water extracts demonstrated cytotoxicity in H460 cells and induced apoptosis.

Effect of *G. bimaculatus* water extracts on cell viability of A549 cells

A549 cells are adenocarcinomic human alveolar basal epithelial cells obtained through removing and culturing cancerous lung tissue from the explanted tumor of a 58-year-old Caucasian male (Giard et al., 1973). A549 is a type II pulmonary epithelial cell model of lung cancer

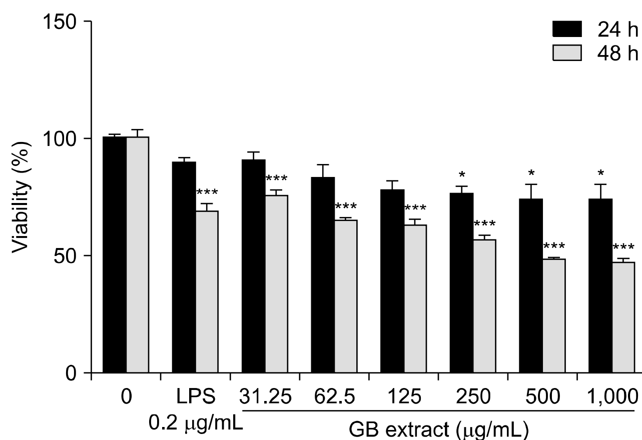

Fig. 1. Cytotoxic effects of *Gryllus bimaculatus* (GB) water extracts on H460 human lung cancer cells. Cells were treated with various concentrations of GB water extracts (31.25, 62.5, 125, 250, 500, and 1,000 μg/mL) for 24 or 48 h, and cell cytotoxicity was assessed using MTT assays. Results are expressed as mean±SD (n=3). One-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test was used to compare differences between multiple groups. Data were considered significantly different at *$P<0.05$ and ***$P<0.001$ vs. non-treated controls. MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide.

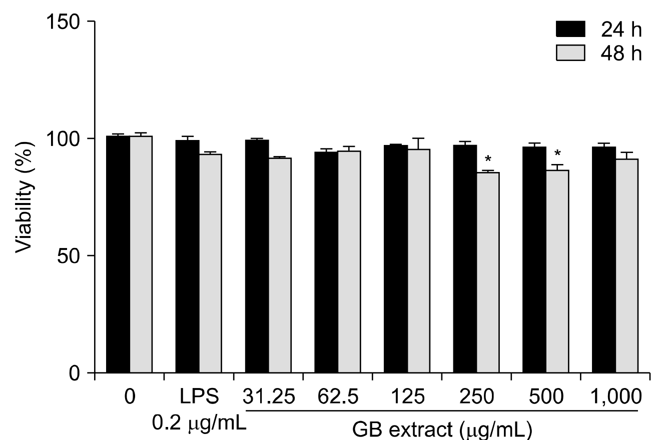

Fig. 2. Cytotoxic effect of *Gryllus bimaculatus* (GB) water extracts on A549 human lung cancer cells. Cells were treated with various concentrations of GB water extracts (31.25, 62.5, 125, 250, 500, and 1,000 μg/mL) for 24 or 48 h, and cell cytotoxicity was assessed using MTT assay. Results are expressed as mean±SD (n=3). One-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test was used to compare differences between multiple groups. Data were considered significantly different at *$P<0.05$ vs. non-treated controls. MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide.

and is widely used in the development of drug therapy for lung cancer (Foster et al., 1998). A549 cells were treated with *G. bimaculatus* water extracts at concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL or LPS at 0.2 μg/mL as a positive control. The results showed that treatment with *G. bimaculatus* water extracts for 24 or 48 h did not inhibit A549 cell proliferation or induce apoptosis. Thus, *G. bimaculatus* water extract did not show a cytotoxicity event in A549 cells (Fig. 2).

Effect of *G. bimaculatus* water extracts on cell death of H460 cells

Cell death is an important regulatory cellular mechanism. H460 cell death was induced by *G. bimaculatus* water extracts (Fig. 3) and assessed by annexin V-FITC/PI staining. Cell death was detected by annexin V-FITC/PI with 500 μg/mL of *G. bimaculatus* water extracts. These data support our findings that *G. bimaculatus* water extract may demonstrate anti-cancer effect in human NSCLC cells, without inducing cell death in healthy cells (data not shown).

Effect of *G. bimaculatus* water extracts on caspase and poly(ADP-ribose) polymerase (PARP) pathways in H460 cells

Cells undergo apoptosis due to extracellular signals, 'death factors', or by internal physical insults such as DNA damage or oxidative stress. Subsequently, two non-exclusive molecular pathways, the extrinsic and the intrinsic pathways, may be activated. Evading apoptosis is a hallmark of human cancer cells. Apoptotic cell death is induced by caspases and several regulatory factors with cancer formation resulting from insufficient caspase activation causing malfunction of apoptosis (Olsson and Zhivotovsky, 2011). Caspases are synthesized as inactive enzymes called procaspases, which are mostly considered inactive. Since caspases cleave at aspartate residues and procaspases are activated by cleavage at aspartate residues, caspases participate in a cascade of activation whereby one caspase activate other caspases in chain reactions. Therefore, regulation of apoptosis is controlled by both initiator and effector caspases. Effector caspases are produced in cells through proteolytic processing by initiator caspases. Caspase 8 is a known initiator caspase and is a key to the extrinsic pathway. Procaspase 8 molecules become activated by self-cleavage since procaspases have low enzymatic activity. This initiates a cascade of caspase activation, with the activated caspase activating executioner caspases. This cascade leads to cleavage of specific protein targets and results in apoptosis (Krelin et al., 2008). Caspase 9 is an initiator caspase key to the intrinsic pathway that is activated by aggregation. Caspase 9 begins a caspase cascade by cleaving and activating downstream caspases 3, 6, and 7. Activation of caspase 3 induces downstream cleavage of cytoplasmic or nuclear substrates, including PARP (Brauns et al., 2005), a marker of apoptosis. These downstream cleavage events show morphological features of apoptosis (Huppertz et al., 1999). PARP is a family of proteins involved in many cellular processes including DNA repair, apoptosis, genomic stability, and is inactivated by caspase cleavage.

Following treatment with *G. bimaculatus* water extracts (250 or 500 μg/mL) or LPS (0.2 μg/mL) for 24 h and suspension in a lysis buffer, expression of cleaved and uncleaved caspases 3, 8, and 9, and PARP in H460 cells were evaluated by western blot analysis. Extracts decreased levels of caspases 3, 8, and 9. With increased extract concentrations, expression levels of cleaved caspase were also significantly increased. However, *G. bimaculatus* water extracts did not affect expression of PARP, which

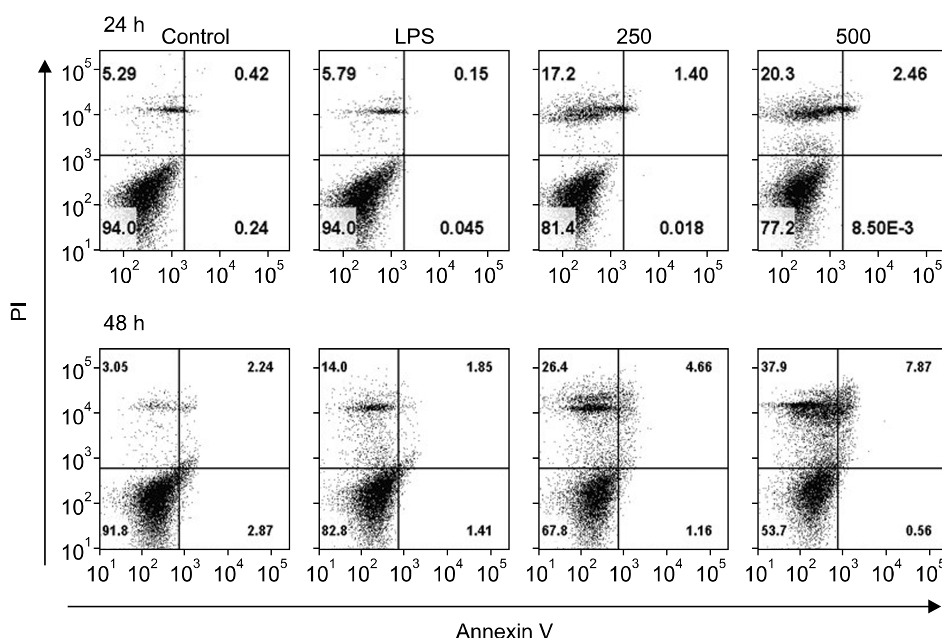

Fig. 3. Cell death induction by *Gryllus bimaculatus* water extracts. H460 cells were treated with lipopolysaccharide (LPS) (100 ng/mL) or *G. bimaculatus* water extracts (250 and 500 μg/mL) for 24 or 48 h. Cell death was determined using annexin V-fluorescein isothiocyanate and propidium iodide (PI) staining and detected by flow cytometry.

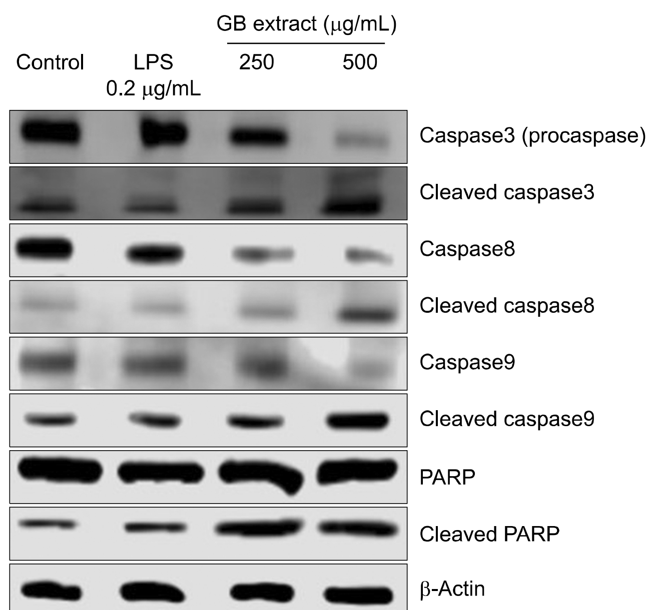

Fig. 4. Effects of *Gryllus bimaculatus* (GB) water extracts on protein expression of various caspases and poly(ADP-ribose) polymerase (PARP) in H460 human lung cancer cells. Cells were treated with lipopolysaccharide (LPS) (0.2 μg/mL) or GB water extracts (250 and 500 μg/mL) for 24 h, and lysed to extract proteins. Protein expression of various caspases and PARP was determined using western blot.

is specifically cleaved during the induction of apoptosis. These data suggests that *G. bimaculatus* water extracts induce apoptosis via caspase pathways in H460 human lung cancer cells (Fig. 4).

Effect of *G. bimaculatus* water extracts on B-cell lymphoma 2 (Bcl-2) family pathways in H460 cells

The two main pathways for apoptosis are the extrinsic receptor-mediated and intrinsic mitochondrial pathways. The Bcl-2 family of proteins modulates apoptosis in mitochondria, and includes proteins that either promote or inhibit apoptosis. Bcl-2 proteins control apoptosis by governing mitochondrial outer membrane permeabilization, which is a key step in the intrinsic pathway of apoptosis (Youle and Strasser, 2008). The ratio of pro- to anti-apoptotic subfamily members existing in cells can be altered by signaling pathways, dependent on cellular stress such as nutrients, DNA damage, and protein processing (Hetz and Glimcher, 2008). Once the executioners are activated, molecules come together to form pores in the outer mitochondrial membrane and stimulate mitochondrial outer membrane permeability (Nechushtan et al., 2001). Some Bcl-2 family protein can release cytochrome c into the cytosol, which activates caspases 9 and 3, leading to apoptosis. The Bcl-2 family are characterized by Bcl-2 homology domains (Warren et al., 2019), which facilitate the interactions and their pro- or anti-apoptotic functions.

To elucidate the molecular pathways responsible for apoptotic effects on cancer cells, we analyzed the induction of apoptotic proteins. Cells were treated with *G. bimaculatus* water extracts at 250 or 500 μg/mL for 24 h and the levels of Bcl-2 family proteins were analyzed by western blot. Expression of Bax was increased in a dose-dependent manner and expression of Bcl-2 was decreased in H460 cells following treatment. Furthermore, expression of Bid did not differ from that of the control group. These data suggest that *G. bimaculatus* water extracts prevent induction of apoptosis by the Bcl-2 family pathway in H460 human lung cancer cells (Fig. 5).

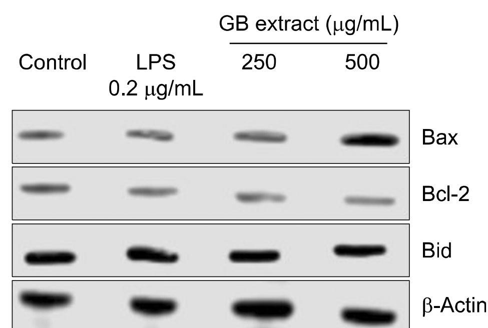

Fig. 5. Effects of *Gryllus bimaculatus* (GB) water extracts on expression of Bcl-2 family proteins in H460 human lung cancer cells. Cells were treated with lipopolysaccharide (LPS) (0.2 μg/mL) or GB water extracts (250 and 500 μg/mL) for 24 h, and lysed to extract proteins. Expression of various Bcl-2 proteins was determined using western blot.

To conclude, we showed that *G. bimaculatus* water extracts are safe and efficient natural materials that may have great potential in the treatment of lung cancer. *G. bimaculatus* water extracts induced apoptosis of lung cancer cells, and may be function via caspase and Bcl-2 family signaling pathways. However, the specific molecular mechanism needs to be further studied. This study was performed to explore novel and valuable uses of insect resources, important sources of natural compounds used in bio-industries. The results may be fundamental for further research into the use of different insects as natural resources.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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