# *Gryllus bimaculatus* extract protects against lipopolysaccharide and palmitate-induced production of proinflammatory cytokines and inflammasome formation

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**Abstract.** Inflammation and the inflammasome complex formation are associated with numerous diseases, and palmitates or lipopolysaccharides (LPS) have been identified as potential links between these disorders. Recently, edible insects such as the *Gryllus bimaculatus* (GB) and the larva of *Tenebrio molitor* have emerged as alternative food sources. In the present study, the effect of GB on LPS- or palmitate-induced production of inflammatory cytokines, the formation of the inflammasome complex, reactive oxygen species (ROS) generation, endoplasmic reticulum (ER) stress and cell death was investigated in RAW264.7 cells. The results revealed that GB extract downregulated the production of inflammatory cytokines (such as TNF-α, IL-1β and IL-6). Since the role of the MAP kinase and NF-κB signalling pathways in the production of inflammatory cytokines is well established, the translocation of p65 into the nucleus and the phosphorylation of IκB and MAP kinases were further examined. Both these processes were upregulated following LPS and palmitate treatment, but they were inhibited by the GB extract. Moreover, GB extract decreased LPS/palmitate-induced inflammasome complex formation (assessed via analysing the levels of the apoptosis-associated speck-like protein containing a caspase-recruitment domain, NOD-like receptor family pyrin domain containing 3, cleaved caspase-1 and IL-1β), the generation of ROS, ER stress and cell death. Treatment with SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor) and pyrrolidinedithiocarbamate ammonium (an NF-κB inhibitor) decreased the production of inflammatory cytokines, as well as helped in the recovery of LPS/palmitate-induced cell death. Overall, GB extract served an inhibitory role in LPS/palmitate-induced inflammation via inhibiting the MAP kinase and NF-κB signalling pathways, inflammasome complex formation, ROS generation, ER stress and cell death.

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*Abbreviations:* ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; ER, endoplasmic reticulum; GB, Gryllus bimaculatus; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family pyrin domain containing 3; ROS, reactive oxygen species

*Key words:* GB, inflammation, MAP kinase, NF-κB, inflammasome, ER stress, ROS, cell death

## Introduction

Inflammation is induced in response to various stimuli such as palmitate and lipopolysaccharides (LPS). Palmitate, a C16 saturated fatty acid, plays an important role in low dose inflammation in obesity and many other metabolic diseases (1). LPS, an important component of the outer membrane of gram-negative bacteria, is the key immune activator against bacterial infection. LPS can bind to TLR4 (toll-like receptor 4) and activate the MAP kinase and NF-κB pathway, which can subsequently increase the expression of various pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and interferon-β. Even though palmitate is not a TLR4 agonist (2), it can activate TLR4, MAP kinase, and the NF-κB pathway (3). Furthermore, palmitate treatment not only increases the generation of reactive oxygen species (ROS) in the mitochondria, but also increases calcium release from the endoplasmic reticulum (ER) leading to mitochondrial dysfunction and cell death (1). LPS also has similar effects that induce ROS generation, mitochondrial dysfunction and cell death (4).

The inflammasome could be triggered by many signals such as potassium efflux, ROS generation in mitochondria, etc. (5). The NOD-like receptor pyrin domain containing 3 (NLRP3), the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and the pro-caspase-1 are assembled, leading to auto-activation of caspase-1 upon its cleavage which subsequently cleaves its substrates, IL-1β and IL-18 (5). IL-1β, a pro-inflammatory cytokine, plays an

important role in inflammatory responses while IL-18 activates inflammatory responses as well as protects against invading various microorganisms (6). The inflammasome complex formation is induced in response to various stimuli such as LPS (7), palmitate (8), and anthrax toxin (9); it is also formed in case of many inflammatory disease such as alcoholic hepatitis (10), liver injury (11), lung injury (12), and several cancers (13). Thus, formation of the inflammasome complex is a novel process involved in regulating many inflammatory responses.

*Gryllus bimaculatus* (GB) is an edible insect that can be used as an alternative protein source. Previously, GB extract has been shown to inhibit inflammation in several disease models such as that of chronic arthritis (14) and alcohol-induced steatohepatitis (15). Furthermore, GB has exhibited glucose lowering effect in streptozotocin-induced diabetic mice (16). Therefore, GB could be used as a functional food source for patients with chronic inflammation or metabolic diseases.

In this study, we evaluated the effects of GB extract on LPS or palmitate-induced production of pro-inflammatory cytokines, inflammasome formation, ER stress, ROS generation, and cell death.

## Materials and methods

*Materials*. The following materials were purchased as indicated: i) LPS, palmitate, SB203580, SP600125, PD98059, 2',7'-dichlorofluorescin diacetate (DCF-DA) and anti-α-tubulin antibody (T9026) from Sigma-Aldrich; Merck KGaA; ii) anti-p65 (8242), anti-phospho-IκB (2859), anti-Lamin B2 (13823), anti-phosphor-p38 (4511), anti-p38 (8690), anti-phospho-JNK (9255), anti-JNK (9252), anti-phospho-ERK (4370), anti-ERK (4695), anti-phospho-protein kinase RNA-like ER kinase (PERK; 3179), anti-PERK (3192), anti-phospho-eukaryotic initiation factor 2α (eif2α; 3597), anti-eif2α (5324), anti-caspase-3 (9664), anti-cleaved-PARP1 (5625) and NLRP3 (15101) antibodies from Cell Signalling Technology, Inc.; iii) anti-caspase-1 (sc-56036) and anti-ACS (sc-22514-R) antibodies from Santa Cruz Biotechnology, Inc.; iv) IL-1β antibody (NB600-633) from Novus Biologicals; v) pyrrolidinedithiocarbamate ammonium (PDTC) from Biovision; vi) Alexa-Fluor 488 antibody (A-11008) from Thermo Fisher Scientific, Inc.; vii) 4',6-Dia midino-2-phenylindole dihydrochloride (DAPI) from Merck (D9542); and viii) anti-mouse-HRP (horseradish peroxidase, 115-036-003) and anti-rabbit-HRP (111-035-003) antibodies from Jackson Laboratory.

*Gryllus bimaculatus extract*. GB extract was generated as per the previous study (15). Briefly, it was dried, ground, extracted overnight using 70% ethanol, and evaporated. Prepared samples were dissolved in PBS and frozen at -20˚C until further use.

*Fatty acid analysis*. GB were snap frozen at -40˚C by lyophilization for three days (Freezone 4.5; Labconco) and ground into fine powder. Heptane (1 ml), 2 ml methylation mixture (MeOH/benzene/dimethoxypropane/sulphuric acid in the ratio of 39:20:5:2, v/v), and 0.2 mg of pentadecanoic acid (internal standard) were added to the GB samples and shaken gently at 80˚C for 2 h (BioFree Co.). Then, the samples were cooled down to room temperature and the supernatant containing fatty acid methyl esters were centrifuged for 1 min. The fatty acid composition in the supernatant was determined using the Agilent 7890A (Agilent Technologies, Inc.) and DB-23 60 mm, 0.25 mm, 0.25 µm (Agilent Technologies, Inc.). The injection volume was 1 µl with 1:50 split mode. The flow rates were as follows: hydrogen flame gas, 35 ml/min; helium carrier gas, 35 ml/min, and mixed gas, 350 ml/min. The corresponding column oven temperatures were as follows: 50˚C, 1 min, 25˚C/min to 200˚C, 3˚C/min to 230˚C, and 18 min as previously described (17).

*In vitro LPS and palmitate treatment*. RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (HyClone). LPS (50 ng/ml) or 250 µM palmitate were used to induce inflammation as previously described (7,18,19) and 50 µg/ml LPS or 2 mM palmitate were used to induce cell death (20, 21).

*Enzyme-linked immunosorbent assay (ELISA)*. At 30 h, after pre-treatment of RAW264.7 cells with 100-200 µg/ml GB extract and another 18 h co-incubation with 50 ng/ml LPS or 250 µM palmitate, TNF-α, IL-1β and IL-6 levels in the culture medium were measured using ELISA kits (TNF-α, IL-1β and IL-6 Mouse ELISA kits; Komabiotech) as per manufacturer's instructions.

*Western blotting*. RAW264.7 cells were lysed using RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). The lysates were then incubated on ice for 30 min and centrifuged (10,000 x g, 10 min, 4˚C); protein levels in the supernatant were measured using the Protein Assay Dye Reagent (Bio-Rad Laboratories). Using SDS-PAGE, 50 µg proteins were separated on 8-15% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked using 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in TBST (TBS with 0.1% Tween-20) for 1 h and incubated with primary antibodies (1:1,000 dilution) overnight at 4˚C. Secondary antibodies were incubated for 1 h at room temperature. Protein bands were detected using the EzWestLumi Plus Reagents (ATTO Corporation) on the Chemidoc MP imaging system (Bio-Rad Laboratories).

*Separation of nuclear and cytoplasmic fractions*. The nuclear and cytoplasmic fractions were separated as previously described (22). Briefly, RAW264.7 cells were lysed using a fractionation buffer (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1% NP-40) containing protease and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). After 30 min incubation on ice, the lysates were centrifuged (4,000 x g, 5 min, 4˚C), and the pellet was further sonicated in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 12.5 mM glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM $Na_3VO_4$, 0.5%, and Triton X-100) containing protease and phosphatase inhibitors to obtain the nuclear fraction. The supernatant was centrifuged (12,500 x g, 5 min, 4˚C) to obtain the cytoplasmic fraction.

*Immunohistochemistry*. RAW264.7 cells were co-treated with GB extract (100 or 200 µg/ml) and palmitate (250 µM) or LPS

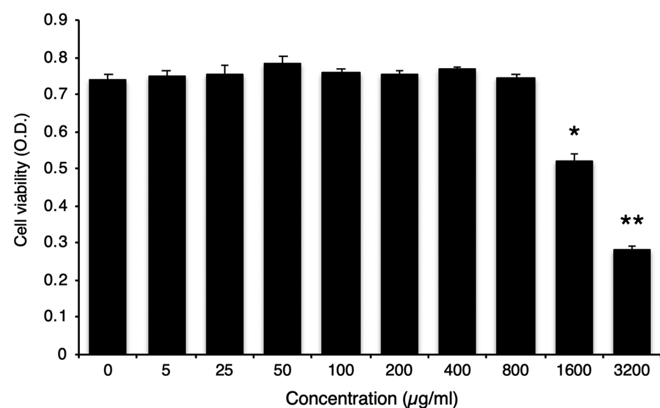


Figure 1. Effect of GB extract on cell viability. After treating the cells with various concentration of GB extract for 48 h, cell viability was examined. The values are expressed as the mean ± SEM (n=3). *P<0.05 and **P<0.01 vs. control (0 $\mu$g/ml). GB, *Gryllus bimaculatus*; OD, optical density.

(50 ng/ml) for 18 h, after which the cells were fixed with cold methanol for 10 min. Fixed cells were blocked with horse serum for 1 h and then incubated with p65 antibody (1:200) overnight at 4˚C. After washing, Alexa‑Fluor 488 secondary antibody (1:500) was incubated with the cells at room temperature in the dark for 1 h. DAPI was used to label nuclei for 10 min, and cells were mounted with vector shield mount media solution (Vector Laboratories). The p65 fluorescence and DAPI signals were viewed using a confocal microscope (LSM 710; Carl Zeiss).

*Reverse transcription‑quantitative PCR (RT‑qPCR).* RT‑qPCR was performed as previously described (23). Briefly, total mRNA was extracted from the RAW264.7 cells using the RNAiso Plus reagent (Takara) and cDNA was synthesized using the PrimeScript™ RT Reagent kit with gDNA Eraser (Takara). qPCR was performed using the SYBR® Premix Ex Taq™ II, ROX Plus (Takara) in a Bio‑Rad CFX96 System (Bio‑Rad). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (24). Primer sequences are listed in Table I.

*MTT assay.* Cell death was evaluated using the MTT (3‑(4,5,‑dimethylthiazol‑2,5‑diphenyl tetrazolium bromide) assay (25). RAW264.7 cells were seeded onto 96‑well plates at a density of $5x10^4$ cells/ well. After treatment with either chemicals (SB203580, SP600125, PDTC) or GB extract, the cells were treated with MTT solution (0.5 mg/ml final concentration) and further incubated for 4 h. The assay was terminated by adding 50 $\mu$l dimethyl sulfoxide (DMSO) to dissolve the purple formazan crystals. Solubilized formazan was quantified spectrophotometrically at 540 nm.

*Detection of ROS.* RAW264.7 cells were pre‑treated with GB extract followed by either LPS or palmitate treatment. To quantify ROS, DCF‑DA was added at a final concentration of 10 $\mu$M and incubated for 30 min in the dark. The fluorescent intensity of DCF was measured at 485 nm (excitation) and 535 nm (emission) using a confocal microscope (LSM 710).

*Statistical analysis.* All the experiments were repeated independently in triplicates and the data was expressed as mean ± standard error of the mean (SEM). Statistical significance was calculated using one‑way ANOVA followed by Tukey's post‑hoc test (GraphPad Prism 6.0; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Table I. Primers used for quantitative PCR.

| Gene | Primer sequences | (Ref.) |
|---|---|---|
| TNF-α (mouse) | F: 5'-CTGTAGCCCACGTCGTAGC-3'<br>R: 5'-TTGAGATCCATGCCGTTG-3' | (22) |
| IL-1β (mouse) | F: 5'-TGTAATGAAAGACGGCACACC-3'<br>R: 5'-TCTTCTTTGGGTATTGCTTGG-3' | (22) |
| IL-6 (mouse) | F: 5'-TCCAGTTGCCTTCTTGGGAC-3'<br>R: 5'-GTACTCCAGAAGACCAGAGG-3' | (22) |
| GAPDH (mouse) | F: 5'-CGACTTCAACAGCAACTCCCACTCTTCC-3'<br>R: 5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3' | (22) |

F, forward; R, reverse.

Table II. Fatty acid composition of the *Gryllus bimaculatus* extract.

| Fatty acid | Concentration (mg/g) |
|---|---|
| Caproic acid (hexanoic acid) | 0.376 |
| Lauric acid (dodecanoic acid) | 0.195 |
| Myristic acid (tetradecanoic acid) | 1.660 |
| Palmitic acid (hexadecanoic acid) | 52.162 |
| Palmitoleic acid | 3.876 |
| Margaric acid (heptadecanoic acid) | 0.708 |
| Stearic acid (octadecanoic acid) | 15.159 |
| Oleic acid | 60.570 |
| Linoleic acid | 60.411 |
| Alpha-linolenic acid (ALA) | 1.170 |
| Arachidic acid (eicosanoic acid) | 1.608 |

## Results

*Fatty acids composition of Gryllus bimaculatus extract.* First, we examined the fatty acid composition of the GB extract. It was found to contain two major saturated fatty acids, palmitic acid (52.2 mg/g) and stearic acid (15.2 mg/g) and two main unsaturated fatty acids, oleic acid (60.5 mg/g) and linoleic acid (60.4 mg/g) as listed in Table II.

*Gryllus bimaculatus extract has an inhibitory effect on LPS or palmitate‑induced production of inflammatory cytokines.* We examined the cell viability *in vitro* using various concentrations of the GB extract; less than 800 $\mu$g/ml of the GB extract did not affect the viability of RAW264.7 cells (Fig. 1). Previous studies showed that 40‑100 $\mu$g/ml of the GB extract could reduce nitrite, TNF‑α, and IL‑6 in the LPS‑treated kupffer cells (15). Therefore, we used 100 and 200 $\mu$g/ml of GB in this study. To examine if GB extract has any anti‑inflammatory effect upon LPS and palmitate treatment, the cells were pre‑treated with GB extract and then with

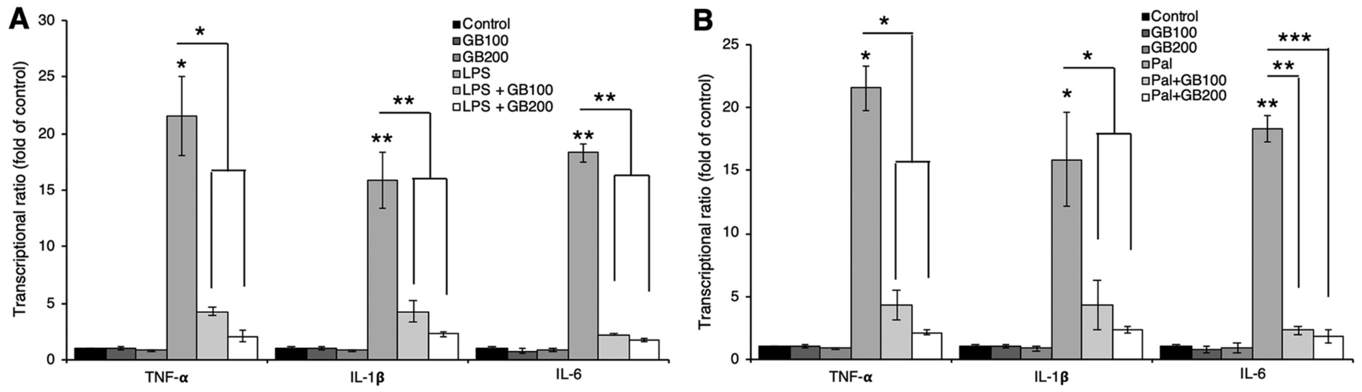


Figure 2. Effect of GB extract on LPS- or Pal-induced production of pro-inflammatory cytokines. Reverse transcription-quantitative PCR of proinflammatory cytokines (TNF-α, IL-1β and IL-6) after co-treatment with (A) LPS (50 ng/ml) and GB extract (100 and 200 µg/ml) or (B) Pal and GB extract. The values are expressed as the mean ± SEM (n=3). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate.

either LPS or palmitate. LPS or palmitate treatment induced pro-inflammatory cytokine production including TNF-α, IL-1β, and IL-6 while GB extract pre-treatment inhibited the pro-inflammatory cytokine mRNA levels (Fig. 2A and B) and their secretion (Fig. 3A-C).

*Gryllus bimaculatus extract has an inhibitory effect on LPS or palmitate-induced activation of NF-κB and MAP kinase*. Previous studies showed that MAP kinases and NF-κB signalling pathways play an important role in the production of LPS or palmitate-induced inflammatory cytokines (18,22,26). Therefore, both these pathways, NF-κB signalling (p65 translocation into nucleus and phosphorylation of IκB) and MAP kinases (p38, JNK, ERK) were examined. LPS or palmitate treatment elevated p65 translocation, IκB phosphorylation (Fig. 4A and B) as well as phosphorylation of MAP kinases (p38, JNK, ERK) (Fig. 4C); though they were reduced upon pre-treatment with GB extract (Fig. 4A-C). To further examine if MAP kinase and NF-κB activation involve any pro-inflammatory cytokine production, we pre-treated the cells with either p38 inhibitor (SB203580), JNK inhibitor (SP600125), ERK inhibitor (PD98059), or NF-κB inhibitor (PDTC). A reversal in the LPS and palmitate induced elevation of pro-inflammatory cytokine production was observed with respect to p38, JNK, and NF-κB inhibitors, but not ERK inhibitor (Fig. 5A-C).

*Gryllus bimaculatus extract has an inhibitory effect on LPS or palmitate-induced NLRP3 inflammasome activation*. Previous studies showed that, both palmitate and LPS induce NLRP3 inflammasome activation (7,27), we therefore examined the inflammasome components in this study- NLRP3, ASC, pro-caspase-1, cleaved-caspase1 (c-caspase-1), and IL-1β. LPS or palmitate treatment increased the expression of all the components while GB extract reduced them, suggesting that the GB extract can reduce inflammasome formation (Fig 6A and B). Interestingly, GB extract did not affect expression of pro-caspase-1.

*Gryllus bimaculatus extract has an inhibitory effect on LPS or palmitate-induced ROS generation and endoplasmic reticulum (ER) stress*. Since both LPS and palmitate are known to increase ROS generation (1,4,28), we examined if GB extract affects ROS generation upon LPS or palmitate treatment. As expected, both LPS and palmitate increased ROS generation, while GB reduced it (Fig. 7A). Furthermore, GB also reduced the LPS or palmitate-induced ER stress markers- PERK and eif2α phosphorylation (Fig. 7B and C).

*Gryllus bimaculatus extract has an inhibitory effect on LPS or palmitate-induced cell death*. Finally, we examined the role of GB extract in cell death upon exposure to high doses of LPS or palmitate. LPS or palmitate treatment induced cell death with increased cleavage of caspase-3 (c-caspase-3) and PARP1 (c-PARP1). Co-treatment with GB extract reduced cell death and also decreased cleavage of caspase-3 and PARP1 (Fig. 8A). Since GB extract reduced phosphorylation of MAP kinases (p38, JNK) and NF-κB pathway, we also treated the cells with these inhibitors and examined cell death. These inhibitors were observed to reduce cell death as well as caspase-3 and PARP1 cleavage (Fig. 8B). Therefore, GB appears to have a protective role in LPS or palmitate-induced cell death.

## Discussion

Inflammation plays an important role in many diseases such as arthritis, steatohepatitis and cancers; macrophages have a central role in inflammation. In the present study, we examined the effect of GB extract on LPS or palmitate-induced production of pro-inflammatory cytokines and formation of the inflammasome complex in RAW264.7 cells. GB extract exhibited a protective role in production of pro-inflammatory cytokines through inhibition of MAP kinase and NF-κB signalling pathway. IκB is phosphorylated by activating IκB kinase in response to an inflammatory stimulus followed by translocation of an activated p65 from the cytosol to the nucleus (29); activated p65 in the nucleus in turn increases the transcription of pro-inflammatory cytokines. MAP kinases (p38, JNK, ERK) play diverse roles in many cellular processes ranging from proliferation to differentiation and cell death. Among the three MAP kinases, only p38 and JNK reduced the production of all the pro-inflammatory cytokines, suggesting that ERK pathway may be playing a minor role in the process. Furthermore, GB also had an inhibitory role in the formation of inflammasome

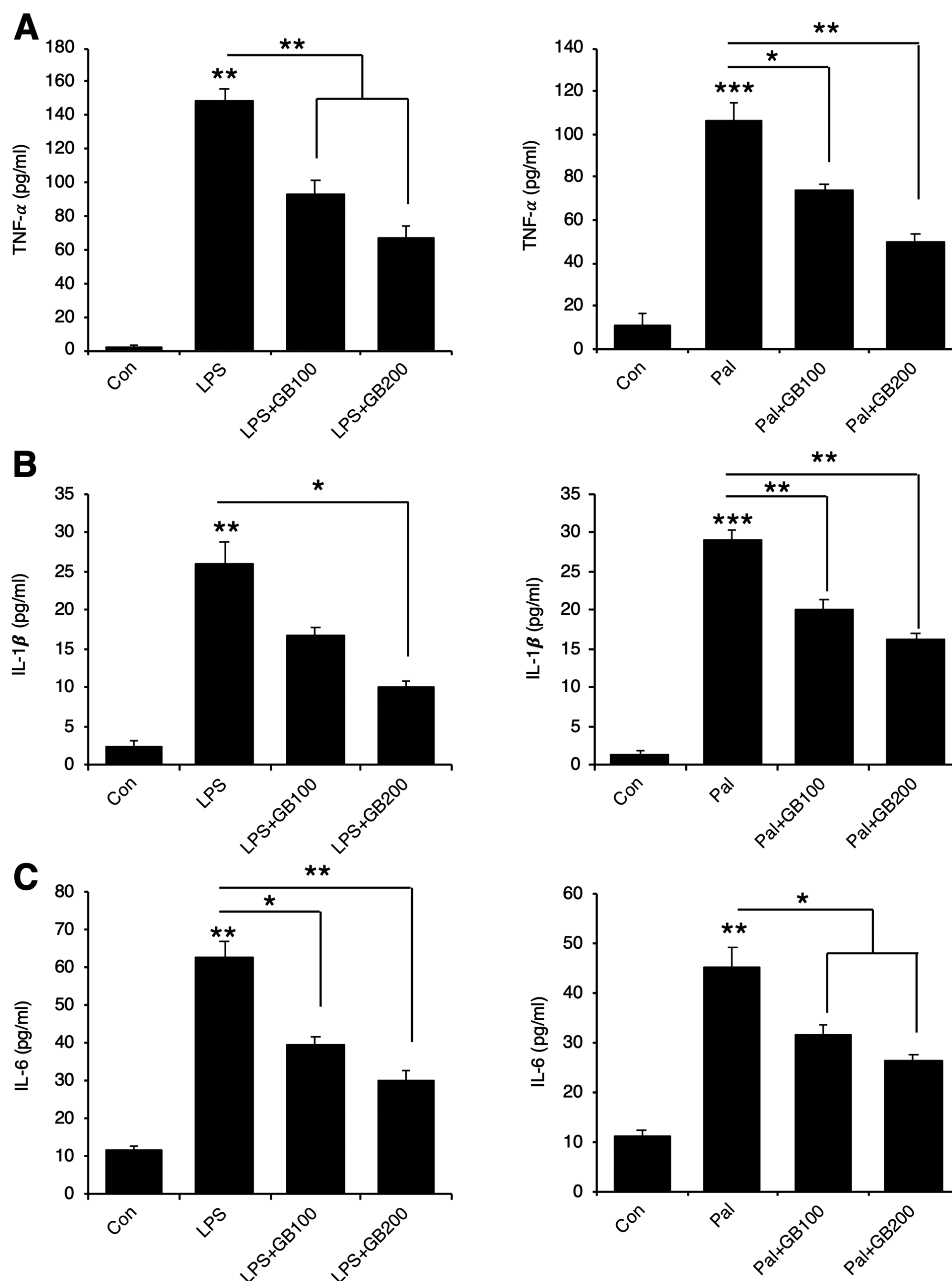


Figure 3. Effect of GB extract on LPS- or Pal-induced release of TNF-α, IL-1β and IL-6. After co-treatment with GB extract (100 and 200 $\mu$g/ml) and Pal (250 $\mu$M) or LPS (50 ng/ml), (A) TNF-α, (B) IL-1β, and (C) IL-6 expression levels were measured in RAW264.7 cell culture medium using ELISA kits. The values are expressed as the mean ± SEM (n=3). *P<0.05; **P<0.01; ***P<0.001. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; Con, control.

complex. Inflammasome is equipped by the formation of NLRP3, ASC and pro-caspase-1 complex (6). GB extract reduced the expression of NLRP3, ASC, c-caspase-1 and IL-1β upon LPS or palmitate treatment. NLRP3 expression is low but can be increased by TLR activation in a NF-κB dependent manner (6). Since GB extract has inhibitory effects on NF-κB pathway, it can also reduce inflammasome formation.

ROS generation upon palmitate treatment is due to the partial inhibition of mitochondrial complexes I and III (1). LPS also inhibited mitochondrial complex I (30) and increased ROS generation (4). GB extract reduced both LPS-induced and palmitate-induced ROS generation. Oleic acid has been shown to downregulate palmitate-induced ROS generation and cell death, in a CD36 dependent manner (31); therefore, oleic acid in GB extract might improve palmitate-induced mitochondrial complex I inhibition and reduce ROS generation. Furthermore, oleic acid and linoleic acid also have protective effects against palmitate-induced ER stress (32-34). These unsaturated fatty acids are known to inhibit inflammation via several mechanisms. For example, linoleic acid and oleic acid prevent inflammation by activating PPAR-γ (35,36) and inhibiting NF-κB signalling (37,38). Therefore, unsaturated fatty acids in the GB extract might prevent LPS or palmitate-induced NF-κB activation, ER stress, and subsequent production of pro-inflammatory cytokines and inflammasome complex formation.

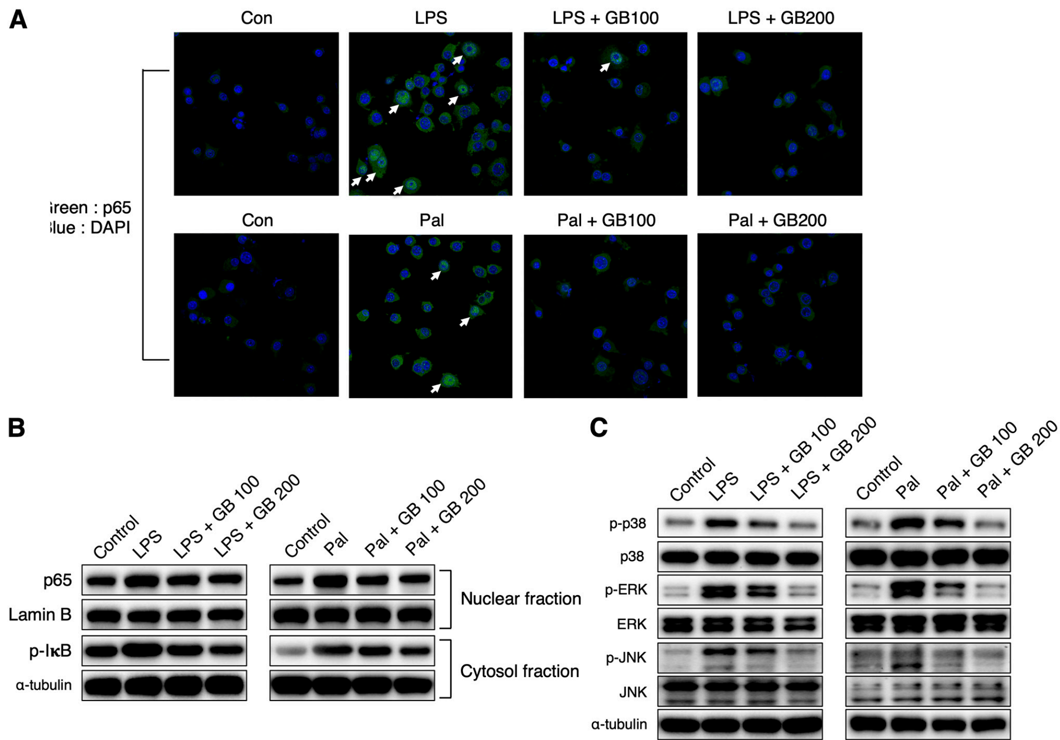


Figure 4. GB extract decreases NF-κB signalling, as well as MAP kinase phosphorylation. (A) After co-treatment with LPS (50 ng/ml), Pal (250 $\mu$M) and GB extract (100 and 200 $\mu$g/ml), translocation of p65 was determined using a p65 antibody and an Alexa-Fluor 488-conjugated anti-rabbit antibody (magnification, x400). Nuclei were counterstained with DAPI. p65 translocation into the nucleus is marked with arrows. Representative western blots were performed for (B) p65 in the nuclear fraction and IκB phosphorylation in the cytosol fraction, and (C) MAP kinase (p38, JNK and ERK) phosphorylation. All the experiments were performed in triplicates. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; Con, control; p-, phosphorylated.

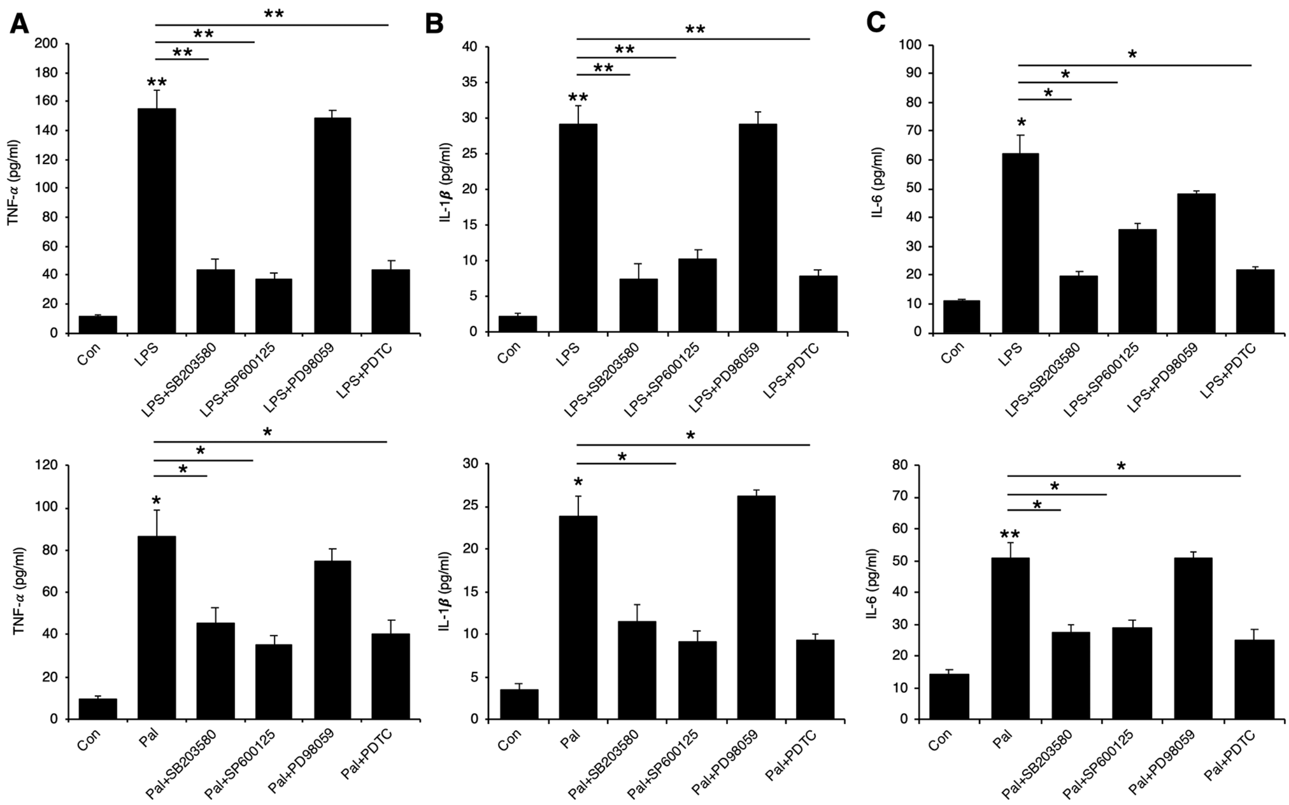


Figure 5. Both MAP kinase activation and NF-κB signalling serve a critical role in LPS- or Pal-induced cytokine secretion. After co-treatment with LPS (50 ng/ml) or Pal (250 $\mu$M) and SB203580 (p38 inhibitor; 10 $\mu$M), SP600125 (JNK inhibitor; 10 $\mu$M), PD98059 (ERK inhibitor; 10 $\mu$M) or PDTC (NF-κB inhibitor; 10 $\mu$M), (A) TNF-α, (B) IL-1β and (C) IL-6 expression levels in RAW264.7 cell culture medium were measured using an ELISA kit. The values are expressed as the mean ± SEM (n=3). *P<0.05; **P<0.01. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; Con, control; PDTC, pyrrolidinedithiocarbamate ammonium.

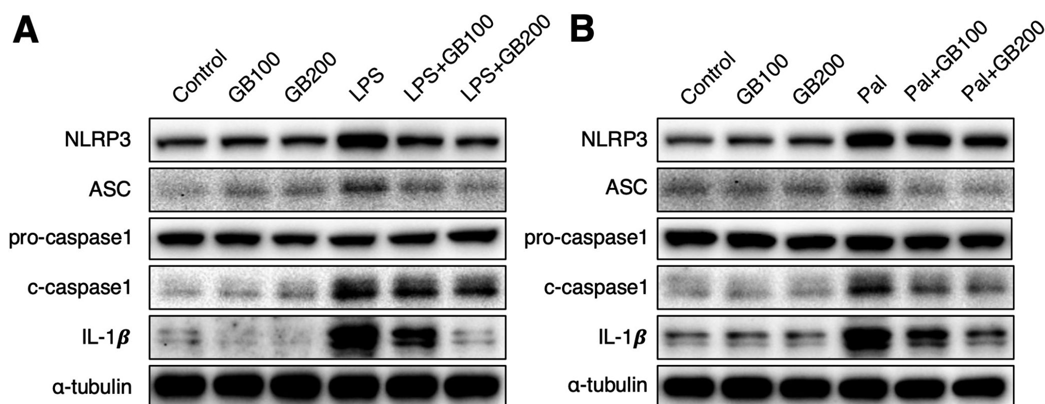


Figure 6. GB extract decreases LPS- or Pal-induced inflammasome complex formation. Representative western blots of the indicated proteins upon (A) LPS (50 ng/ml) or (B) Pal (250 $\mu$M) treatment in RAW264.7 cells co-incubated with GB extract (100 and 200 $\mu$g/ml). All the experiments were performed in triplicates. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; c-, cleaved.

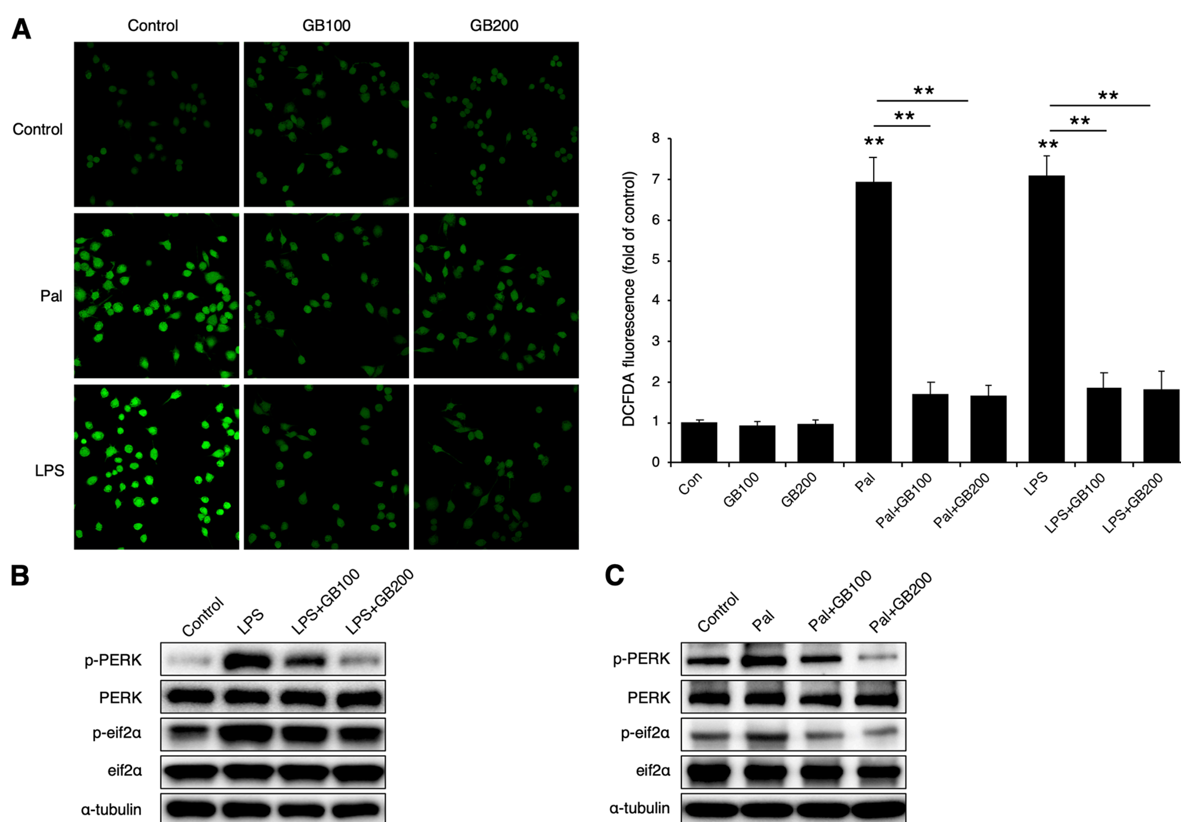


Figure 7. GB extract decreases LPS- or Pal-induced generation of ROS and endoplasmic reticulum stress. (A) After co-treatment with LPS (50 ng/ml), Pal (250 $\mu$M) and GB extract (100 and 200 $\mu$g/ml), ROS generation was evaluated (left panel; magnification, x400) and quantified (right panel). Representative western blots of the indicated proteins in (B) LPS- (50 ng/ml) or (C) Pal-treated (250 $\mu$M) RAW264.7 cells co-incubated with the GB extract (100 and 200 $\mu$g/ml). The values are expressed as the mean ± SEM (n=3). $^{**}$P<0.01. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; Con, control; ROS, reactive oxygen species; p-, phosphorylated; DCFDA, 2',7'-dichlorofluorescin diacetate; PERK, protein kinase RNA-like ER kinase; eif2$\alpha$, eukaryotic initiation factor 2$\alpha$.

Some edible insects have also been known to inhibit inflammation when taken as food. For example, *Tenebrio molitor* and *Allomyrina dichotoma* reduce not only the high fat diet-induced steatohepatitis, but also hepatic inflammation (39). GB also has protective effects against alcohol-induced liver damage (15) and chronic arthritis (14). *Tenebrio molitor* has antioxidant and anti-inflammatory effects, and its composition is well studied; it contains various essential amino acids, unsaturated fatty acids, tocopherol and squalene (40) which may be contributing to these beneficial effects. *Tenebrio molitor*, *Gryllodes sigillatus* and *Schistocerca gregaria* have anti-inflammatory and antioxidant effects (41). Similarly, GB also has high amounts of unsaturated fatty acid, which might affect the anti-inflammatory effect, reduce ROS generation and decrease LPS or palmitate-induced cell death.

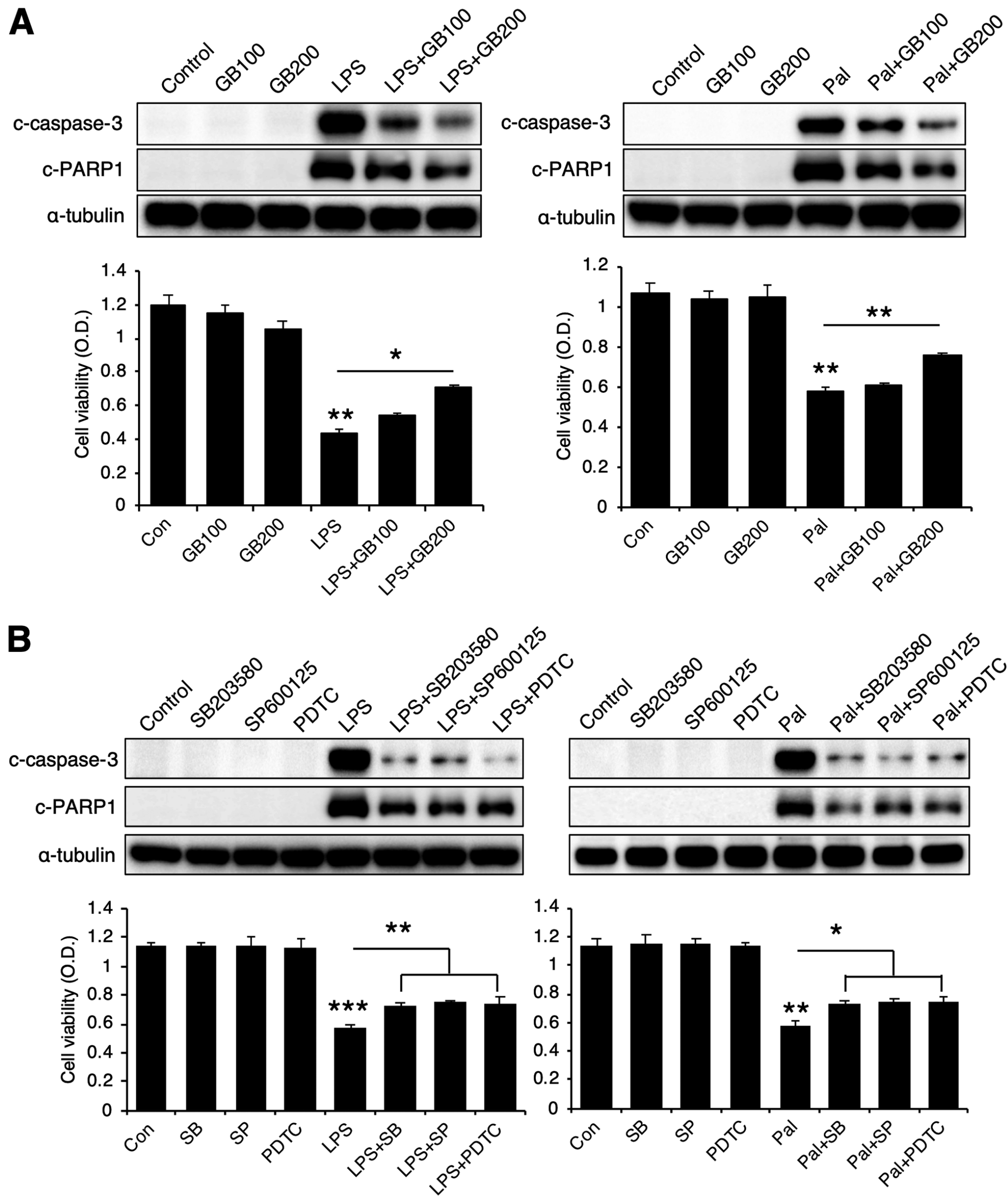


Figure 8. GB extract protects against LPS- or Pal-induced cytotoxicity. (A) Representative western blots of c-caspase-3 and c-PARP1, and MTT cell viability in LPS- (50 $\mu$g/ml) or Pal-treated (2 mM) RAW264.7 cells co-incubated with GB extract (100 and 200 $\mu$g/ml). (B) Representative western blots of c-caspase-3 and c-PARP1, and MTT cell viability in LPS- (5 $\mu$g/ml) or Pal-treated (2 mM) RAW264.7 cells co-incubated with either 10 $\mu$M SB203580 (p38 inhibitor), 10 $\mu$M SP600125 (JNK inhibitor) or 10 $\mu$M PDTC (NF-κB inhibitor). The values are expressed as the mean ± SEM (n=3). $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001. GB, *Gryllus bimaculatus*; LPS, lipopolysaccharide; Pal, palmitate; Con, control; c-, cleaved; PDTC, pyrrolidinedithiocarbamate ammonium; OD, optical density.

In conclusion, GB may potentially be one of the beneficial foods for controlling macrophage inflammatory processes, ROS generation, ER stress and cell death; it may also prevent diseases related to oxidative stress and inflammation. Thus, it can be used as a functional food in conditions of oxidative stress and inflammation.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

WJP and JSH contributed to the conception and design of the study. WJP and JSH performed the experiments and confirmed the authenticity of the raw data. WJP contributed to the acquisition of data and wrote the manuscript. WJP and

JSH reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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