

Anti-inflammatory Action of Indonesian Black Garlic (IBG) Ethanol Extracts in LPS-stimulated RAW 264.7 Macrophage Cells

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ABSTRACT

Indonesian Black Garlic (IBG) ethanol extracts are a new type of fresh garlic product, produced at high a temperature and humidity. IBG has active antioxidant activity. This study aimed to assess the anti-inflammatory actions of IBG in RAW 264.7 macrophage cells. The cells were grown and treated with various concentrations of IBG extracts (50 to 1000mg/L). Cell counting kit-8 assay revealed that IBG was not toxic up to 400mg/L. IBG significantly reduced the expression of inflammation-mediated expression in LPS-stimulated RAW 264.7 macrophage cells. IBG was also found to suppress inflammation cytokines. This is evident in mRNA expression and reduced expression of iNOS, COX-2, IL-6, NF-kB, and TNF- α at protein levels. The results suggest that IBG can be used to prevent or treat diseases associated with inflammation.

Keywords: Indonesian Black Garlic, Anti-inflammatory, RAW 264.7 macrophage cells.

INTRODUCTION

Garlic (*Allium sativum* L.) is part of the onion family, not only has a strong and distinctive flavor but also contains bioactive organosulfur compounds such as allicin, diallyl disulfide, diallyl trisulfide, S-aryl-cysteine, S-aryl-mercatocysteine (Santhosha *et al.*, 2013). The therapeutic effects of garlic that have been observed include anticancer, antibacterial, antiviral, antidiabetic, anti-inflammatory, antioxidant, and immunomodulatory properties (Sasaki, 2006). Recently, a new type of garlic, black garlic (BG) has been discovered. BG is commonly grown in Japan by processing (incubating) fresh white garlic for one month in a controlled room at temperature (70°C) and humidity (75%) without the use of artificial additives (Sasaki, 2006).

BG has a black color, has a mild or harmless sweet, fruity sweetness, and is easily edible when peeled (Sasaki, 2006). Black garlic does not have a strong, unpleasant taste like fresh garlic. The unpleasant odor of black garlic is due to the conversion of pungent allicin compounds into water-soluble antioxidant compounds (Corto-Martinez, 2007). Other studies have shown that black garlic lacks flavor due to reduced allicin

content, which is converted to antioxidant compounds such as bioactive alkaloids and flavonoid compounds during aging (Yuan *et al.*, 2016). It has also been reported that BG extract has antioxidant, anti-allergic, anti-diabetic, cholesterol-lowering, lipid-lowering, anti-cancer, and anti-inflammatory effects (Ishikawa *et al.*, 2006).

During heat treatment, several compounds in fresh garlic are converted into Amadori/Heyns compounds, which are important intermediates in the Maillard reaction (Yuan *et al.*, 2016). BG compounds vary depending on heat treatment conditions and the quality of the product depends on the manufacturing process (Choi *et al.*, 2014). Some researchers reported that during aging, many valuable components of BG, especially polyphenols, flavonoids, and some Maillard reaction mediators, are known as antioxidant due to diseases (Hwang *et al.*, 2011). The increased BG composition compared with raw garlic water-soluble sugar (1.88% increase, Yuan *et al.*, 2016), polyphenol and flavonoid (increased by 4.19% and 4.77% respectively; Choi *et al.*, 2014), Amadori and Heyns (increased by 40-100; Yuan *et al.*, 2016), leucine, isoleucine, and phenylalanine (increased by 1.06%, 1.67%, and 2.43%, respectively; (Choi *et al.*, 2014).

The methyl thiazolyl tetrazolium (MTT) assay showed that BG was not toxic up to 1000 mg/L and was at least four times less cytotoxic than fresh garlic (Kim *et al.*, 2014). Some studies confirmed that Korean Black Garlic decreased inflammatory mediators such as TNF- α and PGE-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The anti-inflammatory activities of BG occur at non-toxic concentrations of up to 250 mg/L (Kim *et al.*, 2014). It was found out that the anti-inflammatory activity in BG decreased cell proliferation and cell cycle progress (Lee *et al.*, 2011). In other studies, BG decreased nitric oxide (NO), TNF- α and IL-6 in LPS-stimulated RAW 264.7 macrophages cells (Kim *et al.*, 2014). The aim of this study was to evaluate the anti-inflammatory action of Indonesian Black Garlic (IBG) in LPS-stimulated RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Preparation of Indonesian Black Garlic (IBG)

IBG was created by maintaining fresh garlic (cloves) at a temperature 70°C and humidity 75%-controlled room for a month without any additional treatments and additives. IBG was made in the Faculty of Agricultural Product Technology, Brawijaya University, Malang, Indonesia. Maillard and Browning reactions changed the color of fresh garlic from white to brown and became black a month later. IBG is softer than fresh garlic and does not have an irritating odor but has a sweet fruit taste. IBG with 6 cloves (first weight; around 30g) was chopped and mashed for heat extraction in 100% ethanol (30g/100mL) at 100°C for 2h. The extracted solution was then centrifuged at 4000 rpm for 20min to isolate the supernatants and frozen at -80°C overnight. Then the solution was freeze-dried (lyophilized) (Freeze Dryer, BFD-2, Nihon Freezer Co. Ltd., Japan), powdered, and stored at -20°C until further analysis. The dried materials were dissolved in media at the indicated concentrations.

DPPH radical scavenging assay

This test is used to demonstrate the free radical scavenging activity of the herb. DPPH (2,1-diphenyl-1-picrylhydrazil) is a stable free radical (absorbed at 517 or 520nm) and turns yellow when the free radical is removed. The DPPH radical is dark purple in solution and becomes colorless or pale yellow when neutralized. This property allows for visual monitoring of the response (Lee *et al.* 2011). Extraction of antioxidant compounds was done by mixing a 50g sample with 500mL of

ethanol and stirring for 1h. 50mL of the supernatant extract was reacted with 150mL of 0.1mM DPPH working solution. The mixture was shaken and incubated for 30min at room temperature in the dark. Absorbance was measured using a UV-Vis spectrophotometer at 517nm. Inhibition was calculated according to the following formula.

$$\text{Scavenging ability (\%)} = 1 - \frac{\text{Absorbance 517nm of sample}}{\text{Absorbance 517nm of control}} \times 100\%$$

Cell culture

The RAW 264.7 macrophage cells (Korean Cell Line Bank, Seoul, Korea) were cultured in Roswell Park Memorial Institute (RPMI-1640, Gibco) medium containing 10% Fetal Bovine Serum (FBS, Gibco), 100 units/mL penicillin/streptomycin (Gibco) and incubated at 37°C under 5% CO₂. The medium was replaced every 2 days. The RAW 264.7 macrophage cells were seeded and treated with different concentrations of IBG extracts (50, 100, 200, 400 mg/L) for 48h. To activate macrophages, 10 mg/L of lipopolysaccharide (LPS, Sigma-Aldrich) was added. The viability of the cells was calculated using a cell counting kit (CCK-8) assay (Dojindo Laboratories, Japan). Then, CCK-8 solution (1mg/L) was added to each well. Optical density was measured at 450nm in a microplate reader. The calculation for treated cells was shown as the percentage of control cells.

Nitrite Oxide (NO) Assays

NO Assays was done to estimate the amount of NO produced by cells. A clean bench and a 70% confluent cell dish were made ready. The cells were washed with 10mL of Phosphate Buffered Saline (PBS, Sigma-Aldrich) and 1mL of trypsin (Gibco) was added then incubated at 37°C for 1min. The cells detached with the remaining 1mL in the dish. 9mL of media was added into a 15mL tube and then 1mL was added from the dish into the tube. Centrifugation at 3000rpm was done for 3min at 37°C. Supernatants were removed, and 9mL of media was added and mixed thoroughly. The volume of the cell solution was calculated as $(1 \times 10^4 \text{ per well/cell count} \times 10^4)$ (assuming that the answer is X mL). X mL of the solution was added to 10mL of FBS. 100 μ L of the solution was added to the 96-well plate. After 24h of incubation, the IBG was added and the cells were stimulated by LPS (1mg/L) and then incubated for 48h. 50 μ L of the supernatant that was mixed with the same amount of Griess reagent (Sigma-Aldrich) was then added and incubated for 30min. The absorbance was read at 540nm.

Table I. List of primers used in the PCR analysis to identify the effect of IBG on anti-inflammatory mediators in RAW 264.7 macrophage cells.

Gene	Primer	Sequence
β-actin	Sense	5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3'
	Antisense	5'-CTC-TCA-GCT-GTG-GTG-AA-3'
IL-6	Sense	5'-AGT-TGC-CTT-CTT-GGG-ACT-GA-3'
	Antisense	5'-TCC-ACG-ATT-TCC-CAG-AGA-AC-3'
iNOS	Sense	5'-GTG-GTG-ACA-AGC-ACA-TTT-GG-3'
	Antisense	5'-GGC-TGG-ACT-TTT-CAC-TCT-GC-3'
COX-2	Sense	5'-CCT-GTG-TTC-CAC-CAG-GAG-AT-3'
	Antisense	5'-CCC-TGG-CTA-GTG-CTT-CAG-AC-3'
NF-κB	Sense	5'-GCC-GTG-GAG-TAC-GAC-AAC-ATC-3'
	Antisense	5'-TTT-GAG-AAG-AGC-TGC-CAG-CC-3'
TNF-α	Sense	5'-GAC-GTG-GAA-CTG-GCA-GAA-GAG-3'
	Antisense	5'-TTG-GTG-GTT-TGT-GAG-TGT-GAG-3'

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Whole-cell RNA was extracted using RNAiso Plus (Takara Shuzo Co.) according to the manufacturer's instructions. CDNA was synthesized from RNA samples using a Thermo fish PCR kit containing 10mM dNTP Mix, 0.2µg/mL random hexamer primer, 40U/µL Ribolock RNase inhibitor, 200U/L transcriptional yeast ReversedAid and 5 times reaction buffer. The Premix Maxime RT kit was used to mix 1mg of cDNA with a 20mL primer tag. The cDNA was grown in a thermal circulator for an initial denaturation cycle of 5min at 95°C and a cycle of 30 amplifications (40s at 95°C, 40s of hybridization at 50-60°C and elongation. last 1min at 72°C). The oligonucleotide primers used in this study (Table I).

Western blot

Protein was extracted from RAW 264.7 macrophages by adding 200µL of protein extraction solution (iNtRON Biotechnology, #17081). The lysate was purified by centrifugation at 15,000 rpm at 40°C for 15min, and the protein concentration was determined using the Bradford assay. A 30µg protein sample was separated by vertical electrophoresis (SDS-PAGE) and transferred to a nitrocellulose transfer membrane. The membrane was blocked with 5% skim milk and hybridized with the following Abcam™ main antibodies: mouse monoclonal anti-β actin (ab6376), rabbit polyclonal anti-iNOS, rabbit polyclonal anti-COX-2, rabbit polyclonal anti-NF-κB, mouse monoclonal anti-TNF-α and mouse monoclonal anti-IL-6. After membrane incubation

with horseradish peroxidase (HRP, AB Frontier #LF-QC0103), the target protein is exposed and detected on radiographic film to enhance chemiluminescence.

Statistical analysis

The experimental results are listed as mean ± standard deviation. One-way analysis of variance and Duncan's multiple range test (DMRT) were used to analyze differences between groups. The statistical significance level was set at P<0.05. The statistical software package SPSS 19.0 (SPSS Inc., Chicago, I11., USA) was used for the analysis.

RESULT AND DISCUSSION

Anti-inflammatory actions of IBG in LPS-stimulated RAW 264.7 macrophage cells

DPPH activity was more effective to show the radical scavenging capacity of high antioxidative components than ABTS with an increasing percentage of negative slope and CUPRAC activity was also more effective than FRAP for BG methanolic extract (Eyupoglu, 2019). The IC₅₀ value of polyphenol extracted from BG for DPPH radical inhibition was 140.79 mg/L (Lu *et al.*, 2017). The power level of antioxidant activity by the DPPH method depends on the IC₅₀ value mg/L and divided into 5 categories, under 50mg/L is highly active, 50-100mg/L is active, 101-250mg/L is moderate, 251-500mg/L is weak, and over 500mg/L is inactive (Marjoni & Zulfisa, 2017). In this study after checking the antioxidant activity (%) of IBG by DPPH in various concentrations (Figure 1), a linear equation can be found. After getting the linear equation, the IC₅₀ value of IBG could be calculated, which was 71.90mg/L. Based

on this value and referring to the literature of Marjoni and Zulfina (2017), the power level of antioxidant activity from IBG by DPPH assay is included in the active category.

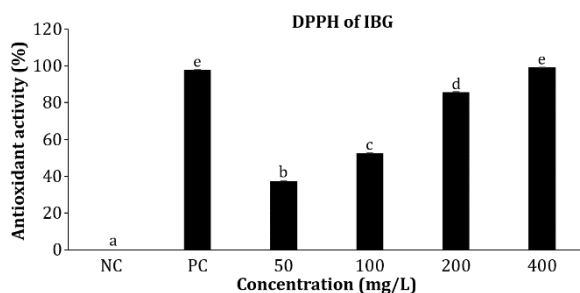


Figure 1. Antioxidant activity (%) of Indonesian Black Garlic (IBG) by DPPH Assay in various concentration. All values are mean \pm SD (n=3). Means with different letter are significantly different (Duncan Multiple Range Test, $p < 0.05$). NC: negative control, only media; PC: positive control, vitamin C (1000 mg/L).

In this study, DPPH is one of the tests used to determine the antioxidant activity of IBG. The antioxidant activity test was used to determine flavonoid content in an extract. The higher the flavonoid content in an extract, the higher the anti-inflammatory activity. This is because flavonoids are anti-inflammatory compounds. In this study, the flavonoid content was not tested further, but the antioxidant activity test carried out and the results showed that IBG had a high enough antioxidant activity. The higher the antioxidant activity, the higher ability to bind free radicals, also known as *Reactive Oxygen Species* (ROS). The imbalance in the immune response and ROS will cause tissue damage and inflammation which can then develop into cancer.

Enzyme superoxide dismutase (SOD) exerts anti-inflammatory effects. This enzyme is an enzymatic antioxidant that is produced by the body to fight free radicals, the formation of these free radicals can cause inflammation and toxicity in the body. Therefore, a test was conducted to determine the toxicity of IBG using RAW 264.7 macrophage cells through CCK-8 assays. In this study, IBG was applied as a treatment on RAW 264.7 macrophage cells <2000mg/L. The results showed that cell viability increased (Figure 2), along with the increases in the concentrations of IBG until 400mg/L, at which point maximum cell viability was reached. The results also suggested that

different concentrations of IBG can affect cell proliferation. IBG showed a certain promotion of macrophage proliferation compared to the control group (naive control) ($p < 0.05$) (Figure 2). The cell viability increased approximately 17-30% at concentrations ranging from 50 to 400mg/L. Based on the study, IBG has a strong antioxidant activity and is not toxic up to 400mg/L.

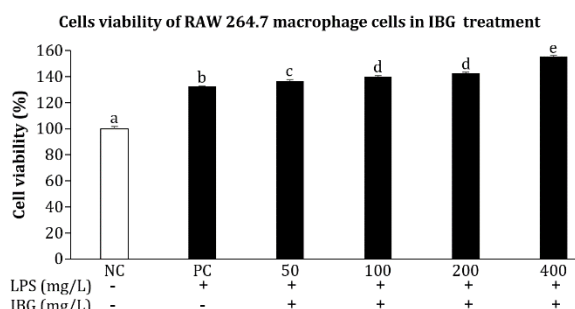


Figure 2. Effects of Indonesian Black Garlic on cells viability in RAW 264.7 macrophage cells. 1×10^4 RAW 264.7 macrophage cell were pre-cultured in RPMI-1640 media containing 10% FBS and 1% Penicillin-streptomycin. Then cells were treated with LPS (1 mg/L) and IBG (50, 100, 200, and 400 mg/L) for 48h. Cell viability assay was undertaken by CCK-8 assay. All values are mean \pm SD (n=5). Means with different letter are significantly different (Duncan Multiple Range Test, $p < 0.05$). NC: negative control, only media; PC: positive control; LPS: Lipopolysaccharide; IBG: Indonesian Black Garlic.

The anti-inflammatory action of IBG is determined by the production of nitrite oxide and the expression of pro-inflammatory cytokines. Studies have shown that NO levels are associated with immune responses in anti-tumor and antiviral processes (Chen *et al.*, 2016). There is growing evidence that an inflammatory environment triggers the formation of nitric oxide synthase (iNOS) followed by the formation of peroxynitrite (Buttery *et al.*, 1996). This fact also explains that garlic has immunomodulatory, anti-inflammatory and antioxidant effects (Reuter *et al.*, 1996). Allicin ((R,S)-diallyl disulfide-S-oxide) and ajoene ((E,Z)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide), it is reported to be active and is done. Components influencing the beneficial effects of garlic preparations on inducible nitric oxide (iNOS) synthesis (Dirsch *et al.*, 1998; Re *et al.*, 1999). BG inhibits NO production of LPS-stimulated RAW 264.7 macrophages (Kim *et al.*, 2017).

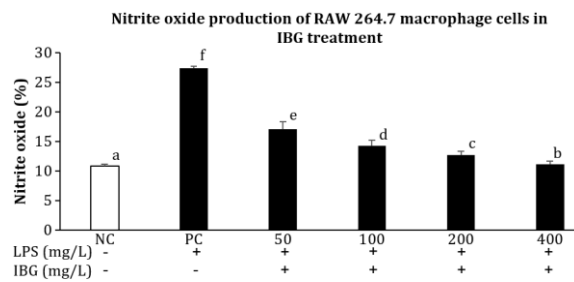


Figure 3. Effects of Indonesian Black Garlic on nitrite oxide in RAW 264.7 macrophage cells. 1X10⁴ RAW 264.7 macrophage cell were pre-cultured in RPMI-1640 media containing 10% FBS and 1% Penicillin-streptomycin. Then cells were treated with LPS (1mg/L) and IBG (50, 100, 200, and 400mg/L) for 48h. Nitrite oxide assay was undertaken by NO assay. All values are mean \pm SD (n=4). Means with different letter are significantly different (Duncan Multiple Range Test, $p < 0.05$). NC: negative control, only media; PC: positive control; LPS: Lipopolysaccharide; IBG: Indonesian Black Garlic.

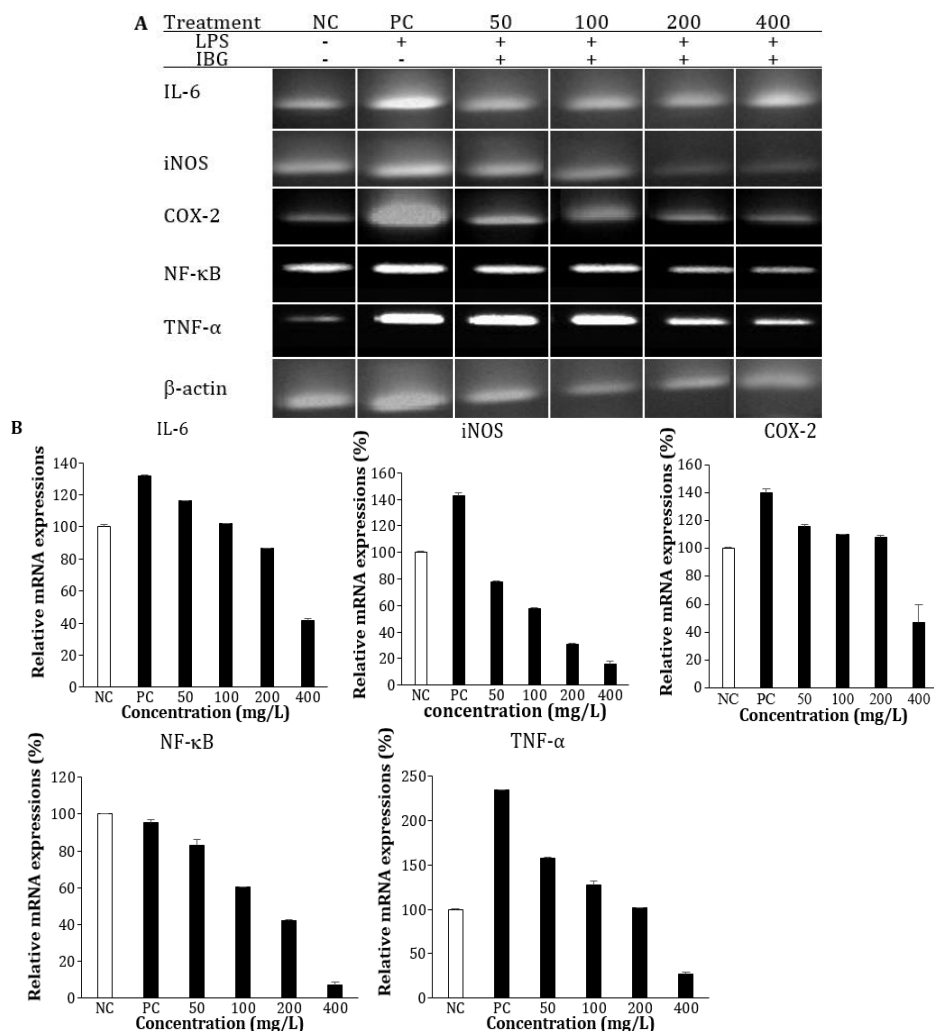


Figure 1 RT-PCR analysis of gene associated with anti-inflammatory activity on RAW 264.7 macrophage cells after treatment with different concentrations of IBG. (A) Representative mRNA bands. (B) Relative mRNA expression (%). All values are expressed as mean \pm SD (n=5). Means with different superscript are significantly different (DMRT, $p < 0.05$).

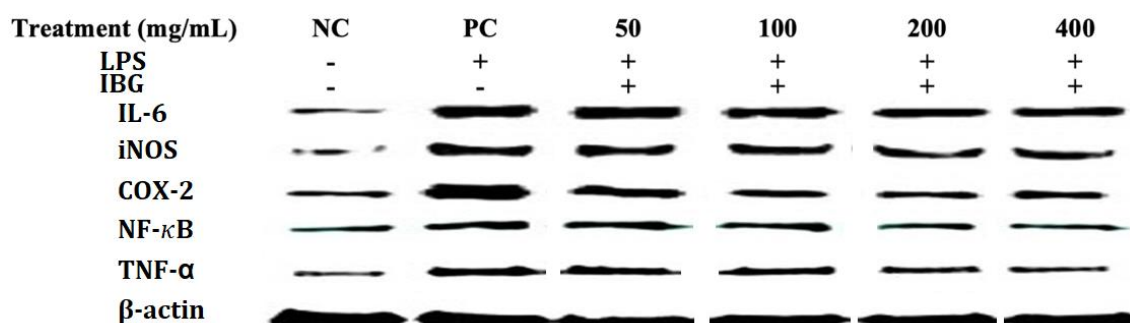


Figure 2. Western blot analysis of proteins associated with anti-inflammatory effect on RAW 264.7 macrophage cells after treatment with different concentrations of IBG.

The results of this study showed that pretreatment of RAW 264.7 macrophages with IBG significantly reduced NO production (Figure 3). These data indicate that IBG inhibits LPS-stimulated NO production in RAW 264.7 macrophages. Its ability shows that IBG can also inhibit inflammatory mediators.

To identify pro-inflammatory cytokines, the evaluation of the IL-6, iNOS, COX-2, NF-κB and TNF-α gene expression were carried out. The mRNA bands representing IL-6, iNOS, COX-2, NF-κB and TNF-α were significantly inhibited after 48h of treatment at various concentrations of IBG (Figure 4). The results showed that IBG suppressed the production of inflammatory cytokines stimulated by LPS (IL-6, iNOS, COX-2, NF-κB and TNF-α) by transcriptionally inhibiting the expression of the mediators. Protein expressions in RAW 264.7 macrophage cells treated with different concentrations of IBG was found to corroborate the results of mRNA expression (Figure 5). All protein-expressing inflammatory cytokines showed decreased expression of IL-6, iNOS, COX-2, NF-κB and TNF-α up to 400mg/L. The results showed that the expression of genes and proteins was significantly affected by different concentrations of IBG.

Regarding the mechanism of anti-inflammatory effects, one possible mechanism may be related to the direct inhibition of the cascade of Toll-like receptor (TLR-4) activation in macrophages (You *et al.*, 2019). It is well known that macrophages are involved in chronic inflammatory diseases because they express TLR4 located on the plasma membrane (You *et al.*, 2019). When TLR4 binds to the LPS endotoxin binding protein complex (LPS binding protein, cluster differentiation antigen 14 and myeloid

differentiation protein 2) (You *et al.*, 2019). TLR4 activates myeloid differentiation factor 88 (MyD88) (O'Neill, 2002). Then, activated MyD88 leads to the production of various inflammatory mediators, such as IL-6, TNF-α and PGE2, which enter the nucleus through NF-κB translocation through activation of mitogen-activated protein kinase (MAPK) (O'Neill, 2002). In addition, released inflammatory mediators trigger severe or chronic inflammatory responses by acquiring other immune cells (You *et al.*, 2019). Thus, activation of NF-κB in LPS-activated macrophages is an important intracellular signaling mediator of the early signaling pathway of the TLR4 signaling cascade (You *et al.*, 2019). To support this, IBG reduces several inflammatory mediators, including IL-6, iNOS, and COX-2 in LPS-activated RAW 264.7 macrophages cells.

The activation of NF-κB leads to the induction of many pro-inflammatory cytokine expressions (Baeuerle, 1991). NF-κB separates from inhibitory protein (IκBα) which releases the p50/p65 heterodimer and translocates owing to the nuclear localization signal and thereby induces the expression of pro-inflammatory genes and proteins (Wandita *et al.*, 2018). TNF-α and interleukin function synergistically to induce the expression of several major pro-inflammatory mediators including prostaglandins, leukotrienes, and NO (Wandita *et al.*, 2018).

Another possible mechanism may be related to the antioxidant activity of IBG. LPS-activated macrophages can produce NO and ROS (O'Neill, 2002). Additionally, inflammatory cytokines, chemicals, and UV light can induce NO and ROS in skin tissue (O'Neill, 2002). Therefore, the regulation of the production of NO and ROS immune cells and skin tissues is another important

event of the anti-inflammatory effect of garlic (You *et al.*, 2019). The antioxidant activity of black garlic (BG10) may be related to components such as phenolics and flavonoids, but BG10 contains sulfur-rich compounds such as diallyl sulfide, diallyl disulfide, and trisulfide of diallyl. Sulfur compounds cannot inhibit the allergic response of IgE/antigen-activated mast cells (You *et al.*, 2019).

IBG also contains active compounds of flavonoids, tannins, saponins, and sterols (Agustina *et al.*, 2020), but in this study, the exact contents of flavonoids and other active compounds were not checked and need to be evaluated in further study. In inflammatory disorders, NO also acts as a cytotoxic agent in some pathological activity because of its free oxygen radical (Bito & Nishigori, 2012). LPS, which is isolated from bacteria, stimulates the macrophage production of NO by iNOS. COX-2 is related to NO production and its overproduction may cause inflammation and carcinogenesis (Park *et al.*, 2014).

In this study, IBG can reduce LPS-stimulated production of inflammatory mediators and cytokines in RAW 264.7 macrophage cells, especially the level production of NO and COX-2 expression decreased. The data clearly demonstrate that IBG may be useful as an anti-inflammatory agent.

CONCLUSION

Since garlic has long been consumed for a long time and recognized as one of the safest food, BG products have no restrictions on additional inventions, especially for IBG food products, functional products, food supplements and medical use. In this study, IBG reduced the levels of NO production in LPS-stimulated RAW 264.7 macrophages, suppressed the NF- κ B pathway, and reduced the expression of the other inflammatory cytokines. In conclusion, IBG has anti-inflammatory effects that reduce inflammation stimulated by LPS.

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The authors' contributions are: all of the authors collaborated on the design of the experiences supervised by Seong Gu Hwang and In Sik Nam; Novita Wijayanti is responsible for the creation of the IBG; Laurentia Adinda Pratidina conducted the experiments and helped write the original manuscript. The authors state that there is no conflict in this work.

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