Bioconversion of *Gentiana scabra* Bunge increases the anti-inflammatory effect in RAW 264.7 cells via MAP kinases and NF-κB pathway

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Abstract Mitogen-activated protein (MAP) kinases play an important role in cell growth and differentiation, as well as the modulation of proinflammatory cytokines. The objective of this study was to examine the increase in the anti-inflammatory effect of *Gentiana scabra* Bunge (GSB), due to bioconversion with the *Aspergillus kawachii* crude enzyme, via inhibition of the NF-κB signaling and MAP kinase pathways in RAW 264.7 cells. The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 in RAW 264.7 cells treated with the GSB ethyl acetate fraction bioconverted with *A. kawachii* crude enzyme (GE-BA), was dramatically suppressed as compared to GSB ethyl acetate fraction non-bioconverted with the *A. kawachii* crude enzyme (GE-UA). The phosphorylation of p38, extracellular signal-regulated kinases, and inhibitory κB in RAW 264.7 cells treated with GE-BA was further suppressed, as compared to exposure to GE-UA. Moreover, the mRNA expression of interleukin 6, interleukin 1-beta, and tumor necrosis factor-α was further suppressed by GE-BA, compared to GE-UA. Similarly, anti-oxidant activities, such as 2,2-diphenyl-1-picrylhydrazyl hydrate and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity, of GE-BA were further increased compared to GE-UA. These observations demonstrate that the anti-oxidant and anti-inflammatory activities of GSB ethyl acetate fraction increases as a result from bioconversion with the *A. kawachii* crude enzyme.

Keywords Anti-oxidant · *Aspergillus kawachii* · *Gentiana scabra* Bunge · Inflammation

Introduction

Inflammation is a protective response to tissue damage resulting from several factors such as infection and burn injury, and usually be associated with tumors, immune response and free radicals. Tissue injury leads to the release of pro-inflammatory mediators such as nitric oxide (NO), cytokines, and leukotrienes [1,2]. The response of chronic inflammatory results in the excessive releases of pro-inflammatory mediators such as interleukin 6 (IL-6), interleukin 1-beta (IL-1β), and tumor necrosis factor-α (TNF-α). Therefore, a disorder in the anti-inflammatory response may cause various diseases including cancer, atherosclerosis, and inflammatory bowel disease [1,3-5]. NO is produced by nitric oxide synthase (NOS) which converts L-arginine to L-citrulline and considered as a regulatory mediator of inflammation. NOS can be classified into constitutive NOS (cNOS) and inducible NOS (iNOS). A high NO concentration stimulates macrophages, which is associated with inflammatory responses and increases the expression of pro-inflammatory cytokines including iNOS and cyclooxygenase-2 (COX-2). Consequently, high NO levels cause inflammation and oxidative stress [6,7].

Overproduction of reactive oxygen species (ROS), free radicals related to oxidative stress and chronic inflammatory response is associated with damaged proteins, lipids, and nucleic acids. ROS, which includes the hydroxyl radical (OH), hydrogen peroxide (H₂O₂), and the superoxide anion (O₂⁻), lead to inflammatory responses by inducing iNOS and COX-2 expression in addition to activating TNF-α and the nuclear transcription factor κB (NF-κB) [8]. Hence, increased oxidative stress can cause various chronic diseases such as arteriosclerosis, diabetes mellitus, and Alzheimer’s disease [9-11]. Nevertheless, consuming anti-oxidant substances
can decrease NO and ROS via the mitogen-activated protein (MAP) kinase and the NF-κB signaling pathways [12-14]. MAP kinases such as the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, are involved in cell proliferation and apoptosis [15]. MAP kinases are activated by oxidative stress [16,17]. In addition, NF-κB is a relevant transcription regulator of inflammatory genes associated with many diseases [18]. Thus, the intake of bioactive substances with anti-oxidant properties could play important roles in various human diseases.

*Gentiana scabra* Bunge (GSB), which belongs to the Gentianaceae family has been used in traditional medicine for its anti-inflammatory, anti-tumor, and anti-oxidant properties. The rhizomes and roots of GSB contain triterpenoids and secoiridoid glycosides. Moreover, GSB roots contain polysaccharides that have immunological effects, as well as anti-coagulant, anti-tumor, and anti-oxidant activity [19-23].

A biocatalysis system called bioprocessing, biosynthesis or biocatalysis lead to the biotransformation of precursors to form new products using an enzyme’s substrate specificity. Bioconversion has many advantages including mild reaction conditions, high specificity, and a low cost [24-26]. Furthermore, anti-oxidative and neuroprotective activities were increased by bioconversion than before it [27].

Therefore, this study was designed to investigate the elevated anti-oxidant and anti-inflammatory activities of a bioconverted GSB. Free radical scavenging activities of the bioconverted GSB were determined using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. We determined the bioconverted GSB’s antioxidant activity was determined using DPPH and ABTS radical scavenging activity. The antioxidant condition was as follows: initial gradient 0% B (0 min) to 100% B (0-10 min). UPLC chromatograms were detected at 254 nm.

### Materials and Methods

#### Preparation of crude enzyme extraction

Ten grams of wheat bran with distilled water (DW, 1:1, w/v) was sterilized at 121 °C for 15 min. After sterilization, *A. kawachii* that was isolated from soybean [27] was inoculated in the sterilized wheat bran and fermented at 30 °C for three days. Afterward, 100 mL of 10 mM sodium phosphate buffer (pH 7.0) was added to the fermented wheat bran and incubated at 4 °C for 18 h. The mixture was filtered (filter paper, Advantec, Tokyo, Japan, No. 1) and centrifuged at 10,000 rpm for 10 min. The supernatant was used as an *A. kawachii* crude enzyme extract. This solution contained 0.276 U/mL (1U is defined as the enzyme activity needed to produce 1 mmol of p-nitrobenzene from p-nitrophenyl-β-D-glucopyranoside per min) of β-glucosidase activity.

#### Sample extraction and bioconversion

*Gentiana scabra* Bunge (GSB) was purchased from Omniherb Co. (Daegu, Korea). Five hundred grams of GSB was extracted twice with 70% ethanol (1:10, v/v) for 3 h using reflux extraction method. The extract was concentrated using a rotary vacuum evaporator (EYELA, N-1110, Tokyo, Japan) at 40 °C. After concentration, the extract was dissolved in DW (10:1, v/v) prior to the bioconversion step. Bioconversion was carried out through an *A. kawachii* enzymatic treatment (0.260 U/mL) [27]. Briefly, the extract solution was incubated with an *A. kawachii* crude enzyme extract (1:1, v/v) at 30 °C for 18 h and fractionated sequentially in n-hexane (He), ethyl acetate (EtOAc), and n-butanol (BuOH). The *A. kawachii* crude enzyme was inactivated by autoclaving for the control group. The fractions were concentrated using a rotary vacuum evaporator and stored at −20 °C before analysis.

#### UPLC chromatogram

UPLC chromatogram was conducted using Waters ACQUITY Ultra Performance LC systems (Waters, Milford, MA, USA), on an ACQUITY UPLC CSH™ C18 column (1.7 μL, 2.1×100 mm). An injection volume was 2 μL and a flow rate 0.3 mL/min were used. The mobile phase was filtered through a nylon membrane filter (0.45 μm). The mobile phase consisted of (A) 0.1% formic acid in DW and (B) 0.1% formic acid in methanol. The gradient condition was as follows: initial gradient 0% B (0 min) to 100% B (0-10 min). UPLC chromatograms were detected at 254 nm.

#### Evaluation of anti-oxidant activity

The antioxidant activity was determined using DPPH and ABTS radical scavenging activity assays. The DPPH assay was performed according to the method of Blois [28]. Briefly, 2 μL of sample was mixed with 190 μL of 150 mM DPPH solution and 8 μL of DMSO. The solution was incubated in the dark at 37 °C for 30 min and measured at 517 nm (Spectrostar Nano, BMG Labtech, Australia). ABTS was determined using the colormetric method with some modifications [29]. 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate (2:1, v/v) and allowed to react for 12-16 h. The reaction solution (ABTS’+) was diluted using 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7±0.02 at 734 nm. One hundred and ninety-eight micro liters of the dilution solution and 2 μL of sample were incubated for 1 min and the absorbance was detected at 734 nm using a microplate reader. DPPH and ABTS radical scavenging activity was calculated using the following equation:

\[
\text{Radical scavenging activity (\text{\%})} = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \right) \times 100
\]

#### Cell culture and cell viability assay

Raw 264.7 cells, mice monocyte macrophage cell line, were
and 5% CO₂ cells were seeded in 96-well plates at a density of 5×10⁴ cells/well and incubated for 24 h in a DMEM media supplemented with 10% FBS and 1% P/S. After 24 h, cells were treated with various sample concentrations (10, 25, 50, 100 ppm) for 1 h and then stimulated with 1 μg/mL of lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) from *E. coli* 0111:B4 for an additional 24 h. After the 24 h incubation with LPS, the cells were added to 100 μL of the MTT solution in each well and allowed to incubate for 4 h. The amount of MTT-formazan crystals was measured at 575 nm using a microplate reader [30]. Cell viability was expressed as percentages of the control group that was untreated with LPS.

**Nitric oxide production assay**

Nitric Oxide (NO) production was measured as previously described using Griess reagents [30]. In brief, RAW 264.7 cells were seeded in 96-well plates at a density of 5×10⁴ cells/well and incubated for 24 h in a DMEM medium containing 10% FBS and 1% P/S. After 24 h, cells were treated with various sample concentrations (10, 25, 50, 100 ppm) for 1 h and then stimulated with 1 μg/mL of lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) from *E. coli* 0111:B4 for an additional 24 h. After the 24 h incubation with LPS, the cells were added to 100 μL of the MTT solution in each well and allowed to incubate for 4 h. The amount of MTT-formazan crystals was measured at 575 nm using a microplate reader [30]. Cell viability was expressed as percentages of the control group that was untreated with LPS.

**Western blot analysis**

The RAW 264.7 cells (5×10⁴ cells/well) were cultured in a 100 mm dish and incubated for 24 h prior to being treated with various concentrations of the sample (50, 100 μg/mL) and with LPS (1 μg/mL) stimulation for 1 h. After incubation for 24 h, the cells were washed with PBS (Gibco BRL, Grand Island, NY, USA), harvested and centrifuged at 12,000 rpm for 1 min at 4 °C. After centrifugation, the supernatant was discarded and 400 μL of pro-prep protein extraction solution (iNtRON Biotechnology Inc., Seoul, Korea) was added. Protein extraction was undertaken at −20 °C for 30 min and centrifuged at 12,000 rpm for 10 min at 4 °C. A BCA protein assay kit (Thermo, Meridian Rd, Rockford, USA) was used to determine the amount of protein in the supernatant. Total protein concentrations (20 μg) were stored at −20 °C before use. The extracted proteins were electrophoretically separated on 10% SDS-PAGE (polyacrylamide gels) and transferred to PVDF membranes (Bio-Rad, Indianapolis, IN, USA). The membranes were blocked with 5% skim milk at room temperature for 1 h. Primary antibodies such as β-actin, iNOS, ERK, p-ERK, p38, p-p38, IKKα, p-IKKα, IκB, p-IκB, NF-κB, and JNK (Cell Signaling Technology, Boston, MA, USA) as well as COX-2 (Abcam, Cambridge, MA, USA) were diluted 1:1,000 in bovine serum albumin (BSA, Amresco, Solon, OH, USA) and incubated for 1 h at room temperature with gentle shaking. After washing with TBST, secondary antibodies (anti-mouse 1:1,000, anti-rabbit 1:1,000, v/v) that were purchased from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA) were added to the membrane for incubating at room temperature for 1 h. The membranes were washed again with tris-buffered saline/tween 20 (TBST). Protein bands were detected using enhanced chemiluminescence (ECL) western substrate solution (EZ west Lumi plus, ATTO, Tokyo, Japan) and measured by Image J 1.48v (NIH, Bethesda, NC, USA) [31,32].

**Nuclear and cytoplasmic protein extraction**

Cells were cultured as previously described in western blot analysis. Nuclear and cytoplasmic protein extraction were undertaken using nuclear extraction kit (Abcam, ab113474, New Territories, Hong Kong) according to the manufacturers protocol. In brief, cells were washed with PBS buffer twice and collected using a scraper. The cells were subsequently centrifuged at 1,000 rpm for 5 min. After discarding the supernatant, 100 μL of a 1X pre-extraction buffer was added and incubated on ice for 10 min. The extraction was vortexed vigorously for 10 sec and centrifuged at 12,000 rpm for 1 min. The supernatant was used as the cytoplasmic extract and the pellet was used as the nuclear pellet. The cytoplasmic extract was quantified using the BCA protein assay kit. An extraction buffer containing DTT and PIC (1:1,000, v/v) was added to the pellet prior to it being incubated on ice for 15 min with vortexing every 3 min. After centrifugation at 14,000 rpm for 10 min at 4 °C, the supernatant was quantified using the BCA protein assay kit. The nuclear and cytoplasmic protein extractions were stored in −20 °C until analysis [33].

**RNA preparation and reverse transcriptase (RT)-PCR**

Cells were cultured at a density of 1×10⁶ cells/well in a 6-well plate and treated as previously described. Total RNA was isolated using a Trizol reagent (Ambion, Austin, TX, USA). Briefly, 1 mL of Trizol was added to the cells and incubated at room temperature for 5 min. Afterward, 250 μL of chloroform was added and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was added to 550 μL of isopropanol and incubated at room temperature for 5 min. This solution was centrifuged again at 13,200 rpm for 20 min at 4 °C, and the supernatant was removed and added to 700 μL of 75% ethanol in DEPC water. The solution was centrifuged again at 9,500 rpm for 5 min at 4 °C after which the supernatant was discarded. Total RNA concentration was dissolved in DEPC water and mixed with PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa, Tokyo, Japan) at a 1:20 (v/v) dilution. The mixture was incubated at 65 °C for 5 min and afterward kept on ice. A polymerase chain reaction (PCR, Analytik, Jena, Germany) was subsequently performed at 42 °C for 45 min and 95 °C for 5 min. Reverse transcriptase (RT)-PCR was performed using a mixture of cDNA, RT-PCR premix and
antisense primers under the following conditions: 30 cycles of 98 °C for 10 min, 55 °C for 30 sec, 72 °C for 1 min. The PCR products were separated using electrophoresis with 1.0% agarose gels at 100 volts for 30 min. β-Actin, iNOS, COX-2, IL-6, IL-1β, and TNF-α were detected using previously described primer pairs. The β-actin primers were 5'-GTGGGCCGCCCTAGGCACCAG-3' (sense) and 5'-GGAGGAAGAGGATGCGGCAGT-3' (antisense), the iNOS primers were 5'-CCCTTCCGAAGTTTCTGGCAGCGC-3' (sense) and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (antisense), the IL-6 primers were 5'-GTACTCCAGAAGACCAGAGG-3' (sense) and 5'-TGCTGGTGACAACCACGGCC-3' (antisense), the IL-1β primers were 5'-CAGGATGAGGACATGACCC-3' (sense) and 5'-CTCTGCAGACTCAAACTCCAC-3' (antisense) [34].

Statistical analysis
Data were expressed as means ± standard deviation (SD). Statistical analyses were conducted using Student’s T-test and p <0.05 was considered statistically significant.

Results

UPLC chromatogram
The UPLC profile of the GSB extracts before and after bioconversion is shown in Fig. 1A. A slight difference was observed between the hexane fractions of A. kawachii-bioconverted GSB (GH-BA, Fig. 1B) and A. kawachii-untreated GSB (GH-UA, Fig. 1A). In addition, no distinct change was seen between the BuOH fractions of A. kawachii bioconverted GSB (GB-BA Fig. 1F) and A. kawachii-untreated GSB (GB-UA, Fig. 1E). However, the ethyl acetate fraction of the A. kawachii-bioconverted GSB showed unknown peaks with remarkable increments (GE-BA, Fig. 1D), as compared to the ethyl acetate fraction of the A. kawachii-untreated GSB (GE-UA, Fig. 1C).

Anti-oxidant activity
Due to the ease of protocols involved, the DPPH and ABTS free radical scavenging activities are widely used in anti-oxidant experimental assays [35]. The free radical scavenging effects of GSB were investigated with the DPPH and ABTS assays, and results are shown in Fig. 2. GSB showed similar free-radical scavenging activity patterns under both DPPH and ABTS assays. Specifically, the free-radical scavenging activity of all the GE-BA concentrations increased remarkably as compared to the GE-UA concentrations, while the other fractions showed no difference between the bioconverted and bio-unconverted extracts. DPPH radical scavenging activity of 200 ppm GE-BA was 87.32±0.75%, while it was 30.87±1.55% for the same GE-UA concentration (Fig. 2A). The DPPH radical scavenging activity of 150 ppm and 200 ppm GE-BA concentrations increased by more than 50%, than seen at the same concentrations of GE-UA.

In addition, ABTS radical scavenging activity was significantly higher at all concentrations. Particularly, GE-BA free-radical scavenging activity was higher (94.43±2.42%) than GE-UA.
Evidence indicates that bioconversion increased the anti-oxidant activity of GSB.

Cell viability and NO production
The effect of GSB on the viability of Raw 264.7 cells is shown in Fig. 3A. Both the bioconverted and the bio-unconverted GSB fractions had similar cell viability as the control group (CNT) at all concentrations tested (10-100 ppm), indicating that GSB had no toxic effect on the RAW 264.7 cells at 10, 25, 50 and 100 ppm (Fig. 3A). As shown in Fig. 3B, at 24 h post exposure, the NO production significantly decreased in a dose-dependent manner after treatment with GE-BA at 10, 25, 50 and 100 ppm (51.65±3.57, 45.66±4.76, 20.15±7.31, and 5.85±7.62%, respectively); however, only a slight decrease was seen after treatment with GE-UA (79.62±8.65, 84.72±5.41, 79.92±6.64, and 79.77±1.50%, respectively), both being compared to the LPS treated group. Particularly, at 100 ppm, GE-BA decreased the NO production by approximately 95%, compared to the LPS-stimulated group. However, the other fractions had no effect on the RAW 264.7 cells.

Effect of bioconversion on the iNOS and COX-2 expression
Based on the previous NO production results, the ethyl acetate GSB fraction was selected for further experiments. The effect of GE-BA on LPS-stimulated iNOS and COX-2 protein expression in RAW 264.7 macrophages was evaluated by Western blot analysis. The analysis proceeded after a 24 h stimulation with LPS (1 μg/mL). As shown in Fig. 4, the expression level of iNOS and COX-2 increased after the LPS treatment. However, GE-BA remarkably inhibited the iNOS and COX-2 protein expressions as compared to GE-UA exposure, in the LPS-stimulated RAW 264.7
cells. Particularly, iNOS and COX-2 proteins expression decreased significantly after treatment with 100 ppm GE-BA ($p<0.001$).

**Effect of bioconversion on the MAPK pathway**

The effect of GE-BA on MAP kinases was examined in LPS-induced RAW 264.7 cells. The level of phospho-p38 (p-p38) decreased in the GE-BA-treated cells as compared to the LPS treated group, by 0.61±0.03-fold by LPS and 0.55±0.03-fold at 50 and 100 ppm, respectively (Fig. 5B). In addition, phospho-JNK (p-JNK) decreased by 0.68±0.04-fold at 100 ppm (Fig. 5C). The level of phospho-ERK (p-ERK) dramatically decreased in the GE-BA-treated cells than in the LPS group (0.38±0.03-fold and 0.19±0.04-fold at 50 and 100 ppm, respectively). The total ERK, p38 and JNK in the GE-BA treated cells had a tendency to increase as much as the CNT group, while the decrease in the GE-UA-treated cells was proportionate to that seen in the LPS group. In other words, the ethyl acetate fraction of the bioconverted GSB was more inhibitory to the activation and phosphorylation of ERK, p38 and JNK, than the bio-unconverted extract.

**Effect of bioconversion on the NF-κB signaling pathway**

RAW 264.7 cells were pretreated with GE-BA and GE-UA (50 and 100 ppm) for 1 h, after which they cells were stimulated with 1 μg/mL of LPS for 1.5 h. Cells were further incubated with IKKα and IκB for 24 h after LPS stimulation. The expression of NF-κB was examined in the nuclear and cytoplasmic fractions. The levels of NF-κB increased in the nuclear fractions after 1.5 h of stimulation with LPS (Fig. 6A). However, treatment with GE-BA decreased the NF-κB levels in the nuclear fraction in a concentration-dependent manner. IκB and IKKα were examined by Western blot analyses after 24 h stimulation with LPS. LPS treatment reduced the IκB levels at 24 h. In addition, 100 ppm
GE-UA concentration also reduced the IκB levels at 24 h. However, GE-BA completely blocked the IκB degradation in a concentration-dependent manner at 24 h. IKKα showed similar results as IκB. After 24 h LPS stimulation, GE-UA and LPS treatment decreased the IKKα levels, while GE-BA blocked IKKα degradation. As shown in Fig. 6C, p-IκB/IκB also decreased after LPS stimulation for 15 min. mRNA expression of iNOS, COX-2 and pro-inflammatory cytokines

RAW 264.7 cells were treated with varying concentrations of GE-BA and GE-UA (50 and 100 ppm). The RAW cells were harvested at 24 h after LPS stimulation, and the levels of the inflammatory cytokines IL-6, IL-1β, and TNF-α were measured by RT-PCR. LPS-induction increases the iNOS, COX-2, IL-6, IL-1β, and TNF-α mRNA levels in RAW 264.7 cells, compared to the CNT group. As shown in Fig. 7B, GE-BA decreased iNOS mRNA expression compared to the LPS group, by 0.51±0.01-fold at 100 ppm concentration. In addition, GE-BA remarkably decreased the IL-6 and IL-1β mRNA expression by 0.30±0.00-fold and 0.45±0.04-fold at 100 ppm, respectively (Fig. 7D, E). Similarly, COX-2 and TNF-α mRNA expression were remarkably decreased.
in the 100 ppm GE-BA-treated group as compared to the LPS group, by 0.50±0.04-fold and 0.53±0.030-fold, respectively (Fig. 7C, F). However, GE-UA had no remarkable effect on the COX-2 and TNF-α levels. These results indicate that the inhibition of inflammatory cytokines is enhanced through bioconversion.

Discussion

The incidence of inflammation, which is relevant in renal, neural, intestinal and pulmonary diseases, has increased in the last decade [36-38]. Recent studies indicate that physiologically active substances such as naringenin, exhibit an anti-nephrotoxic effects by reducing the inflammation and oxidative stress [39]. Moreover, bioconversion mediated increments in the amount of quercetin, the main antioxidant component, enhanced the anti-oxidant activity physiologically [27]. Thus, this study aimed to investigate the improvements in the anti-oxidant and anti-inflammatory activities of GSB through bioconversion, using crude enzyme extract of A. kawachii that belongs to the glycosyl hydrolase family [40].

GSB contains bioactive components such as gentiopicroside and swertiamarin, which are well-known glycosides [41].

In the UPLC profile, the ethyl acetate GSB fractions showed unknown and increased peaks after bioconversion, compared with that of the untreated or inactivated crude enzyme treated fraction. This could be associated with glycosyl hydrolase from A. kawachii, which shows hydrolytic activity toward laminarin, a polysaccharide from seaweed [42,43]. Thus, more compounds might be converted by an enzymatic reaction. Further studies are underway to confirm these results.

Free radicals, including ROS, are molecules containing one or two unpaired electrons that cause damage to tissues and cells. Excess free radicals lead to acute and chronic oxidative stress, resulting in various diseases through oxidized reactions with amino acids, fatty acids, and other bio-macromolecular compounds. However, free radicals are scavenged by antioxidants such as flavonoids, phenolic compounds, vitamin E and rutin [44]. The DPPH assay, which is a simple and stable method, is based on electron and hydrogen transfers. When DPPH radical is scavenged by hydrogen or an electron-donating molecule, absorbance is decreased [45]. However, ABTS assay acts by decolorizing the ABTS cation radical (ABTS⁺) when an antioxidant is present [28]. The DPPH and ABTS radical scavenging activity by phenols, which have high antioxidant activity, yield the same results [46]. Bioconversion using lactic acid fermentation increased the free radical scavenging activity of mulberry juice by improving phenolic and flavonoid constituents [47].

In our study, the ethyl acetate fraction GSB increased the DPPH
and ABTS radical scavenging activity; especially, there was a remarkable increase in the antioxidant activity of the ethyl acetate fraction of *A. kawachii*-bioconverted GSB (GE-BA) than in the *A. kawachii*-untreated GSB (GE-UA) ethyl acetate fraction. These results indicate that the transformation of GSB compounds caused by bioconversion using *A. kawachii* crude enzyme increased their anti-oxidant activity.

NO is a free radical that reacts with oxygen radicals and is synthesized by NOS. Low levels of NO are regulatory in their effects, such as in tumorigenesis and wound repair, etc. However, high levels of NO contribute to DNA damage, cytotoxicity, oxidative and nitrosative stress. NOS has three isoforms, including neuronal NO synthase, inducible NO synthase (iNOS), and endothelial NO synthase. Overproduction of NO results in higher

![Fig. 6 Effect of GSB's bioconversion on NF-κB signaling pathway. (A) Western blot result of NF-κB signaling pathway (B) expression of nucleus NF-κB/cytoplasm NF-κB (C) expression of p-IκB/IκB. All data are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared to LPS group. \#p < 0.05, \#\#p < 0.01, \#\#\#p < 0.001 compared to bioconversion of the EtOAc fraction.](image_url)
production of iNOS, which is highly expressed during inflammation. iNOS regulates the inflammatory cytokines such as IL-1β and TNF-α [6,48]. Likewise, COX catalyzes the biosynthesis of prostaglandins, which are involved in edema, inflammation, and tumors from arachidonic acid, and has two isoforms: COX-1 and COX-2. [48,49]. NO production as well as iNOS and COX-2 expression increase after stimulation with LPS [50-52]. Also, previous studies have reported that iNOS and COX-2 expression were suppressed by bioactive substance [31,32].

This study also found that GE-BA inhibited the COX-2 expression, while LPS treatment increased it. In addition, NO production and iNOS expression were increased after stimulation with LPS, while GE-BA remarkably decreased the expression of both isoforms. These results suggest that low-level NO led to a reduction in iNOS expression, and this effect was enhanced through bioconversion.

MAP kinases (including ERK, JNK and p38) are activated by various stimuli, including inflammation, osmotic shock, apoptosis and oxidative stress [16,53]. Obesity activates MAP kinases, while quercetin suppresses their phosphorylation [13]. In this study, we observed that LPS increased the expression of MAP kinases. However, GE-BA treatment inhibited the activation and phosphorylation of MAP kinases, including ERK, JNK, and p38.

Activation of p38 is related to NF-κB signaling [55]. NF-κB is a major transcription factor for the expression of pro-inflammatory
mediators such as iNOS, COX-2, and IL-1\(\beta\), when stimulated by LPS [56,57]. Inactive NF-\(\kappa\)B combines with IkB, and is present in the cytoplasm. The IKK\(\alpha\) complex, which induces the phosphorylation of IkB and NF-\(\kappa\)B, translocates to the nucleus and regulates the transcription of iNOS and COX-2 [18,58]. LPS stimulation remarkably increases the phosphorylation of IKK\(\alpha\), with an equivalent increase in the phosphorylation and degradation of IkB [59]. Finally, activated NF-\(\kappa\)B increases the production of inflammatory cytokines [57]. According to a study by Hu TY [60] bioactive component such as xanthone was decreased phosphorylation of NF-\(\kappa\)B.

In the present study, we found that LPS stimulation led to the accumulation of NF-\(\kappa\)B in the nucleus, which may be due to the translocation of NF-\(\kappa\)B from the cytoplasm to the nucleus. In GE-BA-treated cells, the level of NF-\(\kappa\)B significantly decreased in the nucleus. Furthermore, the level of IKK\(\alpha\) and IkB (at 24 h) inhibited the phosphorylation and degradation, as compared to GE-UA. This result suggests that GE-BA blocked the LPS stimulated IKK\(\alpha\) phosphorylation and inhibited the IkB degradation. Eventually, GE-BA inhibited the traslocation of NF-\(\kappa\)B from the cytoplasm to the nucleus. As a result, we also observed that GE-BA inhibited the mRNA expression of inflammatory cytokines, such as iNOS, COX-2, TNF-\(\alpha\), IL-6, and IL-1\(\beta\). Our study indicates that bioconversion, which involves the enzymatic activity of \textit{A. kawachii} crude enzyme on GSb, decreases the production of inflammatory mediators through IKK\(\alpha\), IkB, and NF-\(\kappa\)B signaling pathways in RAW 264.7 cells, than in the \textit{A. kawachii} crude enzyme-ununtreated groups.

In summary, we propose that the ethyl acetate fraction of GSb enhances anti-oxidant and anti-inflammatory activities through the \textit{A. kawachii} crude enzyme mediated bioconversion. Specifically, the anti-inflammatory activity is associated with the inhibition of MAP kinases and the NF-\(\kappa\)B signaling pathway. As a result, the iNOS, COX-2, and inflammatory cytokines were remarkably reduced. These findings suggest that bioconversion of GSb with \textit{A. kawachii} crude enzyme may have potential benefits for the treatment of inflammation and related oxidative stress diseases.

**References**


