Anti-Inflammation Assay of Black Soybean Extract and Its Compounds on Lipopolysaccharide-Induced RAW 264.7 Cell

W Widowati1*, S Prahasuti1, N L W Ekayanti2, U Z Munshy3, H S W Kusuma2, S H B Wibowo3, A Amalia2, W S Widodo2 and R Rizal2

1Faculty of Medicine, Maranatha Christian University, Jl Surya Sumantri No. 65, Bandung 40164, West Java, Indonesia
2Biomolecular and Biomedical Research Center, Aretha Medika Utama, Jalan Babakan Jeruk II No. 9, Bandung 40163, West Java, Indonesia

*Corresponding author’s email: wahyu_w60@yahoo.com

Abstract. Inflammation response is related with various diseases. One of the useful therapeutic method to suppress inflammatory mediator synthesis is by application of compounds isolated from herbal medicine as treatment for inflammatory diseases. The aim of this study was to analyse the anti-inflammatory activity of black soybean extract (BSE), daidzein, and genistein through in vitro analysis of inflammatory mediators such as prostaglandin 2 (PGE2) and cytokines interleukin 1β (IL-1β), and tumor necrosis factor α (TNF-α). Safety of samples was determined by viability test using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Concentration tested for viability assay were 40, 200, 1000 µg/mL for BSE, daidzein, and genistein. Anti-inflammation activity of samples was determined by ELISA quantification of PGE-2, TNF-α, and IL-1β in conditioned medium (CM) of supplemented pro-inflammatory activated RAW 264.7 cell. Inflammation on cells were induced by Lipopolysaccharide (LPS). BSE 1000 µg/mL, daidzein 1000 µg/ml, and genistein 1000 µg/ml treatments shows <80% cell viability average compared to control cell, indicating the treatments have cytotoxicity effect on RAW 264.7 cells. Hence, concentration used for treatments are 40 and 200 µg/mL for each sample. Genistein with concentration of 40 µg/ml treatment result shows highest anti-inflammatory activity which indicated from PGE-2, TNF-α, and IL-1β concentration. This study suggests that BSE, daidzein, and genistein with concentration of 40 and 200 µg/ml were safe to use for RAW 264.7 cell and genistein with concentration of 40 µg/ml have the best anti-inflammatory activity compared to daidzein and BSE.

Keywords : inflammation, black soybean, RAW 264.7 Cell, PGE-2, TNF-α, IL-1β

1. Introduction

Inflammation is a biological response of body tissues to perilous stimuli, such as pathogens, irritants, and damaged self-cell. This response relates with various diseases such as rheumatoid arthritis, Alzheimer’s, inflammatory bowel disease, atherosclerosis, and has a role in various cancer developments [1]. Many cells are involved in this response including macrophage which is the primary cells of chronic inflammation. During inflammation, macrophage is activated by exposure to interferon-γ, pro-inflammatory cytokines, or bacterial lipopolysaccharide (LPS) [2]. Activated macrophage releases amounts of inflammatory mediators such as nitric oxide (NO), reactive nitrogen species (RNS),...
inflammatory mediator nitric oxide (NO) and prostaglandin 2 (PGE2), and also pro-inflammatory cytokines such as interleukin (IL-12, IL-1β, IL-6) and tumor necrosis factor alpha (TNF-α) [2]. Bacterial endotoxin lipopolysaccharides (LPS) exposure can increase mRNA expression of those inflammatory cytokines and mediators which can stimulates innate immunity [3–5].

One of the useful therapeutic method in the treatment of inflammatory diseases is suppression of inflammatory mediator synthesis. Recently, application of compounds isolated from herbal medicine for the treatment of inflammatory diseases has been gaining interest. Herbal medicine also had several other properties such as non-toxic, Antioxidant (scavenge free radicals) and pharmacologically safe to use due to its pleiotropic immune modulatory properties [4,6,7].

Black soybean (Glycine max L. Merr) is not only used as protein source, but also functional food due to its isoflavone content that could prevent degenerative disease. Isoflavone in black soybean consist of four forms which are malonyl-glicoside, acetyl-glicoside, glicoside, and aglicon. The most significant biological activity is shown by aglicon form of isoflavone, genistein (5,7,4ꞌ-dihydroxy isoflavone), daidzein (7,4ꞌ-dihydroxy isoflavone) and glycitein (6-metoxo-7,4ꞌ dihydroxy isoflavone) [8,9]. Daidzein is an isoflavone mainly extracted from soy plants. Daidzein can be used as anti-inflammation by inhibition of signal transducer and activator of transcription 1 (STAT-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activations [10], meanwhile genistein has numerous antioxidative and anti-cancer effects and is known to restrict tyrosine specific protein kinases [11].

The aim of this study was to analyze the anti-inflammatory activity of black soybean extract (BSE), daidzein, and genistein on the in vitro production of inflammatory mediators such as, PGE2 and cytokines IL-1β and TNF-α.

2. Materials and Methods

2.1. Extract preparation

Black soybeans were obtained from Unit Pengelolaan Benih Sumber (UPBS) Balai Penelitian Tanaman Aneka Kacang dan Umbi, Malang, East Java. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. A total of 250 g of black soybean simplicia was added to the maserator for extraction. The solvent used was 70% ethanol. The filtrate was collected every 24 h and ethanol was added until the resulting filtrate was colorless. The filtrate was then evaporated using a rotary evaporator (Zhengzhou Well-known, RE-201D) at 50 °C until a paste extract was formed. The extract from black soybeans was then stored at -20 °C [12–14].

2.2. Cell culture

RAW 264.7 macrophages cell line (ATCC® TIB- 71™) was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Biowest L0060) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10082147) and 1% penicillin-streptomycin (Biowest L0022-100), and then incubated at 37 °C and 5% CO₂ until the cells were confluent. The cells then washed using Phosphate Buffer Saline (PBS) and harvested using trypsin-EDTA (Biowest, L0931-500) [12,15,16].

2.3. Pro-inflammatory activation of RAW 264.7 cells

The activation of inflammatory condition of the macrophage cells was performed according to previous study [12]. The cells with density of 5x10⁵ cells/well were plated in 6-well plate, incubated for 24 hr at 37 °C in a humidified atmosphere and CO₂ 5%. After the culture medium was discharged, 1,600 µM of fresh medium were supplemented to each well along with 200 µl of sample with various concentration (40 and 200 µg/ml of each sample: BSE, daidzein (Chengdu Biopurify Phytochemical Ltd, BP0445), genistein (Chengdu Biopurify Phytochemical Ltd, BP0635). Based on the viability assay. 200 µl of LPS from Escherichia coli (Sigma Aldrich, L2880) (1 µg/ml) was added into each well two hours following the addition of extract or compound, and the plate was incubated for 24 h at 37 °C, humidified
atmosphere, 5% CO₂. The medium then was collected and centrifuged at 2000xg for 10 min. The supernatant was collected and stored at -80 °C for further assay [12,15,16].

2.4. RAW 264.7 cell viability assay

The assay was conducted using Proliferation Assay Kit (Abcam, ab197010). This assay is based on the conversion of yellow tetrazolium salt to form a purple formazan product. Cell with density 5 x 10³ cell/well is seeded and incubated for 24 h (37°C, 5% CO₂). Briefly 20 µL of sample with various concentration (40, 200, 1000 µg/mL of each sample: BSE, daidzein, genistein) was added into each well and incubated for 24 h (37°C, 5% CO₂). After incubation, 20 µL of MTS 3 - (4,5-dimethylthiazol – 2 – yl) – 5 - (3 - carboxymethoxyphenyl) – 2 - (4-sulfoophenyl) - 2H tetrazolium) Cell Proliferation Assay Kit (Abcam, ab197010) was added into each well. After 3 h of incubation (37 °C, 5% CO₂), absorbance was measured by Multiskan-GO Microplate Reader at 490 nm [12,15,16].

2.5. Total protein assay

Bovine Standard Albumin (BSA) standard solution was made from dilution series of BSA stock. The stock was obtained by dissolving 2 mg of BSA (Sigma, A9576, Lot. SLB2412) in 1000 µL ddH₂O, briefly 20 µL of standard solutions and 200 µL Quick Start Dye Reagen 1X (Biorad, 5000205) was added into each well plate. After 5 min of incubation in room temperature, absorbance was measured by microplate reader at 595 nm [17].

2.6. PGE-2, IL-1β, and TNF-α assay

PGE-2, IL-1β, and TNF-α concentration was measured using Mouse ELISA Kit (E-EL-M0052/0049/0037). Briefly 100 µl of each standard solution was added into well. The plate was sealed and incubated for 90 min 37 °C. The solution in well was discarded and 100 µL/well of Biotinylated Detection Ab was added. The plate was closed and incubated for 1 hr 37 °C. The solution was discarded and each well was washed with Wash Buffer and settled for 1-2 min. The washing process was repeated for three times. After that on each well was added 100 µL/well of HRP Conjugate. The plate was sealed incubated in 37 °C for 30 min and followed by five times washing. 90 µL/well of substrate was added and incubated for 15 -30 min 37 °C. After the color change 50 µL/well of stop solution was added and Optical Density (OD) was measured by Multiskan GO Microplate Reader at 450 nm [12,15,16].

2.7. Statistical Analysis

All the experiment was done in triplicate. Statistical analysis was conducted using SPSS software (version 20.0). Data were presented as mean ± standard deviation. The Significant differences within the groups were determined using One-Way ANOVA followed by Tukey’s HSD Post-hoc Test.

3. Results and Discussion

Inflammation is a dynamic process involving pro-inflammatory cytokines, and it acts as an important biological response towards perilous stimuli [18,19]. In this study, we examined the anti-inflammatory properties of daidzein, genistein, and black soybean (Glycine max) extract on LPS-induced RAW 264.7 murine macrophage cell line which has been widely used as an inflammatory model in vitro [12,15,16,18,20,21]. During inflammation induced by LPS, RAW 264.7 cell release PGE2 and pro-inflammatory cytokines such as IL-1β and TNF-α [12,16,20,21,25].

3.1. RAW 264.7 cell viability

Cell viability was measured by MTS assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. The viability of the RAW 264.7 cells can be seen on table 1. BSE 1000 µg/ml, daidzein 1000 µg/mL, and genistein 1000 µg/mL treatments shows <80% cell viability compared to control cell, indicating the treatments have cytotoxicity effect on RAW 264.7 cells. Meanwhile, other treatment used were nontoxic and safe to the cells with cell viability 90% [12], this concentration to be
applied for the next assay. Hence, concentration used for treatment are 40 and 200 µg/mL for each sample.

Table 1. RAW 264.7 cell viability assay of BSE, daidzein and genistein.

| Concentration (µg/mL) | BSE Cells viability (%) | Daidzein Cells viability (%) | Genistein Cells viability (%) |
|-----------------------|-------------------------|------------------------------|-----------------------------|
| 0 (Control cell)      | 100.00 ± 3.54           | 100.00 ± 3.54                | 100.00 ± 3.54               |
| 40                    | 98.86 ± 7.63b           | 104.31 ± 10.56b              | 89.94 ± 4.87b               |
| 200                   | 87.76 ± 1.75b           | 99.95 ± 13.20b               | 87.56 ± 3.40b               |
| 1000                  | 67.20 ± 0.60a           | 78.61 ±2.12a                 | 71.92 ± 4.63a               |

Data are presented as mean ± SD. Different superscript letters a, b for BSE, a,ab,b for daidzein, a,b for genistein show significant differences among concentrations based on Tukey HSD post hoc test (P-value < 0.05).

3.2. PGE-2, TNF-α, and IL-1β assay

Measurement of standard curve for total protein determination is presented. The linearity of the curve is in acceptable range. Total protein measured is 125.30 ± 4.79 (µg/mL). The results of PGE-2, TNF-α, IL-1β quantification which is shown in figure 1, 2 and 3 reveal that BSE had concentration dependent manner inhibition activity towards mentioned inflammatory mediators production in LPS induced RAW 264.7. Meanwhile, figure 1, 2 and 3 also reveal that daidzein and genistein also had inhibition activity towards mentioned inflammatory mediators but treatment with lower concentration which is 40 µg/ml resulted in better activity compared to dose of 200 µg/mL. PGE-2, TNF-α, and IL-1β level in positive control are significantly higher compared to the level of negative control which prove that LPS can increase mentioned inflammatory mediators production in RAW 264.7 cell.

Figure 1. Effect BSE, daidzein, and genistein toward PGE-2 level, inhibition activity on LPS-induced RAW 264.7 cells.

The data are presented as histogram, of mean±standard deviation.
BSE 4 = Black Soybean Extract 40 µg/mL, BSE 20 : Black Soybean Extract 200 µg/mL, DZ4 : Daidzein 40 µg/mL, DZ20 : Daidzein 200 µg/mL, GN4 : Genistein 40 µg/mL, G20 : Genistein Daidzein 200 µg/mL, CTR+ : Positive control, CTR- : Negative control

1A. Effect BSE, daidzein, and genistein toward PGE-2 level (pg/mL), different superscript letters (a,b,c,d,e) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

1B. Effect BSE, daidzein, and genistein toward PGE-2 level (pg/mg protein), different superscript letters (a,b,c,d,e) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

1C. Effect BSE, daidzein, and genistein toward inhibition activity over positive control (%), different superscript letters (a,b,c,d,e) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

PGE2 the most abundant PGs produced in the body, has a central role in inflammation and both cyclooxygenase-2 (COX-2). In inflammation, the expression of COX-2 and inducible microsomal prostaglandin E synthase-1 (mPGES-1) are enhanced by bacterial products and cytokines, resulted in increased PGE2 formation in macrophages and tissue cells [10]. PGE2 involves important mediators of many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility in physiological regulation. PGE2 Deregulated synthesis or degradation has been associated with a wide range of pathological conditions [32]. PGE2 indicating process of inflammation, leading to the classic signs of inflammation such as redness, swelling, and pain [33]. PGE2 has a role and its receptors in modulating the inflammatory response has been observed in atherosclerosis [34]. In this study, daidzein, genistein, and black soybean could inhibit PGE2, TNF-α, and IL-1β production in RAW 264.7 cell lines which suggest that they have anti-inflammatory effect through down regulation of those pro-inflammatory cytokines. Daidzein plays a vital role in the regulation of mammary tumor cell invasion induced by TNF-α, related to nuclear factor-kappaB (NF-κB) signaling pathway. Daidzein treatment can inhibit breast cancer cells MDA-MB-231 migration through suppression of TNF-α induced NF-κB and AP-1, followed by a reduction in the secretion of uPA from breast cancer cells [35]. Estrogen receptor-mediated response of Daidzein treatment on Alzheimer’s disease can decrease mRNA expression of IL-1 which is a key inducer of the expression inflammatory process related molecule in astrocytes cultured in the presence of LPS. These results further increase the possibility that daidzein may have potential to ameliorate the inflammatory process and also alleviate the risk of Alzheimer’s disease progression [36].

Figure 2. Effect BSE, daidzein, and genistein toward TNF-α level, inhibition activity on LPS-induced RAW 264.7 cells.
The data are presented as histogram, of mean±standard deviation.

BSE 4 = Black Soybean Extract 40 µg/mL, BSE 20 : Black Soybean Extract 200 µg/mL, DZ4 : Daidzein 40 µg/mL, DZ20 : Daidzein 200 µg/mL, GN4 : Genistein 40 µg/mL, G20 : Genistein Daidzein 200 µg/mL, CTR+ : Positive control, CTR− : Negative control

2A. Effect BSE, daidzein, and genistein toward TNF-α level (pg/mL), different superscript letters (a,ab,b,c,d) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

2B. Effect BSE, daidzein, and genistein toward TNF-α level (pg/mg protein), different superscript letters (a,ab,b,c,d) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

2C. Effect BSE, daidzein, and genistein toward inhibition activity over positive control (%), different superscript letters (a,b,bc,c,d) show significant differences among treatments based on Tukey HSD post hoc test (P-value < 0.05).

TNF-α is also a cytokine that plays a significant role in inflammation. This cytokine is produced chiefly by activated macrophages and play role during inflammatory response activating adhesion molecule inducer and nuclear factor kappa-light-chain-enhancer of activated cells (NF-κB) (25–27). TNF, being an endogenous pyrogenic, is able to induce fever, apoptotic cell death, cachexia, inhibit tumor genesis and viral replication, respond to sepsis via IL-1 and IL-6 producing cells. Deregulation of TNF production has been implicated in a variety of human diseases including Alzheimer’s disease, cancer, major depression, psoriasis and inflammatory bowel disease (IBD) (28–30). With all effects caused, TNF-α inhibitory activity measurement is important in anti-inflammatory potential agent screening since this cytokine is an important mediator of inflammation (31).

IL-1β is important for the initiation and increase the inflammatory response to microbial infection during inflammation process due to its role to induces secretion of proinflammatory cytokines such as IL-6 and IL-8 [19,22]. IL-6 has a wide range effect on immune system cells resulted in the acute...
inflammation response. Increasing of IL-6 level are reported in rheumatoid arthritis, psoriasis, and encephalomyelitis individuals [22–24]. Therefore, inhibition of IL-1β synthesis would indirectly be useful for autoimmune disease and inflammation treatment [12,15,16,20,21,25].

Genistein as the major isoflavone in soybean has advantageous effects in metabolic disorders and inflammatory diseases. Genistein in dietary concentrations of (1, 5, and 10 µM) can inhibit LPS-induced IL-6 and TNF-α overproduction in plasma and liver of NASH model rats [36,37]. This isoflavone also significantly reduced LPS-induced NO production, increased antioxidant enzyme activity, and suppressed NF-κB activation in RAW264.7 macrophages. Major isoflavones contained in black soybean (genistein, daidzein, and glycitein) reported to have inhibition activity of LPS-induced NO production and decrease iNOS activity and gene expression in RAW264.7 macrophages. The administration of genistein significantly decreased the IL-β expression in the dry-eye rat model group due to ovariectomy IL-1β involved conjunctiva inflammation which occurs in more than 80% of patients with dry eye syndrome [31-33, 40-42]. IL-1β are fundamental in the acute phase proteins synthesis and are produced in large quantities [43]. Genistein could suppress NF-κB activation in LPS-treated macrophages, which is in agreement with observations that genistein inhibits NF-κB that play role as pleiotropic regulator of many proinflammatory cytokines and has been found to be activated by a variety of stimuli. [42,44,45]. The results suggest that genistein’s ability to inhibit LPS-induced TNF-α and IL-6 release may be explained in part by blocking NF-κB activation [46]. By more than 50% COX 2 mRNA expression is inhibited in Murine J774 macrophages by genistein. As measured by quantitative RT PCR. All three isoflavones studied, daidzein, genistein, and genistein, inhibited PGE2 production and COX 2 mRNA accumulation [10]. Genistein has earlier been shown to down regulate PGE2 production and COX 2 expression in macrophages [47,48]. In breast cancer cell line MCF-7, both daidzein and genistein inhibited phorbl 12-myristate 13-acetate (PMA)-induced COX 2 expression [49].

The inhibition activity of BSE is due to its major isoflavone content, daidzein and genistein. Other than those two substance, BSE also contain other isoflavone such as glycitein which is the least studied isoflavone, due to the fact that it is the least abundant isoflavone found in soy. Glycitein has been studied for some therapeutic effects including anti-oxidant, hypocholesterolemic, antitumor activity and was also tested for its anti-inflammatory activity both in vitro and in vivo [8,9]. Based on the results of this study and that has been reported, daidzein, genistein, and BSE may have potential to be used as safe anti-inflammatory agent to prevent chronic disease related to inflammation according to the result of viability assay.

4. Conclusion
This study suggests that BSE, daidzein, and genistein with concentration of 40 and 200 µg/mL were safe on RAW 264.7 cell and genistein with concentration of 40 µg/mL have the best anti-inflammatory activity compared to daidzein and BSE by inhibiting PGE-2, TNF-α, IL-1β.

5. Conflict of interest
All contributing authors declare no conflicts of interest.

6. Acknowledgement
The authors gratefully acknowledge to Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for research grant, research methodology and laboratory facilities. We are thankful to Ika Adhani Sholihan, Dewani Tediana Yusepany, Dwi Surya Artie, Rizki Amalia from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for their valuable assistance.

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