Anti-inflammatory effect of aerial bulblets of *Dioscorea japonica* Thunb extract through inhibition of NF-κB and MAPK signalling pathway in RAW 264.7


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Abstract

**Background:** *Yam* (*Dioscorea japonica* Thunb) is a well-known health food in Korea and is widely distributed in the temperate and tropical regions. Although various medical effects of yam have been demonstrated, there is little current knowledge on the efficacy of Youngyeoja (YYJ; the aerial bulblets of the yam plant), their physiological effects, and their mechanism of action.

**Methods:** To investigate the anti-inflammatory effects of YYJ, we examined the level of inflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells treated with YYJ extract. Nitric oxide (NO) and prostaglandin E₂ (PGE₂) levels were determined using enzyme-linked immunosorbent assays. Expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were evaluated using real-time polymerase chain reaction and western blotting. In addition, activation of nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase (MAPK) was detected using western blotting.

**Results:** Treatment of macrophages with LPS markedly induced the production of NO and PGE₂. YYJ treatment inhibited the induction of inflammatory mediators and the expression of iNOS and COX-2. More importantly, LPS-induced phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) was suppressed by treatment with YYJ, suggesting YYJ inhibited NF-κB activation. Furthermore, YYJ inhibited the LPS-induced phosphorylation of MAPKs.

**Conclusion:** YYJ was shown to have a potent anti-inflammatory effect in LPS-stimulated RAW 264.7 cells, which may be attributed to its inhibitory effect on NF-κB and MAPK activation, consequently blocking the production of inflammatory factors. Therefore, these results suggest that the YYJ extracts could be used as anti-inflammatory agents.

**Keywords:** Inhibition; Macrophages; Phosphorylation; Western blotting; Yam

**1. INTRODUCTION**

Macrophages are a major component of the mononuclear phagocytic system and play important roles in the innate and adaptive immune responses. If the host immune response does not respond appropriately, these inflammatory mediators induce an inflammatory response, leading to inflammatory diseases such as neurodegenerative diseases, metabolic syndrome, and cancer. Macrophages are involved in mediators such as nitric oxide (NO), prostaglandins (PG), and proinflammatory cytokines in the immune response. Activated macrophages can express inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) that can produce much larger amounts of NO and prostaglandin E₂ (PGE₂). They also secrete various inflammatory mediators, such as cytokines and reactive oxygen, to cause an inflammatory reaction. Therefore, inhibition of proinflammatory mediators and inhibition of relevant pathways can effectively alleviate inflammatory symptoms. Aspirin is one of the most commonly used nonsteroidal anti-inflammatory drugs (NSAIDs) to resolve inflammation, exhibiting anti-inflammatory action by inhibiting COX activity. However, nonselective NSAIDs and selective COX-2 inhibitors have been associated with side effects, including renal dysfunction, heart failure, gastrointestinal toxicity, and increased risk of cardiovascular side effects. Therefore, many studies focus on the applications of natural plants for treating an inflammation.

*Yam* (*Dioscorea japonica* Thunb) is a perennial plant belonging to the family Dioscoreaceae and has long thick underground roots. It is widely distributed in temperate (eg, Korea, China) and tropical regions. *Yam* is an alkaline food and has been used in the treatment of gastritis, pulmonary tuberculosis, and diabetes. Saponins in yam are known to reduce arteriosclerosis, hypertension, and blood cholesterol content. However, the majority of studies have focused on the root, and very few studies have been conducted on the aerial bulblets of Yam, known as ’Youngyeoja (YYJ)’ in Korea. YYJ has been used for medicinal purposes but not as a food ingredient. The YYJ is kept as a seed for planting the following year, and most of it is discarded. However, it is expected that the utilization will increase, because it is registered as a food ingredient by the Ministry of Food and Drug Safety in Korea. Therefore, it is important to evaluate various functionalities of.
YYJ and to examine its applicability. Accordingly, we evaluated the anti-inflammatory activity of YYJ through the expression and secretion of proinflammatory mediators.

2. METHODS

2.1. Preparation of extracts

YYJ was obtained from the Andong-si Agricultural Center, Andong, Gyeongsangbuk-do, Korea. YYJ was cleaned, sliced, and dried at 50°C for 48 hours. Dried plant material (20g) was extracted twice with 70% ethanol (200mL) at 70°C for 6 hours in a shaking water bath (WSB-30; DAIHAN Scientific, Wonju, Korea). The final ethanol extract was filtered (No. 42, Whatman International Ltd., Maidstone, UK) and evaporated (EVELA CCA-110; Tokyo Rikakikai Co., Tokyo, Japan) at 40°C. The concentrated extract was lyophilized in a freeze dryer (Ilshin Lab, Suwon, Korea), and the lyophilized powder was stored at −20°C. YYJ extract yielded 22% ethanol extract from 20g dried plant material. For further analysis, the extracts were reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 100mg/mL and diluted to the final concentration for application with phosphate buffered saline (PBS).

2.2. Cell culture

The RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GenDEPOT, Barker, TX, USA) and 1% penicillin-streptomycin antibiotics (Gibco) at 37°C in 5% CO₂.

2.3. Cell viability

We investigated the cell viability of YYJ extracts by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1 × 10⁴ cells were seeded in a 96-well plate and incubated at 37°C for 2 hours. Various concentrations (0, 100, 200, and 300 μg/mL) of YYJ extracts were applied to cells for 24 hours with or without stimulation with lipopolysaccharide (LPS) (1 μg/mL). After 24-hour incubation, MTT reagent (5 mg/mL) was added to each well and incubated for another 4 hours at 37°C. The medium was removed, and DMSO was added to each well. The absorbance was measured at 540nm.

2.4. Nitrite oxide measurement

NO accumulation in cells was determined using Griess reagent (Promega, Madison, WI, USA). The cells were treated with various concentrations of YYJ extracts and stimulated with LPS. After 24-hour incubation, the cell supernatant was collected and measured using a sodium nitrite standard curve. The NO content was calculated using a sodium nitrate standard curve.

2.5. PGE2 production

After treating RAW 264.7 cells with LPS and YYJ extracts, cell supernatants were obtained. PGE2 was measured using an ELISA kit according to the manufacturer’s instructions (Abcam, Cambridge, MA, USA). Briefly, diluted cell supernatant was added to a 96-well plate and incubated for 2 hours. Then, the plate was washed using washing buffer and the color was developed by adding p-nitrophenyl phosphate substrate. After 45 minutes, stop solution was added and the PGE2 content was calculated using a PGE2 standard curve.

2.6. Real-time reverse transcription polymerase chain reaction analysis

To determine iNOS and COX-2 mRNA expression levels, real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using a Rotor-Gene Q real-time thermal cycler (Qiagen, Valencia, CA, USA). RAW 264.7 cells were treated with LPS and YYJ extracts. After incubation for 24 hours, RNA was isolated using an RNA extraction kit (Qiagen). cDNA was synthesized using M-MLV Reverse Transcriptase (Promega). The real-time PCR reaction was performed using 2X SYBR® Green Master Mix (Qiagen) and specific primers for the inflammatory-related genes iNOS and COX-2. All results were normalized to those of the housekeeping gene GAPDH.

2.7. Western blot analysis

YYJ extract-treated cells were lysed in lysis buffer (50mM Tris-HCl, 150mM sodium chloride, 0.5% NP-40, 0.5% Triton X-100, 0.1% sodium deoxycholate, and 1mM ethylenediaminetetraacetic acid) on ice for 40 minutes. The culture was centrifuged at 12,000g for 20 minutes at 4°C. The cell lysates were separated by 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), and incubated with the appropriate antibody, such as iNOS, COX-2, p-IκB, p-nuclear factor-kappaB (NF-κB), NF-κB, p-e-Jun N-terminal kinases (p-JNK), p-Akt, p-p38, p-extracellular-signal-regulated kinase (p-ERK), and β-actin (Abcam). Immunodetection was performed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA); the immune-signals were captured using the ChemiDoc image detector (Bio-Rad).

2.8. Determination of cytokine levels

Tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β levels in the culture medium of RAW 264.7 cells were quantified using ELISA kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

2.9. Measurement of NF-κB activity

RAW 264.7 cells were transfected with a p-NF-κB-luciferase vector (Promega) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). RAW 264.7 cells (5 × 10⁴) were seeded in a 24-well plate and incubated for 24 hours to grow to 80% to 90% confluence. The cells were treated with DNA-TransFast reagent mixture. After 16 hours of incubation, the media in each well was replaced with growth media and the transfection was allowed to proceed for 48 hours. After transfection, cells were treated with YYJ extracts and stimulated with LPS for 1 hour. Luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined by normalizing the firefly luciferase activity to that of the Renilla luciferase.

2.10. Statistical analysis

Statistical analyses were performed with SPSS v12.0 (SPSS, Chicago, IL, USA). All data are expressed as mean ± SEM from three independent experiments, except where indicated. Statistical analysis was determined using the Student’s t-test with p < 0.05 considered as significant.

3. RESULTS

3.1. Effect of YYJ extract on cell cytotoxicity

The cytotoxicity of YYJ extracts was investigated using RAW 264.7 cells, which play a major role in inflammation, as shown in Figure 1. To confirm the cytotoxicity of YYJ extract, RAW 264.7 cells were cultured in a 96-well plate, and each extract was added at various concentrations (0, 100, 200, or 300 μg/mL). After 24 hours, the inhibition of growth was measured using MTT. The maximum concentrations of the YYJ extracts were within a range that did not show cytotoxicity up to 200 μg/mL. Therefore, subsequent experiments were performed at concentrations where no cytotoxicity was observed.

3.2. Effect of YYJ extracts on LPS-stimulated NO and PGE2 production

Next, we investigated whether YYJ extracts might have anti-inflammatory activities in LPS-stimulated cells. The results

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showed that LPS treatment for 24 hours significantly increased NO and PGE2 production as compared to that in the untreated group, whereas in the RAW 264.7 cells treated with YYJ extracts, NO and PGE2 production gradually decreased (Fig. 2), indicating that treatment with YYJ extracts effectively reduced the production of NO and PGE2 when inflammation was induced.

3.3. Effects of YYJ extracts on iNOS, COX-2 protein, and mRNA expression level

Real-time RT-PCR and western blotting assays were performed to confirm the inhibitory activity of YYJ extracts on LPS-induced iNOS and COX-2 expression. RAW 264.7 cells were treated with LPS (1 μg/mL) to induce iNOS and COX-2 expression and treated with 100 and 200 μg/mL of YYJ extracts. LPS stimulation increased protein expression of iNOS and COX-2, as well as their mRNA levels. However, a reduction in iNOS and COX-2 expression was observed in the groups treated with YYJ extracts (Fig. 3). Therefore, YYJ extracts appeared to suppress NO and PGE2 production in LPS-stimulated RAW 264.7 cells by reducing expression of their encoding genes at the transcriptional level.

3.4. Effects of YYJ extracts on LPS-induced cytokine production

We confirmed the effects of YYJ extracts on the production of proinflammatory cytokines in LPS-induced RAW 264.7 cells using ELISA. LPS stimulation significantly increased production of proinflammatory cytokines. However, YYJ extracts significantly reduced TNF-α, IL-6, and IL-1β production in a dose-dependent manner (Supplementary Fig. 1).

3.5. Effect of YYJ extracts on NF-κB activation

NF-κB signaling plays an important role in regulating the expression of iNOS and COX-2 genes. We investigated whether the inhibitory activity of YYJ extracts on iNOS and COX-2 expression could be induced by the inactivation of NF-κB. As shown in Figure 4A, YYJ extracts significantly inhibited the NF-κB luciferase activity induced by LPS in RAW 264.7 cells at the 200 μg/mL concentration, suggesting that YYJ extracts inhibit LPS-induced NF-κB activation.

A series of upstream regulatory signaling factors, such as interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6), transforming growth factor beta-activated kinase 1 (TAK1), and IkB kinase (IKK), mediate LPS-induced NF-κB activation. Activated IKK can directly phosphorylate IkB, leading to its degradation, to allow translocation of NF-κB into the nucleus to initiate target gene transcription. Therefore, we investigated whether YYJ extracts could inhibit LPS-induced IkB phosphorylation. As shown in Figure 4B, YYJ extracts inhibited LPS-induced IkB phosphorylation. LPS-induced RAW 264.7 cells had increased protein levels of phospho-NF-κB compared to those of LPS-untreated cells, without affecting total NF-κB expression. In contrast, YYJ extract treatment decreased phospho-NF-κB protein, also without any YYJ-mediated changes in total NF-κB protein expression (Fig. 4C). In addition, we confirmed the expression of upstream regulatory signaling factors, such as IRAK1, TRAF6, TAK1, and IKK. As shown in Supplementary Figure 2, YYJ extracts did not change the upstream regulatory signaling factors. These results...
suggested that YYJ extracts inhibit LPS-induced NF-κB activation through inhibiting IκB phosphorylation.

3.6. Effect of YYJ extracts on mitogen-activated protein kinase signaling

Mitogen-activated protein kinase (MAPK) pathways, which include Akt, ERK, p38, and JNK, are also involved in LPS-induced production of inflammatory mediators in macrophages. Therefore, the effect of YYJ extracts on the activation of MAPK pathways was examined by evaluating the phosphorylation of these kinases. As shown in Figure 5, YYJ extracts significantly suppressed LPS-induced phosphorylation of JNK, Akt, and ERK in RAW264.7 cells at the concentration of 200 μg/mL, indicating that YYJ extracts can suppress LPS-induced activation of MAPK pathways.

4. DISCUSSION

Inflammatory reactions are beneficial defense mechanisms that restore tissue structure and function to biological or inanimate invasion or tissue damage. However, uncontrolled and persistent inflammation is the cause of tissue damage in many diseases, including rheumatoid arthritis. Therefore, there have been many attempts to target substances involved in inflammatory reactions in the development of therapeutic methods for this disease. Yam is one of the most soothing medicines to cool rhythms and is effective for weakness, loss of appetite, and physical fatigue. It is also used as a nourishing tonic and is known as an effective herbal medicine for back pain, frostbite, burns, diarrhea, and enuresis. The reported constituents of yam include saponin, dioscin, vitamin C, mannan, amylase, and amino acids. It also has reported effects on gastrointestinal motility, antioxidant activity, inhibition of mutation, hypoglycemic activity, and anti-inflammatory processes. Because of these various effects, yam has been suggested to be effective as an anti-inflammatory. However, studies on the physiological activities of YYJ, aerial bulblets of yam, have never been conducted. Therefore, in the present study, we report the anti-inflammatory effects of YYJ for the first time.

Among the inflammatory mediators secreted in macrophages in response to LPS stimulation, the release of NO and PGE2 is an important factor in the inflammatory process. In this study, YYJ treatment prevented the LPS-stimulated production of NO and PGE2 in RAW 264.7 macrophages. NO and PGE2 production was decreased. In addition, YYJ is a potent inhibitor of iNOS and COX expression, which are related to NO and PGE2 production. In the present study, iNOS and COX-2 are
representative genes regulated by NF-κB and MAPK. NF-κB is inactivated by forming a complex with IκB, an inhibitory protein, and is present in the cytoplasm. Inflammatory stimulation, including LPS, activates IκB kinase, thereby phosphorylating the serine residue in the N-terminal region of IκB. Phosphorylation of IκB induces multisubunit formation of the lytic residues of IκB, thereby promoting its degradation by the proteasome complex.

On the degradation of IκB, the nuclear translocation sequence of the NF-κB complex hidden in the NF-κB/IκB complex is exposed. NF-κB is targeted to the nucleus, binding to the target gene promoter and inducing the expression of various genes including inflammation-mediated genes. The activation of NF-κB in macrophages is synergistically influenced by the activation of various sensing molecules in the cell. MAPK is the most representative signaling molecule that affects the activation of NF-κB. MAPK has been reported to have three members, including inflammation-mediated genes. The activation of NF-κB by LPS stimulation, nuclear accumulation of NF-κB, and phosphorylation of MAPKs were observed. Activation of many NF-κB and MAPK signaling molecules by LPS was inhibited by the YYJ extracts, but that of p-p38 was not. This suggests that YYJ can influence the expression of inflammatory mediators by regulating the activity of NF-κB and MAPK signaling (except p38), which are activated by inflammatory signals.

In conclusion, our results suggest novel physiological effects of YYJ extracts, which are available as a foodstuff and partially elucidate the molecular mechanism involved. If subsequent studies on the efficacy of the YYJ extracts in vivo are promising, it is expected that YYJ extract will be used for the treatment of various inflammatory diseases as a new anti-inflammatory agent. If subsequent studies on the efficacy of the YYJ extracts in vivo are promising, it is expected that YYJ extract will be used for the treatment of various inflammatory diseases as a new anti-inflammatory agent. This would lead to an increase in the utilization of YYJ extracts.

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APPENDIX A. SUPPLEMENTARY DATA
Supplementary data related to this article can be found at http://links.lww.com/JCMA/A22.

REFERENCES