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Anti-Inflammatory Effects of Grasshopper Ketone from *Sargassum fulvellum* Ethanol Extract on Lipopolysaccharide-Induced Inflammatory Responses in RAW 264.7 Cells

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This study evaluated the anti-inflammatory potential of a grasshopper ketone (GK) isolated from the brown alga *Sargassum fulvellum* on lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophage cell line. GK was isolated and purified from the *n*-hexane fraction and its structure was verified on the basis of NMR spectroscopic data. GK up to 100 μg/ml is not cytotoxic to RAW 264.7, and is an effective inhibitor of LPS-induced NO production in RAW 264.7 cells. The production of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α was found significantly reduced in 0.1−100 μg/ml dose ranges of GK treatment ($p < 0.05$). We confirmed the dose-dependent and significant inhibition of iNOS and COX-2 proteins expression. In addition, it has been shown that GK induces anti-inflammatory effects by inhibiting MAPKs (ERK, JNK, and p38) and NF-κB p65 phosphorylation. Our results show that the anti-inflammatory properties of GK may be due to the inhibition of the NF-κB and MAPKs pathways, which are associated with the attenuation of cytokine secretion.

Keywords: Grasshopper ketone, anti-inflammation, *Sargassum fulvellum*, nuclear transcription factor kappa-B, mitogen-activated protein kinases

Introduction

Inflammation is a complex biological response of the body for protection of damaged tissue against bacterial infection or pathogens [1]. In a general inflammatory response, pro-inflammatory mediators are decreased after antigens are removed, while anti-inflammatory mediators are increased. However, chronic inflammatory disease occurs when there is a remarkable imbalance between pro-inflammatory and anti-inflammatory processes [2]. Chronic inflammation may cause several diseases, like periodontitis, hay fever, atherosclerosis, rheumatoid arthritis, and even cancer [3].

Macrophages play an important role in the inflammatory response and are the essential cellular mediators of the innate immune system. Lipopolysaccharide (LPS) is an outer membrane component of gram-negative bacteria that activates macrophages via Toll-like receptor 4 (TLR 4) stimuli signaling. Once activated, several intracellular signaling pathways such as nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs) are stimulated. The activated macrophages release excessive amounts of NO and prostaglandins (PGs) which are generated by iNOS (inducible nitric oxide synthase) and COX (cyclooxygenase)-2, respectively, as well as pro-inflammatory cytokines like interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α). These signaling pathways are able to induce inflammatory reactions, resulting in the development of various inflammation-related diseases [1].

It has been reported that marine organisms account for 80% of all living organisms on Earth [4]. Marine algae have been used for many years in many countries around the world, including Korea and Japan, due to the enormous amount of minerals that can be derived from the unique underwater environment with high salinity and high

pressure levels compared to the soil environment [5].

In the present study, *Sargassum fulvellum*, which is widely distributed in Southeast Asia, was used as a natural resource. *S. fulvellum* is a kind of perennial brown algae and about 20 different species exist in South Korea [6]. It is particularly known as edible seaweed and is composed of 30–60% polysaccharide, including cellulose, fucoidan, laminaran, alginic acid, 15.8% protein, 5% fat, and 27.5% ash [7]. According to the *Donguibogam* (or, *Principles and Practice of Eastern Medicine*), *S. fulvellum* is used for the treatment of wens (trichilemmal cysts), edema, and urination disturbance [8]. So far, *S. fulvellum* has been reported to have anti-cancer [9], anti-microbial and anti-oxidant [10], and anticoagulation [11] properties. However, there is currently no research on the anti-inflammatory effects of fractions isolated and purified from *S. fulvellum*.

Therefore, in this study, we showed that a grasshopper ketone (GK) was isolated and purified from the fractions of *S. fulvellum* ethanol extract and was examined to verify its anti-inflammatory activity and develop potential therapeutic material for inflammation-related diseases.

Materials and Methods

Chemicals

Specific antibodies against β-actin, iNOS, COX-2, NF-κB p65, p-p38, p-ERK, p-JNK, and anti-mouse immunoglobulin G (IgG)-conjugated horseradish peroxidase were obtained from Santa Cruz (USA). LPS, dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagents were purchased from Sigma-Aldrich Co., LLC. (USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-6, and IL-1β were purchased from BD Biosciences (USA) and Dulbecco's Modified Eagle's Medium (DMEM) from GIBCO (USA). BCA protein assay kit and enhanced chemiluminescence kit (ECL kit) were from Pierce (USA), and fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone (USA).

Plant Material

S. fulvellum purchased from Jindo, Jeollanam-do, Korea (2013) was used in this study.

Extraction and Isolation of Active Compound

Powdered *S. fulvellum* (1.3 kg) was extracted with 95% ethanol for 24 h at room temperature with an agitator (Dongwon Science Co., Korea). Then, the extract was centrifuged at 2,090 × g for 10 min and the supernatant was filtered and concentrated using a rotary evaporator (Yamato Co., Japan). *S. fulvellum* ethanol extract (125 g) was suspended in water and *n*-hexane. The active *n*-hexane fraction (52 g) was subjected to silica gel column chromatography and successively eluted with stepwise gradient ($CHCl_3$:MeOH, 50:1-5:1; v/v). The active fraction was subsequently subjected to Sephadex LH-20 column chromatography (2.5 × 90 cm column; $CHCl_3$:MeOH, 1:1; v/v). Finally, the fraction was purified using preparative ODS HPLC (25% MeOH, 3 ml/min), yielding an active compound (6 mg). The structure of the compound (Fig. 1) was determined from its NMR spectroscopic data (data not shown) and was compared with those identified in the literature as GK. This purified compound was subjected to evaluation of its cytotoxicity and anti-inflammatory effects.

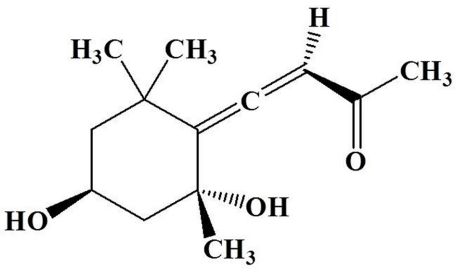

Fig. 1. Chemical structure of grasshopper ketone.

Cell Culture

The murine macrophage RAW 264.7 cells were purchased from Korean Cell Line Bank (KCLB 40071). The cells were cultured in plastic dishes containing DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator (Sanyo, Japan) at 37°C. All cells were sub-cultured when they grew at the density of about 80–90% in the experimental process. Only cells that did not exceed 20 passages were used.

Cell Viability Assay

RAW 264.7 cells (1×10^6 cells/ml) were seeded on 96-well plates and pre-incubated for 20 h. Then, cells were cultured with GK (0.1, 1, 10, 50, and 100 μg/ml) for 22 h at 37°C and 5% CO_2. The 5 mg/ml MTT reagent was added, and the cells were incubated for 2 h. The medium was then discarded and DMSO was added to each well and absorbance was measured at 540 nm with a microplate reader (Bio-Rad Laboratories, USA). The cell proliferation ability was calculated according to the following formula:

Proliferation index (%)
= absorbance of the sample/ absorbance of the control × 100.

Nitric Oxide Determination

RAW 264.7 cells were pre-incubated in 24-well plates (2.5×10^5 cells/ml) for 20 h. After this initial incubation, LPS (1 μg/ml) and GK (0.1, 1, 10, 50, and 100 μg/ml) were added and the culture incubated for 24 h. Then, 100 μl of supernatant was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthalene diamine dihydrochloride in 5% phosphoric acid) and the culture

was again incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (Bio-Rad) and the quantity of nitrite was calculated with standard curves of sodium nitrite ($NaNO_2$).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of pro-inflammatory cytokine were determined using an ELISA kit (Mouse ELISA set). Briefly, RAW 264.7 cells (2.5×10^5 cells/ml) were stimulated with LPS (1 μg/ml) and the indicated concentration of GK for 24 h. Then, the levels of TNF-α, IL-6, and IL-1β in the culture medium were measured by ELISA using anti-mouse TNF-α, IL-6, and IL-1β antibodies and biotinylated secondary antibodies, as per the manufacturer's instructions.

Western Blot Analysis

RAW 264.7 cells treated with various concentrations of GK (0.1, 1, 10, 50, and 100 μg/ml), followed by treatment with LPS (1 μg/ml) for 24 h, were lysed with lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% Triton X-100, and 0.1% NP-40. The cell lysates were centrifuged at 15,520 ×*g* for 20 min to remove cell membrane components. Protein concentration was quantified with a Pierce BCA protein assay kit (USA). Protein samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) at 200 mA for 1 h. After blocking nonspecific sites with 5% skim milk (Fluka, Switzerland) in 0.1% Tris-buffered saline (TBS)-Tween 20 for 2 h, the membranes were incubated with anti-mouse iNOS, COX-2, NF-κB p65, p-JNK, p-ERK and p-p38 antibodies in TBS (1:500) for 2 h. The membranes were further incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (1:2000). The immune-active proteins were detected using an enhanced chemiluminescence (ECL) detector. The signal intensity of each protein band was measured by densitometry, employing the Gene Tools from Syngene software.

Statistical Analysis

Data are expressed as mean ± standard error of the mean ($n = 3$). Statistical evaluation was carried out using analysis of variance with SAS software (SAS Institute, USA), according to Duncan's multiple range test ($p < 0.05$).

Results

Cell Viability

The cytotoxicity of GK in RAW 264.7 cells was determined based on MTT assay. Cells were cultured with 0.1, 1, 10, 50, and 100 μg/ml of GK for 24 h. Treatment with 0.1 to 100 μg/ml GK for 24 h did not cause any changes in MTT-based cell viability (data not shown). Thus, in the subsequent experiments, GK was used at concentrations between 0.1 and 100 μg/ml.

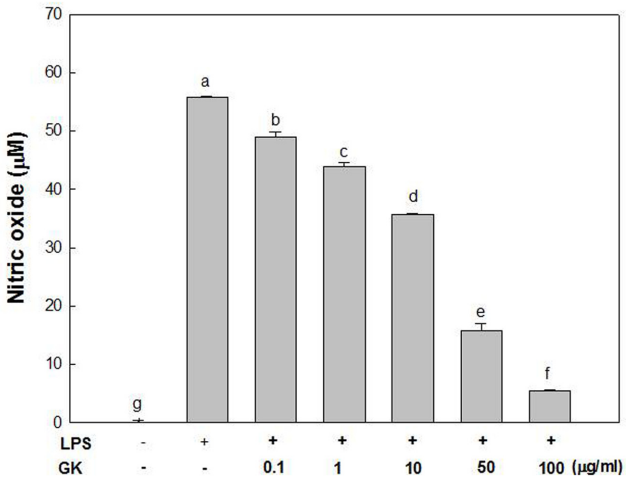

Fig. 2. Inhibitory effect of grasshopper ketone (GK) on the production of nitric oxide in RAW 264.7 cells.
RAW 264.7 cells were treated with the indicated concentrations of GK (0.1, 1, 10, 50, and 100 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h. Culture supernatants were then isolated and analyzed using the Griess reagent for nitric oxides. [a-g]Means with different superscripts are significantly different ($p < 0.05$).

Effects of GK on LPS-Induced NO Production

To examine whether GK could modulate NO production, we measured NO secretion in LPS-induced RAW 264.7 cells after GK treatment. LPS treatment group induced significantly NO secretion compared with control group (Fig. 2). However, GK treatment suppressed NO production in a dose-dependent manner, in particular, treatment with 100 μg/ml GK reduced NO release to 5.47 ± 0.07 μM; nearly basal levels. These results suggest that concentrations of 0.1–100 μg/ml GK inhibit NO secretion in LPS-induced RAW 264.7 cells.

Effects of GK on LPS-Induced Pro-Inflammatory Cytokine Production

During the inflammatory response, there were excessive amounts of mediators like pro-inflammatory cytokines and NO. Several pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α play a key role in LPS-stimulated RAW 264.7 cells. These pro-inflammatory cytokines are secreted at an early stage in the inflammatory response against pathologic stimuli [12].

To investigate the anti-inflammatory activity of GK, we

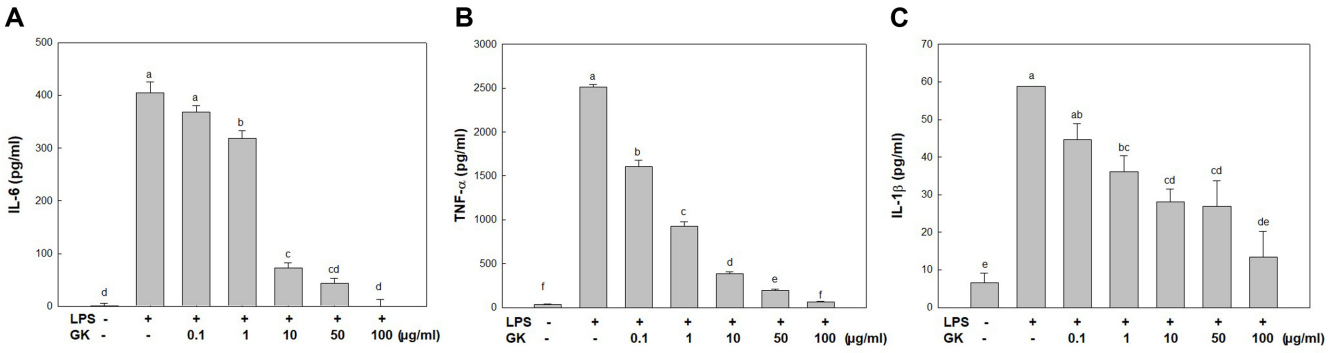

Fig. 3. Inhibitory effect of grasshopper ketone (GK) on the production of IL-6 (**A**), TNF-α (**B**), and IL-1β (**C**) in RAW 264.7 cells.
RAW 264.7 cells were treated with the indicated concentrations of GK (0.1, 1, 10, 50, and 100 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h. Culture supernatants were then isolated and analyzed using the ELISA kit for cytokines. [a-f]Means with different superscripts are significantly different ($p < 0.05$).

measured pro-inflammatory cytokine production in LPS-induced inflammatory response. As a result, it was shown that the levels of pro-inflammatory cytokines were reduced in a dose-dependent manner (Fig. 3). In particular, the secretion of IL-6 was decreased by 90% (33.7 pg/ml) at 50 μg/ml and reached nearly basal levels when treated with a GK concentration of 100 μg/ml (Fig. 3A). In addition, the secretion of TNF-α and IL-1β was inhibited by 97% (75.3 pg/ml) and 78% (13.0 pg/ml) at a GK concentration of 100 μg/ml, respectively (Figs. 3B, 3C).

Effects of GK on LPS-Induced iNOS, COX-2, and NF-κB p65 Expressions

In macrophages, the expression of pro-inflammatory cytokines and inflammatory gene is regulated by NF-κB [13]. NF-κB serves as an inducer of transcription of target

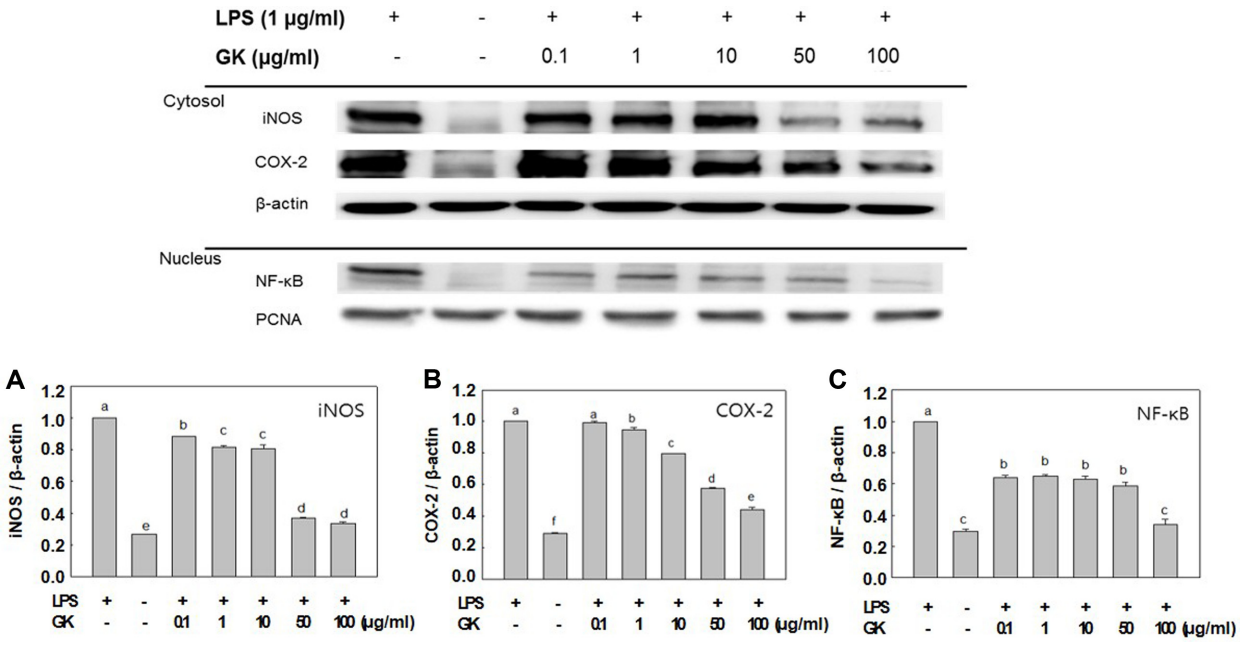

Fig. 4. Inhibitory effect of grasshopper ketone (GK) on the protein expression of iNOS (**A**), COX-2 (**B**), and NF-κB p65 (**C**) in RAW 264.7 cells.
The levels of iNOS and COX-2 in the cytosolic protein and the p65 subunit of NF-κB in nuclear protein were determined by a western blot analysis. RAW 264.7 cells were treated with indicated concentrations of GK (0.1, 1, 10, 50, and 100 μg/ml) and LPS (1 μg/ml) for 24 h or 30 min and the proteins were detected using specific antibodies. [a-f]Means with different superscripts are significantly different ($p < 0.05$).

genes, such as IL-6 inflammatory enzymes like COX-2 and iNOS [14]. Also, the activity of NF-κB is regulated by MAPKs including extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 kinase [15, 16]. For this reason, we examined the effect of GK on LPS-stimulated expressions of iNOS, COX-2 and NF-κB subunit NF-κB p65 via western blot analysis. The expressions of iNOS and COX-2 were diminished over 60% at a concentration of 100 μg/ml (Figs. 4A, 4B). In addition, the expression of phosphorylated p65 was decreased by approximately 60% at a concentration of 100 μg/ml (Fig. 4C).

Effects of GK on LPS-Induced p-JNK, p-ERK, and p-p38 Expressions

Phosphorylation of the MAPK family is an important pathway influencing the secretion of LPS-induced inflammatory factors. It has been known that MAPKs, such as ERK1/2, JNK, and p38 subfamilies, play an essential role in the signaling pathways and induce activation of NF-κB [17]. To investigate the effect of GK on phosphorylation of MAPKs, phosphorylation of JNK, ERK and p38 was determined by western blot analysis. The phosphorylation of those proteins was reduced dose-dependently compared to the group treated with LPS only (Fig. 5).

Discussion

In this study, we first isolated GK from *S. fulvellum*, an ethanol extract of brown algae, and demonstrated that GK significantly inhibited major proinflammatory cytokines and NO production and had an effective anti-inflammatory activity. Moreover, we demonstrated that GK can regulate the expression of pro-inflammatory cytokines, COX-2 and iNOS via the NF-κB signaling pathway.

NO has been known as a signaling molecule involved in inflammation via iNOS upregulation, and high levels of NO induce various inflammatory disorders. LPS-induced activation of RAW 264.7 cells initiates the secretion of various inflammatory products, such as NO. Elevated production of NO induces severe inflammatory responses such as asthma, and in blood vessels, from the NO response itself or from a toxic metabolite related to peroxynitrite ($ONOO^-$) even though NO plays a key role in some diseases such as tumors and viruses [18]. In addition, NO leads PGE_2 production resulting in accelerated inflammatory responses since it activates COX-2 [19]. Thus, inhibition of NO production in the LPS-stimulated RAW 264.7 cell system is an excellent target for the anti-inflammatory therapeutic field.

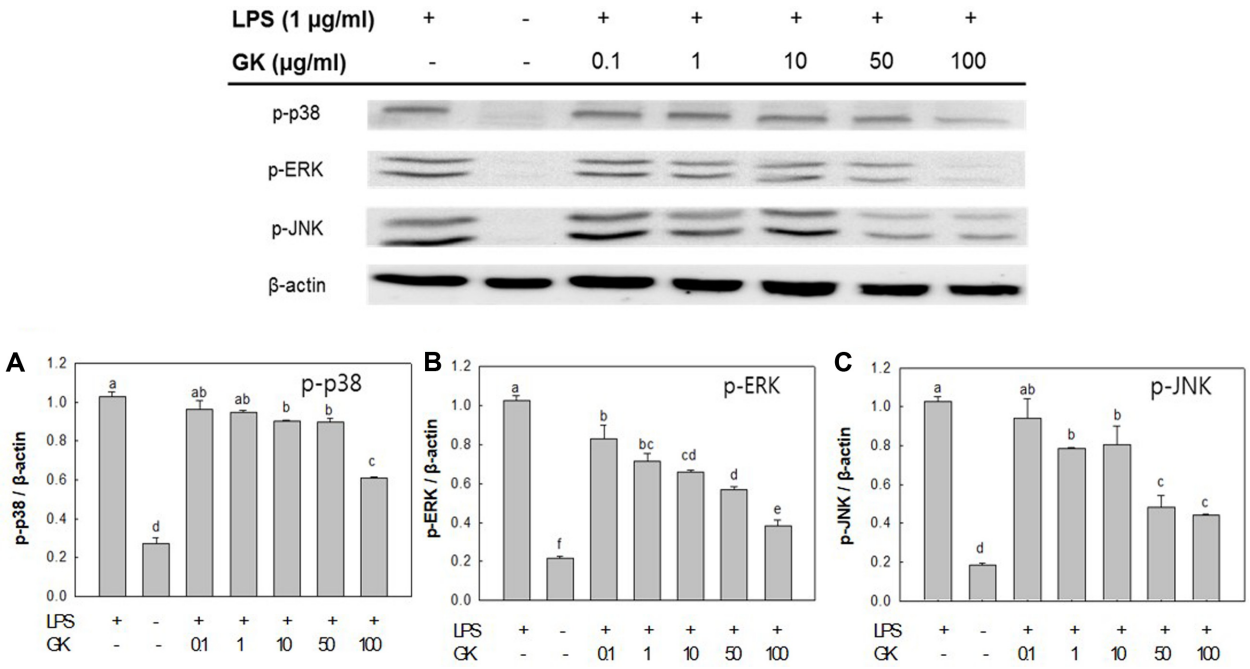

Fig. 5. Inhibitory effect of grasshopper ketone (GK) on the phosphorylation of MAPKs in RAW 264.7 cells.
The levels of phosphorylated-p38 (**A**), p-ERK (**B**), and p-JNK (**C**) were determined by a western blot analysis. RAW 264.7 cells were treated with indicated concentrations of GK (0.1, 1, 10, 50, and 100 μg/ml) and LPS (1 μg/ml) for 30 min and the proteins were detected using specific antibodies. [a-f]Means with different superscripts are significantly different ($p < 0.05$).

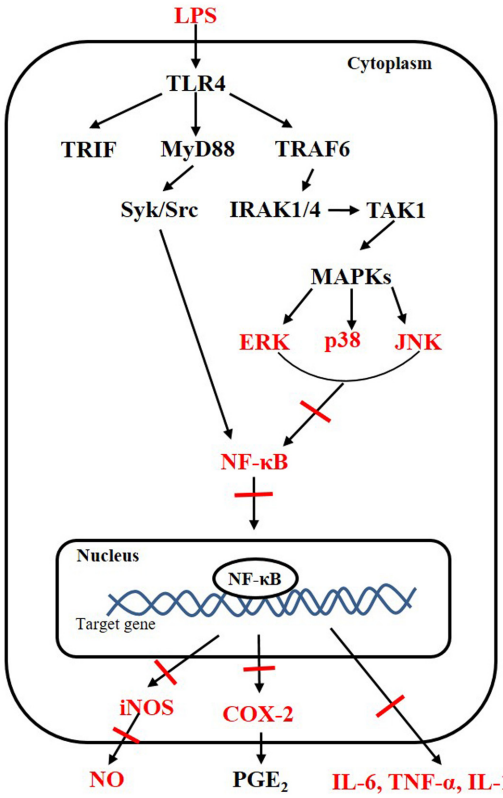

Fig. 6. Schematic diagram of a potential inhibitory pathway used by grasshopper ketone (GK) during the LPS-induced inflammatory response in RAW 264.7 cells.

Furthermore, binding of TNF-α and TNF-α receptors in the membrane of a macrophage causes the activation of NF-κB and other factors down stream during the inflammatory response [20]. In addition, IL-1β is known as a cytokine induced by caspase-1 in activated macrophages, and is critical for the response to infection as it influences the production of NO [21]. For this reason, the development of therapies targeting these pro-inflammatory cytokines is an important therapeutic strategy for treating a variety of inflammatory diseases.

This study showed that GK inhibited the production of iNOS, COX-2 and inflammatory cytokines through the NF-κB signaling pathway in LPS-induced RAW 264.7 cells (Fig. 4). In the previous study, sargachromanol purified from *S. micracanthum* showed 61.6% inhibitory activity of NO production when treated with 100 μM [22]. In addition, fucoxanthin derivatives isolated from the brown alga species *S. siliquastrum* inhibited production of IL-6 and TNF-α by 37.9% and 42.1%, respectively, at a concentration of 60 μM [23]. Compared to these compounds, GK has a more outstanding level of anti-inflammatory activity (Figs. 2, 3).

LPS-stimulated NF-kB and MAPK are known to modulate the expression of pro-inflammatory cytokines and enzymes such as TNF-α, IL-1β, IL-6, iNOS, and COX-2. Apo-9'-fucoxanthinone isolated from *S. muticum* has been shown to have potent anti-inflammatory activity by inhibiting the expression of iNOS and COX-2 via blockade of the NF-κB signaling pathway [24]. Therefore, we confirmed the effect of GK on the phosphorylation of MAPKs (ERK, JNK, and p38) and the NF-κB subunit NF-κB p65. We found that phosphorylation of NF-κB p65, p38, ERK, and JNK were significantly reduced in LPS-induced RAW 264.7 cells by GK treatment (Fig. 5). Therefore, these results suggest that GK treatment not only inhibits the production of NO and cytokines but also inhibits the activation of MAPK and NF-kB, thereby reducing the inflammatory response.

In summary, anti-inflammatory activity occurs primarily through down-regulation of MAPKs and NF-κB phosphorylation in their signaling pathway. GK treatment significantly ($p < 0.05$) inhibited the expression of iNOS and COX-2, and phosphorylation of NF-κB and MAPKs. These results suggest that GK has anti-inflammatory activity and contributes to the treatment of inflammatory diseases.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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