

Anti-Inflammatory Activity of *Sonchus oleraceus* Extract in Lipopolysaccharide-Stimulated RAW264.7 Cells

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The anti-inflammatory activity and non-toxicity of *Sonchus oleraceus* extract (J6) were tested by measuring its effect on the levels of nitric oxide (NO), prostaglandin E₂ (PGE₂), and the pro-inflammatory cytokines, interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. We treated the RAW264.7 cells with various concentrations (50, 100, or 200 μ g/mL) of J6. Our results showed that J6 inhibited the production of NO, PGE₂, and pro-inflammatory cytokines in a concentration-dependent manner, without compromising cell viability. In addition, we provided supporting evidence that the inhibitory activity of J6 on the production of NO and PGE₂ occurred via the downregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Our findings suggest that J6 is a new source for anti-inflammatory drugs and ingredients for healthcare products that include functional cosmetics.

Keywords: *Sonchus oleraceus*, anti-inflammation, iNOS, COX-2, TNF- α , PGE₂.

Lipopolysaccharide (LPS), a well-known endotoxin, is found in the outer membrane of Gram-negative bacteria. LPS induces the expression of inflammatory mediators in macrophages, such as RAW264.7 cells, and monocytes¹ and increases the production of nitric oxide (NO) and cytokines in the early stage of inflammation².

Nitric oxide synthase (NOS), which mediates inflammation, exists as three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). eNOS is mainly expressed in endothelial cells and nNOS expression

is restricted mostly to the nervous system and pancreatic beta cells. These two isoforms are constitutively expressed and play a key role in homeostasis, including vasodilation, blood flow control, and nerve signal transmission³. iNOS, in contrast, is induced by LPS and enzymatically generates the pro-inflammatory mediator, NO^{4,5}. Another inflammatory enzyme, cyclooxygenase (COX), exists as two isoforms, namely COX-1 and COX-2. COX-1 is expressed in almost every tissue and is involved in homeostasis and protection of body organs. COX-2 is mostly involved in

inflammatory responses^{6,7}, and its expression is induced by LPS and inflammatory cytokines⁸. The expression of iNOS and COX-2, their production of NO and prostaglandin E₂(PGE₂), respectively, and the generation of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6, are common markers of inflammation⁹.

Sonchus oleraceus is a biennial, conical-shaped plant that belongs to the Asteraceae family. It can grow up to 1 m high and has been utilized as a medicinal compound and food source for livestock and humans. Its seedling leaves can be eaten uncooked. This plant contains a high content of flavonoid compounds, such as kaempferol, luteolin, quercetin, quercimeritrin, and chrysanthemins¹⁰⁻¹³.

Previous studies mainly investigated the polyphenol content and antioxidant activity of *S. oleraceus* when grown at different locations, with seasonal changes, and after exposure to various environmental factors¹⁰⁻¹³. However, there has been little study on the pharmacological and functional properties of *S. oleraceus* and its use as an ingredient in functional cosmetics. Some widely used synthetic materials such as oxybenzone, mineral oil, and parabens can reportedly cause adverse effects such as liver damage, abnormal body growth, and gastrointestinal bleeding²⁰. As a result, interest in biotic materials with minimal side effects is increasing. Thus, the aim of the present study was to investigate the inhibitory activity of J6 *S. oleraceus* extraction, the expression of inflammatory mediators in LPS-stimulated macrophages and to explore its potential as a biotic material.

MATERIALS AND METHODS

J6 procurement and cell culture

J6 was obtained from the Jeju Biodiversity Institute¹⁹. The murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 100 units/mL penicillin-streptomycin and 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO₂.

Cytotoxicity assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was implemented to assess the cytotoxicity of the J6 extract. RAW264.7 cells were plated at 1.8×10^5 cells/200 μ L/well in a 96-well plate and incubated for 18 h prior to the 24 h-treatment of 1 μ g/mL LPS and different concentrations of J6 extract (50, 100, and 200 μ g/mL) at 37°C and 5% CO₂. Fifty microliters of MTT reagent was then added and incubated for 4 h. The spent culture medium was completely removed and 200 μ L dimethylsulfoxide (DMSO, Sigma, MO, USA) was added to dissolve the formazan precipitate. The absorbance at 540 nm was then measured using a microplate reader (Bio-Tek Instrument Inc., Vermont, WI, USA). The average absorbance for each sample group was used to evaluate cell viability.

NO assay

RAW264.7 cells were plated at 1.8×10^5 cells/200 μ L/well in a 24-well plate and incubated for 18 h prior to the 24 h-treatment of 1 μ g/mL LPS and different concentrations of J6 extract (50, 100, and 200 μ g/mL) at 37°C and 5% CO₂. One hundred microliters of culture medium from each well and an equal volume of Griess reagent were mixed and incubated at room temperature for 10 min¹⁵ followed by the measurement of absorbance at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The Griess reagent (1% (w/v) sulfanilamide, 0.1% (w/v) N-(1-naphthyl) ethylenediamine in 2.5% (v/v) phosphoric acid) reacts with nitrite in the culture medium to estimate the amount of NO produced. Values were calculated by using a standard curve of sodium nitrite (NaNO₂).

PGE₂, IL-1 β , IL-6, and TNF- α ELISAs

RAW264.7 cells were cultured as described above and treated for 24 h with 450 μ L of 1 μ g/mL LPS and 50 μ L of 10-times-concentrated 1 mg/mL J6 extract. The culture medium was then centrifuged at 12,000 rpm for 3 min and the resulting supernatant was used to measure the content of PGE₂, IL-1 β , IL-6, and TNF- α . All samples were kept at 20°C until quantification. PGE₂ and the other three pro-inflammatory cytokines were quantified using specific ELISA kits (R&D Systems Inc., Minneapolis, MN, USA); the R-squared values for the standard curves were 0.99.

Western blot analysis

As previously described, RAW264.7 cells were cultured and treated with LPS and J6 extract. After washing twice in PBS, proteins were isolated in lysis buffer [1X RIPA buffer (Upstate Cell Signaling Solution, Lake Placid, NY, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin] for 1 h, followed by centrifugation. The protein-containing supernatant was subjected to 10% SDS-PAGE and electrophoretically transferred to

a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 200 mA for 2 h. The resulting membrane was blocked at room temperature with 5% skim milk in 0.05% Tween-20/Tris-buffered saline (T/TBS) followed by incubation with the primary antibody at 4°C overnight. The primary antibodies were iNOS antibody (1:5000, Calbiochem, USA), COX-2 antibody (1:1000, BD Biosciences Pharmingen, USA), and β-actin antibody clone AC-74 (1:10,000, Sigma, USA). After washing four times in T/TBS, the membrane was incubated with 5,000- or

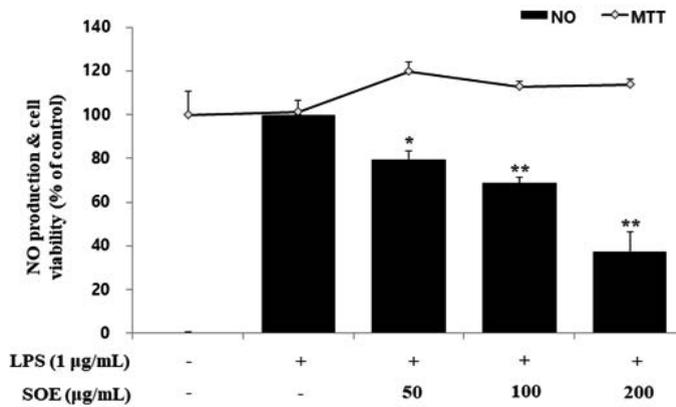


Fig. 1. Effect of J6 extract on nitric oxide production in LPS-stimulated RAW264.7 cells. Cells were stimulated with 1 μg/mL LPS alone or in combination with various concentrations (50, 100, or 200 μg/mL) of J6 for 24 h. Nitric oxide production was determined by the Griess reagent method. Cell viability was determined using an MTT assay from the 24-h culture of cells stimulated with LPS (1 μg/mL) in the presence of J6. All data are expressed as the means ± SD of triplicate experiments. **p* < 0.005, ***p* < 0.001 versus LPS alone. IC₅₀ = 158.6 μg/mL

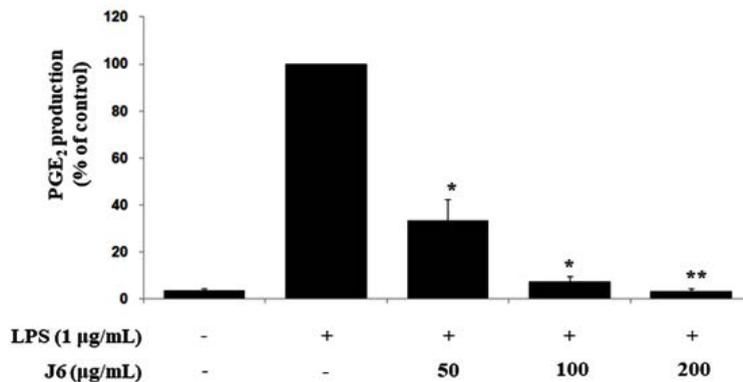


Fig. 2. Effect of J6 on PGE₂ production in LPS-stimulated RAW264.7 cells. Cells were stimulated with 1 μg/mL LPS with or without various concentrations (50, 100, or 200 μg/mL) of J6 for 24 h. PGE₂ production was measured by ELISA. The data represent the means ± SD of triplicate experiments. **p* < 0.005, ***p* < 0.001 versus LPS alone. IC₅₀ = 18.3 μg/mL

10,000-fold diluted secondary antibody (Jackson ImmunoResearch, East Grove, PA, USA), followed by washing thrice in T/TBS. The proteins of interest were detected and quantified using an ECL kit and an imaging densitometer (Model GS-700; Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are expressed as means \pm standard deviations (SDs). Statistical differences between samples were resolved by Student's t-tests.

RESULTS AND DISCUSSION

Effect of J6 on cell viability

For the cytotoxicity assay, murine RAW264.7 macrophage cells were treated for 24 h with 1 $\mu\text{g}/\text{mL}$ LPS alone or in combination with various concentrations of J6 extract (50, 100, or 200 $\mu\text{g}/\text{mL}$). An increase in cell viability and no toxicity were observed in the J6-treated samples relative to that of the control group (Fig. 1, line plot), which was consistent with the results of a previous study

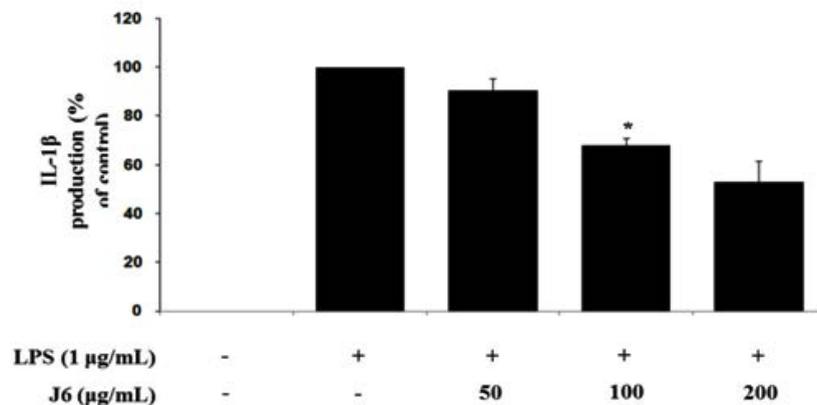


Fig. 3. Effect of J6 on IL-1 β production in LPS-stimulated RAW264.7 cells

Cells were stimulated with 1 $\mu\text{g}/\text{mL}$ of LPS only or with various concentrations (50, 100, and 200 $\mu\text{g}/\text{mL}$) of J6 for 24 h. IL-1 β production was measured by ELISA. The data represent the means \pm SD of triplicate experiments. * $p < 0.005$, ** $p < 0.001$ versus LPS alone

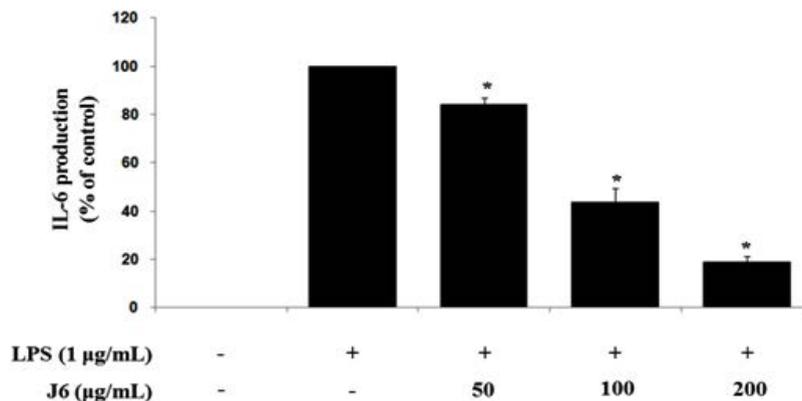


Fig. 4. Effect of J6 on IL-6 production in LPS-stimulated RAW264.7 cells

Cells were stimulated with 1 $\mu\text{g}/\text{mL}$ of LPS with or without various concentrations (50, 100, and 200 $\mu\text{g}/\text{mL}$) of J6 for 24 h. IL-6 production was measured by ELISA. The data represent the means \pm SD of triplicate experiments. * $p < 0.005$, ** $p < 0.001$ versus LPS alone. $\text{IC}_{50} = 114.5 \mu\text{g}/\text{mL}$

on the anti-inflammatory effect of *Scutellariaeradix* extract¹⁶. Up to 200 µg/mL of J6 extract, near the cytotoxic threshold concentration, was used further to assay the inhibitory activity of J6 on the

generation of NO, PGE₂, and pro-inflammatory cytokines.

Effect of J6 on NO generation

The effect of J6 extract on the generation

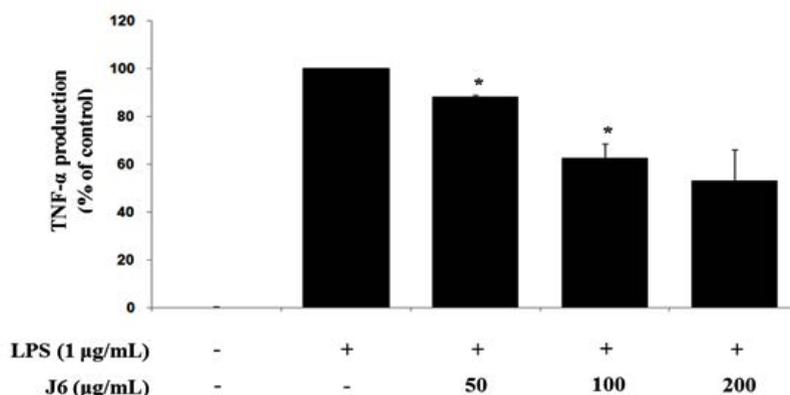


Fig. 5. Effect of J6 on TNF-α production in LPS-stimulated RAW264.7 cells
Cells were stimulated with 1 µg/mL LPS only or with various concentrations (50, 100, or 200 µg/mL) of J6 for 24 h. TNF-α production was measured by ELISA. The data represent the means ± SD of triplicate experiments. *p < 0.005, **p < 0.001 versus LPS alone

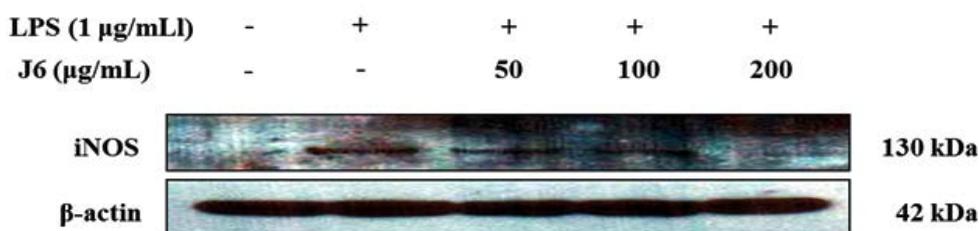


Fig. 6. Effect of J6 on protein level of iNOS in LPS-stimulated RAW 264.7 cells
RAW 264.7 cells (6.0 × 10⁵ cell/mL) were stimulated with LPS (1 µg/mL) in the presence of J6 (50, 100, or 200 µg/mL) for 24h. Whole-cell lysates (30 µg) were prepared and subjected to 10% SDS-PAGE; the expression level of iNOS and β-actin were determined by western blotting. β-Actin was used as a loading control. Representative images are shown

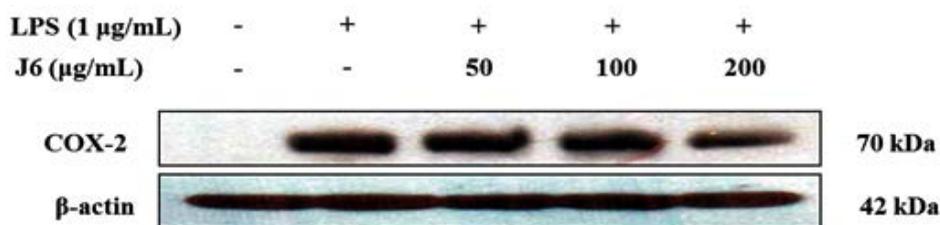


Fig. 7. Effect of J6 on protein level of COX-2 in LPS-stimulated RAW 264.7 cells
RAW 264.7 cells (6.0 × 10⁵ cell/mL) were stimulated with LPS (1 µg/mL) in the presence of J6 (50, 100, or 200 µg/mL) for 24h. Whole-cell lysates (30 µg) were prepared and subjected to 10% SDS-PAGE; the expression level of COX-2 and β-actin were determined by western blotting. β-actin was used as a loading control. Representative images are shown

of NO, a critical mediator of the inflammatory response, was examined (Fig. 1, bar graph). RAW264.7 cells were treated with LPS and J6 extract as described above and the generated NO was quantified using a Griess assay. Relative to that of the LPS-only-treated group, NO production was decreased by 64% at the peak concentration of 200 $\mu\text{g}/\text{mL}$ J6 extract. This confirmed the potent inhibitory activity of J6 on NO production and its potential to inhibit iNOS expression.

Effect of J6 extract on PGE₂ production in LPS-stimulated RAW264.7 cells

RAW264.7 cells were plated and treated with LPS and J6 extract as described in the NO assay and PGE₂ generation was then assessed using a PGE₂ ELISA kit. The cells were treated with J6 at various concentrations of 50, 100, and 200 $\mu\text{g}/\text{mL}$. Relative to that of the cells treated with LPS only, PGE₂ production was substantially decreased over the J6 concentration range of 50–200 $\mu\text{g}/\text{mL}$; this reduction reached 95.5% at the peak concentration of 200 $\mu\text{g}/\text{mL}$ J6 (Fig. 2). Therefore, J6 extract inhibited LPS-induced PGE₂ production concentration-dependently.

Effect of J6 extract on pro-inflammatory cytokine (IL-1 β , IL-6, and TNF- α) generation

Cytokines act as mediators in the activation, proliferation, and differentiation of immune cells and typical pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , are well recognized as modulators of inflammatory responses both *in vitro* and *in vivo*¹⁷. The effect of J6 extract on the production of these three pro-inflammatory cytokines in the presence and absence of LPS was examined. The secretion of IL-1 β , IL-6, and TNF- α was increased in the medium of LPS-treated cells (Fig. 3, 4, 5). Relative to that of the LPS-only-treated group, IL-1 β and IL-6 production were concentration-dependently decreased by J6 extract, reaching 43.5% (Fig. 3) and 80% (Fig. 4) reduction, respectively, at 200 $\mu\text{g}/\text{mL}$ J6. Similarly, TNF- α production was decreased in a concentration-dependent manner (Fig. 5). This confirmed the anti-inflammatory effect of J6 extract on secretion of the three pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α , in LPS-stimulated RAW264.7 cells.

Effect of J6 on protein expression of iNOS and COX-2

iNOS and COX-2 are known as key players in the synthesis of NO and PGE₂ during inflammation¹⁸. Based on our findings that J6 inhibits the production of NO and PGE₂ in a concentration-dependent manner, we used western blotting to examine whether the reduced production of NO and PGE₂ was associated with reduced protein expression of iNOS and COX-2. Our results demonstrate that J6 extract had a suppressive effect on the LPS-induced expression of iNOS and COX-2 in a concentration-dependent manner (Fig. 6, 7). This also showed that J6 inhibits NO and PGE₂ generation via down regulation of iNOS and COX-2 expression, respectively.

CONCLUSIONS

S. oleraceus has been valued for animal and human consumption but there have been few studies on its pharmacological activity and toxicity. Our study confirmed that J6 extract was not toxic and exerted an inhibitory effect on the production of NO, PGE₂, and pro-inflammatory cytokines in LPS-stimulated macrophages. We found that during LPS-stimulation J6 extract inhibited the generation of NO and PGE₂ in a concentration-dependent manner. We also examined whether J6 was involved in modulating the production of pro-inflammatory cytokines and found it to concentration-dependently inhibit the secretion of three pro-inflammatory mediators (i.e., IL-1 β , IL-6, and TNF- α), suggesting that it has potent anti-inflammatory effects. Moreover, the protein level of both iNOS and COX-2 was drastically decreased with increasing concentrations of J6 extract. Further research is required to clarify that the down-regulation of iNOS and COX-2 expression is through the downregulation of NF- κ B activation and MAPK phosphorylation^{21,22}. These results provide strong support that J6 is a potential new source for anti-inflammatory drugs and ingredients for healthcare products, including functional cosmetics.

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