Leaves of *Raphanus sativus* L. Shows Anti-Inflammatory Activity in LPS-Stimulated Macrophages via Suppression of COX-2 and iNOS Expression.

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ABSTRACT: *Raphanus sativus* L. (RS) is a cruciferous vegetable that is widely consumed in Korea. The anticancer activity of leaves of RS (RSL) extract has been investigated; however, no studies focused on its anti-inflammatory effects. Therefore, the aim of the current study was to evaluate the anti-inflammatory effects of RSL extract. In brief, RSL powder was fractionated into *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water-soluble fractions. Lipopolysaccharide (LPS)-stimulated RAW264.7 cells were treated with each fraction for initial screening. It was found that the chloroform fraction significantly inhibited nitric oxide release in LPS-stimulated RAW264.7 cells with a half maximal inhibitory concentration value of 196 μg/mL. In addition, the mRNA and protein expression levels of inducible nitric oxide synthase, measured using reverse transcriptase-polymerase chain reaction and western blotting, respectively, were reduced in a concentration-dependent manner. Moreover, the inflammatory cyclooxygenase-2 enzyme expression decreased. Furthermore, the expression of nuclear factor-kappa B (NF-κB), the key regulator of the transcriptional activation of the inflammatory cytokine genes, was reduced by the RSL chloroform fraction. Therefore, the results of our study suggest that RSL exhibits anti-inflammatory effects in LPS-stimulated macrophages via NF-κB inactivation.

Keywords: *Raphanus sativus* L., nitric oxide, anti-inflammatory, COX-2, NF-κB

INTRODUCTION

Inflammation is a complex host defense mechanism against noxious substances and microorganisms. Inflammatory responses play an important role in the host survival and homeostasis. However, in several cases, excessive inflammatory responses can lead to chronic inflammatory diseases, such as colitis, neuroinflammation, asthma, atopic dermatitis, arthritis, and allergic rhinitis (1). Current therapies for inflammatory diseases generally focus on alleviating pain and suppressing inflammatory cell activation and migration (2,3). However, these treatments result in undesirable side effects and cannot prevent recurrence (4). Recently, natural products with substantial ethnopharmacological activities have received great attention as treatments for inflammatory diseases. Many pharmacologically active compounds isolated from natural products were developed as medicines (5). In particular, food-based compounds with anti-inflammatory activities have gained widespread attention owing to their safety (6-8).

Raphanus sativus L. (RS) is a widely consumed cruciferous vegetable in Korea. Its root and leaves are the main materials for the traditional Korean food, kimchi. RS has been used in traditional medicine to treat digestive related diseases such as indigestion, digestive inflammation, diarrhea, and abdomen illness in East Asia (9). Its extracts are known to show an inhibitory effect on tumor growth. For example, the root of RS extracts showed a pro-apoptotic activity in human colon carcinoma cells and breast cancer cells (10,11). In addition, they inhibited metastasis in melanoma cells (12). Recently, several studies reported that the seeds of RS showed anti-inflammatory effects (9,13,14). However, no studies investigated the anti-inflammatory activity of the leaves of RS (RSL). Therefore, the purpose of this study was to evaluate the anti-inflammatory properties of the RSL by investigating its effects on the inflammatory mediator levels and to elucidate the underlying mechanism. The components of RSL are known as phenolic compounds

(caffeic, *p*-coumaric, ferulic, hydroxycinnamic, *p*-hydroxybenzoic, vanillic, salicylic, and gentisic acids) and glucosinolates (benzyl isothiocyanate). The roots of RS contain alkaloid, nitrogen compounds (pyrrolidine, phenethylamine, and arabinogalactan proteins), enzyme (isoperoxidases), oil seed (alkenylglucosinolates), and organic acids (oxalic, malic, malonic, and erythorbic acids) (15).

Inflammation is mediated by several factors and cellular signaling molecules that are released from macrophages (6,16). When the macrophages are stimulated by endotoxins, such as lipopolysaccharides (LPS), they release various proinflammatory mediators and cytokines, such as nitric oxide (NO), prostaglandin E_2 (PGE$_2$), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (16). NO is an inflammatory mediator that acts as an important cellular second messenger as well as a regulator of inflammatory responses (17). Cyclooxygenase-2 (COX-2) is a predominant enzyme that is induced by various stimuli, including inflammation, growth factors, and cytokines. Increased expression of inducible NO synthase (iNOS) and COX-2 are related to the activation of the nuclear factor-kappa B (NF-κB) pathway. NF-κB, which is activated in inflammatory responses, is a transcriptional activator of iNOS and COX-2 genes (18,19).

This is the first study to report the anti-inflammatory effects of the RSL in LPS-activated macrophages. The anti-inflammatory activity was investigated via determination of the levels of pro-inflammatory cytokines, as well as iNOS, COX-2, and NF-κB proteins levels.

MATERIALS AND METHODS

Radish extracts preparation

RSL was purchased from Kyungdong pharmaceutics (Seoul, Korea) and freeze-dried using liquid nitrogen. A voucher specimen (kucari 201210) was deposited at the herbarium of the Plant Extract Bank at Gachon University. A dried sample (2 kg) was ground and dissolved in methanol (6 L, 3 times a day for 3 days) at 24°C in a dark room. After filtration, the methanol extracts were concentrated under reduced pressure using a rotor evapora-
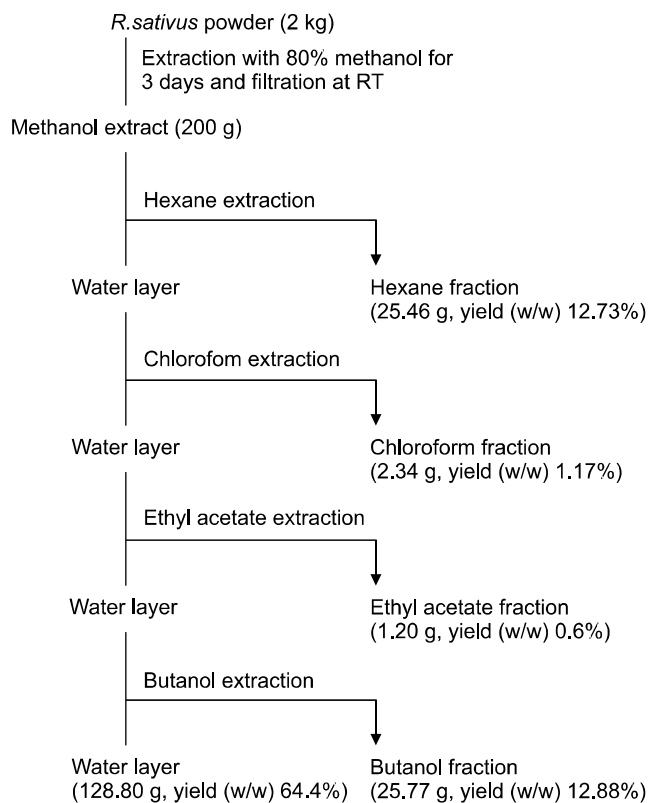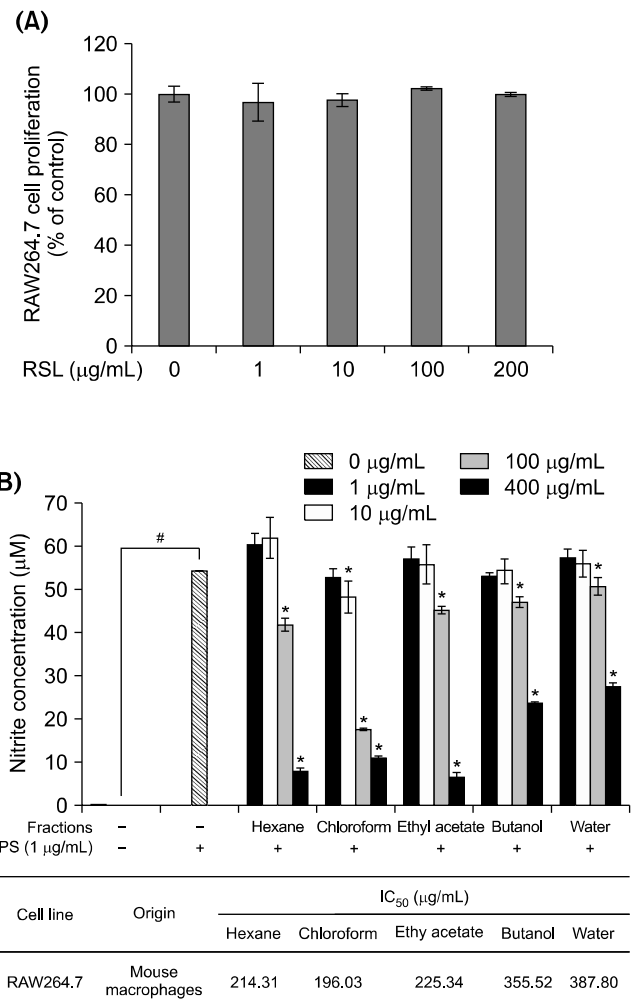
Fig. 2. (A) Cytotoxicity of leaves of *Raphanus sativus* L. (RSL) at different concentrations (0, 1, 10, 100, and 200 μg/mL). (B) Effect of RSL fractions (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water) on lipopolysaccharide (LPS)-stimulated nitrite production in RAW264.7 macrophages and IC$_{50}$ values. Each value represents the mean±SD. Nitrite concentration in the absence and presence of LPS was compared and statistical difference was denoted as $^{\#}P<0.001$. Nitrite concentration under each fraction treatment was compared to LPS stimulated control and statistical difference was denoted as $^{*}P<0.05$. All experiments were performed in triplicate.

Fig. 1. The extraction procedure of leaves of *Raphanus sativus* L. (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water) fractions.

tor (EYELA, Tokyo, Japan), and the extract (644 g) was collected. The methanol extract (200 g) was re-suspended in distilled water (1 L), and further fractionated with n-hexane, chloroform, ethyl acetate, n-butanol, and water based on its polarity. The dried weights and ratios of the n-hexane, chloroform, ethyl acetate, n-butanol, and water fractions were 25.46 g (12.73%), 2.34 g (1.17%), 1.20 g (0.60%), 25.77 g (12.88%), and 128.80 g (64.40%), respectively (Fig. 1).

Macrophage culture and cell viability by CCK-8 assay

RAW264.7 macrophages were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in the media consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Invitrogen), and 100 U/mL penicillin-streptomycin (Sigma, St. Louis, MO, USA). RAW264.7 cell viability in the presence of different concentrations of RSL was measured with the cell counting kit-8 (CCK-8) assay (Daelillab Co., Seoul, Korea). Cells were placed onto 96-well plates (5×10^3 cells/well) and treated with RSL (0, 1, 10, 100, and 200 μg/mL) for 48 h. CCK-8 solutions were added, and viable cells were detected using a microplate reader at 450 nm (Model550; Bio-Rad Laboratories, Hercules, CA, USA).

NO and PGE$_2$ assay

Cells were plated and treated with each RSL fraction (n-hexane, chloroform, ethyl acetate, n-butanol, and water) for 1 h followed by LPS (1 μg/mL; Sigma). After 18 h incubation, the supernatants were collected. For the NO assay, the supernatants were reacted with Griess solution (1% sulfanilamide in 5% phosphoric acid, 0.1% N-1-naphthyl ethylenediamine in water) to measure nitrite levels, and the absorbance was determined at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek Instruments Inc., Winooski, VT, USA). PGE$_2$ production was measured with ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), and final absorbance was determined at 470 nm.

RNA isolation and real-time RT-PCR

For quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, cells were plated onto 6-well plate at 5×10^5 cells/well and treated with the RSL chloroform fraction (0, 1, 10, and 100 μg/mL) followed by a 1 h LPS stimulation. After 4 h, the total RNA was isolated using an RNA extraction kit (Toyobo Co., Ltd., Osaka, Japan). qPCR was performed using an ABI7500 thermal cycler (Applied Biosystems, Foster City, CA, USA). Specific primers for iNOS (QT00100275), TNF-α (QT00104006), IL-6 (QT00098875), and GAPDH
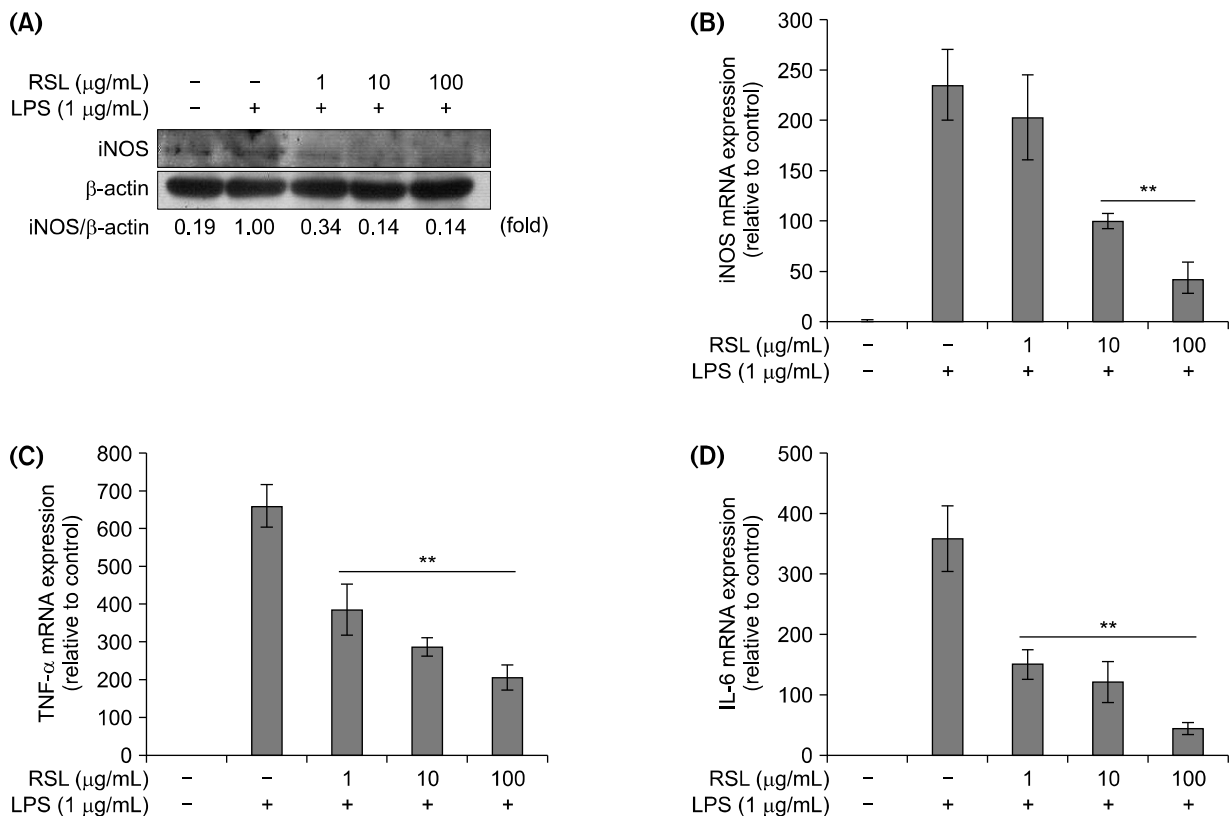

Fig. 3. (A) iNOS protein expression by western blot on leaves of *Raphanus sativus* L. (RSL) chloroform fraction. Relative mRNA expression of iNOS (B), TNF–α (C), and IL–6 (D). Each value represents the mean±SD. mRNA level in the absence and presence of RSL was compared and expressed as **$P<0.01$. All experiments were performed in triplicate.

(QT01658692) were purchased from Qiagen (Venlo, the Netherlands). The relative iNOS mRNA levels were normalized to those of GAPDH and calculated using the $2^{\Delta\Delta Ct}$ method.

Western blot analysis

Cells were plated and treated with each RSL chloroform fraction for 1 h and LPS (1 μg/mL; Sigma) was added. After 18 h incubation, cells were lysed with ice-cold lysis buffer with protease inhibitors (Sigma). The protein concentration was measured with the Bradford protein assay reagent (Bio-Rad Laboratories). Equal amounts of proteins were loaded to each well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blocked in 5% non-fat milk. Samples were probed with the following primary antibodies: mouse anti-iNOS (Abcam, Cambridge, MA, USA), rabbit anti-COX-2 (Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-β-actin (Santa Cruz Biotechnology) antibodies. The secondary antibodies used were either goat anti-mouse IgG or anti-rabbit IgG-peroxidase conjugates (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology).

Statistical analysis

All data were expressed as the mean±standard deviation. The significance of the differences was analyzed using analysis of variance (ANOVA) (version 18, SPSS, IBM, New York, NY, USA). Differences at $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The effect of RSL on RAW 264.7 cell viability was investigated using the CCK-8 assay. Treatment with different concentrations of RSL extract (0, 1, 10, 100, and 200 mg/mL) did not change cell viability (Fig. 2A). As shown in Fig. 2B, NO production significantly increased upon LPS stimulation (55 μM) compared to the control group (0 μM). Chloroform, ethyl acetate, and water RSL fractions significantly inhibited the LPS-induced NO production in a concentration-dependent manner. The half-maximal inhibitory concentration (IC$_{50}$) values were 214.31 μg/mL, 196.03 μg/mL, and 387.8 μg/mL in *n*-hexane, chloroform, and water fraction-treated cells, respectively. Among these fractions, the chloroform fraction showed the strongest inhibitory effects, and thus, it was selected for further studies. NO is an inflammatory mediator that acts as an important cellular second messenger as well as a regulator of inflammatory responses (3,7). The iNOS

protein expression decreased, which confirmed the suppressive effect of the RSL chloroform fraction on NO production (Fig. 3A). Activation of iNOS can promote NO production. As shown in Fig. 3B, the RSL chloroform fraction suppressed iNOS mRNA expression in a concentration-dependent manner. The blocking effect of the RSL chloroform fraction in LPS-stimulated RAW264.7 macrophages might have resulted from transcriptional inhibition of the iNOS gene. In addition, mRNA expression of inflammatory related cytokines such as TNF-α and IL-6 was suppressed by RSL in a concentration-dependent manner (Fig. 3C and 3D).

The effects of the RSL chloroform fraction on COX-2 protein expression were investigated. As shown in Fig. 4A, LPS treatment significantly increased COX-2 protein expression; however, it was suppressed by the RSL chloroform fraction treatment in a concentration-dependent manner. In inflammatory responses, PGE$_2$ is an important inflammatory mediator, which contributes to tissue
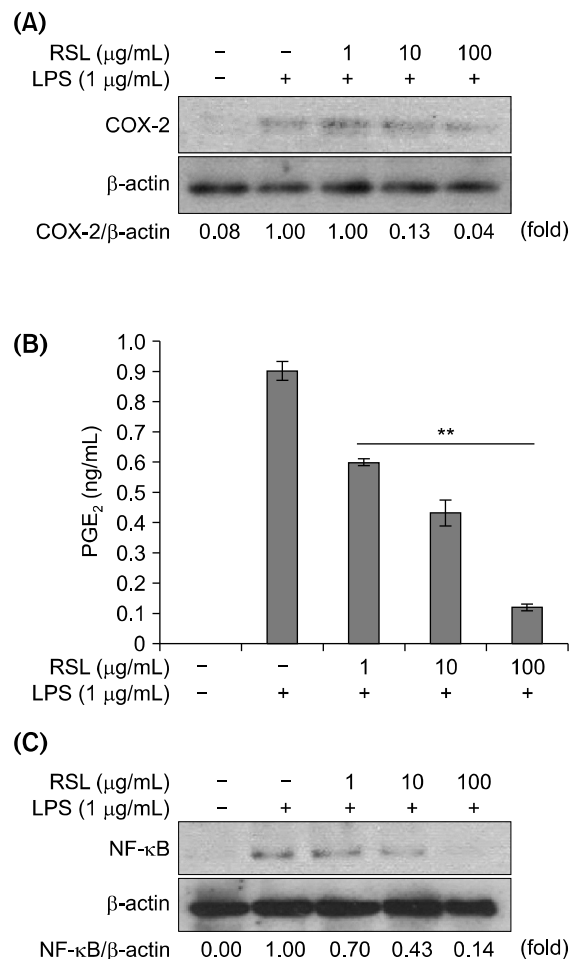

(A)

| RSL (μg/mL) | − | − | 1 | 10 | 100 |
| LPS (1 μg/mL) | − | + | + | + | + |

COX-2

β-actin

| COX-2/β-actin | 0.08 | 1.00 | 1.00 | 0.13 | 0.04 | (fold) |

(B)

| RSL (μg/mL) | − | − | 1 | 10 | 100 |
| LPS (1 μg/mL) | − | + | + | + | + |

(C)

| RSL (μg/mL) | − | − | 1 | 10 | 100 |
| LPS (1 μg/mL) | − | + | + | + | + |

NF-κB

β-actin

| NF-κB/β-actin | 0.00 | 1.00 | 0.70 | 0.43 | 0.14 | (fold) |

Fig. 4. The effect of leaves of *Raphanus sativus* L. (RSL) chloroform fraction on LPS-stimulated RAW264.7 cells at (A) COX-2 protein expression, (B) PGE$_2$ level on different RSL concentration. PGE$_2$ levels in the absence and presence of RSL was compared and expressed as **$P<0.01$. (C) NF-κB protein expression when treated on different concentrations (0, 1, 10, and 100 μg/mL).

edema, increased blood flow, hyperalgesia, and pain sensitization at the sites of inflammation (20). PGE_2 is mainly produced by the action of COX-2 on arachidonic acid. In this study, it was found that RSL significantly inhibited COX-2 protein expression in a concentration-dependent manner, which reduced PGE_2 production as well (Fig. 4B). Thus, the anti-inflammatory effects of RSL might be attributed to its inhibitory effects on PGE_2 production via inhibition of COX-2 protein expression.

The release of various inflammatory cytokines and mediators by LPS-stimulated macrophages is related to NF-κB (21-24). NF-κB is activated in inflammatory responses, where it is involved in the transcriptional activation of iNOS, TNF-α, IL-6, and COX-2 expressing genes (12). Because the RSL chloroform fraction showed suppressive effects of those genes as shown in Fig. 3, the expression level of NF-κB was investigated. As seen in Fig. 4C, the result showed that LPS-induced NF-κB activation in RAW264.7 cells was suppressed by RSL treatment. The chloroform fraction of RSL inhibited NF-κB expression, and subsequently inhibited NF-κB nuclear translocation. Treatment with the RSL chloroform fraction might lead to transcriptional suppression of iNOS- and COX-2-encoding genes because NF-κB is the key regulator of the transcriptional activation of these genes. To our knowledge, this is the first study to show that the RSL chloroform fraction inhibits inflammatory responses in macrophages via inhibition of NF-κB signaling pathways.

In conclusion, we showed that the RSL chloroform fraction suppressed LPS-induced production of pro-inflammatory mediators in RAW264.7 macrophages via modulation of the signal transduction cascades. The chloroform fraction of RSL inhibited the release of several inflammatory mediators from the macrophages, including iNOS, COX-2, and pro-inflammatory cytokines. These findings suggest that the RSL extract can be used as a potential cost-effective alternative for treatment and/or prevention of inflammatory diseases. However, further studies on inflammatory disease animal models and more mechanism studies are needed to provide a better understanding of the preventive effects of RSL in inflammatory disorders.

ACKNOWLEDGEMENTS

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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