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Antioxidant Activities of *Peucedanum insolens* Kitagawa Root Extracts and Their Anti-inflammatory Effects on LPS-treated RAW264.7 Cells

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This study was performed to investigate the antioxidant activities of subfractions of *Peucedanum insolens* Kitagawa root in various organic solvents and their anti-inflammatory effects on LPS-treated RAW264.7 cells. First, *P. insolens* Kitagawa roots were dried at room temperature for one week, chopped, and extracted with 70% ethanol. The resulting extracts were successively sub-fractionated with hexane, chloroform, ethyl acetate, and water. The antioxidant potential of the fractions was evaluated using a DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay and by measuring total polyphenol and flavonoid contents. The anti-inflammatory potency of the fractions was evaluated by measuring the inhibition levels of the expressions of inflammatory-mediated genes and proteins (e.g., iNOS, COX-2, IL-1β, and IL-6) in RAW264.7 cells. The results clearly showed that the ethyl acetate fraction of the *P. insolens* Kitagawa root contained relatively high total flavonoid (34.08±1.68 μg of quercetin equivalents per mg) and total polyphenol (154.1±3.2 μg of gallic acid equivalents per mg) contents. The DPPH assay results showed that the *P. insolens* Kitagawa root possessed strong free radical scavenging activity in the ethyl acetate fraction. Both the ethyl acetate and hexane fractions showed strong inhibitory potencies to nitric oxide production induced by lipopolysaccharide (1 μg/ml) treatment for 24 hr in RAW264.7 cells. The results also showed that both the hexane and ethyl acetate fractions of the *P. insolens* Kitagawa root strongly inhibited mRNA levels of iNOS, IL-1β, and IL-6, which were overexpressed by LPS treatment for 24 hr in the RAW264.7 cells. These results suggest that *P. insolens* Kitagawa root may contain compounds that possess strong potency for anti-inflammatory activity. Further studies are needed to discover more detailed modes of action of *P. insolens* Kitagawa root fractions against inflammation modulation, such as the regulation of cytokine signaling and inflammatory signaling pathways.

Key words : Inflammatory-mediated genes, *Peucedanum insolens* Kitagawa, RAW264.7, total flavonoid, total polyphenol

Introduction

Oxidative stress leads to an excessive accumulation of reactive oxygen species (ROS) and reactive nitrogen species, such as superoxide and nitric oxide, in activated macrophages that has been observed in both acute and chronic inflammation in a number of disease conditions [5, 27]. The generation of highly ROS with a lone unpaired electron induce oxidative stress and plays a key role in the pathogenesis of numerous physiological conditions [23, 24]. Environmental pollutants, radiation, chemicals, toxins, as well as physical stress are responsible for generating reactive oxygen radicals that induce the formation of abnormal proteins, leading to the depletion of antioxidants in the immune system [1]. Even though there are a number of endogenous antioxidant enzymes, such as glutathione peroxidase, catalase and superoxide dismutase, which can deactivate free radicals and therefore maintain optimal cellular functions, endogenous antioxidants may not be sufficient to maintain optimal cellular functions under increased oxidative stress. Therefore, additional dietary antioxidants may be necessary [35].

Inflammation has been associated with various human diseases including carcinogenesis, obesity, and diabetes [22, 28, 32]. In addition, inflammatory enzymes (COX-2 and iNOS), cytokines (IL-1b and IL-6, TNF-a) and ROS contribute to cell death and the pathology of various human diseases

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[3, 12]. Thus, regulation of these factors is important for inflammation and inflammation related diseases.

Lipopolysaccharide (LPS) is a well-known gram-negative bacterial outer membrane component, which triggers the inflammatory response and production of pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), cytokines (IL-1b and IL-6), tumor necrosis factor-alpha (TNF-a) and ROS. These inflammatory mediators are closely associated with the pathogenesis of various inflammatory diseases [3, 13, 17, 46].

Phenolic compounds are thought to be the active ingredients in many dietary plants and traditional medicines used for the treatment of disorders related to oxidative stress and inflammation [42]. Natural phenolic and flavonoid compounds are plant secondary metabolites that hold an aromatic ring bearing at least one hydroxyl group [41]. Phenolic compounds are good electron donors because their hydroxyl groups can directly contribute to antioxidant action [2]. Furthermore, some of them stimulate the synthesis of endogenous antioxidant molecules in the cell [10]. According to multiple reports in the literature, phenolic compounds exhibit free radical inhibition, peroxide decomposition, metal inactivation or oxygen scavenging in biological systems and prevent oxidative disease [31].

Peucedanum insolens Kitagawa is a plant of the umbelliferous type. This plant is a perennial herb distributing in middle Korea and chiefly related to *Peucedanum cervaria* (L.) Cusson. It is also called "Wangsan Bang Poong" or "Deokwoo Oil herb" because it was first discovered in Deokwoo Mountain, Wangsan-myeon, Myeongju-gun, Gangwon Province, South Korea [6]. The plant mainly grows in the limestone areas of Gangwon Province, and also grows naturally in nearby areas of Mungyeong and Andong. Korean umbelliferous plants, "Bang Poong", are important herbal medicines used in oriental medicine for antipyretic and pain relief, colds, chills, and sore throats [8]. *Siler divaricatum* in China and *Glehnia littoralis* in Korea are used as a substitute of "Bang Poong" in fork medicine. The dried roots "wang-san-fang-feng" have been used for medicinal purposes in Korea for the treatment of diaphoresis, sedation and antipyresis [6, 7, 29]. In the present study, we have investigated antioxidant capacity and anti-inflammatory activities of the three different organic solvent-fractions of *P. insolens* Kitagawa root in LPS-stimulated RAW264.7 macrophage cells.

Materials and Methods

Reagents and chemicals

Folin-Ciocalteu reagent, gallic acid, DPPH, and quercetin standards were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Aluminum chloride hexahydrate, methanol, and sodium carbonate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified using a Milli-Q system (Millipore, Billerica, USA). All chemicals and solvents used were of analytical grade.

Preparation of root extracts of *Peucedanum insolens* Kitagawa

P. insolens Kitagawa were collected in May 2016 from Jeongseon area, Gangwon-do. *P. insolens* Kitagawa roots were formally identified by Sik-Je Cho, a specialist for plant classification. *P. insolens* Kitagawa roots were first dried at room temperature and cut to about 0.5 cm or less in length. Three different organic solvent-fractions of *P. insolens* Kitagawa root were prepared according to the scheme shown in Fig. 1. Briefly, the cut roots (100 g) were extracted with 70% ethanol (2.5 l) for 5 days at room temperature with frequent agitation. The extract was filtered using a Buckner funnel and Whatman No. 1 filter paper. The filtrate was concentrated to dryness in a rotary vacuum evaporator (EYELA N-1200 Tokyo Rikakikai Co., Ltd., Japan) under reduced pressure and controlled temperature (40-50℃). The concentrated root extract was stored at -20℃ in an airtight container until further use. As shown in Fig. 1, the ethanol extracts were successively sub-fractionated with hexane, chloroform, ethyl acetate, and water.

Determination of total polyphenolic contents in the organic solvent-fractions of *P. insolens* Kitagawa root

The total phenolic contents of the organic solvent-fractions were determined by Folin-Ciocalteu method as described by Singleton and Rossi [37] with slight modifications. Briefly, 5 µl of the fraction (1 mg/ml) was mixed with 100 µl of 2% (w/v in water) sodium carbonate solution in a 96-well plate. After 5 min, 5 µl of 50% Folin-Ciocalteu reagent was added to the mixture and allowed to stand in the dark for 30 min at room temperature. After centrifuging, the absorbance of blue color from the reaction mixtures was measured using a microplate reader (Biotek EL808, Winooski, VT, USA) at 630 nm against the reagent blank without extract. The content of total phenolic compounds was ex-

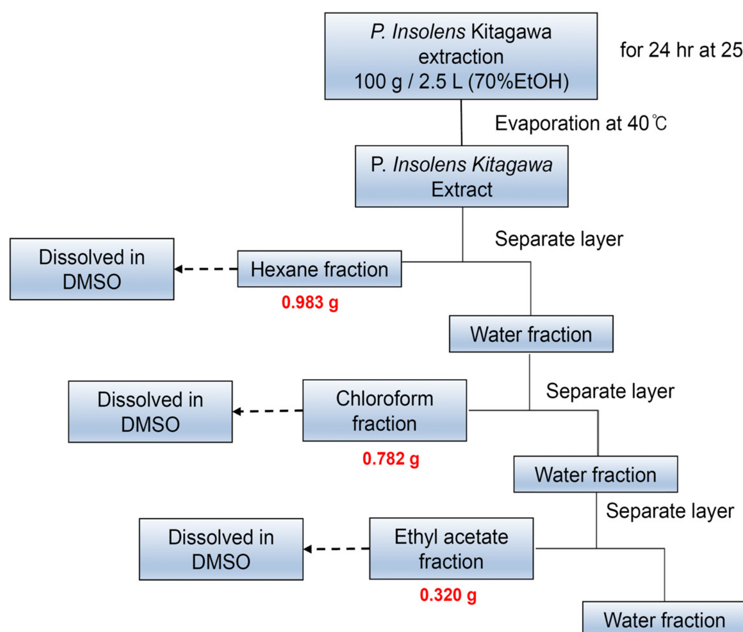

Fig. 1. Scheme for preparation of organic solvent-fractions of *Peucednum insolens* Kitagawa root.

pressed as mg of gallic acid equivalents per g dry plant extract (mg GAE/g DE) on the basis of a standard curve of gallic acid (0~20 μg/ml). All the experiments were run in triplicate. The mean values and standard deviations were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

Determination of total flavonoid content in the organic solvent-fractions of *P. insolens* Kitagawa root

Total flavonoid content was determined in all the fractions using the colorimetric Davis method [11] with slight modifications. Briefly, after mixing of 10 μl of the fraction and 100 μl of diethylene glycol in a 96-well plate, 10 μl of 1 N NaOH was added to the mixture and reacted in a water bath at 37℃ for 1 hr. Afterwards, the mixture was cooled and the absorbance was measured at 405 nm against the reagent blank without extract using a microplate reader (Biotek EL808, Winooski, VT, USA). In this method, fractions reacting with diethylene glycol in alkaline solution produce a yellow chalcone, which was measured at a wavelength of 405 nm. The measurement was compared with a standard curve of prepared quercetin solution. The content of total flavonoid in the organic solvent-fractions was expressed as mg of quercetin equivalents per g of dry plant extract (mg QE/g DE) on the basis of a standard curve of quercetin (0~2 mg/ml). All the experiments were run in triplicate. The mean values and standard deviations were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

Measurement of DPPH radical scavenging activity of organic solvent-fractions of *P. insolens* Kitagawa root

The antioxidant activity was determined by the DPPH radical scavenging assay described by Brand-Williams et al., [4] with slight modifications. Briefly, 2 μl of sample solution was mixed with 198 μl of 0.15 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) solution in a 98-well plate, and then reacted for 30 min at room temperature while blocking the light. Absorbance was measured at 540 nm using a microplate reader (Biotek EL808, Winooski, VT, USA). Ascorbic acid solution (0.0-10.0 μg/ml) was used as a standard. The radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 540 nm. When the reading was complete, the percentage of DPPH radical scavenging activity of samples was calculated using the equation:

% Scavenging of DPPH radical = $[(A_0 - A_1)/A_0] \times 100$

where A_0 = absorbance of the control and A_1 = absorbance of the test extracts.

All the experiments were performed in triplicate.

RAW264.7 Cell culture and treatment of organic solvent-fractions of *P. insolens* Kitagawa root

Murine RAW264.7 macrophage-like cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and routinely

cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco by Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37℃ and 5% CO_2 in humidified air. Cells were plated and cultured in plastic culture dishes and cells reached to 60% confluency were exposed to LPS. Cells were treated with LPS (1 µg/ml) and organic solvent-fractions of *P. insolens* Kitagawa root at the indicated concentrations and incubated for the indicated time periods. Following incubation, the cells were dissociated from dishes by scraping. Dissociated cells were collected by centrifugation (500×g, 5 min), washed twice with ice-cold PBS, and was stored at -80℃ until further analyses.

MTT assay for the measurement of cell viability

Cell viability was determined colorimetrically by measuring the reduction of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Briefly, murine RAW264.7 cells were seeded at a density of 1×10^5 cells/well in 96-well plates (Falcon, Germany) in DMEM supplemented with 10%(v/v) fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin and cultured the cells for 24 hr. After cell attachment, culture media were freshly changed, and various concentrations of organic solvent-fractions of *P. insolens* Kitagawa root were added. Cells were additionally cultured for 24 hr and then MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells. After 3 hr incubation, the medium was removed, and 100 µl of DMSO was then added to dissolve the formazan crystals produced by the cells. The optical density of formazan solution was measured at 540 nm with a microplate reader (Biotek EL808, Winooski, VT, USA). Cell viability was expressed as a percentage of the value against the non-treated control group.

Measurement of NO_2^- and NO_3^- concentration by Griess assay

Nitrite plus nitrate (NOx) in RAW264.7 cells was spectrophotometrically determined by assaying the culture supernatants for nitrite using the Griess reagent. To do this, RAW264.7 cells (5×10^4 cells/well) were seeded into a 96-well plate and allowed to grow for 24 hr. Cells, in the presence or absence of 1 µg/ml LPS, were treated with organic solvent-fractions of *P. insolens* Kitagawa root in the various concentrations. After additional 24 hr incubation, 100 µl of cell-free supernatant was mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl) ethylenediamine in distilled water and 1% sulfanilamide in 5% phosphoric acid, 1:1 ratio). After a 10 min incubation period, absorbance was measured at 540 nm on a microplate reader (Biotek EL808, Winooski, VT, USA). The $NaNO_2$ was used as a standard. Three replicates were performed for each of the different treatments.

RNA extraction, first strand cDNA synthesis

Total RNA was isolated from cultured RAW264.7 cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The concentration of RNA was measured using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All RNA samples were stored at -80℃ until further analysis. First strand cDNA synthesis was performed using AccuPower RocketScript™ Cycle RT PreMix (Bioneer, Korea), according to the manufacturer's instructions. Briefly, one µg of total RNA was added into the AccuPower RocketScript Cycle RT PreMix tubes. Oligo $(dT)_{20}$ primer and RocketScript reverse transcriptase were included in the premix. After adjusting the total volume to 20 µl by adding DEPC-water the reaction was performed under the following conditions: 10 reaction cycles of 37℃ for 0.5 min, 50℃ for 4 min and 60℃ for 0.5 min. The reverse transcription reaction was terminated by heat inactivating at 95℃ for 5 min.

Determination of mRNA expression by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed using an Applied Biosystems 7500 Real-Time PCR system and software (Applied Biosystems, Carlsbad, CA, USA). For real-

Table 1. Primer sequences and lengths used for qRT-PCR

Gene	Accession number	Forward primer	Reverse primer	Size (bp)
iNOS	nm_010927.4	5′-TCTCCCTTTCCTCCCTTCTT-3′	′5′-CTTCAGTCAGGAGGTTGAGTTT-3′	116
IL-1β	nm_008361.4	5′-ATGGGCAACCACTTACCTATTT-3′	5′-GTTCTAGAGAGTGCTGCCTAATG-3′	94
IL-6	nm_031168.2	5′-GATAAGCTGGAGTCACAGAAGG-3′	5′-TTGCCGAGTAGATCTCAAAGTG-3′	105
GAPDH	nm_001289726	5′-TCTCCCTCACAATTTCCATCC-3′	5′-GGGTGCAGCGAACTTTATTG-3′	100

time PCR, cDNA was mixed with 1X SYBR Green PCR Master Mix (Applied Biosystems) and the forward and reverse primers were added to a final volume of 15 µl. Sequences and concentrations of primers are listed in Table 1. Real-time PCR was carried out on an ABI 7500, Applied Biosystems with Sequence Detection Software v1.4. The cycling conditions included a hot start at 95℃ for 10 min, followed by 40 cycles at 95℃ for 15 s and 60℃ for 1 min. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Each sample was analyzed in triplicate for quantification. Relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method and transcript levels were normalized to that of GAPDH. Amplification efficiency for each set of primers was near 98%. RNA samples incubated without reverse transcriptase during cDNA synthesis, as well as PCR reactions using water instead of template showed no amplification.

Statistical analysis

Student's *t*-test and one-way ANOVA were used to determine the statistical significance of the difference between values for the various experimental and control groups. Experimental data were expressed as means ± standard deviation (SD), and the results were obtained from at least three independent experiments performed in triplicate. A *p*-value of 0.05 or less was considered statistically significant.

Results

Total polyphenolic and total flavonoid contents in the organic solvent-fractions of *P. insolens* Kitagawa root

Three different organic solvent (hexane, chloroform, and ethyl acetate)-fractions of *P. insolens* Kitagawa root were prepared as shown in Fig. 1. For this, 70% ethanol extract was first prepared from the 100 g of dried *P. insolens* Kitagawa root. From the 70% ethanol extracts, fractions of hexane, chloroform, and ethyl acetate were successively recovered as 0.983, 0.782, and 0.320 g, respectively.

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity [38]. The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging. As a basis, phenolic content was measured using the Folin–Ciocalteu reagent in each extract. The results were derived from a calibration curve ($y = 0.0345x + 0.0235$, $R^2 = 0.9931$) of gallic acid (0-20 µg/ml) and expressed in gallic acid equivalents (GAE) per gram dry extract weight (Table 2). The contents of phenolic compounds in the hexane-, chloroform- and ethyl acetate-fractions of the extracts were determined as 11.9±4.4, 21.4±2.7 and 154.1±3.2 µg GAE/mg, respectively, suggesting that the ethyl acetate-fraction possess the remarkably high contents of total polyphenolic compounds. Total flavonoid content was also determined through a linear quercetin standard curve and are expressed as µg QE/mg. As shown in Table 2, The contents of total flavonoids in the hexane-, chloroform- and ethyl acetate-fractions of the extracts were determined as 6.8±0.8, 7.1±1.5 and 340.8±16.7 µg QE/mg, respectively, suggesting that the content of total flavonoids was the remarkably high at the ethyl acetate- fraction. Phenolic compounds in medicinal plants have been reported to be associated with antioxidant activity, anticancer effects, and other biological functions, and may prevent other diseases associated with aging [38, 40, 43]. Thus, our study results suggest that ethyl acetate-fraction of *P. insolens* Kitagawa root extracts with ethanol might have high antioxidant activities.

Table. 2. Total polyphenols and total flavonoids in the organic solvent extracts of *P. insolens* Kitagawa root

Organic solvent-Fractions	Contents	
	Total polyphenol[a] (µg GAE/mg extracts)	Total flavonoid[b] (µg QE/mg extracts)
Hexane (RH)	11.9±4.4	6.8±0.8
Chloroform (RC)	21.4±2.7	7.1±1.5
Ethyl acetate (RE)	154.1±3.2	340.8±16.7

[a]Total polyphenolic content expressed in µg of gallic acid equivalent (GAE) per mg of dry weight of plant extracts.
[b]Total flavonoid content expressed in µg of quercetin equivalent (QE) per mg of dry weight of plant extracts.

Antioxidant capacities of the organic solvent-fractions of *P. insolens* Kitagawa root.

The radical scavenging capacities of the organic solvent-fractions of *P. insolens* Kitagawa root as determined by DPPH assay are shown in Fig. 2. The DPPH radical scavenging activities were found to be 65, 83, 88, and 90% at 100, 200, 300, and 400 µg/ml of ethyl acetate-fraction of *P. insolens* Kitagawa root, respectively. However, hexane- and chloroform-fractions of the extracts showed relatively low DPPH radical scavenging activities, compared to that of ethyl acetate-fraction. Therefore, it was confirmed that ethyl ace-

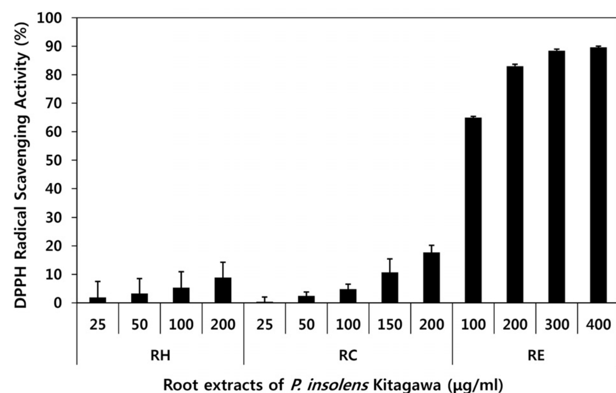

Fig. 2. DPPH radical-scavenging activities of organic solvent-fractions of *P. insolens* Kitagawa root. RH, RC and RE represent the fractions of hexane, chloroform, and ethyl acetate of *P. insolens* Kitagawa root, respectively.

tate-fraction of *P. insolens* Kitagawa root has the best electron donating ability among the organic solvent fractions. According to Hwang et al. [18], antioxidant activity highly correlates with the total phenolic content. Therefore, as observed in the DPPH assay in the present study, the excellent antioxidant activity of ethyl acetate-fraction of *P. insolens* Kitagawa root indicates high phenolic flavonoid contents of the extract.

Cytotoxicity of organic solvent-fractions of *P. insolens* Kitagawa root in RAW264.7 cells

As a first step, in order to identify non-cytotoxic dose ranges of the organic solvent-fractions of *P. insolens* Kitagawa root to RAW264.7 cells, cytotoxicity was determined after exposure to the various concentrations of the organic solvent-fractions up to 400 μg/ml. Our results showed that, when cultured for 24 hr, all the three organic solvent-fractions had cytotoxic effect on RAW264.7 cells dose-dependently (Fig. 3). For RAW264.7 cells, the concentration of hexane- and chloroform-fractions with a cell viability of 70% or more was 50 μg/ml or less, whereas the ethyl acetate-fraction had a cell viability of 70% or more at a concentration of 200 μg/ml or less. The hexane-, chloroform- and ethyl acetate-fractions showed strong cytotoxicity to RAW 264.7 cells at concentrations higher than 100, 150 and 300 μg/mg, respectively. Based on this result, 50, 50, and 200 μg/ml of the hexane-, chloroform-, and ethyl acetate-fraction was determined as the maximal doses for other experiments in this study, respectively. The concentrations did not affect the growth or morphological features of RAW264.7 cells. On the other hand, all the three organic solvent-fractions at their

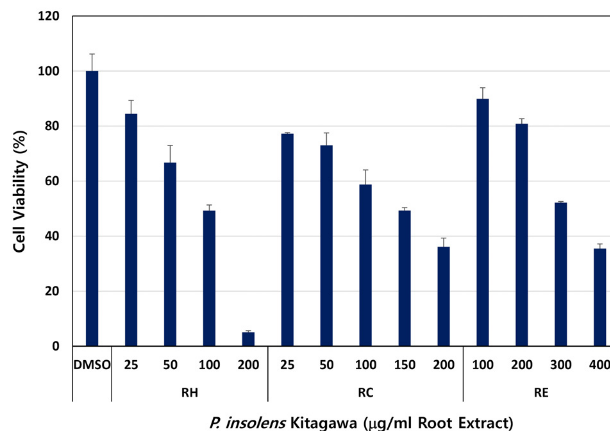

Fig. 3. Cytotoxicity of organic solvent-fractions of *P. insolens* Kitagawa root in RAW264.7 cells. Cells were treated with various concentrations of hexane (RH), chloroform (RC) or ethyl acetate (RE)-fraction of *P. insolens* Kitagawa root extracts. After treatment for 24 hr, cell viability was measured with the MTT assay. Values are the mean ± SD of experiments in triplicate (n=3).

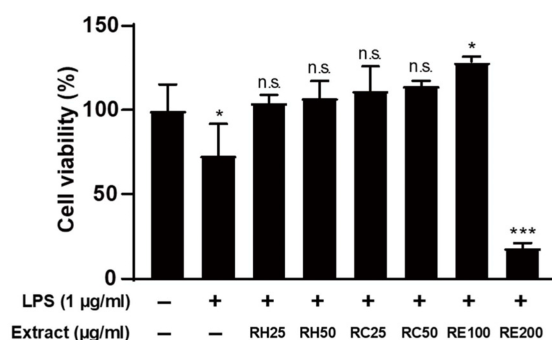

Fig. 4. Protective effects of organic solvent-fractions of *P. insolens* Kitagawa root against cytotoxicity induced by LPS treatment in RAW264.7 cells. RAW264.7 cell were treated with both LPS (1 μg/ml) and either hexane (RH), chloroform (RC) or ethyl acetate (RE)-fraction of *P. insolens* Kitagawa root extracts simultaneously for 24 hr. All experiments were performed in triplicate and results are expressed as mean ± SD. *$p<0.05$, **$p<0.01$ (untreated group vs each treated group); n.s., not significant.

selected low concentrations showed protective effects against the cytotoxicity induced by LPS treatment, especially at RE 100 significantly (Fig. 4). However, RW264.7 cells treated with LPS showed unexpectedly very strong cytotoxicity in 200 μg/mg of ethyl acetate-fraction (RE200). Based on this result, 200 μg/mg of ethyl acetate-fraction was excluded from the analysis of the expression of inflammatory-related genes in LPS-treated RW264.7 cells.

Effects of organic solvent-fractions of *P. insolens* Kitagawa root on Nitric oxide production in LPS-Stimulated RAW264.7 cells

Nitric oxide (NO) is one of the key inflammatory mediators linked with various acute and chronic inflammation-associated diseases. In macrophages, NO production is significantly increased upon LPS-stimulation. The level of NO production was estimated in cell culture supernatant after treatment of LPS-stimulated macrophages with organic solvent-fractions of *P. insolens* Kitagawa root using Griess reagent. As shown in Fig. 5, NO production in RAW264.7 cells was greatly increased by LPS (1 µg/ml) treatment for 24 hr. But when hexane- or ethyl acetate-fraction was simultaneously treated with LPS the increase of NO production by the LPS treatment was significantly reduced. On the other hand, it is suggested that the decrease in the amount of nitrite produced in the cells simultaneously treated with LPS and RE200 is also due to the cytotoxicity of the concentration. The chloroform-fraction of *P. insolens* Kitagawa root also showed inhibitory potency to nitrite production, but the inhibitory potency was weak compared to other two organic solvent-fractions. These results suggest that the three organic solvent-fractions of *P. insolens* Kitagawa root possess the significant anti-inflammatory potential.

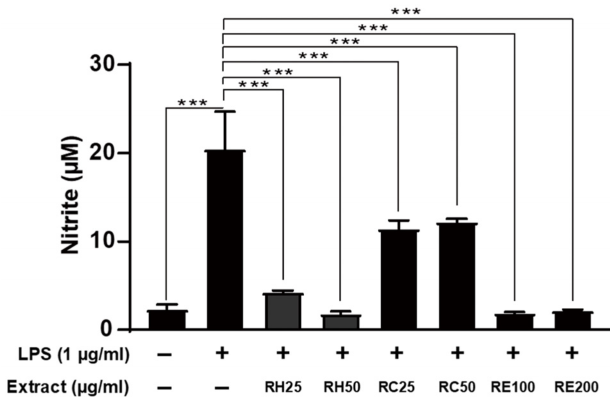

Fig. 5. Nitrite-scavenging activities of organic solvent-fractions of *P. insolens* Kitagawa root in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with either hexane (RH), chloroform (RC) or ethyl acetate (RE)-fractions at indicated concentrations in the absence or presence of LPS (1 µg/ml) for 24 hr. After the incubation period, cell culture supernatants were harvested and nitrite production was determined by Griess reagent. Values are expressed as described in the Materials and Methods section. All experiments were performed in triplicate and results are expressed as mean ± SD. ***$p<0.001$ (untreated group vs each treated group).

Organic solvent-fractions of *P. insolens* Kitagawa root inhibit LPS-induced mRNA expression of the inflammatory-related genes in RAW264.7 cells

The effect of organic solvent-fractions of *P. insolens* Kitagawa root on the mRNA expression levels of pro-inflammatory cytokines such as IL6 and IL1β and pro-inflammatory enzymes such as COX-2 and iNOS in LPS-induced RAW264.7 cells were evaluated using qRTPCR. As shown in Fig. 6, mRNA levels of iNOS and the inflammatory cytokines were significantly increased ($p<0.001$) following LPS (1 µg/ml) treatment compared with the untreated control group. The mRNA expression levels of IL6, IL1β and iNOS genes were selectively inhibited by organic solvent-fractions of *P. insolens* Kitagawa root in the LPS-stimulated RAW264.7 cells (Fig. 6). The *P. insolens* Kitagawa root in hexane-fraction at 50 µg/ml and ethyl acetate-fraction at 100 µg/ml significantly inhibited mRNA expression levels of IL6, IL1β and iNOS genes in the LPS-stimulated RAW264.7 cells (Fig. 6). In contrast, chloroform-fraction of the root extract showed weak or no inhibitory potency to mRNA expression levels of IL6, IL1β and iNOS genes in the LPS-stimulated RAW 264.7 cells (Fig. 6). Meanwhile, organic solvent-fractions of *P. insolens* Kitagawa root did not

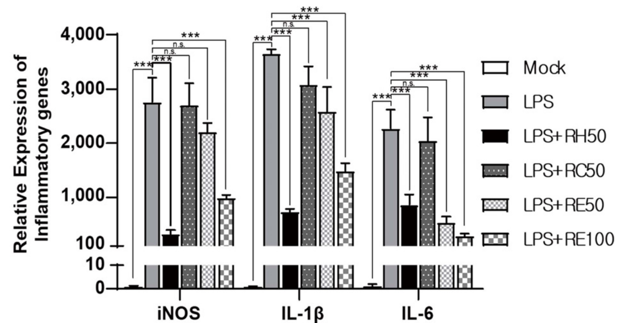

Fig. 6. Real time inhibitory effects of organic solvent-fractions of *P. insolens* Kitagawa root on the expressions of iNOS (A), IL-1β (B) and IL-6 (C) genes in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with 1 µg/ml LPS in the absence or presence of either hexane (RH), chloroform (RC) or ethyl acetate (RE) fractions of *P. insolens* Kitagawa roots at indicated concentrations (50 or 100 µg/ml) for 24 hr. After the incubation period, total RNAs were extracted from the treated cells and cDNAs were reverse transcribed using first strand cDNA synthesis kit as described in Materials and Methods. Expression levels of iNOS, IL-1β and IL6 mRNA were amplified by real-time PCR analysis. All experiments were conducted in triplicate and results are expressed as mean ± SD. ***$p<0.001$ (untreated group vs each treated group); n.s., not significant.

show any inhibitory potency to COX-2 mRNA expression in the LPS-stimulated RAW264.7 cells (data not shown). The results suggest that organic solvent-fractions of *P. insolens* Kitagawa root may possess selective inhibitory potency to the expressions of pro-inflammatory cytokines and pro-inflammatory enzymes at the transcriptional level in LPS-induced RAW264.7 cells depending on the organic solvent-fractions.

Discussion

Plant extracts are attracting greater interest in antiinflammatory drug discovery due to their low side effects and effective mode of action [33]. The various types of phytochemicals present in plants such as phenols, flavonoids and alkaloids, are responsible for the effective biological activities of plants, including antioxidant and anti-inflammatory effects [25]. However, to the best of our knowledge, no previous studies have reported antioxidant and anti-inflammatory effects of organic solvent-extracts of *P. insolens* Kitagawa roots on macrophage cells for their biological activities. The present study examined antioxidant capacities of organic solvent-extracts of *P. insolens* Kitagawa root and their anti-inflammatory effects on LPS-stimulated RAW264.7 cells. Our results showed that ethyl acetate fraction of *P. insolens* Kitagawa root contained relatively high contents of total flavonoid (340.8±16.7 μg of quercetin equivalents per mg dry extract) and total polyphenol (154.1±3.2 μg of gallic acid equivalents per mg dry extract). DPPH assay result showed that *P. insolens* Kitagawa possessed strong free radical scavenging activity in the ethyl acetate fraction. This result is thought to be due to the fact that the ethyl acetate-fraction contains a fairly high amount of total flavonoid and total polyphenol. Both ethyl acetate- and hexane-fractions showed strong inhibitory potencies to NO production induced by LPS (1 μg/ml) treatment for 24 hr in RAW264.7 cells. Both the two organic solvent-fractions of *P. insolens* Kitagawa root also strongly inhibited mRNA levels of iNOS, IL-1β and IL-6 in the LPS-stimulated RAW264.7 cells for 24 hr. From the results of this study, it is suggested that *P. insolens* Kitagawa contains ingredients that exhibit antioxidants and anti-inflammatory activities.

NO, a reactive free radical gas, has been widely recognized as a key mediator of metabolic homeostatic processes, host defense mechanisms, and oxidant tissue injury (1). NO is produced from L-arginine through nitric oxide synthases (NOS) that affects immune functions by eliciting intracellular signals [19, 20]. The high level of NO causes inflammatory damage to target tissue during infection [39]. Hence, the regulation of NO release via the inhibition of iNOS expression is helpful to alleviate inflammatory damage. In the present study, we also showed that hexane- and ethyl acetate-fractions of *P. insolens* Kitagawa root significantly suppressed LPS-induced iNOS expression at the transcriptional and translational levels in RAW264.7 cells.

Endotoxin LPS is a major cell wall component of the outer membrane of Gram-negative bacteria, and stimulates immune response in mammalian cells, particularly macrophages. It plays a critical role in the induction of systemic inflammation in RAW264.7 cells by inducing the production of host inflammatory mediators such as tumor necrosis factor-α, interferon ɣ (INF-ɣ), and interleukin-1β, which in turn cause an increase in the expression of iNOS [45]. LPS binds to Toll-like receptor 4 (TLR4) and activates the cascade of pro-inflammatory mediators, transcription factors, and enzymes involved in NF-κB, mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) signaling pathways [21]. Hence, inhibitors of this signaling cascade have been considered as effective anti-inflammatory therapeutic agents. In terms of this aspect, it is necessary to further check the regulatory effects of the organic solvent-fractions of *P. insolens* Kitagawa root on the NF-κB, MAPK and JNK signaling pathways.

Macrophages stimulated by LPS overexpress various primary inflammatory mediators such as COX-2 and iNOS, which are involved in the synthesis of NO. Excessive production of NO can trigger numerous acute and chronic inflammation-associated diseases [16, 30]. This molecule is a key mediator of endotoxin or microbial-induced inflammation; therefore, suppression of the activity can alleviate acute and chronic inflammation. In the present investigation, we observed a concentration-dependent decrease in the level of NO in LPS-stimulated macrophages when treated with hexane- and ethyl acetate- fractions of *P. insolens* Kitagawa root. The results suggested that the two organic solvent-fractions played a key role in suppressing NO production during inflammation.

Among pro-inflammatory cytokines, the multifunctional IL-1β and IL-6 are highly expressed and are crucial in the pathogenesis of many inflammatory diseases/disorders. Their expression is predominantly regulated by the key transcription factor, NF-κB [9]. In this investigation, we found

that production of the two pro-inflammatory cytokines, IL-1β and IL-6, was strongly inhibited by treatment with hexane- and ethyl acetate- fractions of *P. insolens* Kitagawa root in a concentration-dependent manner in LPS-stimulated RAW264.7 cells, suggesting that the two organic solvent-fractions may contain the components that mediate NF-κB signaling pathway.

Nuclear factor-κB is one of the key transcription factors in LPS-stimulated inflammation, which regulates a number of inflammatory mediators such as iNOS, COX-2, TNF-α, IL-6, IL-8, and IL-1β [34], and plays crucial roles in the early stages of inflammatory response. Activated NF-κB through a multistep process induced via numerous signal transduction pathways results in an excessive production of inflammatory mediators, particularly iNOS and COX-2 [26]. Both the two inducible enzymes are responsible producing NO and PGE2, respectively, and these mediators are closely related with the initiation of the early stage of inflammatory pathways [36]. Our result showed that hexane- and ethyl acetate-fractions of *P. insolens* Kitagawa root can mediate the inflammatory response by effectively inhibiting the production of nitric oxide in LPS-stimulated RAW264.7 cells. In the present study, treatment of LPS-stimulated macrophages with the hexane- and ethyl acetate-fractions of *P. insolens* Kitagawa root also effectively suppressed the expression of iNOS mRNA, which led to a decrease in the production of NO, suggesting the inhibition of the early stage of specific inflammatory pathways [43]. In order to further understand the mechanism of anti-inflammatory efficacy, further studies are necessary to investigate the efficacy of the organic solvent-fractions of *P. insolens* Kitagawa root on the expression of COX-2.

To investigate the molecular mechanism of the anti-inflammatory action of the organic solvent-fractions of *P. insolens* Kitagawa root, we observed their modulatory effects on the expressions of the primary inflammatory mediators such as iNOS and pro-inflammatory cytokines such as IL-1β and IL-6 in LPS-stimulated RAW264.7 cells. From the qRT-PCR analysis, it was confirmed that the iNOS mRNA level over-expressed by LPS-treatment was down-regulated upon co-treatment with the organic solvent-fractions except the chloroform-fraction. The present findings suggest that the hexane- and ethyl acetate-fractions that interfere with the activation of iNOS may be effective for treating various inflammation-associated diseases.

The release of pro-inflammatory cytokines, such as TNFα, IL1β and IL6, by activated macrophages at the site of infection is an important target for anti-inflammatory therapeutic strategies [14, 15]. Consistent with the qRT-PCR results, hexane- and ethyl acetate-fractions of *P. insolens* Kitagawa root significantly down-regulated the mRNA expression levels of IL1β and IL6, suggesting that hexane- and ethyl acetate-fractions of *P. insolens* Kitagawa root exerted anti-inflammatory effects via inhibition of the proinflammatory cytokines.

In conclusion, the results of the present study suggest that organic solvent-extracts of *P. insolens* Kitagawa root contain antioxidant capacities and exert antiinflammatory effects in RAW264.7 cells *in vitro* by suppressing NO production, and by inhibiting the expressions of the inflammatory mediators iNOS and the proinflammatory cytokines IL1β and IL6. Further investigation into the isolation and identification of responsible antioxidant components and their action mechanism of anti-inflammation is necessary to better understand their ability to control inflammatory-related diseases.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 왕산방풍의 뿌리로부터 제조한 유기용매 분획물에서의 항산화 활성 및 RAW264.7 세포주에서의 항염증 효능

김진익[1] · 최용원[1] · 최근준[1] · 강지안[1] · 이인영[1] · 나란투야 난딘셋세그[1] · 오명석[1] · 조식제[2] · 문자영[1]*
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본 연구는 *Peucedanum insolens* Kitagawa 뿌리의 여러 유기용매 분획물에 대한 항산화 활성과 LPS 처리된 RAW264.7 세포에서 항염증 효과를 조사하기 위해 수행되었다. 이 연구를 위해 *P. insolens* Kitagawa 뿌리를 먼저 실온에서 1 주일 동안 건조하여 잘게 썰고 70% 에탄올로 추출한 다음 hexane, chloroform, ethyl acetate 및 물로 연속적으로 분획하였다. DPPH (1,1-diphenyl-2-picrylhydrazyl) 라디칼 소거능 분석과 total polyphenol 및 total flavonoid 함량을 측정하여 유기용매 분획물의 항산화 활성을 평가했다. RAW264.7 세포에서 iNOS, COX-2, IL-1β 및 IL-6과 같은 염증 매개 유전자의 발현 억제 수준을 측정하여 분획의 항 염증 효능을 평가했다. 본 연구 결과는 *P. insolens* Kitagawa 뿌리의 ethyl acetate 분획물이 상대적으로 높은 함량의 total flavonoid (34.08±1.68 μg QE/mg)와 total polyphenol (154.1±3.2 μg의 GE/mg)을 함유하고 있음을 분명히 보여주었다. DPPH 분석 결과 *P. insolens* Kitagawa는 ethyl acetate 분획에서 강력한 자유 라디칼 소거 활성을 가지고 있음을 보여주었다. Ethyl acetate 분획 및 hexane 분획은 모두 RAW264.7 세포에서 24시간 동안 LPS (1 μg/ml) 처리에 의해 유도된 산화질소 생성에 대해 강력한 억제력을 나타냈다. 본 연구결과는 또한 *P. insolens* Kitagawa의 hexane 분획 및 ethyl acetate 분획 모두 RAW264.7 세포에서 24시간 동안 LPS 처리에 의해 과발현된 iNOS, IL-1β 및 IL-6의 mRNA 수준을 강력하게 억제하는 것으로 나타났다. 이러한 결과는 *P. insolens* Kitagawa가 항 염증 활성에 강한 효능을 가진 화합물을 포함할 수 있음을 시사한다. 염증 조절에 대한 *P. insolens* Kitagawa 분획물의 더 자세한 효과를 이해하기 위하여 이 분획물이 cytokine 신호 전달 경로 및 염증 신호 경로에 대한 조절작용과 같은 추가적인 작용기전 연구가 필요하다.