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In Vitro Anti-Inflammatory Effects of the Essential Oil of *Artemisia iwayomogi* and Its Main Component, Vulgarone B

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Abstract – The essential oil fraction was obtained from the leaves and flowers of *Artemisia iwayomogi* (Compositae) by steam distillation, and its main component, vulgarone B, was isolated by column chromatography. RAW 264.7 cells were used to investigate the anti-inflammatory properties of *A. iwayomogi* and vulgarone B. Cell viability was determined by MTT assay after treatment with various dilutions of the compounds. In addition, several assays were used to determine the effects of *A. iwayomogi* essential oil components on immune stimulation. Nitric oxide production in cells activated with lipopolysaccharide (LPS) was evaluated by reaction with Griess reagent. Both vulgarone B and the essential oil fraction of *A. iwayomogi* inhibited the production of nitric oxide. The effects on various cytokines released from the cells were also measured using ELISA. The production of prostaglandin E_2 was significantly decreased by treatment with *A. iwayomogi* oils. LPS-induced IL-1β and IL-6 production were also decreased in a dose-dependent manner, but no significant effect on TNF-α was observed at the concentrations tested. Finally, Western blot analysis revealed that *A. iwayomogi* oils reduced the levels of COX-2 and iNOS.

Keywords – *Artemisia iwayomogi,* Essential oil, Vulgarone B, Anti-inflammatory effect, Nitric oxide, Prostaglandin E_2, Cytokine

Introduction

Artemisa iwayomogi Kitamura is (Compositae) the representative Korean plant source for "Korean Injin", which is used in traditional therapy in Korea to alleviate various symptoms related to liver diseases, including hepatitis (Park *et al.,* 2000; Lee *et al.*, 2008). The anti-inflammatory, anti-allergic, anti-oxidative, anti-bacterial, and molluscicidal activity of *A. iwayomogi* extracts and some of its components have been reported in the last decades (Yu *et al.*, 2003; Meepagala *et al*., 2004; Kim *et al.*, 2005; Shin *et al.*, 2006; Ahn *et al.*, 2009).

In this study, the essential oil fraction was obtained from the leaves and flowers of this plant by steam distillation, and its main component, vulgarone B, was isolated by column chromatography. The effects of these components on mediators of inflammation were assayed to evaluate their potential as a source of natural anti-inflammatory agents.

Nitric oxide (NO), prostaglandins (PG), and various cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), are present in macrophages and are known to be important mediators of inflammatory processes (Tewtrakul *et al.*, 2009; Zhao *et al.*, 2009). Nitric oxide synthase (NOS) and cyclooxygenase (COX) catalyze the formation of NO and PG, respectively. COX-2 and iNOS are induced by cytokines or other immune stimulating factors, including lipopolysaccharide (LPS). In recent decades, a number of reports have demonstrated that these molecules participate in inflammatory disorders, including hepatitis (Ahn *et al.*, 2003; Ryu *et al.*, 2003).

To investigate the anti-inflammatory properties of *A. iwayomogi* oil and its components, we examined their effects on RAW 264.7 cell survival and immune status. Cell viability was determined by an MTT assay after treatment with various dilutions of the compounds. Inhibition of nitric oxide production in cells treated with lipopolysaccharide (LPS) was evaluated by reaction with Griess reagent. The effects of *A. iwayomogi* oil components on immune stimulation were examined using multiple assays. Levels of TNF-α, IL-6, IL-1β and PGE_2 released from the cells were measured by ELISA, and the enzyme expression levels of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were investigated by Western blot analysis which revealed that *A. iwayomogi*

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oils reduced the levels of COX-2 and iNOS.

Experimental

Reagents – Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, fetal bovine serum (FBS), tris-glycine-PAG pre-cast gel containing 8% (for iNOS) and 10% (for COX-2) SDS, assay kits for cytokines, and secondary antibodies were purchased form Komabiotech (Seoul, Korea). Anti-COX-2 was obtained from Millipore. Anti-iNOS and anti-β-actin antibodies were from Santa Cruz Biotechnology (USA). Lipopolysaccharide (LPS) was provided from Sigma-Aldrich Co. (USA).

Extraction and analysis of the essential oil fraction by gas-chromatography and mass spectrometry – Essential oils were obtained from flowers and leaves (1 : 1) of *A. iwayomogi* cultivated in Youngchun, Korea by steam distillation for five hours in a simultaneous steam distillation-extraction apparatus. The plant was identified by comparing its morphology with the authentic sample growing in medicinal plant garden of Korea Forest Service. A voucher specimen was deposited at the herbarium of Duksung Women's University (No. COMAC2). The composition of the oil fraction was analyzed by a previously described method. The essential oil fraction of *A. iwayomogi* was analyzed by a Hewlett-Packard 6890 GC and a Hewlett-Packard 5973 MSD apparatus (Agilent 5973 network mass selective detector, 280 °C) with a fused silica capillary column (HP-5 MS, 30 m × 250 μm × 0.25 μm). The injector was adjusted to 260 °C and the oven temperature was programmed as follows: the initial temperature was 50 °C for 5 min, increased 2 °C/min up to 180 °C, sustained at 180 °C for 5 min, and then increased 20 °C/min up to 220 °C.

Isolation of vulgarone B – Vulgarone B was isolated from the essential oil fraction (2 g) of *A. iwayomogi* by silicagel column chromatography with hexane-ethylacetate (5 : 95). Fractions 22 - 31 were subjected to rechromatography with hexane-methylene chloride 8 : 2 - 2 : 8 gradient), yielding 0.28 g of 98.8% vulgarone B (a sesquiterpene ketone, MW 218). The chemical structure was elucidated using UV, MS, ^{1}H-NMR, and ^{13}C-NMR data and confirmed by comparison with previously reported spectral data of vulgarone B (Uchio, 1978; Meepagala *et al*., 2003).

Vulgarone B – Colorless oil, $C_{15}H_{22}O$; EI-MS m/z: 218 $[M]^{+}$; UV (CH_2Cl_2) λ_{max} nm: 250. 322; IR (KBr) ν_{max} cm^{-1}: 3038, 2867, 1676, 1374; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.75 (1H, br s, H-3), 2,78 (1H, d, *J* = 6.6, H-5), 2.56 (1H, d, *J* = 6.6, H-1), 2.04 (1H, s, H-7), 2.08 (3H, s, H-8), 2.02-1.43 (6H, m, H-10, H-11, H-12), 0.96 (6H, s, H-14, H-15), 0.87 (3H, s, H-9); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 205.4 (C-4), 173.1 (C-2), 122.9 (C-3), 67.2 (C-5), 58.3 (C-7), 55.3 (C6), 50.4 (C-1), 42.0 (C-12), 38.9 (C-10), 34.2 (C-13), 28.2 (C-15), 27.1 (C-14), 25.1 (C-9), 23.7 (C-8), 21.7 (C-11).

Culture of RAW264.7 cells – RAW264.7 cells provided by the Korean Cell Line Bank were cultured in DMEM supplemented with penicillin (10 U/ml), streptomycin (10 μg/ml), and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. Cells were subcultured to generate cells for successive experiments.

Cell viability measurements using the MTT assay – 100 μl of the cell suspension was seeded in 96 well plates in triplicate at a density of 5×10^4 cells/well as determined using a hematocytometer. After a 24 h incubation to allow cell to adhere, 5 μl of medium was removed from each well. Cells were treated with 5 μl of vulgarone B or the *A. iwayomogi* essential oil fraction (dissolved in DMEM containing 2% Tween 80) at various concentrations (0.5~1000 μg/ml). After a 2 h incubation, MTT solution (25 μl, 5 mg/ml in PBS) was added, and the cells were incubated for an additional 4 h at 37 °C in 5% CO_2. The medium was then discarded, and isopropanol containing 0.04 M HCl was added to dissolve the produced formazan. The absorbance was measured at 570 nm with microplate reader. Results are expressed as the percentage of viable cells compared to the untreated control.

Assay for inhibition of nitric oxide production – The effects of vulgarone B and the essential oil fraction on the production of NO and PGE_2 were determined following LPS (0.1 μg/ml) treatment. 200 μl of the cell suspension was added to each well of 96 well plates in triplicate at a density of 10^4 cells/well. After a 24 h incubation, the medium was discarded, and the cells were treated with 2.5 μl of the compounds at various concentrations (6~100 μg/ml), incubated for 1 h at 37 °C, treated with 2 μl of LPS (0.1 μg/ml), and then incubated for 18 h at 37 °C in 5% CO_2. The supernatant (100 μl) of each well was mixed with 100 μl of Griess reagent (1 : 1 mixture of 1% sulfanilamide and 0.1% naphthylethylene dihydrochloride in 5% H_3PO_4), followed by incubation for 10 min at room temperature. The absorbance was measured at 540 nm with a microplate reader. Inhibition of NO production was demonstrated by comparing the measured absorbance of the treated wells and the control (untreated).

Assay for inhibition of PGE_2, TNF-α, IL 1β, and IL-6 production – To quantitatively determine PGE_2 levels,

an assay kit was purchased from R&D systems, USA. Cell cultures were treated with *A. iwayomogi* products and LPS in a method similar to that for NO measurements, and supernatants were diluted 3 fold to generate 150 µl samples for the assay. 200 µl and 150 µl of Calibrator Diluent RD5-39 were added to the NSB (non-specific binding) wells, respectively. PGE_2 levels were then assayed according to the manufacturer's instructions (KGE004).

To measure TNF-α, IL 1β, and IL-6 production, 200 µl of the cell suspension (5×10^4/ml) was plated in each well of a 96-well plate, treated with test components (0.007~0.1 mg/ml in DMSO) for 1 h, and then incubated for 18 h at 37 °C in 5% CO_2. 100 µl of the supernatant was then assayed using complete ELISA kits (Komabiotech, Seoul) according to the manufacturer's recommendations.

Western blots for COX-2 and iNOS – Sub-cultured cell suspensions (10 ml/plate) were adjusted to 5×10^4/ml and then transferred to seven petri-dishes [1, cells only; 2, treated with LPS alone; 3-6, treated with LPS and various concentrations of the *Artemisia* oil fraction (0.006~0.1 mg/ml in DMEM containing 2% tween 20)]. After the oil treatment for 1 h and incubation with LPS (1 µg/ml) for 24 h, RAW 264.7 cells were washed with cold PBS and centrifuged.

Pro-PREP Protein Extraction Solution (Intron Biotechnology, Inc.) was used to lyse the cells according to the protocol provided by the manufacturer. After the protein concentration for each aliquot was determined by the Bradford method, suspensions were boiled in SDS-PAGE loading buffer. 20 µg of protein from each sample was subjected to gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in distilled water on a shaker at room temperature for 90 min. The membranes were then incubated in primary antibody (1 : 1000, anti-iNOS, anti-COX-2, or anti-actin antibodies) for 3 h at room temperature. After washing three times with tris buffered saline containing 0.5% Tween20 (TBS-T), the blots were incubated with HRP-conjugated secondary antibody solution for 90 min at room temperature and washed again three times. The corresponding bands were detected using enhanced chemiluminescence reagent (ECL) followed by exposure to photographic film (Kodak) for 1 min.

Results and Discussion

A. iwayomogi, or "Korean Injin", is a representative herbal drug used to treat liver disease, including early stage hepatitis. In this study, the effect of the essential fraction of *A. iwayomogi* and its main component, vulgarone B, on NO, PGE_2, and some pro-inflammatory cytokines was evaluated. Vulgarone B (19.07%), camphor (10.22%), and borneol (6.67%) were identified as main components of this oil by GC-MS analysis. Vulgarone B (Fig. 1) was isolated by silicagel column chromatography, identified by its spectral data, and used for further experiments.

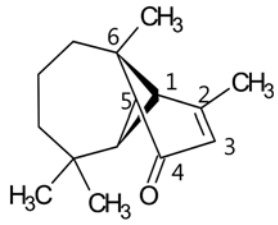

Fig. 1. Structure of vulgarone B.

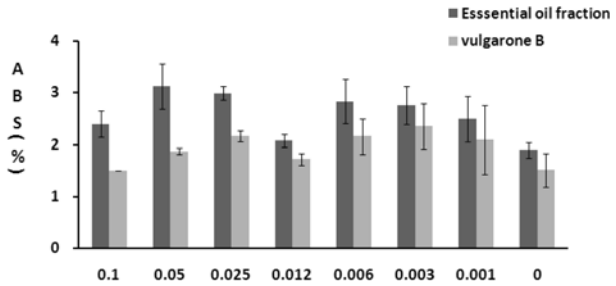

Fig. 2. The survival rate (%) of RAW 264.7 cells compared to the control (0) as determined by MTT assay following treatment with the total essential oil fraction of *A. iwayomogi* or vulgarone B.

To exclude possible effects of the components on cell viability, we first treated RAW264.7 cells with various concentrations of the *A. iwayomogi* essential oil and determined the survival rate using an MTT assay. As demonstrated in Fig. 2, treatment with the essential fraction of *A. iwayomogi* or vulgarone B at concentrations between 0.1 and 0.001 mg/ml did not significantly reduce cell viability.

Nitric oxide (NO) is an important inflammatory mediator released by activated macrophages. Significant increases in NO levels have been observed in many inflammatory diseases, including hepatitis. As shown in Fig. 3, both the essential oil fractions of *A. iwayomogi* and vulgarone B exhibited a dose-dependent inhibitory effect on NO release in RAW264.7 cells treated with 0.1 µg/ml of lipopolysaccharide (LPS). Most concentrations tested suppressed NO release, and the rate of suppression was over 50% compared to untreated samples exposed to LPS

for 24 hours.

Highly increased production of PGE_2 and other cytokines, such as IL-1β, IL-6, and TNF-α, results from inflammatory macrophage stimulation (Yun *et.al.*, 2008). To estimate the effects of the essential oil fraction of *A. iwayomogi* and vulgarone B on these inflammatory mediators, the production of PGE_2, IL-1β, IL-6, and TNF-α was measured by ELISA assay following LPS treatment. Treatment with either component at concentrations ranging from 0.006~0.1 mg/ml markedly reduced the production of PGE_2, IL-1β, and IL-6 in a dose-dependent manner (Fig. 4). The inhibitory activity of the total essential oil fraction was higher than that of vulgarone B. In contrast, treatment with *A. iwayomogi* essential oil induced a slight increase of TNF-α production compared to the control and vulgarone B treated samples.

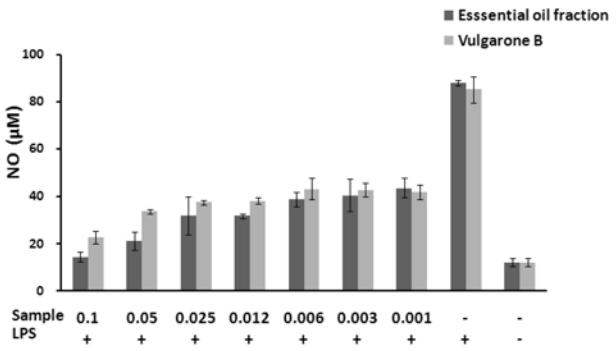

Fig. 3. Inhibitory effects of *A. iwayomogi* oils at various concentrations on the production of nitric oxide (NO) in RAW 264.7 cells induced with LPS (1 μg/ml).

Among the nitric oxide synthases (NOS), inducible NOS (iNOS) contributes most to the highly increased production of NO that occurs during inflammatory responses. In addition, inhibiting COX-2 expression is the target of many non-steroidal anti-inflammatory agents (Won *et al.*, 2006). The inhibitory activity of the *A. iwayomogi* essential oil fraction on iNOS and COX-2 expression was examined by Western blot analysis. As demonstrated in Fig. 5, the intensity of the iNOS band was significantly reduced by treatment with 0.1 and 0.025 mg/ml of the *A. iwayomogi* essential oil fraction. The levels of COX-2 were also reduced by the oil, but not to the same degree as iNOS.

Thus, we confirmed that both the essential oil fraction of *A. iwayomogi* and its central component vulgarone B

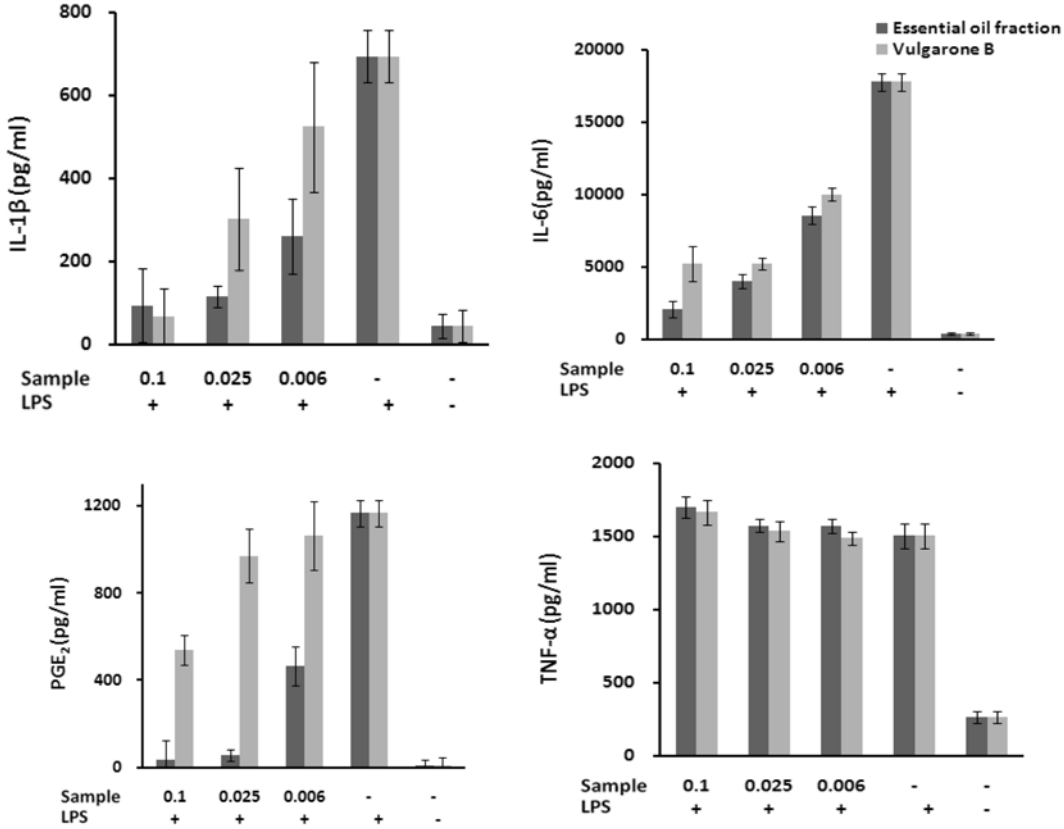

Fig. 4. Effects of *A. iwayomogi* essential oils on IL-1â, IL-6, PGE_2, and TNF-α release following LPS treatment as measured using ELISA.

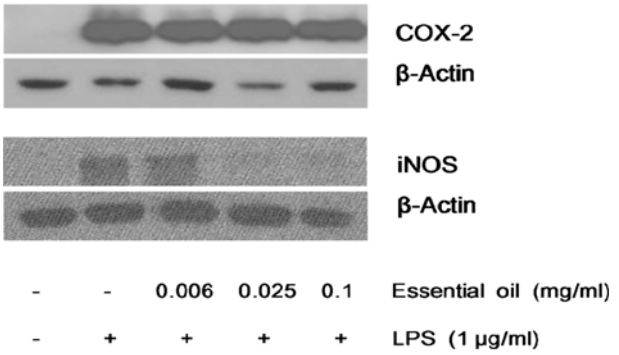

Fig. 5. Western blot analysis of the effects of *A. iwayomogi* essential oil fraction on the levels of iNOS and COX-2.

possess significant anti-inflammatory effects. The activity of the essential oil fraction may not be only due to vulgarone B, which comprises ca. 20% of the total fraction, but also to other minor components that may potently contribute to its activity. The effects of *A. iwayomogi* oils might be related to their ability to repress PGE_2, IL-1β and IL-6 release, but are not due to their effects on TNF-α directly.

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