EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

Original article:

HYPOCHOERIS RADICATA ATTENUATES LPS-INDUCED INFLAMMATION BY SUPPRESSING P38, ERK, AND JNK PHOSPHORYLATION IN RAW 264.7 MACROPHAGES

Min-Jin Kim[1,2], Se-Jae Kim[1], Sang Suk Kim[3], Nam Ho Lee[2], Chang-Gu Hyun[2,4,*]

[1] Department of Biology, Jeju National University, Ara-1-dong, Jeju 690-756, Korea
[2] Cosmetic Science Center, Department of Chemistry, Jeju National University, Ara-1-dong, Jeju 690-756, Korea
[3] Citrus Research Station, National Institute of Horticultural and Herbal Science, RDA, Jeju 699-946, Korea
[4] LINC Agency, Jeju National University, Ara-1-dong, Jeju 690-756, Korea

* Corresponding author: Chang-Gu Hyun, Cosmetic Center Science and LINC Agency, Jeju National University, Ara-1-dong, Jeju 690-756, Korea; E-mail: cghyun@jejunu.ac.kr; Tel: +82-64-754-4419; Fax: +82-64-751-3127

ABSTRACT

Hypochoeris radicata, an invasive plant species, is a large and growing threat to ecosystem integrity on Jeju Island, a UNESCO World Heritage site. Therefore, research into the utilization of *H. radicata* is important and urgently required in order to solve this invasive plant problem in Jeju Island. The broader aim of our research is to elucidate the biological activities of *H. radicata*, which would facilitate the conversion of this invasive species into high value-added products. The present study was undertaken to identify the pharmacological effects of *H. radicata* flower on the production of inflammatory mediators in macrophages. The results indicate that the ethyl acetate fraction of *H. radicata* extract (HRF-EA) inhibited the production of pro-inflammatory molecules such as NO, iNOS, PGE_2, and COX-2, and cytokines such as TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells. Furthermore, the phosphorylation of MAPKs such as p38, ERK, and JNK was suppressed by HRF-EA in a concentration-dependent manner. In addition, through HPLC and UPLC fingerprinting, luteolins were also identified and quantified as extract constituents. On the basis of these results, we suggest that *H. radicata* may be considered possible anti-inflammatory candidates for pharmaceutical and/or cosmetic applications.

Keywords: *Hypochoeris radicata***,** alien plant invader, inflammation, mitogen-activated protein kinases (MAPKs)

INTRODUCTION

Hypochoeris radicata, also known as hairy cat's ear, spotted cat's ear, flatweed, common cat's ear, frogbit, gosmore, rough cat's ear, or false dandelion, is a perennial member of the *Hypochoeris* genus in the family *Compositae*. *H. radicata* grows mainly in pastures, but is also found in lawns, and the plant prefers nutrient-poor, slightly acidic soils. A typical flower head consists of 50 to 100 single yellow florets. They are known to produce achenes (hereafter called seeds) that have a pappus and

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

are adapted to long-distance dispersal by wind (Soons and Heil, 2002). While the plant is native to Europe, it has also been introduced in America, Japan, Australia, New Zealand, and Korea, where it can be considered an invasive weed. It has also been listed as a harmful weed in the state of Washington, USA.

On Jeju Islands, a UNESCO World Heritage site in Korea, several invasive non-native species have altered both plant and insect communities. Currently, the environmental weed *H. radicata*, a representative invader plant, threatens the biodiversity of the Jeju Island ecosystem, including the Hallasan National Park and the adjacent natural area. For this reason, research into the utilization of *H. radicata* is urgently needed in order to address the problems related to invasion of non-native plants in Jeju Island. The aim of this research was to elucidate the biological activities of *H. radicata*, which would facilitate the conversion of this invader plant into high value-added products for pharmaceutical and/or cosmetic materials.

Inflammation is the response of an organism to invasion by foreign pathogens such as parasites, bacteria and viruses. Activation by inflammatory stimuli produces a variety of inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines including TNF-α, IL-1β and IL-6. However, if left uncontrolled, the inflammatory mediators become involved in the pathogenesis of many inflammatory disorders (Park et al., 2013; Yang et al., 2013). For this reason, regulation of the production of NO and PGE_2 in macrophages are current research topics for the development of new anti-inflammatory agents. There have been many attempts to derive new anti-inflammatory agents from natural compounds (Chen et al., 2013; Lee et al., 2013; Yang et al., 2013). Therefore, this study was designed to explore the anti-inflammatory effects of *H. radicata* extracts by measuring their effects on the production of pro-inflammatory factors (TNF-α, IL-1β, IL-6, iNOS, cyclooxygenase (COX)-2, and prostaglandin [PG]E_2). Furthermore, we sought to elucidate the mechanism of these anti-inflammatory effects by investigating the role of mitogen-activated protein kinase (MAPK) pathways in murine macrophage RAW 264.7 cells. To the best of our knowledge, this is the first report of the anti-inflammatory biological activity of *H. radicata*.

MATERIALS AND METHODS

Materials and solvent extraction

H. radicata flowers were collected from Namwon (a region on Jeju Island, Korea) in May 2009. The voucher specimen was identified by Dr. Gwanpil Song, of the Jeju Biodiversity Research Institute (JBRI), Jeju, Korea, and deposited in the herbarium of that institution. The materials for extraction were freeze-dried and then ground into a fine powder by using a blender. The dried powder (2 kg) was extracted with 80 % ethanol (EtOH; 30 L) at room temperature for 3 days and then evaporated under a vacuum. The evaporated EtOH extract (5 g) was suspended in water (1 L) and fractionated with ethyl acetate (EtOAc; 1 L). The yield and recovery of EtOAc fractions were 0.80 g and 16 %, respectively.

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). ELISA kits for PGE_2, TNF-α, IL-1β, and IL-6 were purchased from R & D Systems, Inc. (St. Louis, MO, USA) and BD Biosciences (San Diego, CA, USA). Anti-JNK, anti-phosphorylated JNK (anti-p-JNK), anti-ERK1/2, anti-phosphorylated ERK1/2 (anti-p-ERK1/2), anti-p38, and anti-phosphorylated p38 (anti-p-p38) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB, was purchased from Calbiochem (San Diego, CA, USA).

All other reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

RAW 264.7 cell culture

RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea) and maintained at sub-confluence in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. The medium for routine sub-cultivation was DMEM supplemented with FBS (10 %), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were counted with a hemocytometer, and the number of viable cells was determined by trypan blue dye exclusion. The cell line was mechanically passaged by dissociation every 2 days.

LDH assay for measuring cell toxicities

RAW 264.7 cells were (1.8×10^5 cells/mL) plated in 24-well plates and pre-incubated for 18 h. They were then treated with LPS (1 µg/mL), as well as with aliquots of the 80 % EtOH extract and each solvent fraction at 37 °C for 24 h. The release of lactate dehydrogenase (LDH) from the cells was used to assess cytotoxicity, by using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA) to determine LDH activity from the production of NADH during the conversion of lactate to pyruvate. The optical density of the solution was measured at a wavelength of 490 nm, by using an ELISA plate reader (Bio-TEK Instruments Inc., Vermont, WI, USA). The cytotoxicity percentage was determined relative to the control group. All experiments were performed in triplicate.

MTT assay for measuring cell proliferation

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells (1.8×10^5 cells/mL) were plated onto 96-well plates for 18 h, followed by treatment with various concentrations of extract samples. After incubation for 24 h, MTT was added to the medium for 4 h. Finally, the supernatant was removed and the formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity was determined relative to the control group.

Assay for NO production by activated macrophages

After pre-incubating RAW 264.7 cells (1.8×10^5 cells/mL) with LPS (1 µg/mL) for 18 h, they were then treated with LPS (1 µg/mL) as well as aliquots of the 80 % EtOH extract and each solvent fraction at 37 °C for 24 h. The quantity of nitrite in the culture medium was measured and used as an indicator of NO production. Briefly, 100 µL of the cell culture medium was mixed with 100 µL of Griess reagent (1 % sulfanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured using a microplate reader (Bio-TEK Instruments Inc., Vermont, WI, USA). The quantity of nitrite was determined on the basis of a sodium nitrite standard curve. All experiments were performed in triplicate.

Measurement of PGE_2 and cytokine assays

The levels of cytokines TNF-α, IL-1β, and IL-6 in the cell culture supernatant were measured using an ELISA kit (R&D Systems Inc., Minneapolis, MI, USA) as described previously (Yoon et al., 2009a, b). RAW 264.7 cells were plated in a 24-well cell culture plate at a density of 1.8×10^5 cells/well in the presence of various concentrations of extract samples and 1 µg mL^{-1} LPS, and then incubated for 24 h. The culture supernatant was collected and assayed for PGE_2, TNF-α, IL-1β, and IL-6 according to the manufacturer's instructions.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

Western blot analysis

RAW 264.7 cells were pre-incubated for 18 h in a 6-well cell culture plate at a density of 1.5×10^6 cells/well before being stimulated with LPS (1 μg/mL) in the presence of extracts for 24 h. After incubation, the cells were collected and washed twice with cold phosphate-buffered saline (PBS). The cells were lysed in a lysis buffer (1XRIPA [Upstate USA Inc., NY, USA], 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin) and kept on ice for 1 h. The cell lysates were centrifuged at 15,000 rpm and 4 °C for 15 min, and the supernatants were stored at -70 °C until use. Protein concentrations were then determined using a Bradford Assay (Bio-Rad, Richmond, CA, USA). Aliquots of the lysates (30~50 μg of protein) were separated on an 8~12 % SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to polyvinylidene fluoride (PVDF) membranes (BIO-RAD, HC, USA), blocked with 5 % non-fat milk in TBS-T buffer and blotted with each primary antibody (1:1,000, except for iNOS (1:5,000) and β-actin (1:10,000)) and its corresponding secondary antibody (1:5,000 or 1:10,000) according to the manufacturer's instructions. Immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, NJ, USA).

High and ultra-performance liquid chromatography (HPLC and UPLC) fingerprint of H. radicata extracts

The various *H. radicata* extracts were redissolved to a 1 mg/mL concentration and filtered through a 0.22 μm membrane filter (Millipore). A luteolin analysis was carried out using high-performance liquid chromatography (Waters 2695). The analysis was performed on a 100 × 4.6 mm i.d. XTerra RP8 column with 3.5 μm particles (Waters). Eluents were ACN/H_2O [10:90, by vol. (A) and 90:10, by vol. (B). The flow rate was 0.8 mL/min. Sample volumes of 20 μL were injected. Gradient conditions were as follows: 0–30 min, linear from 10 % to 50 % A; 30–35 min, linear from 50 % to 100 % A; 35–40 min, linear 100 % to 10 % A; and 40–50 min, isocratic 10 % A. For the calibration curve, luteolin was dissolved with methanol, and 4 concentration levels (10, 25, 50, 100 mg/mL) were analyzed. For quantification, peak areas were correlated with the concentrations according to the calibration curve. The Ultra Performance Liquid Chromatography (LCQ-Fleet) analyses were performed using a Hypersil Gold column (2.1 mm × 50 mm, 1.8 μm, C_{18}) with a binary phase at a flow rate of 0.2 mL/min. Eluent A was acetonitrile and eluent B was water/formic acid (99.9/0.1, v/v). The elution gradient was 0-8 min, 5-100 % A. Mass spectrometry was performed on a Thermo mass spectrometer operating in negative ion electrospray mode. The capillary and spray voltage were set at 42 V and 5 kV for negative electrospray mode. The capillary temperature was set to 275 °C and collision energy was set at 35 eV.

Statistical analysis

Differences between conditions were assessed using a one-way analysis of variance (ANOVA) and the Student's *t*-test, with $p < 0.05$ considered to be statistically significant. In all instances, the means of data from three independent experiments were analyzed.

RESULTS

Effect of H. radicata extracts on NO synthesis in activated macrophages

To evaluate whether extracts of *H. radicata* could modulate NO production by activated macrophages, we examined the effects of 80 % EtOH extracts of each part (flower, leaf, stem, root, aerial part, or whole plant) of *H. radicata*, as well as 2-amino-4-methylpyridine (10 μM) and dexamethasone (20 μM) on NO production by the murine macrophage cell line, RAW

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

264.7. As shown in Figure 1A, among the six parts, the flower extract (100 µg/mL) markedly inhibited LPS-induced RAW 264.7 cell NO production by 47 %. 2-amino-4-methylpyridine (10 µM), a NO inhibitor and positive control, also inhibited LPS-induced NO production by 88 %. There was no basal NO production after treatment with only the crude extract from *H. radicata* without LPS (data not shown). As shown in Figure 1B, the numbers of viable activated macrophages were not altered by either each part of *H. radicata* (flower, leaf, stem, root, aerial part, and whole plant extracts), 2-amino-4-methylpyridine, or dexamethasone as determined by LDH and MTT assays, indicating that the inhibition of NO synthesis by each of the treatments was not due to cytotoxic effects.

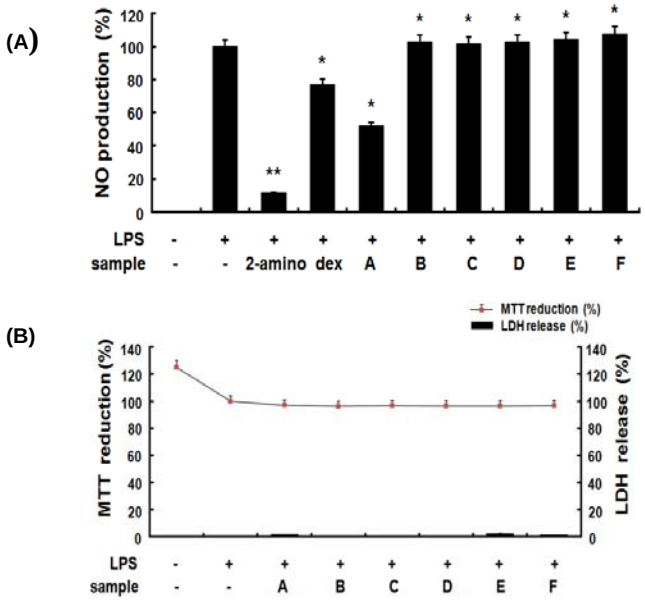

Figure 1: Effects of 80 % EtOH extract of *H. radicata* L. on cytotoxicity and NO production in LPS-stimulated RAW 264.7 cells. Cells (1.8 × 10^5 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 µg/mL) for 24 h in the presence of 80 % EtOH extracts of each part of *H. radicata* L. (100 µg/mL A: flower; B: leaf; C: stem; D: root; E: aerial part; F: whole plant), 2-amino (10 µM 2-amino-4-methylpyridine), or dex (20 µM dexamethasone). **(A)** Cytotoxicity was determined by MTT and LDH assay. **(B)** NO production levels. Data are presented as means ± S.D. of three independent experiments. *$p < 0.05$; **$p < 0.01$ compared to positive control

Comparison of the anti-inflammatory effects of the H. radicata flower (HRF) and the T. platycarpum flower (TRF)

H. radicata is also known as "false dandelion," as it is commonly mistaken for true dandelions *(Taraxacum)*, since both carry similar flowers that form windborne seeds. In particular, the shape of *H. radicata*, especially the flower part, is similar to that of *T. platycarpum*, a Korean medicinal plant. Therefore, we compared the anti-inflammatory activity of 80 % ethanol extracts of the *H. radicata* flower (HRF) and the *T. platycarpum* flower (TRF). This comparison was performed using RAW 264.7 murine macrophage cells, which can produce both NO and PGE_2 upon stimulation with LPS. Cells were pre-incubated with HRF and TRF for 1 h, after which they were stimulated with 1 µg/mL LPS for 24 h. Neither LPS nor extract samples were present in the control group. Cell culture media were collected and nitrite and PGE_2 levels determined. Though both HRF and TRF were found to dose-dependently inhibit the production of NO and PGE_2 (Figure 2), HRF showed a higher level of inhibition than TRF. At concentrations of 100 µg/mL, 200 µg/mL, and 400 µg/mL, HRF decreased the production of NO by LPS-treated macrophages by 35 %, 51 %, and 82 %, respectively, as compared with LPS-treated macrophages not exposed to HRF. The same concentrations of HRF also inhibited PGE_2 production by 30 %, 43 %, and 76 %, respectively. 2-amino-4-methylpyridine (10 µM) also markedly reduced NO levels in RAW 264.7 macrophages. In contrast, dexamethasone (20 µM) significantly reduced PGE_2 levels, but did not affect NO production. The potential cytotoxicity of HRF and TRF was evaluated by LDH assay after incubating cells for 24 h in the absence or presence of LPS. However, cell viability was negligibly affected at the concentrations used (100, 200, or 400 µg/mL) to inhibit NO and PGE_2 (Figure 2A). Thus, the inhibitory effects of HRF and TRF were not attributable to cytotoxicity.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

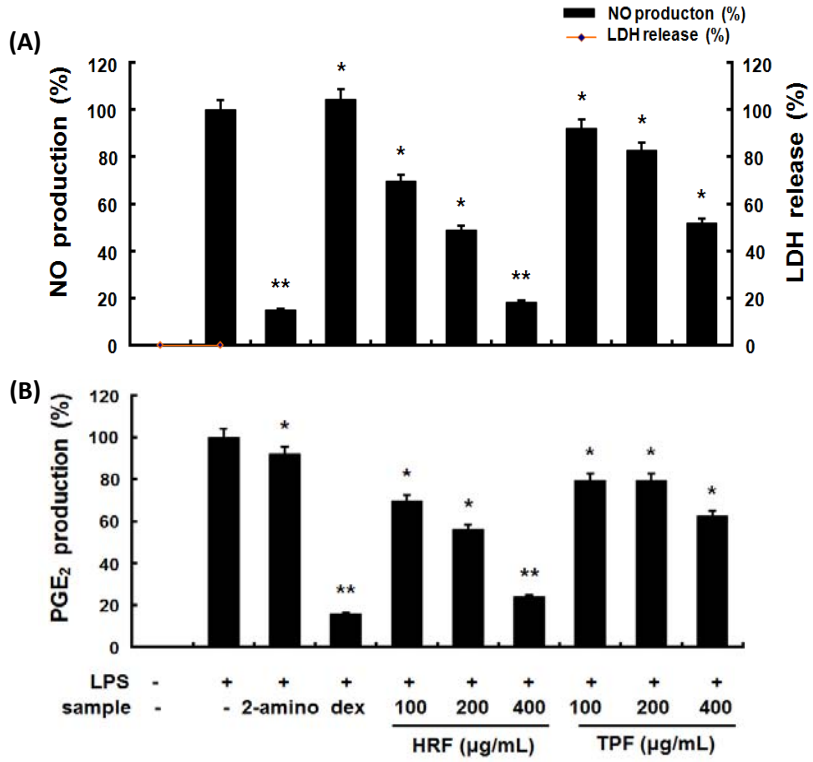

Figure 2: Effects of 80 % EtOH extracts of *H. radicata* flower (HRF) or *T. platycarpum* flower (TRF) on NO and PGE_2 production, and on cytotoxicity in LPS-stimulated RAW 264.7 cells. Cells (1.8×10^5 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence of each of the 80 % EtOH extracts (100, 200, or 400 μg/mL), 2-amino (10 μM 2-amino-4-methylpyridine), or dex (20 μM dexamethasone). **(A)** NO and **(B)** PGE_2 production levels. Data are presented as means ± S.D. of three independent experiments. *$p < 0.05$; **$p < 0.01$ compared to positive control

Effects of HRF and TRF on LPS-induced expression of iNOS and COX-2 proteins

Western blot analyses were performed to determine whether the inhibitory effects of HRF and TRF on the pro-inflammatory mediators NO and PGE_2 were related to a modulation of iNOS and COX-2 expression. We did not detect iNOS and COX-2 protein expression in unstimulated RAW 264.7 cells. However, iNOS and COX-2 protein levels were markedly upregulated by LPS, an effect that was inhibited by co-treatment with HRF, but not TRF (Figure 3). On the other hand, HRF and TRF did not affect the expression of β-actin, a housekeeping gene. In general, these results suggest that the inhibitory effects of HRF on LPS-induced NO and PGE_2 production are the result of the suppression of iNOS and COX-2 protein expression.

Effects of 80 % EtOH extract and solvent fractions of H. radicata flower on cytotoxicity and the production of NO and PGE

Since HRF was observed to modulate macrophage-mediated inflammatory functions such as the overproduction of NO and PGE_2, extracts of differing polarities were prepared by extraction of HRF and ethyl acetate for the further study. To assess the effect of HRF, ethyl acetate, and water fractions on LPS-induced NO and PGE_2 production, RAW 264.7 cells were treated with LPS using the process described above in the presence or absence of various concentrations of HRF, ethyl acetate, and water fractions (12.5, 25, or 50 μg/mL). As shown in Figure 4, NO production was induced by treatment with LPS, which was inhibited by 16, 44, and 60 % by the addition of the ethyl acetate fraction (HRF-EA) at 12.5, 25, 50 μg/mL, respectively. Furthermore, LPS-induced PGE_2 production was also dose-dependently decreased by the ethyl acetate fraction (9, 25, and 47 % decrease after treatment with 12.5, 25, and 50 μg/mL, respectively). However, the HRF and water fractions did not exert a strong influence on either NO or PGE_2 production. Potential cytotoxic effects were also assessed in the presence or absence of LPS via an LDH assay. HRF-EA did not influence cytotoxicity in RAW 264.7 cells at the tested concentrations (12.5, 25, or 50 μg/mL) that had been observed to inhibit NO and PGE_2.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

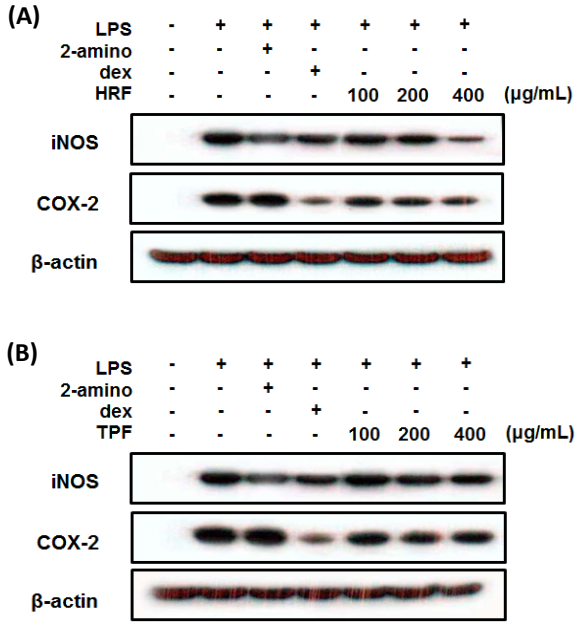

Figure 3: Effects of 80 % EtOH extract of *H. radicata* flower (HRF) and *T. platycarpum* flower (TRF) on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. Cells (1.0 × 10^6 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence of each of the 80 % EtOH extracts (100, 200, or 400 μg/mL), 2-amino (10 μM 2-amino-4-methylpyridine), or dex (20 μM dexamethasone). The protein levels were determined using Western blotting. 80 % EtOH extract of **(A)** HRF and **(B)** TPF.

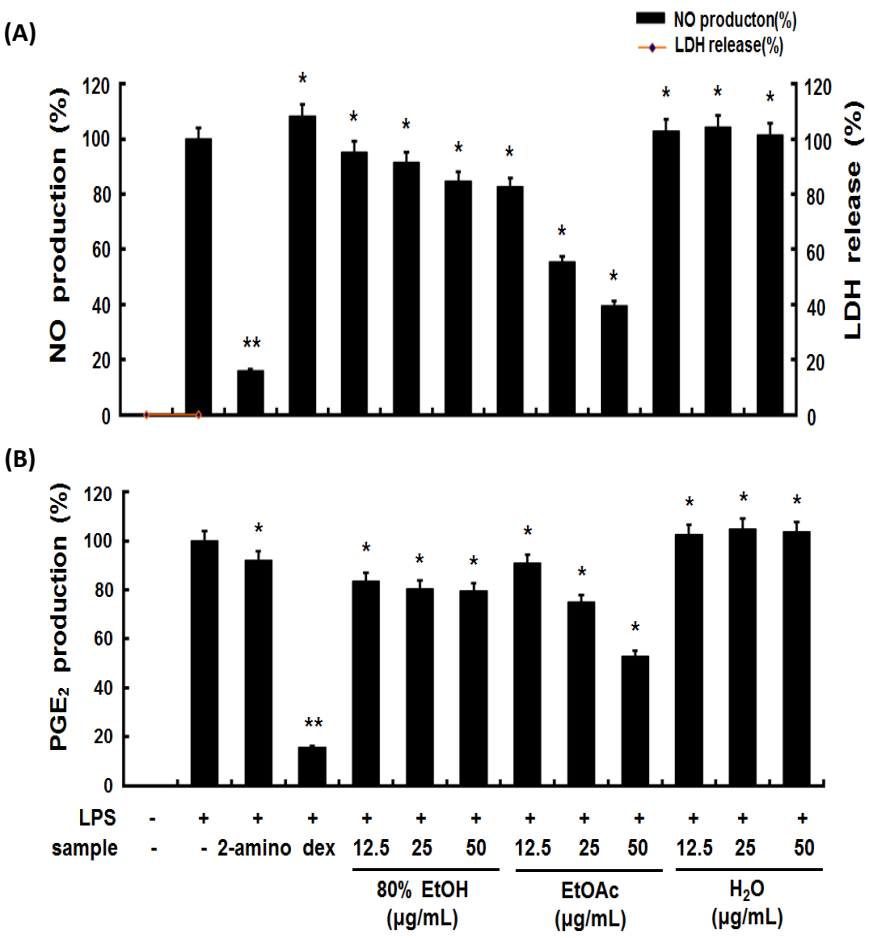

Figure 4: Effects of *H. radicata* flower (HRF) and ethyl acetate extract of *H. radicata* flower (HRF-EA) on NO and PGE_2 production, and on cytotoxicity in LPS-stimulated RAW 264.7 cells. Cells (1.8 × 10^5 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence of 80 % EtOH extract and solvent fractions of *H. radicata* L. (12.5, 25, or 50 μg/mL), 2-amino (10 μM 2-amino-4-methylpyridine), or dex (20 μM dexamethasone). **(A)** NO and **(B)** PGE_2 production levels. Cytotoxicity was determined using the LDH method. Data are presented as means ± S.D. of three independent experiments. *p < 0.05; **p < 0.01 compared to positive control

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

Effect of HRF-EA on the production of iNOS and COX-2

Because iNOS and COX-2 are the key enzymes for the production of NO and PGE_2, respectively, we analyzed the expression level of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 cells by Western blotting. As shown in Figure 5, HRF-EA strongly suppressed the expression of iNOS and COX-2 proteins in a dose-dependent manner ($p < 0.05$). These results suggest that HRF-EA-mediated inhibition of NO and PGE_2 production is associated with down-regulation of iNOS and COX-2 protein expressions.

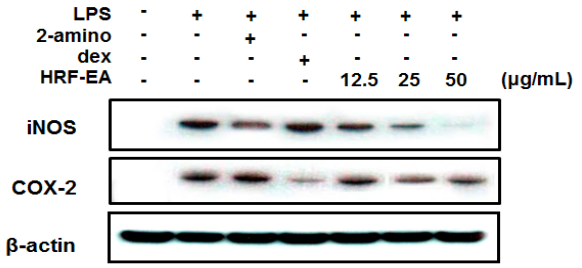

Figure 5: Effects of ethyl acetate extract of *H. radicata* flower (HRF-EA) on the protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. Cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence of HRF-EA (12.5, 25, or 50 μg/mL), 2-amino (10 μM 2-amino-4-methylpyridine), or dex (20 μM dexamethasone). The protein levels were determined using Western blotting.

Effect of HRF-EA on the production of pro-inflammatory cytokines

Because TNF-α, IL-1β, and IL-6 are early phase pro-inflammatory cytokines and elevated levels of these molecules can be detected in a variety of acute and chronic inflammatory diseases, we determined the effects of HRF-EA on secreted levels of these cytokines in LPS-treated cells using an enzyme immunoassay (EIA). As expected, RAW 264.7 cells treated with LPS displayed notably increased levels of TNF-α, IL-1β, and IL-6. The induction of pro-inflammatory cytokines was significantly decreased by HRF-EA in a dose-dependent manner ($p < 0.05$). As shown in Figure 6, TNF-α, IL-1β, and IL-6 production was decreased to 61 %, 86 %, and 62 %, respectively. This result indicates that HRF-EA efficiently suppressed LPS-induced TNF-α, IL-1β, and IL-6 production, suggesting that HRF-EA inhibits the initial phase of the LPS-stimulated inflammatory response.

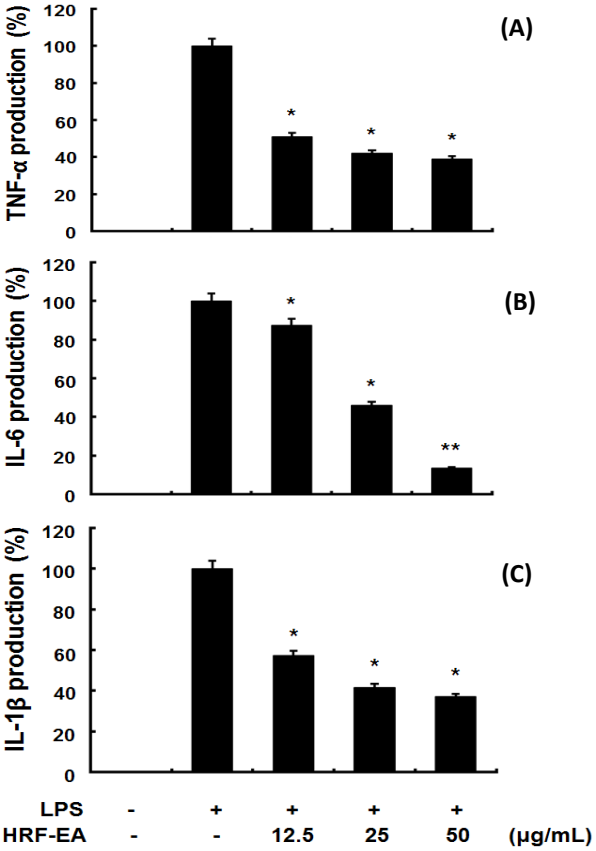

Figure 6: Effect of ethyl acetate extract of *H. radicata* flower (HRF-EA) on production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Cells (1.8×10^5 cells/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence of HRF-EA (12.5, 25, or 50 μg/mL). **(A)** TNF-α, **(B)** IL-6, and **(C)** IL-1β production were analyzed by ELISA kit. The data are expressed as means ± S.D. of three determinations. *$p < 0.05$; **$p < 0.01$ compared to positive control.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

Effect of HRF-EA on the phosphorylation of MAPKs in LPS-stimulated RAW 264.7 cells

MAPKs play critical roles in the regulation of cell growth and differentiation as well as in the control of cellular responses to cytokines and stressors. To further investigate whether HRF-EA regulates MAPKs, we used Western blotting to evaluate the effects of HRF-EA on LPS-induced phosphorylation of ERK1/2, p38, and JNK MAPKs in RAW 264.7 cells. As shown in Figure 7, LPS (1 µg/mL) significantly promoted the phosphorylation of ERK1/2, JNK, and p38 MAPKs in RAW 264.7 cells. HRF-EA (12.5, 25, and 50 µg/mL) treatment dramatically reduced the phosphorylation of ERK1/2, p38, and JNK MAPKs in a dose-dependent manner. However, the expressions of unphosphorylated ERK1/2, p38, and JNK MAPKs were unaffected by either LPS alone, or LPS in combination with HRF-EA. These results suggest that phosphorylation of MAPKs may be involved in the inhibitory effects of HRF-EA on LPS-stimulated inflammation in RAW 264.7 cells.

Quantification of luteolin (3,4,5,7-tetrahydroxyflavone) in H. radicata extracts

Although the constituents in extracts of *H. radicata* have not been previously reported, several natural products such as quercetin, naringenin, luteolin, and catechin have been previously described as constituents of extracts of members of the Compositae/Asteraceae family. Identification of the flavonoids quercetin, naringenin, luteolin, catechin, etc., was performed by comparison of HPLC retention times. Figure 8 shows the HPLC chromatogram of HRF-EA, HRF extract, and TPF extract. The bibliographical data, the UV/Vis spectra, and the retention times permitted the identification of luteolin. The HPLC analysis showed the presence of luteolin in HRF-EA, HRF extract, and TPF extracts. The luteolin content in the individual extracts was calculated from the calibration curve and expressed as the mass percentage of the dry flower. In order to further confirm the structures of the constituents, luteolin and HRF-EA were analyzed by UPLC/MS (Figure 9). The detection of the fragment corresponding to luteolin (*m/z* 284.9) corroborated its identification. Moreover, the fragmentation of this base peak provided a fragment corresponding to m/z 150.9, 174.9, 198.9, 216.9, 256.9, and 284.9, typical of luteolin. The main peak provided a fragment of *m/z* 284.9, and was tentatively assigned to luteolin, as it is shown in Figure 9. An MS bases peak ([M-H]-) of *m/z* 284.9 was detected, as well as a fragment of *m/z* 150.9, 175, 198.9, 216.9, 242.9, 257, and 284.9, thus clearly indicating the presence of luteolin. The content of luteolin in the individual extracts was calculated from the calibration curve and expressed as the mass percentage of the dry flower. The luteolin contents of HRF-EA, HRF extracts, and TPF extracts were 70 mg/g, 29 mg/g, and 16 mg/g, respectively.

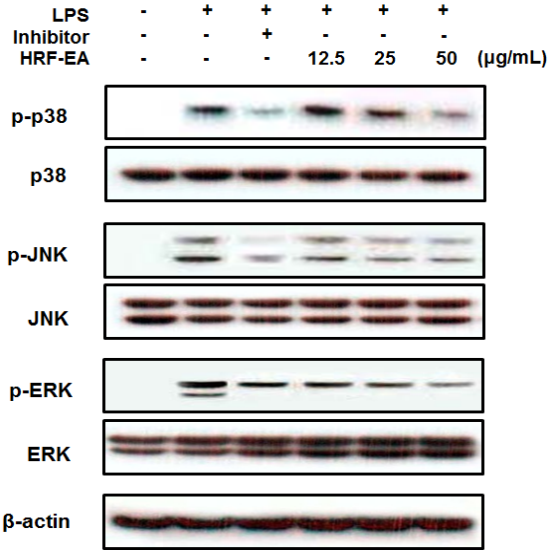

Figure 7: Effects of ethyl acetate extract of *H. radicata* flower (HRF-EA) on p38, JNK, and ERK protein levels in LPS-stimulated RAW 264.7 cells. Cells (1.5×10^6 cells/mL) were pre-incubated for 18 h, incubated with HRF-EA (12.5, 25, and 50 µg/mL) and each of the indicated inhibitors (SB [10 µM SB203580], SP [20 µM SP600125], and PD [20 µM PD98059]) for 2 h, and then stimulated with LPS (1 µg/ml) for 30 min. The protein levels were determined using Western blot.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

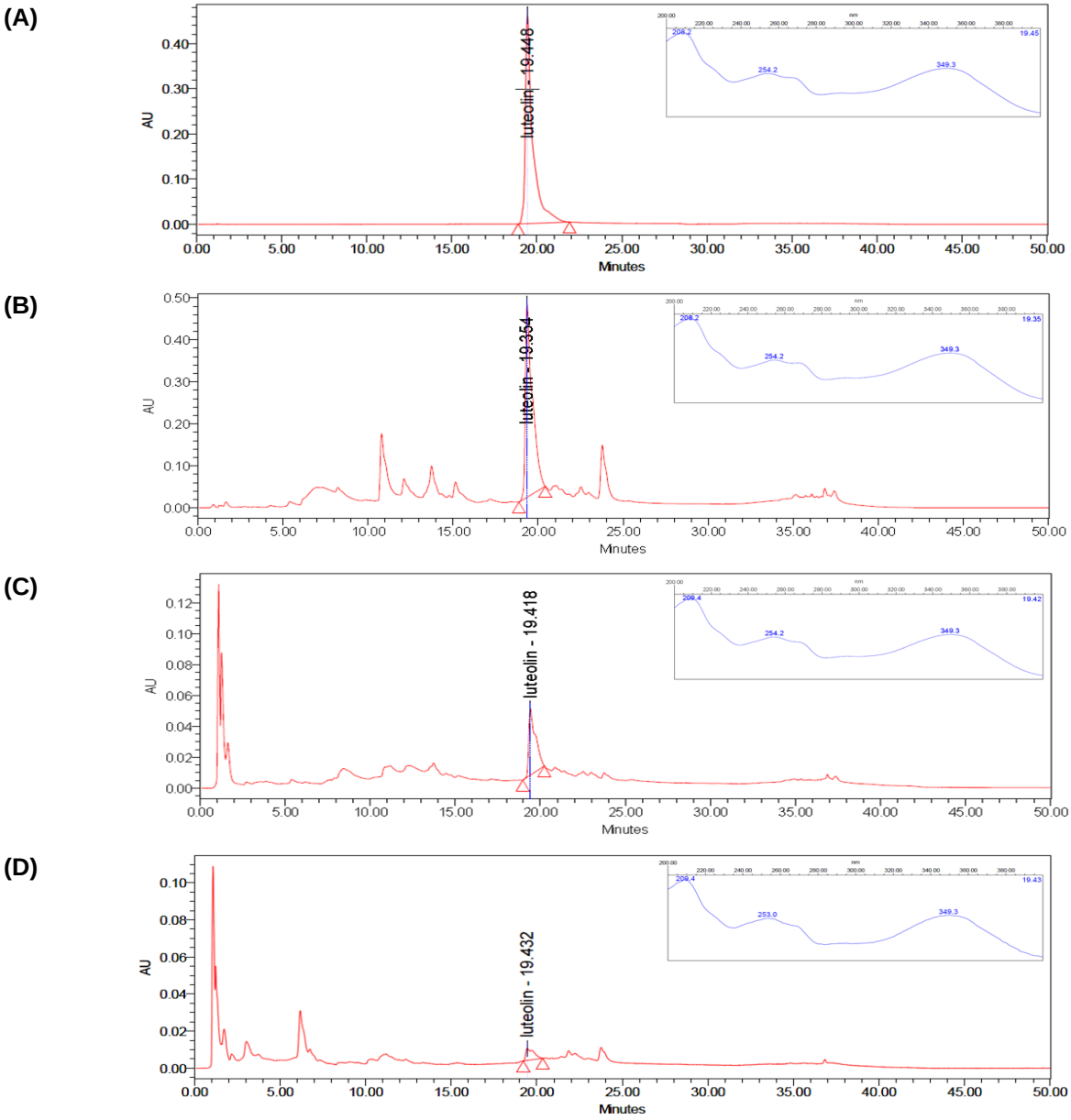

Figure 8: HPLC chromatogram of luteolin, ethyl acetate extract of *H. radicata* flower (HRF-EA), and the 80 % EtOH extracts of *H. radicata* flower (HRF) or *T. platycarpum* flower (TRF). **(A)** luteolin, **(B)** HRF-EA, and the 80 % EtOH extracts of **(C)** HRF and **(D)** TPF.

EXCLI Journal 2014;13:123-136 – ISSN 1611-2156
Received: December 10, 2013, accepted: February 07, 2014, published: February 17, 2014

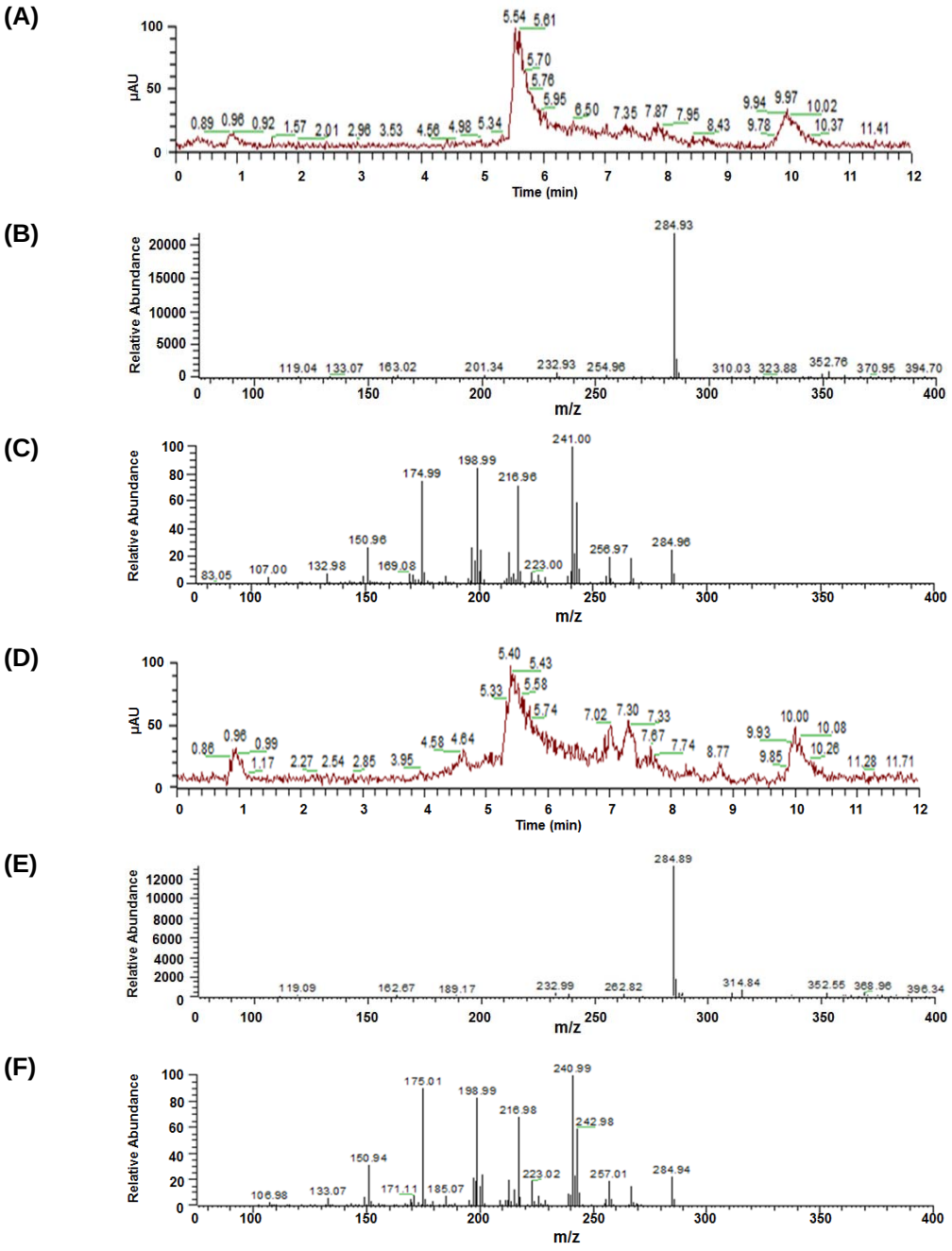

Figure 9: UPLC/MS of luteolin and ethyl acetate extract of *H. radicata* flower (HRF-EA). An HPLC chromatogram of luteolin **(A)**, precursor ions **(B)**, and their corresponding fragments **(C)** obtained by a UPLC/MS (negative mode) analysis chromatogram of luteolin. An HPLC chromatogram of the HRF-EA **(D)**, precursor ions **(E)**, and their corresponding fragments **(F)** obtained by a UPLC/MS (negative mode) analysis chromatogram of HRF-EA

DISCUSSION

H. radicata, an alien plant invader, is a large and growing threat to ecosystem integrity in Jeju Island, a UNESCO World Heritage site in Korea, with negative consequences for the conservation of biodiversity. Consequently, billions of dollars have been spent controlling this invasive species. This point highlights the urgent need for new approaches to deal with invasive alien

species, particularly as a strategy to inhibit new invasion and expansion. To help eliminate the invasive plant *H. radicata*, we focused our studies on the plant's flower, which forms windborne seeds during breeding.

The present study was designed to elucidate the pharmacological and biological effects of the flower of *H. radicata* on the production of pro-inflammatory cytokines and inflammatory mediators in macrophages. We discovered that *H. radicata* inhibits NO and PGE_2 production in LPS-stimulated RAW264.7 macrophages. *H. radicata* attenuated LPS-induced synthesis of iNOS and COX-2, in parallel, and inhibited LPS-induced TNF-α, IL-1β, and IL-6 production. The mechanism of the anti-inflammatory action of *H. radicata* — suppression of MAPK activation—was also investigated.

Inflammation is the first response of the immune system to infection or irritation. Macrophages play a salient role in these inflammatory responses, and become activated by a variety of stimuli, such as LPS (Zhang et al., 2011). A variety of studies have shown that the pleiotropic inflammatory mediators NO and PGE_2, which are generated by iNOS and COX-2, are involved in the pathogenesis of infection and inflammation, including sepsis and arthritis. Thus, the inhibition of pro-inflammatory cytokines, or iNOS and COX-2 expression in inflammatory cells offers a new therapeutic strategy for the treatment of inflammation (Moon et al., 2011; Yoon et al., 2010a-d). In order to validate the use of *H. radicata* flower (HRF) as an anti-inflammatory material, we investigated the effects of HRF on the production of NO and PGE_2 in LPS-activated RAW 264.7 macrophages. We found that HRF and its ethyl acetate extract (HRF-EA) inhibit PGE_2 and NO production in macrophage cells, and that it probably does so by acting at transcriptional and/or translational levels, as evidenced by dose-dependent reductions in protein expression.

Owing to the fact that the therapeutic actions of herbal extracts are based on the integral interaction rational combinations of multiple ingredients, chromatographic methods are essential for developing molecular fingerprints of traditional oriental medicines and their raw materials. Therefore, a simple HPLC and UPLC fingerprint of this plant was generated, and is presented in this paper. Luteolin is a common dietary flavonoid widely distributed in plants. Its beneficial effect attenuating inflammation has been previously demonstrated in other human gestational tissues and LPS-induced microglia (Huang et al., 2011; Wall et al., 2013). The potent photoprotective and antioxidative properties of luteolin has recently been demonstrated in solar simulator irradiated human skin fibroblasts (Wölfle et al., 2013). Since luteolins were reported as ingredients in the *dandelion* plant, and were also shown to be effective inhibitors of inflammation (Park et al., 2010, 2011a, b), we investigated their presence as standard substances in HRF. The HPLC analysis showed the presence of luteolin in HRF-EA, HRF extract, and TPF extract. Furthermore, the structures of the constituents of luteolin and HRF-EA were analyzed by UPLC/MS (Figure 9). The luteolin content of HRF-EA was more abundant than HRF or TPF extracts (70 mg/g vs. 29 mg/g vs. 16 mg/g).

One of the most extensively investigated transduction pathways involved in the inflammatory process is the mitogen-activated protein kinase (MAPK) pathway. Previous studies have shown that MAPKs have a significant role in the regulation of COX-2 and iNOS, and in pro-inflammatory cytokine production in LPS-stimulated macrophages (Yoon et al., 2012; Kim et al., 2013a, b). Furthermore, specific MAPK inhibitors suppress NO, PGE_2, and pro-inflammatory cytokine production. Therefore, a number of anti-inflammatory drugs target MAP kinases to control the transcriptions of COX-2, iNOS, and pro-inflammatory cytokines since the promoters

of these pro-inflammatory genes possess an active NF-κB binding site (Yamamoto and Gaynor, 2001; Kaminska, 2005). In agreement with these previous observations, our data demonstrated that HRF-EA inhibited JNK, ERK, and p38 phosphorylation in LPS-stimulated RAW264.7 cells in a dose-dependent manner (Figure 7), suggesting that p38, ERK, and JNK MAP kinase may be involved in the suppression of LPS-stimulated inflammation by HRF-EA.

In summary, the findings of the present study suggest that *H. radicata* flower is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α, IL-1β, and IL-6 production in macrophage cells. Moreover, the inhibitory effects of *H. radicata* flower were found to be associated with an inactivation of MAPKs that resulted from a blockade of JNK, ERK, and p38 phosphorylation. Since MAPK is a transcription factor that regulates the transcription of many genes associated with inflammation, its inhibition by *H. radicata* flower offers a possible approach to the treatment of severe inflammatory diseases for pharmaceutical and/or cosmetic applications.

ACKNOWLEDGEMENTS

This research was financially supported by the Fostering Program of regionally specialized industries through the Korea Institute for Advancement of Technology (KIAT) and Jeju Institute for Regional Program Evaluation funded by the Ministry of Trade, Industry and Energy (2013-R0002288).

REFERENCES

Chen TY, Sun HL, Yao HT, Lii CK, Chen HW, Chen PY et al. Suppressive effects of *Indigofera suffruticosa* Mill extracts on lipopolysaccharide-induced inflammatory responses in murine RAW 264.7 macrophages. Food Chem Toxicol 2013;55: 257-64.

Huang F, Zhu MM, Deng HM, Zhang ZJ, Yang L, Xiao F et al. The inhibitory effect of luteolin on inflammation in LPS-induced microglia. Zhong Yao Cai 2011;34:1746-9.

Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 2005;1754:253-62.

Kim KN, Ko YJ, Kang MC, Yang HM, Roh SW, Oda T et al. Anti-inflammatory effects of trans-1,3-diphenyl-2,3-epoxypropane-1-one mediated by suppression of inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. Food Chem Toxicol 2013a;53:371-5.

Kim KN, Ko YJ, Yang HM, Ham YM, Roh SW, Jeon YJ et al. Anti-inflammatory effect of essential oil and its constituents from fingered citron (*Citrus medica* L. var. sarcodactylis) through blocking JNK, ERK and NF-κB signaling pathways in LPS-activated RAW 264.7 cells. Food Chem Toxicol 2013b;57:126-31.

Lee J, Yang G, Lee K, Lee MH, Eom JW, Ham I et al. Anti-inflammatory effect of *Prunus yedoensis* through inhibition of nuclear factor-kappaB in macrophages. BMC Complement Altern Med 2013;13: 92.

Moon JY, Yang EJ, Kim SS, Kang JY, Kim GO, Lee NH et al. *Sasa quelpaertensis* phenylpropanoid derivative suppresses lipopolysaccharide-induced nitric oxide synthase and cyclo-oxygenase-2 expressions in RAW 264.7 cells. Yakugaku Zasshi 2011; 131:961-67.

Park CM, Park JY, Song YS. Luteolin and chicoric acid, two major constituents of dandelion leaf, inhibit nitric oxide and lipid peroxide formation in lipopolysaccharide- stimulated raw 264.7 cell. J Food Sci Nutr 2010;15:92-7.

Park CM, Jin KS, Lee YW, Song YS. Luteolin and chicoric acid synergistically inhibited inflammatory responses via inactivation of PI3K-Akt pathway and impairment of NF-κB translocation in LPS stimulated RAW 264.7 cells. Eur J Pharmacol 2011a;660: 454-9.

Park CM, Park JY, Noh KH, Shin JH, Song YS. *Taraxacum officinale* Weber extracts inhibit LPS-induced oxidative stress and nitric oxide production via the NF-κB modulation in RAW 264.7 cells. J Ethnopharmacol 2011b;133:834-42.

Park HH, Kim MJ, Li Y, Park YN, Lee J, Lee YJ et al. Britanin suppresses LPS-induced nitric oxide, PGE_2 and cytokine production via NF-κB and MAPK inactivation in RAW 264.7 cells. Int Immunopharmacol 2013;15:296-302.

Soons MB, Heil GW. Reduced colonization capacity in fragmented populations of wind-dispersed grassland forbs. J Ecol 2002;90:1033-43.

Wall C, Lim R, Poljak M, Lappas M. Dietary flavonoids as therapeutics for preterm birth: luteolin and kaempferol suppress inflammation in human gestational tissues in vitro. Oxid Med Cell Longev 2013; 2013:485201.

Wölfle U, Haarhaus B, Schempp CM. The photoprotective and antioxidative properties of luteolin are synergistically augmented by tocopherol and ubiquinone. Planta Med 2013;79:963-5.

Yamamoto Y, Gaynor RB. Role of the NF-kappaB pathway in the pathogenesis of human disease states. Curr Mol Med 2001;1:287-96.

Yang EJ, Ham YM, Lee WJ, Lee NH, Hyun CG. Anti-inflammatory effects of apo-9'-fucoxanthinone from the brown alga, *Sargassum muticum*. Daru 2013;21:62.

Yoon WJ, Kim SS, Oh TH, Lee NH, Hyun CG. *Cryptomeria japonica* essential oil inhibits the growth of drug-resistant skin pathogens and LPS-induced nitric oxide and pro-inflammatory cytokine production. Pol J Microbiol 2009a;58:61-8.

Yoon WJ, Kim SS, Oh TH, Lee NH, Hyun CG. *Abies koreana* essential oil inhibits drug-resistant skin pathogen growth and LPS-induced inflammatory effects of murine macrophage. Lipids 2009b; 44:471-6.

Yoon WJ, Kim DS, Yang EJ, Moon JY, Kim MJ, Lee WJ et al. Inhibitory effects of *Enteromorpha prolifera* on the production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines in RAW 264.7 cells. Lat Am J Pharm 2010a;29:165-70.

Yoon WJ, Lee NH, Hyun CG. Limonene suppresses lipopolysaccharide-induced production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines in RAW 264.7 macrophages. J Oleo Sci 2010b;59:415-21.

Yoon WJ, Moon JY, Kang JY, Kim GO, Lee NH, Hyun CG. *Neolitsea sericea* essential oil attenuates LPS-induced inflammation in RAW 264.7 macrophages by suppressing NF-kappaB and MAPK activation. Nat Prod Commun 2010c;5:1311-6.

Yoon WJ, Moon JY, Song G, Lee YK, Han MS, Lee JS et al. *Artemisia fukudo* essential oil attenuates LPS-induced inflammation by suppressing NF-kappaB and MAPK activation in RAW 264.7 macrophages. Food Chem Toxicol 2010d;48:1222-9.

Yoon WJ, Heo SJ, Han SC, Lee HJ, Kang GJ, Kang HK et al. Anti-inflammatory effect of sargachromanol G isolated from *Sargassum siliquastrum* in RAW 264.7 cells. Arch Pharm Res 2012;35:1421-30.

Zhang X, Xiong H, Li H, Yu L, Deng X. Effects of florfenicol on LPS-induced nitric oxide and prostaglandin E_2 production in RAW 264.7 macrophages. Fund Clin Pharmacol 2011;25:591-8.