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약용식물 추출물에 의한 면역세포 산화질소 생성 억제 활성 분석

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Inhibitory Effects of Methanolic Extracts of Medicinal Plants on Nitric Oxide Production in Activated Macrophage RAW 264.7 Cells

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ABSTRACT : A variety of herbs and plants have been traditionally used in oriental folk medicine for the treatment of inflammatory diseases. In our attempt to search for anti-inflammatory agents from natural products, we investigated 64 methanol extracts from 42 medicinal plants belonging to 10 families which were evaluated for inhibitory activities of NO production in lipopolysaccharide (LPS)-stimulated macrophage RAW 264.7 cells. Among them, 16 extracts exhibited inhibitory activities of NO production (IC_{50} values ranging from 59.6 to 94.7 ㎍/㎖). Only the extract from aerial parts of *Hosta lancifolia* (*H. lancifolia*) did not exert cytotoxic effects at the concentrations tested. The extract from *H. lancifolia* decreased the mRNA and protein levels of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines in activated macrophage RAW 264.7 cells in dose-dependent manner. The results suggest that the extract may contain bioactive compounds that suppress expression of pro-inflammatory cytokines, which may prove beneficial with regard to the development of natural agents for prevention and treatment of inflammatory diseases.

Key Words **:** Plant Extracts, inducible Nitric Oxide Synthase (iNOS), Nitric Oxide (NO), Inflammation, *Hosta lancifolia.*

INTRODUCTION

Herbal medicines that have been used in Korea for thousands of years are now being manufactured as drugs containing ingredients of standardized quality and quantity. Numbers of medicinal herbs have been reported to contain variety of pharmacological activities such as antioxidant, anti-inflammatory, anti-tumor and anti-allergenic activity (Chen *et al*., 2007; Jin *et al*., 2007; Kumarasamy *et al*., 2007; Lee *et al*., 2006; Yoo *et al*., 2007). Despite the fact that there have been many efforts to screen and develop anti-inflammatory agents, there are still large demands for developing new agents and there are large numbers of medicinal plants grown in Korea yet to be screened.

Nitric Oxide (NO) is synthesized from L-arginine by three types of nitric oxide synthase (NOS) in various cells and tissues; endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS) (Moncada, 1999; Nathan, 1992). The small amount of NO produced by constitutive eNOS and nNOS plays roles as an important regulator of physical homeostasis but the large amount of NO produced by iNOS in activated macrophages has been implicated in variety of diseases such as arthritis, inflammatory bowel disease, and other types of tissue injury (Bogdan *et al*., 2000; Raghav *et al*.,2007). Hence, therapeutic inhibition of iNOS would provide new targets for prevention and treatment of inflammatory diseases (Ahn *et al*., 2005; Korhonen *et al*., 2005; Moncada, 1999; Ricciardolo *et al*., 2004).

In this study, 64 methanolic extracts from medicinal plants grown in Korea were screened for their inhibitory effect on NO release in activated macrophage RAW 264.7 cells. Our investigation showed that methanol extract of *H. lancifolia*

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exhibited significant suppression of NO production in activated macrophage RAW 264.7 cells without cytotoxicity at the concentrations tested. This study is designed to the mechanism of action of the plant extracts leading to potential anti-inflammatory effects.

MATERIALS AND METHODS

Materials

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT, Sigma, USA), Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA), 10% fetal bovine serum (10% FBS, Invitrogen, USA), Lipopolysacharide (LPS, Sigma, USA), Griess reagent (Sigma, USA), Dimethyl sulfoxide (DMSO, Sigma, USA), RIPA buffer (Pierce, Rockford, IL, USA), protein assay kit (Bio-Rad Laboratories, USA), Rabbit anti-iNOS (Santa Cruz Biotechnology, USA), X-ray film (FUJI Photo Film Co., Ltd).

Plant extracts of the fruits, seeds, stems, leaves, aerial parts or roots (whichever parts of the plants are used in traditional medicine) of 42 plant species from 10 families were provided by the Biogreen 21 program [20050301034393 & 20070301034045] of Rural Development Administration, Korea. Filtered methanol extracts were air dried using a rotary evaporator and powdered. The dried materials were dissolved in DMSO to the concentration of 100 ㎍/㎖.

Cell culture

Murine macrophage RAW 264.7 cells (ATCC) were cultured at 37℃ in Dulbecco's modified Eagles's medium containing 10% fetal bovine serum, 2 mM glutamate, 100 unit/㎖ of penicillin, and 100 ㎍/㎖ of streptomycin in a humidified incubator with 5% CO_2. Cells were incubated with 1 ㎍/㎖ LPS along with various concentrations of plant extracts for 24 hrs as indicated.

Measurements of NO and cell viability

Nitric oxide was determined by measuring the amount of nitrite, a stable oxidized product, in cell culture supernatants, as previously described (Jin *et al*., 2007). To test the effect of the plant extracts on iNOS activity, RAW 264.7 cells (1×10^4 cells/well) were grown in serum-free medium for 18 hrs and treated with 1 ㎍/㎖ LPS (Sigma, USA) in the presence of various concentrations of the plant extracts for 24 hrs. 100 ㎕ of cell culture supernatant was mixed with 100 ㎕ of Griess reagent (Sigma, USA) in a 96-well plate, and absorption was read at 550 ㎚ with a spectrophotometer (Sigma, USA). Nitrite concentrations were determined by comparison with a sodium nitrite standard curve. The cells remaining after the Griess assay were used to determine cell viability by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-thetazoliumbromide (MTT)-based colorimetric assay (Park *et al*., 2006).

Reverse transcriptase-polymerase chain reaction analysis

Total RNA was prepared from RAW 264.7 cells with a Trizol Reagent kit as previously described (Lee *et al*., 2007). The following primers were used for PCR amplification: iNOS, 5'-TCTTCGAAATCCCACCTGAC-3' (forward) and 5'-CCATG ATGGTCACATTCTGC-3' (reverse), TNF-α, 5'-TTGACCTCAGCGCTGAGTTG-3' (forward) and 5'-CCTG TAGCCCACGTCGTAGC-3' (reverse), IL-6, 5'-GAGGATAC CACTCCCAACAG-3' (forward) and 5'-TTCACAGAGGATA CCACTCC-3' (reverse), IL-1β, 5'-GAAGCTGTGGCAGCTAC CTATGTCT-3' (forward) and 5'-CTCTGCTTG TGAGGTGCT GATGTAC-3' (reverse) (Raghav *et al*., 2004). β-actin mRNA levels were measured as an internal control.

Western blot analysis

Raw 264.7 cells were lysed by RIPA buffer containing proteinase inhibitors. Protein concentrations were quantified with a protein assay kit. The proteins (20 ㎍/lane) were resolved by SDS-polyacrylamide gel electrophoresis, and western blot analysis was performed as described previously (Lee *et al*., 2006). Rabbit anti-iNOS was used as primary antibodies and peroxidase conjugated antibody as secondary antibody. The membranes were developed with an enhanced chemiluminescence system from Amersham, and exposed to X-ray film for 30 s.

Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's t-test.

RESULTS AND DISCUSSION

We investigated 64 methanolic extracts of 42 medicinal

Table 1. Medicinal plant extracts which do not affect NO production in LPS-activated macrophage RAW264.7 cells.

Scientific name	Part	Scientific name	Part	Scientific name	Part
Juglans sinensis Dode	Leaves	*Atractylodes macrocephala*	Fruit	*Inula helenium L.*	Aerial parts
Juglans sinensis Dode	Fruit	*Atractylodes macrocephala*	Root	*Inula helenium L.*	Root
Lycium chinense Mill.	Leaves	*Helianthus annuus L.*	Stem	*Aster scaber Thunb.*	Aerial parts
Physalis alkekengi var. francheti (Mast) Hort.	Leaves	*Artemisia argyi H. Lev. & Vaniot*	Leaves	*Aster scaber Thunb.*	Root
Physalis alkekengi var. francheti (Mast) Hort.	Root	*Artemisia argyi H. Lev. & Vaniot*	Bamboo	*Cichorium intybus Nakai*	Root
Datura datura L.	Seeds	*Verbesina alternifolia Britton*	Aerial parts	*Prunella vulgaris for. Lilacina.*	Aerial parts
Datura datura L.	Aerial parts	*Cirsium setidens (Dunn) Nakai.*	Root	*Ricinus communis L.*	Leaves
Datura datura L.	Root	*Calendula officinalis L.*	Flower	*Hosta lancifolia*	Flower
Physalis angulata L.	Fruit	*Calendula officinalis L.*	Aerial parts	*Impatiens balsamina L. (red)*	Aerial parts
Physalis angulata L.	Aerial parts	*Artemisia gmelini Weber ex Stechm*	Aerial parts	*Sium suave walter*	Aerial parts
Coniogramme intermedia Hieron	Leaves	*Leibnitzia anandria Turcz*	Aerial parts	*Sium suave walter*	Stem
Aster ageratoides Turcz. Var. ageratoides	Aerial parts	*Silybum marianum*	Flower	*Silene armeria L.*	Advance post
Xanthium strumarium L.	Leaves	*Achillea millefolium*	Flower	*Gypsophila oldhamiana Miq.*	Root
Rudbeckia laciniata var. hotensis Bailey	Leaves	*Achillea millefolium*	Aerial parts	*Dianthus longicalyx Miq.*	Aerial parts
Lactuca indica var. laciniata (Kunze) H. Hara	Aerial parts	*Arctium lappa L.*	Stem	*Boehmeria spicata thumb.*	Flower
Eupatorium makinoi var. oppisitifolium (Koidz.) Kawahara & Yahara	Leaves	*Arctium lappa L.*	Root	*Mosla dianthera var. nana (Hara) Ohwi Prunella vulgaris for. Lilacina*	Leaves

Table 2. Effects of medicinal plant extracts on NO production and cell viability.

Scientific name	Family	Plant part	Traditional indications/uses[a]	IC50 (㎍/㎖)[b]	LD50 (㎍/㎖)c
Impatiens balsamina L.	Balsaminaceae	Aerial parts	*Antidote, diuretic, Dysphagia, emetic*	59.6 ± 1.7	83.8 ± 3.0
Impatiens balsamina L.	Balsaminaceae	Root	*Antidote, diuretic, Dysphagia, emetic*	60.5 ± 1.1	82.1 ± 3.8
Inula britannica var. japonica (Thunb.) Franch & Sav	Compositae	Flower	*Diuretic, Expectorant, Stomachic*	86.0 ± 1.6	93.2 ± 1.9
Inula britannica var. japonica (Thunb.) Franch & Sav	Compositae	Leaves	*Diuretic, Expectorant, Stomachic*	80.9 ± 2.1	91.3 ± 4.5
Inula helenium L.	Compositae	Leaves	*Alterative, Bronchitis*	78.2 ± 2.6	84.5 ± 6.5
Leibnitzia anandria Turcz	Compositae	Root	*Rheumatismus, Detoxication*	88.6 ± 3.1	95.5 ± 3.2
Prunella vulgaris for Lilacina	Labiatae	Root	*Alterative, Antiseptic, Astringent*	92.0 ± 2.0	93.2 ± 4.3
Hibiscus mutabilis	Malvaceae	Aerial parts	*Anodyne, Antidote, Cancer, Cough*	62.1 ± 0.9	80.4 ± 3.0
Hydrangea macrophylla (Thunb.) Ser	Saxifragaceae	Leaves	*Diuretic, Febrifuge, Malaria*	67.6 ± 2.5	68.6 ± 4.0
Hyoscyamus niger L.	Solanaceae	Aerial parts	*Ache(Tooth), Alcoholism, Anodyne*	94.7 ± 6.0	80.8 ± 3.5
Hyoscyamus niger L.	Solanaceae	Flower	*Ache(Tooth), Alcoholism, Anodyne*	81.4 ± 3.4	87.6 ± 3.5
Physalis alkekengi var. francheti (Mast) Hort.	Solanaceae	Stem	*Absces, Aperient, Detergent, Diabetes, Rheumatism*	94.5 ± 6.8	96.5 ± 2.5
Physalis angulata L.	Solanaceae	Root	*Analgesic, Antidote, Antiseptic, Asthma*	73.5 ± 4.4	92.3 ± 4.3
Ledebouriella seseloides (Hoff.) H. Wolff	Umbelliferae	Root	*Ache(Head), Anodyne, Arthritis, Ophthalmia*	64.7 ± 3.2	77.2 ± 2.2
Boehmeria longispica steud	Urticaceae	Root	*Unknown*	74.6 ± 4.0	78.5 ± 1.3
Hosta lancifolia	Liliaceae	Aerial parts	*Anodyne, Toothache*	68.1 ± 4.0	-d

[a] Information summarized from Phytochemical and Ethnobotanical Databases (2006): www.ars-grin.gov
[b] IC_{50} indicates that the concentration at which activity is inhibited by 50%
[c] LD_{50} indicates that the dose at which 50% of test subjects die
[d] (–) indicates no effect of plant extracts on cell viability up to the concentration of 100 ìg/ml

plants belonging to 10 families for inhibitory activity of NO production in LPS-stimulated macrophage RAW 264.7 cells. The inhibitory activity of 64 extracts from these plants was assessed quantitatively by measuring the level of nitrite, a stable metabolite of NO. Among 64 plant extracts tested, 48 samples did not exhibit the inhibitory activities of nitric oxide production in the activated macrophage cells (Table 1).

Plant extracts from 16 samples exhibited significant inhibition of NO production in the LPS-stimulated macrophage RAW 264.7 cells with IC_{50} values in the ranges from 59.6 to 94.7 ㎍/㎖, which are shown in Table 2 with the parts used and medicinal uses. Next we evaluated cytotoxic effect of the extracts and found that only *H. lancifolia* extract did not exert cytotoxic effects at the concentrations tested, of which LD_{50} values were in the ranges from 0 to 100 ㎍/㎖ (Table 2). The effects of *H. lancifolia* extract on NO production and cell viability test are shown in Fig. 1.

We further investigated the effect of the *H. lancifolia* extract on the expression of iNOS by PCR analysis. These inhibitory activities were compared well with the known inhibitor of NO production; ammonium pyrrolidinedithiocarbamate (PDTC) with IC_{50} of 3.5 μM. High level of NO is produced by iNOS of which expression is induced in LPS-stimulated macrophage RAW 264.7 cells. As shown in Fig. 2, mRNA and protein levels of iNOS are markedly increased by LPS treatment. Co-treatment of macrophage cells with LPS and various concentrations of plant extract from aerial parts of *Hosta lancifolia* resulted in inhibition of mRNA (Fig. 2A) and protein (Fig. 2B) levels induced by LPS in dose-dependent manner. *H. lancifolia* extract inhibited iNOS expression in similar fashion as shown in LPS-induced nitric oxide synthesis.

A number of iNOS inhibitors have been known to suppress the production of inflammatory cytokines such as TNF-α, IL-6 and IL-1β. We tested inhibitory effects of *H. lancifolia* extract on LPS-induced pro-inflammatory cytokines mRNA expression levels by RT-PCR analysis. *H. lancifolia* extract inhibits IL-6 and IL-1β mRNA levels in dose-dependent, while *H. lancifolia* extract only slightly suppresses TNF-α mRNA in 50 ㎍/㎖ (Fig. 3), indicating that the *H. lancifolia* extract contains some anti-inflammatory compounds.

In this study, we investigated the effects of 64 plant extracts on NO production in LPS-activated macrophage RAW 264.7 cells, and found that *Hosta lancifolia* exhibited

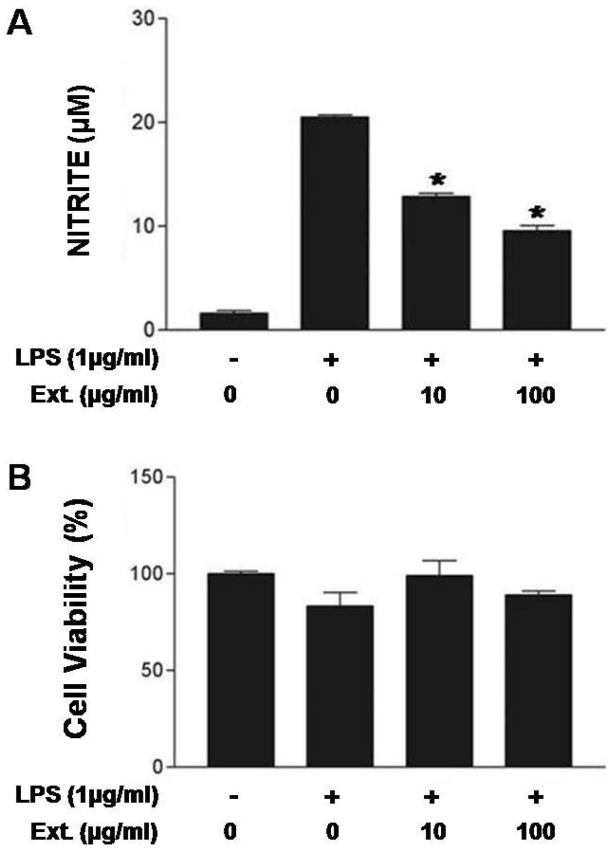

Fig. 1. Effects of *H. lancifolia* extract on LPS induced NO production and viability. **A.** RAW 264.7 cells were treated with LPS (1 ㎍/㎖) in the presence of various concentrations of *H. lancifolia* extract for 24 h. **B.** Effect of *H. lancifolia* extract on cell viability. Values represent the means and standard errors and all experiments were carried out in triplicates. * $p < 0.05$ versus LPS alone.

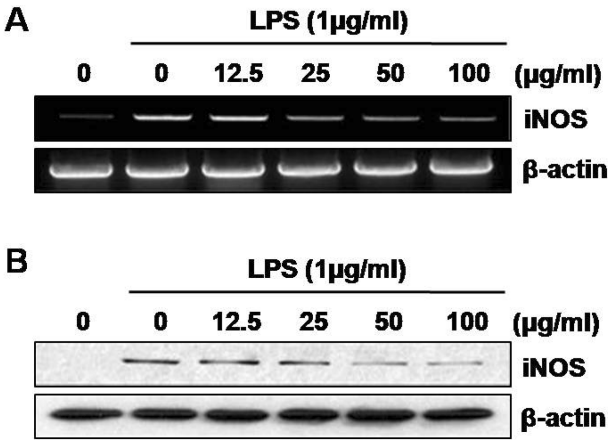

Fig. 2. Inhibitory effect of *H. lancifolia* extract on LPS induced iNOS mRNA and protein level. **A.** Levels of iNOS mRNA were determined by RT-PCR analysis. **B.** Levels of iNOS protein were measured by western blot using polyclonal antibodies against murine iNOS. β-actin was used as internal control.

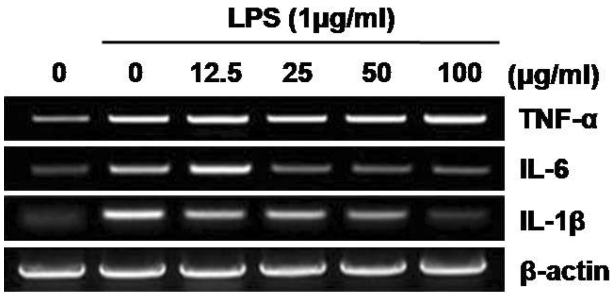

Fig. 3. Inhibitory effect of *H. lancifolia* extract on LPS induced pro-inflammatory cytokines mRNA level. Levels of pro-inflammatory cytokines mRNA were determined by RT-PCR analysis. RAW 264.7 cells were treated with LPS (1 ㎍/㎖) in the presence of various concentrations of *H. lancifolia* extract for 24 h. RT-PCR analysis were carried out at the temperature of 57℃. β-actin was used as internal control.

strong inhibitory activity of iNOS activity without affecting cell viability at the concentrations up to 100 ㎍/㎖. The *H. lancifolia* extract similarly inhibited the mRNA and protein levels of iNOS in a dose-dependent manner, indicating that the extract might suppress the expression of iNOS at the transcriptional level. The *H. lancifolia* extract also exhibited inhibition of inflammatory cytokines such as IL-6 and IL-1β.

Nitric oxide is generated by immune-activated macrophages and at inflammatory sites, and performs a pivotal role as neurotransmitter, vasodilator and immune regulator in variety of tissues (Bogdan *et al*., 2000, Jin *et al*., 2007). Thus, inhibition of nitric oxide production may constitute an effective therapeutic strategy for prevention of inflammatory reaction and diseases.

It has been reported that several plants related to *Hosta lancifolia* exhibited various biological activities such as anti-microbial activity (Rojas *et al*., 2003), antiprotozoal activity, (Fournet *et al*., 2007), anti-HIV-1 activity (Xiao *et al*., 2005a; Xiao *et al*., 2005b; Xiao *et al*., 2006a), and anti-inflammatory activity (Kim *et al*., 2004a; Kim *et al*., 2004b; Zúñiga *et al*., 2005). A number of previous studies revealed the presence of triterpenoids, nortriterpenoids, uleine and demethosyaspidospermine in the extracts of *lancifolia*-related plants (França *et al*., 2000; Xiao *et al*., 2006a; Xiao *et al*., 2006b). However, phytochemical information about *Hosta lancifolia* has not been elucidated yet. Thus, fractionation and identification of active compounds from these extracts would lead to discovery of a number of natural anti-inflammatory agents that may be potentially useful for prevention and treatment of inflammatory diseases such as rheumatic arthritis, atherosclerosis and cancer (Park *et al*., 2007, Yin *et al*., 2007). In conclusion, our present study reveals that several medicinal plants grown in Korea can be of use as easily accessible sources of natural drugs and possible food supplements.

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