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Effects of Natural Products on the Inhibition of Lipopolysaccharide-Inducible Nitric Oxide Synthase Activity in RAW264.7 Cell Culture System

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Abstract – Nitric oxide (NO) is a free radical synthesized from L-arginine by nitric oxide synthase (NOS). It is believed that NO is an important mediator in numerous physiological and inflammatory responses. Particularly, a large amount of NO released from the inducible nitric oxide synthase (iNOS) is mostly associated with inflammatory processes. Overproduction of NO in these processes including sepsis and autoimmune diseases can have deleterious consequences and pathophysiologic relevance. Therefore, for the discovery of new inhibitory agents against iNOS activity, we have evaluated about 100 kinds of natural products after partition into three layers (*n*-hexane, ethyl acetate and aqueous) from 100% methanol extracts to study inhibitory effects on iNOS activity induced by lipopolysaccharide (LPS) in RAW264.7 cells culture system. As a positive control, curcumin, which is known as an anti-tumor promotor, anti-inflammatory agent as an iNOS inhibitor, was used and showed the dose-dependent inhibitory effect (IC_{50}, 2.5 μg/ml). Among tested fractions, the *n*-hexane fraction of *Cimicifuga heracleifolia* (IC_{50}: 9.65 μg/ml), *Forsythiae fructus* (IC_{50}: 6.36 μg/ml), *Saposhnikovia divaricata* (IC_{50}: 5.92 μg/ml), and the ethyl acetate fraction of *Chrysanthemum sibiricum* (IC_{50}: 2.56 μg/ml), *Gastrodia elata* (IC_{50}: 3.46 μg/ml), and the aqueous fraction of *Dianthus chinensis* (IC_{50}: 6.73 μg/ml), *Euonymus alatus* (IC_{50}: 6.78 μg/ml), *Mechania urticifoloria* (IC_{50}: 8.01 μg/ml) showed strong inhibitory activity against LPS-stimulated iNOS. Especially, the ethyl acetate fraction of *Chrysanthemum sibiricum* (IC_{50}: 2.56 μg/ml), which exhibited the strongest inhibition against iNOS, was fractionated with silica-gel column chromatography. These subfractions exhibited dose-dependent inhibition against iNOS activity in the range of 2.59-5.6 μg/ml except for fraction No. 3, 4, 5, 6, 8, 9, and 16. Our study shows that *Chrysanthemum sibiricum* has the strongest inhibitory effect against iNOS activity and has similar effect to curcumin. Therefore, further studies for the identification of active principles from *Chrysanthemum sibiricum* and investigation for the mechanism of the inhibition of iNOS by active principles will be performed.
Key words – Nitric oxide, Inducible nitric oxide synthase, Lipopolysaccharide, RAW264.7 cells

Introduction

Nitric oxide (NO) is a readily diffusible, short-lived unstable free radical and an important physiological mediator. NO originates from oxidation of a guanidino nitrogen of L-arginine (Schmidt *et al.*, 1988; Palmer *et al.*, 1988) and this reaction is catalyzed by nitric oxide synthase (NOS) (EC1.14.13.39) and all NO synthases depend on NADPH, flavin nucleotides, tetrahydrobiopterin and calmodulin as cofactors (Kwon *et al.*, 1989; Mayer *et al.*, 1991; Stuehr *et al.*; 1991 Pollock *et al.*, 1993). To date three different isoforms of NOS have been characterized and distinguished by their Ca^{2+}/calmodulin dependence: two of them are termed neuronal constitutive (ncNOS, or type I) and endothelial constitutive (ecNOS, or type III). These enzymes are always present (thus named constitutive), transiently produce small amounts of

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NO, which is important in both intercellular and intracellular signalling (Bredt *et al.*, 1990; Moncada *et al.*, 1991). Type I in nerve cells (Mayer *et al.*, 1990; Bredt *et al.*, 1991; Schmidt *et al.*, 1991) and type III in endothelial cells (Pollock *et al.*, 1991; Janssens *et al.*, 1992; Sessa *et al.*, 1992) regulate homeostasis of blood flow, platelet function and signal transduction in the central and peripheral nervous systems.

The third NOS isoform, the inducible NOS (iNOS, or type II), produces larger amounts of NO when the cells are stimulated with bacterial products such as lipopolysaccharide (LPS) and cytokines (TNF, IL-1β, IFN-γ). This enzyme is expressed in nearly all the body cells (Nathan *et al.*, 1992; Forstermann *et al.*, 1994; Knowles *et al.*, 1994), particularly in macrophages/monocytes (Hibbs *et al.*, 1988; Weinberg *et al.*, 1995), neutrophils (Wright *et al.*, 1989; Wheeler *et al.*, 1997), and hepatocytes (Curran *et al.*, 1989; Nussler *et al.*, 1992).

Although NO expressed by constitutive NOS (cNOS or type I, III) appears to be beneficial for many physiological processes, the excess of NO expressed by inducible NOS (iNOS or type II) has been implicated in the pathogenesis of various inflammatory and immunologically mediated diseases, including graft-versus-host disease (Langrehr *et al.*, 1992), diabetes (Corbett *et al.*, 1991; Kolb *et al.*, 1991), viral infections (Zheng *et al.*, 1993), and arthritis (Farrell *et al.*, 1992; Ialenti *et al.*, 1993).

The purpose of this study is to find iNOS inhibitors with high potency and efficacy, which might have cancer chemopreventive activity and anti-inflammatory process by using murine macrophages (RAW264.7) cell culture system stimulated with LPS (Mukhopadhyay *et al.*, 1982; Strivastava *et al.*, 1985; Huang *et al.*, 1988; Conney *et al.*, 1991; Azuine *et al.*, 1992; Ammom *et al.*, 1993; Tanaka *et al.*, 1994).

Experimental

General chemicals – Lipopolysaccharide (LPS), curcumin, sulfanilamide, naphythyl-ethylenediamine dihydrochloride, sodium nitrite, and Dulbecoo's modified Eagle's medium (DMEM) were purchased from Sigma Chem. Co., USA.

Plant materials – Plant materials were purchased from a herb market in Seoul and boucher specimens were deposited in the Natural Products Resource Depository of Natural Products Research Institute, Seoul National University. Each of dried plant parts were sliced, and then extracted 3 times with methanol at room temperature and were concentrated under reduced pressure below 40°C, and then the concentrated methanol extracts were partitioned into *n*-hexane, ethyl acetate, and water layers.

Cell culture – The murine macrophage cell line (RAW264.7) was purchased from American Type Culture Collection, USA. Cells were grown at 37°C in a humidified atmosphere (5% CO_2) in Dulbecoo's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, and 10% fetal bovine serum.

Determination of inducible Nitric Oxide Synthase (iNOS) activity – RAW264.7 cells were cultured in 150 cm^2 tissue culture flask until confluent. Cultured cells were plated at 2×10^4 cells per well in a 96-well microtiter plate and preincubated for 24 hr at 37°C in a humidified atmosphere (5% CO_2). After 24 hr preincubation period, cells were treated with LPS (1 ug/ml) and test samples (final 0.5% DMSO) in fresh medium containing 10% fetal bovine serum for 24 hr. Nitrite concentrations produced in the medium were measured by an automated colorimetric assay based on the Griess reaction with some modification (Ding *et al.*, 1988). Griess reagent (0.25% naphthylethylenediamine, 2.5% sulfanilamide in 10% H_3PO_4) was added into each well and the plates were shaked for 5 min at 37°C. The nitrite concentration was determined with a microtiter plate reader at 540 nm (THERMOmax, Molecular Device Co., USA). The inhibitory effects of tested samples were represented by IC_{50} value. IC_{50} represents the concentration required to inhibit LPS-induced production of NO by 50%, calculated on the basis of nitrite concentrations released into the culture medium treated with vehicle solvent (DMSO).

Results and Discussion

Nitric oxide (NO) is an important physiological and pathophysiological mediator synthesized by nitric oxide synthase (NOS). Because the nitric oxide play a role in inflammation and also possibly in the multistage process of carcinogenesis, this study is specially focused on the nitric oxide generation enzyme, which is induced by inflammatory mediated endotoxins. In order to investigate the inhibitory effects of natural products on iNOS activity from RAW264.7 cells induced by LPS, we evaluated plant extracts with NO

Table 1. Inhibition of plant extracts against LPS-induced iNOS activity in RAW264.7 cell culture system.

Scientific name / Family name	Plant part[a]	Inhibition of iNOS activity (IC_{50}, ug/ml)		
		Hexane fraction	Ethyl acetate fraction	Aqueous fraction
Acanthopanax senticosus / Araliaceae	Wp	>10	>10	>10
Aconitum koreanum / Araceae	Rt	>10	>10	>10
Aconitum kusnezoffii / Ranunculaceae	Rt	>10	>10	>10
Adenophora trachelioides / Campanulaceae	Wp	>10	>10	>10
Ainsliaea acerifolia / Compositae	Wp	>10	>10	>10
Albizzia julibrissin / Leguminosae	Rt	>10	>10	>10
Alisma orientale / Alismataceae	Rh	>10	>10	>10
Amomum cardamomum / Zingiberaceae	Wp	>10	>10	>10
Anemarrhena asphodeloides / Liliaceae	Rh	>10	>10	>10
Angelica dahurica / Umbelliferae	Rt	>10	>10	>10
Angelica gigas / Umbelliferae	Rt	>10	>10	>10
Angelica gigas / Umbelliferae	Rt	>10	>10	>10
Angelica tenuissima / Umbelliferae	Rt	>10	>10	>10
Anthriscus sylvestris / Umbelliferae	Wp	>10	>10	>10
Aralia continentalis / Araliaceae	Rt	>10	>10	>10
Artemisia argyi / Compositae	Lf	>10	>10	>10
Asparagus cochinchinensis / Liliaceae	Bk	>10	>10	>10
Aster tataricus / Compositae	Wp	>10	>10	>10
Atractylodes japonica / Compositae	Rt	>10	>10	>10
Benicasa cerifera / Cucurbitaceae	Fr	>10	>10	>10
Boswellia carteriz / Bruseraceae	Wp	>10	>10	>10
Bupleurum chinense / Umbelliferae	Wp	>10	>10	>10
Bupleurum falcatum / Umbelliferae	Rt	>10	>10	>10
Cadamine leucantha / Cruciferae	Wp	>10	>10	>10
Caltha paluotris var. membranacea / Ranunculaceae	Wp	>10	>10	>10
Caragana sinica / Legumonisae	Wp	>10	>10	>10
Cassia tora / Leguminosae	Sd	>10	>10	>10
Caulophyllum robustum / Berberidaceae	Wp	>10	>10	>10
Chrysanthemum sibiricum / Compositae	St,Lf	>10	2.56	>10
Cichorium intybus / Compositae	Rt	>10	>10	>10
Cimicifuga heracleifolia / Ranunculaceae	Rh	9.65	>10	>10
Cirsium japonicum var. ussuriense / Compositae	Wp	>10	>10	>10
Citrus Unshiu / Rutaceae	Fb	>10	>10	>10
Clematis chinensis / Ranunculaceae	Wp	>10	>10	>10
Cnidium officinale / Umbelliferae	Ap	>10	>10	>10
Codonopsis pilosula / Campanulaceae	Fr	>10	>10	>10
Corydalis ternata / Papaveraceae	Rh	>10	>10	>10
Crataegus pinnatifida / Rosaceae	Fr	>10	>10	>10
Dianthus chinensis / Caryophyllaceae	Wp	>10	>10	6.73
Dioscorea batata / Dioscoreaceae	Wp	>10	>10	>10
Dryopteris crassirhigoma / Aspidaceae	Ap	>10	>10	>10
Epimedium koreanum / Berberidaceae	Lf,St	>10	>10	>10
Euonymus alatus / Celastraceae	Ap	>10	>10	6.78
Foeniculum vulgare / Umbelliferae	Wp	>10	>10	>10
Forsythiae fructus / Oleaceae	Wp	6.36	>10	>10
Gardenia jasminoides / Rubiaceae	Fr	>10	>10	>10
Gastrodia elata / Orchidaceae	Rh	>10	3.46	>10
Gleditschia japonica / Leguminosae	Fr	>10	>10	>10
Hydnocarpus anthelmintica / Flacourtiaceae	Sd	>10	>10	>10
Kalopanax pictum / Leguminosae	Bk	>10	>10	>10
Lilium lancifolium / Liliaceae	Wp	>10	>10	>10
Loncicera japonica / Caprifoliaceae	Fl	>10	>10	>10
Lonicera japonica / Caprifoliaceae	Fl	>10	>10	>10
Lycium chinense / Solanaceae	Fr	>10	>10	>10

Table 1. Continued

Scientific name / Family name	Plant part[a]	Inhibition of iNOS activity (IC_{50}, ug/ml)		
		Hexane fraction	Ethyl acetate fraction	Aqueous fraction
Lycium chinense / Solanaceae	Fl	>10	>10	>10
Malva verticillata / Malavaceae	Wp	>10	>10	>10
Mechania urticifoloria / Labiatae	Wp	>10	>10	8.01
Nepeta japonica / Labiatae	Ap	>10	>10	>10
Paeonia obovata / Ranunculaceae	Rt	>10	>10	>10
Paris veiticillata / Liliaceae	Ap	>10	>10	>10
Perilla sikokiana / Labiatae	Sd	>10	>10	>10
Phlomis umbrosa / Dipsacaceae	Rt	>10	>10	>10
Plantago asiatica / Plantaginaceae	Sd	>10	>10	>10
Polustichum tripteron / Aspidaceae	Ap	>10	>10	>10
Polygonatum falcatum / Liliaceae	Rh	>10	>10	>10
Polygonum multiflorum / Polygonaceae	Rb	>10	>10	>10
Poncirus trifoliata / Rutaceae	Fr	>10	>10	>10
Poria cocos / Polygonaceae	Rb	>10	>10	>10
Prunella vulgaris / Labiatae	Fl	>10	>10	>10
Prunus armeniaca var. ansu / Rosaceae	Sd	>10	>10	>10
Prunus humilis / Rosaceae	Sd	>10	>10	>10
Prunus persica / Rosaceae	Sd	>10	>10	>10
Psoralea corylifolia / Leguminosae	Wp	>10	>10	>10
Pulsatilla chinensis / Ranunculaceae	Wp	>10	>10	>10
Raphanus sativus var. raphanistroides / Cruciferae	Sd	>10	>10	>10
Rehmania glutinosa / Scrophulariaceae	Rt	>10	>10	>10
Rehmania glutinosa / Scrophulariaceae	Rt	>10	>10	>10
Rehmania glutinosa var. purpurea / Scrophulariceae	Rt	>10	>10	>10
Rheum undulatum / Polygonaceae	Rh	>10	>10	>10
Rhus verniciflua /	St	>10	>10	>10
Salvia miltiorrhiza / Labiatae	Rt	>10	>10	>10
Sambucus williamsii var. coreana / Caprifoliaceae	Ap	>10	>10	>10
Santalum album / Santalaceae	Wd	>10	>10	>10
Saposhnikovia divaricata / Umbelliferae	Wp	5.92	>10	>10
Schizandra chinensis / Magnoliaceae	Fr	>10	>10	>10
Scrophularia buergeriana / Scrophulariaceae	Rt	>10	>10	>10
Siegesbeckia pubescens / Compositae	Wp	>10	>10	>10
Sinocalamus beecheyanus / Gramineae	Wp	>10	>10	>10
Smilax glabra / Liliaceae	Wp	>10	>10	>10
Sophora flavescens / Leguminosae	Rt	>10	>10	>10
Sophora japonica / Leguminosae	Wp	>10	>10	>10
Strychnos ignatii / Loganiaceae	Sd	>10	>10	>10
Taraxacum ohwianum / Compositae	Wp	>10	>10	>10
Taraxacum platycarpum / Compositae	Rt	>10	>10	>10
Torilis japonica / Umbelliferae	Sd	>10	>10	>10
Torreya nucifera / Taxaceae	Fr	>10	>10	>10
Trlia volatile oils / Trliaceae	Wp	>10	>10	>10
Tussilago farfara / Compositae	Wp	>10	>10	>10
Vaccinium koreanum / Ericaceae	Lf	>10	>10	>10
Valeriana fauriei / Valerianaceae	Wp	>10	>10	>10
Veratum patulum / Liliaceae	Ap	>10	>10	>10
Vitex rotundifolia / Verbenaceae	Fr	>10	>10	>10
Xanthium strumarium / Compositae	Fr	>10	>10	>10
Zanthoxylum bungeanum / Rubiaceae	Wp	>10	>10	>10
Zingiber officinale / Zinggiberaceae	Rh	>10	>10	>10

[a] Ap: aerial parts; Bk: bark; Fb: fruits bark; Fl: flower; Fr: fruits; Lf: leaves; Rb: root bark; Rh: rhizome; Rt: roots; Sb: stem bark; Sd: seeds; St: stem; Tu: tuber; Wd: wood; Wp: whole plants.

Table 2. Inhibition of column chromatographic subfractions from ethyl acetate fraction of *Chrysanthemum sibiricum* against LPS-induced iNOS activities.

Subfraction No.	Inhibition of iNOS activity (IC_{50}, ug/ml)
Fraction no. 1	3.89
Fraction no. 2	3.52
Fraction no. 3	>10
Fraction no. 4	>10
Fraction no. 5	>10
Fraction no. 6	>10
Fraction no. 7	4.62
Fraction no. 8	>10
Fraction no. 9	>10
Fraction no. 10	2.77
Fraction no. 11	4.8
Fraction no. 12	4.96
Fraction no. 13	2.59
Fraction no. 14	5.6
Fraction no. 15	5.15
Fraction no. 16	>10

assay cell culture system. With this assay system, approximately 150 kinds of natural products were evaluated, and the results were summarized in Table 1 and Table 2. Curcumin, which is known as an anti-tumor promotor, anti-inflammatory agent as an iNOS inhibitor, was used as a positive control and showed the dose-dependent inhibitory effect (IC_{50}: 2.5 μg/ml) against iNOS activity in RAW264.7 cells stimulated with LPS (Figure 1).

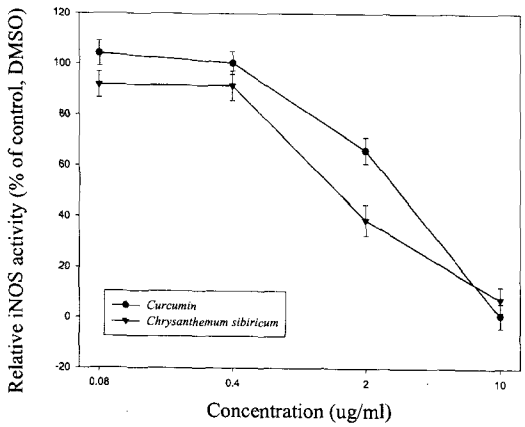

Fig. 1. Inhibition of curcumin (●) and the ethyl acetate fraction of *Chrysanthemum sibiricum* (▼) against iNOS activity. The inhibitory effects against iNOS were represented by the relative LPS-induced iNOS activity compared with control group (DMSO-treated). The results were represented mean ±S.D. performed in duplicate.

Among the 3000 kinds of solvent fractionated plant extracts, the hexane fractions of *Cimicifuga heracleifolia* (IC_{50}: 9.65 μg/ml), *Forsythiae fructus* (IC_{50}: 6.36 μg/ml), *Saposhnikovia divaricata* (IC_{50}: 5.92 μg/ml), and the ethyl acetate fractions of *Chrysanthemum sibiricum* (IC_{50}: 2.56 μg/ml), *Gastrodia elata* (IC_{50}: 3.46 μg/ml), and the aqueous fractions of *Dianthus chinensis* (IC_{50}: 6.73 μg/ml), *Euonymus alatus* (IC_{50}: 6.78 μg/ml), *Mechania urticifoloria* (IC_{50}: 8.01 μg/ml) showed inhibitory activity aganist LPS-induced iNOS.

Especially, the ethyl acetate fraction of *Chrysanthemum sibiricum* (IC_{50}: 2.56 μg/ml), which exhibited the strong inhibition against iNOS (Figure 1), was fractionated with silica-gel column chromatography to find active fractions in this system and was evalulated. The results of the inhibitory effects against iNOS with column chromatographic subfractions from ethyl acetate fraction of *Chrysanthemum sibiricum* are summarized in Table 2. These column chromatographic subfractions exhibited dose-dependent inhibition on iNOS activity in LPS-induced RAW264.7 cells. These active fractions are under investigation by using activity-guided fractionation method with column chromatography to find active principles.

After treatment by certain cytokines or endotoxin, iNOS produces large quantities of NO with cytotoxic and bacteriotoxic effects. But high amounts of NO, synthesized systematically and intra-articularly, play

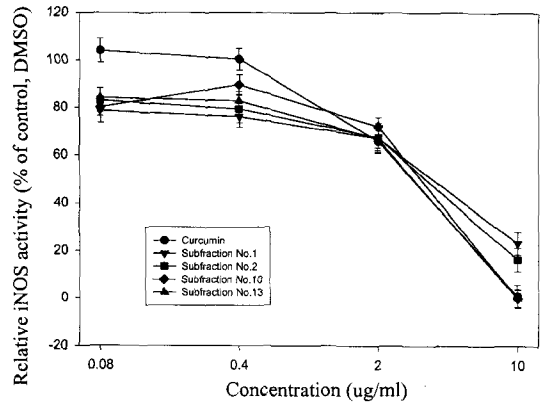

Fig. 2. Inhibition of the column chromatographic subfractions No. 1 (●), No. 2 (▼), No. 10 (■), and No. 13 (◆) from the ethyl acetate fraction of *Chrysanthemum sibiricum* against iNOS activities. The inhibitory effects against iNOS were represented by the relative LPS-induced iNOS activity compared with control group (DMSO-treated). The results were represented as mean ±S.D. performed in duplicate.

an important role in inflammatory joint diseases. In experimental arthritis, administration of NOS inhibitors profoundly reduced disease activity. In humans, beneficial effects of NO synthesis inhibition are inferred from indirect evidence. Glucocorticoids, inhibiting induction of the iNOS, reduced NO synthesis and disease activity. Thus, selective inhibition of the pathologically enhanced NO synthesis emerges as a new experimental therapeutic approach in the treatment of inflammatory joint diseases.

Curcumin, used as positive control, has been reported to inhibit protein kinase C by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), TPA-induction of ornithine decarboxylase, *in vitro* cyclooxygenase/lipoxygenase and TPA-induced xanthine dehydrogenase/oxidase. Also curcumin decreases the activity and protein levels of iNOS by reducing the expression of iNOS mRNA. But exact mechanism for the inhibition of iNOS induction by curcumin is not known. Several reports suggest that inhibition of iNOS induction by curcumin can be mediated through inhibition of iNOS transcription by suppression of c-Jun/AP-1 activation or can be mediated through inhibition of protein kinase C and tyrosine protein kinase. Among tested plant extracts, the ethyl acetate of *Chrysanthemum sibiricum* has the strongest effect for the inhibition of iNOS and has similar effect to curcumin. Our study recommends further studies for finding active principles from *Chrysanthemum sibiricum* and investigation for the mechanism of the inhibition of iNOS induction by the active principles.

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