Arch Pharm Res Vol 27, No 12, 1220-1225, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

A Curcuminoid and Two Sesquiterpenoids from *Curcuma zedoaria* as Inhibitors of Nitric Oxide Synthesis in Activated Macrophages

Mi Kyung Jang, Hwa Jin Lee, Ji Sun Kim, and Jae-Ha Ryu
College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

(Received September 18, 2004)

The overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is known to be responsible for vasodilation and hypotension observed in septic shock and inflammation. Inhibitors of iNOS, thus, may be useful candidates for the treatment of inflammatory diseases accompanied by overproduction of NO. In the course of screening oriental anti-inflammatory herbs for the inhibitory activity of NO synthesis, a crude methanolic extract of *Curcuma zedoaria* exhibited significant activity. The activity-guided fractionation and repetitive chromatographic procedures with the EtOAc soluble fraction allowed us to isolate three active compounds. They were identified as 1,7-bis (4-hydroxyphenyl)-1,4,6-heptatrien-3-one (**1**), procurcumenol (**2**) and epiprocurcumenol (**3**) by spectral data analyses. Their concentrations for the 50% inhibition of NO production (IC_{50}) in lipopolysaccharide (LPS)-activated macrophages were 8, 75, 77 μM, respectively. Compound **1** showed the most potent inhibitory activity for NO production in LPS-activated macrophages, while the epimeric isomers, compound **2** and **3** showed weak and similar potency. Inhibition of NO synthesis by compound **1** was very weak when activated macrophages were treated with **1** after iNOS induction. In the immunoblot analysis, compound **1** suppressed the expression of iNOS in a dose-dependent manner. In summary, 1,7-bis (4-hydroxyphenyl)-1,4,6-heptatrien-3-one from *Curcuma zedoaria* inhibited NO production in LPS-activated macrophages through suppression of iNOS expression. These results imply that the traditional use of *C. zedoaria* rhizome as anti-inflammatory drug may be explained at least in part, by inhibition of NO production.

Key words: Nitric oxide synthase, Inhibitor, *Curcuma zedoaria*, Curcuminoid, Sesquiterpene

INTRODUCTION

Nitric oxide (NO), an intracellular mediator, is synthesized by the oxidation of terminal guanidine nitrogen of L-arginine, and this reaction is catalyzed by three types of nitric oxide synthase (NOS) enzyme. The constitutive NOS (cNOS) found in neuronal tissues (type I) and in vascular endothelium (type III) is Ca^{2+}-dependent and release small amounts of NO required for physiological functions (Bredt and Snyder, 1990), whereas inducible NOS (iNOS, type II) can be induced by several stimuli and leads to the micromolar levels of NO (Lowenstein *et al.*, 1992). NO produced by activated macrophages serves as an important signaling molecule in immune systems and may exerts the anti-viral and anti-tumor activity (Thomsen *et al.*, 1992).

NO produced in large amounts by iNOS and its derivatives, such as peroxynitrite and nitrogen dioxide, plays a role in inflammation and also possibly in the multistage process of carcinogenesis (Oshima and Bartsch, 1994). NO is also known to be responsible for the vasodilation and hypotension observed in septic shock (Thiemermann and Vane, 1990). So, the inhibitors of iNOS may serve as therapeutic agents for treatment of septic shock and inflammaion. There are many plant-derived constituents that can affect the NO signaling pathway (Achike and Kwan, 2003) and most of these compounds showed their inhibitory activity of NO production through the inhibition of iNOS expression (Ryu *et al.*, 2002; Hong *et al.*, 2002; Kim *et al.*, 2001; Chi *et al.*, 2001). In order to find new iNOS inhibitors from medicinal plants, we have screened inhibitory activity of NO production by measuring the NO production in LPS-activated RAW 264.7 cells (Ryu *et al.*, 2003).

Correspondence to: Jae-Ha Ryu, College of Pharmacy, Sookmyung Womens University, Seoul 140-742, Korea
Tel: 82-2-710-9568, Fax: 82-2-714-0745
E-mail: ryuha@sookmyung.ac.kr

Among the screened medicinal plants, rhizome of *C. zedoaria* has been used as a gastrointestinal remedy in oriental medicine. Several cytotoxic curcuminoids (Syu *et al.*, 1988) and sesquiterpenes (Yoshioka *et al.*, 1998) that showed inhibitory activity of prostaglandin E_2 and NO production (Hong *et al.*, 2002; Matsuda *et al.*, 2001a, 2001b) have been reported from this plant. From *C. zedoaria* we purified three NOS inhibitors and identified their chemical structures.

MATERIALS AND METHODS

General procedures

NMR spectra were recorded on a Brucker AMX 400 NMR and Bruker Avance-600 spectrometer with TMS as internal standard. EI-MS were measured with a Hewlett-Packard 5890-JMS AX505WA spectrometer. IR spectra were recorded on a Jasco FT-IR-430 spectrometer in $CHCl_3$ solution. UV spectra were obtained on a Pharmacia Biotech Ultraspec 4000 UV-VIS spectrophotometer. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter and optical density was measured with a Dynatech MR 5000 microplate reader.

Reagents and materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories (Detroit, MI) and lipopolysaccharide (LPS, *Escherichia coli*, 0127:B8), bovine serum albumin, sodium nitrite, naphthylethylene diamine, sulfanilamide, aminoguanidine, L-arginine, *N*-(1-naphthyl) ethylenediamine and N^G-monomethyl-L-arginine (L-NMMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-mouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and anti-β-actin monoclonal antibody from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Murine macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/mL) and streptomycin (10 μg/mL). Cells were grown at 37°C, 5% CO_2 in fully humidified air, and were split twice a week. RAW 264.7 cells were seeded at 3×10^5 cells/mL in 24 well plates and activated by the incubation in 1% FBS medium containing LPS (1 μg/mL) and various concentrations of test compounds dissolved in DMSO (final 0.1% in media). The supernatant was collected as a source of secreted NO.

Nitrite assay

NO released from macrophages was assessed by the determination of NO_2^- concentration in culture supernatant. Samples (100 μL) of culture media were incubated with 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplate (Green *et al.*, 1982). Absorbance at 540nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

Cell viability

Cell viability was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma)-based colorimetric assay (Mosmann, 1983). Cells in 96-well plates (5×10^3 cells/well) were exposed to various concentrations of sample at 37°C, 5% CO_2 in air for 24 h. The 10 μL MTT solution (5 mg/mL in phosphate buffered saline) was added and further incubated for 4 h at 37°C. After aspirating the supernatant from the wells, 100 μL of extraction buffer (10% SDS in 0.01 M HCl) was added for the dissolution of formazan crystals. The absorbance of each well was then read at 570 nm using an ELISA plate reader.

Immunoblot analysis

Confluent monolayers of RAW 264.7 cells in 75 cm^2 culture flasks (1×10^7 cells) were incubated for 18 hr in DMEM with either LPS or in combination with compound **1**. The cells were rinsed with cold phosphate buffered saline and lysed by boiling with lysis buffer (1% SDS, 1.0 mM sod. vanadate, 10 mM Tris, pH 7.4) for 5 min. The protein concentration of cell lysates was determined using Bio-Rad protein assay kit. Thirty μg protein of cell lysates was applied on 8% SDS-polyacrylamide gels and transferred to PVDF membrane by the standard method. The membrane was probed with antibody for anti-mouse iNOS and anti-β-actin. The Western blot was visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham bioscience, Piscataway, NJ) according to the manufacturers instruction.

Extraction and isolation

The rhizomes of *Curcuma zedoaria* were purchased from the Kyungdong oriental drug market in Seoul, and authenticated by Prof. K. S. Yang at the College of Pharmacy, Sookmyung Womens University. A voucher specimen (No. SPH 97008) was deposited in the herbarium of the Sookmyung Womens University. The rhizomes of *C. zedoaria* (4 kg) were extracted three times with MeOH and the combined extracts (215 g) were concentrated and partitioned between EtOAc and H_2O to yield EtOAc soluble fraction (70 g). The EtOAc soluble fraction was subjected to column chromatography on silica gel (70-230 mesh, 1500 g), *n*-hexane/EtOAc (10:1) as eluents,

to yield bioactive fr. 6 (17.7 g, elution volume 4500-5200 mL). Fraction 6 was further chromatographed on silica gel (500 g) using *n*-hexane/acetone (20:1, 10:1, 5:1, 1:1, 1000 mL each) as eluents and afforded two bioactive subfractions fr. 6-4 (1.6 g) and fr. 6-7 (0.4 g). Fr. 6-7 containing compound **1** was applied onto reversed-phase HPLC (μ-Bondapak C-18 column, 10 x 300 mm; 60% MeOH, 2.0 mL/min; UV 254 nm) to yield **1** (3.5 mg, t_R 14.7 min). Portion of Fr. 6-4 (290 mg) was also applied onto reversed-phase HPLC (μ-Bondapak C-18 column, 10 x 300 mm; 75% MeOH, 2.0 mL/min; UV 254 nm) to yield **2** (32.9 mg, t_R 34 min) and **3** (1.5 mg, t_R 28 min).

1,7-Bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (1)

Yellow powder; EI-MS m/z: 354 $[M]^+$ ($C_{19}H_{16}O_3$); UV (EtOH) λ_{max} (log): 248 (4.04), 398 (4.54) nm; IR (KBr): 3300, 1653 cm^{-1}; ^{1}H-NMR (acetone-d_6, 400 MHz) δ: 6.63 (1H, *d*, *J*=15.3 Hz, H-4), 6.87 (2H, *d*, *J*=8.7 Hz, H-3" and H-5"), 6.90 (2H, *d*, *J*=8.7 Hz, H-3' and H-5'), 6.93 (1H, *dd*, *J*=15.3, 10.3 Hz, H-6), 7.04 (1H, *d*, *J*=15.3 Hz, H-7), 7.06 (1H, *d*, *J*=15.8 Hz, H-2), 7.46 (2H, *d*, *J*=8.7 Hz, H-2" and H-6"), 7.53 (1H, *dd*, *J*=15.3, 10.3 Hz, H-5), 7.60 (2H, *d*, *J*=8.7 Hz, H-2' and H-6'). 7.61 (1H, *d*, *J*=15.8 Hz, H-1); ^{13}C-NMR (acetone-d_6, 100 MHz) δ: 116.16 (C-3" and C-5"), 116.25 (C-3' and C-5'), 123.03 (C-2), 124.83 (C-6), 127.13 (C-1"), 128.57 (C-1'), 128.62 (C-4), 129.33 (C-2" and C-6"), 130.66 (C-2' and C-6'), 141.47 (C-7), 142.39 (C-1), 143.42 (C-5), 159.09 (C-4'), 160.18 (C-4"), 188.17 (C-3).

Procurcumenol, (1α,4β,5β) 4-hydroxy-7(11), 9-guaiadien-8-one (2)

Oil; EI-MS m/z: 234 $[M]^+$ ($C_{15}H_{22}O_2$); $[\alpha]_D^{29.7}$ +222.3(*c*=1.34, $CHCl_3$); UV (EtOH): λ_{max} (log): 248 (4.11), 275 (3.97) nm; IR ($CHCl_3$): 3421, 1645, 1440, 1376 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): 1.21 (3H, *s*, H-15), 1.67 (1H, *m*, H-2a), 1.72 (3H, *s*, H-12), 1.74 (3H, *s*, H-13), 1.85 (3H, *s*, H-14), 1.87 (2H, *m*, H-3), 1.89 (1H, m, H-5), 1.94 (1H, m, H-2b), 2.15 (1H, *dd*, *J*=15.8, 12.3 Hz, H-6a), 2.34 (1H, *dd*, *J*=18.8, 9.08 Hz, H-1), 2.58 (1H, *d*, *J*=15.8 Hz, H-6b), 5.84 (1H, *q*, *J*=1.2 Hz, H-9); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 21.6 (C-12), 22.8 (C-13), 23.8 (C-14), 24.6 (C-15), 27.3 (C-2), 29.0 (C-6), 40.2 (C-3), 50.9 (C-1), 54.2 (C-5), 80.6 (C-4), 129.5 (C-9), 136.7 (C-11), 137.0 (C-7), 155.6 (C-10), 199.5 (C-8).

Epiprocurcumenol, (1β,4β,5β) 4-hydroxy-7(11), 9-guaiadien-8-one (3)

Oil; EI-MS m/z: 234 $[M]^+$ ($C_{15}H_{22}O_2$); $[\alpha]_D^{29}$ -58(*c*=0.09, EtOH); UV (EtOH) λ_{max} (log): 253 (2.46) nm; IR ($CHCl_3$): 3420, 1645, 1456, 1376 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.12-3.25 (8H, *m*, H-1~H-6), 1.38 (3H, *s*, H-14), 1.84 (3H, *s*, H-12), 1.92 (3H, *s*, H-13) 1.94 (3H, *s*, H-15), 5.94 (1H, *br s*, H-9); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 21.9 (C-12), 23.1 (C-13), 25.2 (C-14), 26.8 (C-15), 26.9 (C-2), 28.8 (C-6), 38.5 (C-3), 46.7 (C-1), 54.9 (C-5), 82.3 (C-4), 129.4 (C-9), 134.8 (C-7), 141.1 (C-11), 155.2 (C-10), 196.5 (C-8).

Statistics

The results were expressed as mean±S.D. of three experiments, and statistical analysis was performed by the Students *t*-test, and a P value of <0.05 was considered to indicate a significant difference.

RESULTS AND DISCUSSION

Among the tested plants that have been used for the treatment of inflammation in oriental medicine, the methanol extract from rhizomes of *Curcuma zedoaria* (Zingiberaceae) showed 78% inhibition of LPS-induced NO production from RAW 264.7 cells at 50 μg/mL in culture media. The activity-guided fractionation and repetitive chromatographic procedures with the EtOAc soluble fraction of *C. zedoaria* resulted in the isolation of three active compounds **1**, **2**, and **3**.

Compound **1** was purified as yellow powder and positive to $FeCl_3$ reagent that indicated the presence of phenol group in the structure. The NMR spectral data showed that **1** has fourteen sp_2 methine carbons, four quaternary carbons and one carbonyl carbon. All the peaks of proton NMR spectrum were observed at lower field than 6.5 ppm. Two spin systems of para-disubstituted aromatic rings were identified. These are typical spectral pattern of diarylheptanoids. From the one pair of isolated *trans* coupling and two conjugated *trans* couplings in ^{1}H-^{1}H COSY spectrum, we confirmed the presence of carbonyl group at 3 position in the structure. The structure of **1** were identified as 1,7-bis (4-hydroxyphenyl)-1,4,6-heptatrien-3-one, and all the NMR spectral data were identified by the analysis of ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, DEPT, and HSQC spectrum. This structure was reported from *Curcuma domestica* (Nakayama *et al.*, 1993) (Fig. 1). This is the first report of this structure from *C. zedoaria*.

Compound **2** and **3** were purified as oil and gave the same molecular formula from the mass data as $C_{15}H_{22}O_2$. Their NMR spectral data showed the presence of four methyl groups, and three of them were confirmed as vinyl methyl groups from the long rage coupling between methyl protons and olefinic carbons in HMBC spectrum. Overall NMR data of **2** and **3** were similar together and they showed the typical pattern of guaiane-sesquiterpenoids. The difference of chemical shift values of carbon 1, 4, 7, 8, and 15 were observed. In the ^{13}C-NMR spectral data of **3**, the chemical shift of C-1, 3, 7, 8 were shifted to

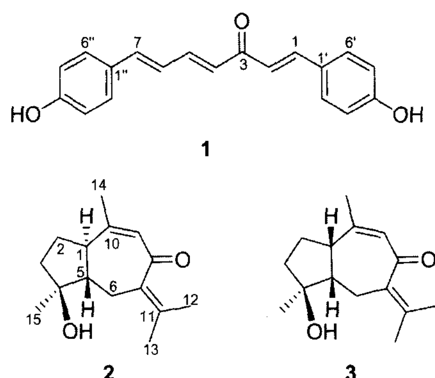

Fig. 1. *Chemical structures of compounds* **1**-**3** *from Curcuma zedoaria*

higher field, and those of C-4, 11, 14, 15 were shifted downfield compared with those of **2**. From these patterns of NMR spectral data, structure of **2** was elucidated as procurcumenol (Yoshihara *et al.*, 1986) and **3** as epiprocurcumenol (Kuroyanagi *et al.*, 1990) that is the epimer of **2** at C-1 as reported previously. The optical rotation data also supported these structural conclusion of **2** and **3** as shown in "extration and isolation" section.

RAW 264.7 cells were stimulated with 1 μg/mL of LPS and the production of NO was increased by the enzymatic reaction of iNOS. When compounds **1**-**3** obtained from *C. zedoaria* were added to the culture media at the time of stimulation of cells, they inhibited the production of NO in a dose-dependent manner. Inhibitory potency of NO production by **1** was strong, and those of **2** and **3** were weak and no difference between. The concentrations required for 50% inhibition of NO production (IC_{50} value) by compounds **1**-**3** were calculated on the basis of concentrations of nitrite released into the culture media (Fig. 2 and Fig. 3), and were found to be 8, 75, 77 μM, respectively. Cell viabilities were assessed to be above 89% by MTT method at the treated concentrations of compound **1** and **2** for nitrite assay. The accumulation of NO was also inhibited by the treatment with 0.1 mM of L-NMMA, an inhibitor of NOS, and the production of NO was restored by addition of excess amount of arginine (1 mM) into the culture media (Fig. 4). But the inhibition of NO production by compound **1** was not restored by addition of arginine (Fig. 4). These data implied that the inhibition of NO production by **1** was not due to the substrate competition for NOS like L-NMMA. When compound **1** (15 μM) was treated after 18 h activation of RAW 264.7 cells, the concentration of NO in the media was 29.1±0.2 μM, while the co-treatment of **1** with LPS resulted in much lower concentration of NO, 5.1±0.1 μM (Fig. 5). The treatment of L-NMMA (0.1 mM) and aminoguanidine (1 mM) after 18 h activation of RAW 264.7 cells resulted in strong inhibition of NO production. These results showed that NOS inhibitors-L-NMMA, an arginine analog, and

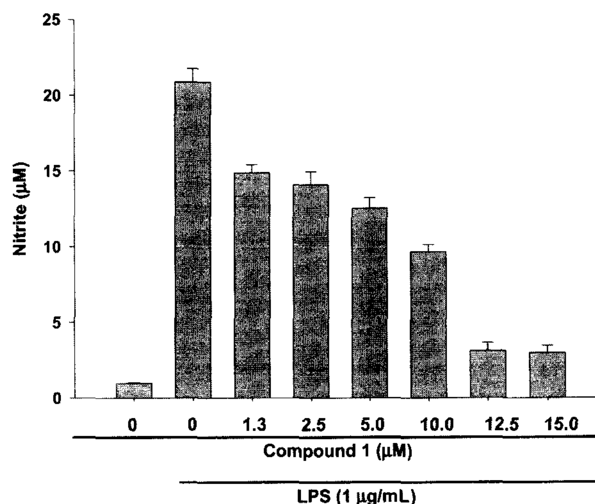

Fig. 2. Compound **1** inhibits the production of NO released into the media of LPS-activated RAW 264.7 cells. Media treated with compound **1** (1.3~15.0 μM) were collected after 18 h activation and the NO_2^- levels were assayed as described *in the materials and methods* section. Results are expressed as mean±S.D. of three experiments.

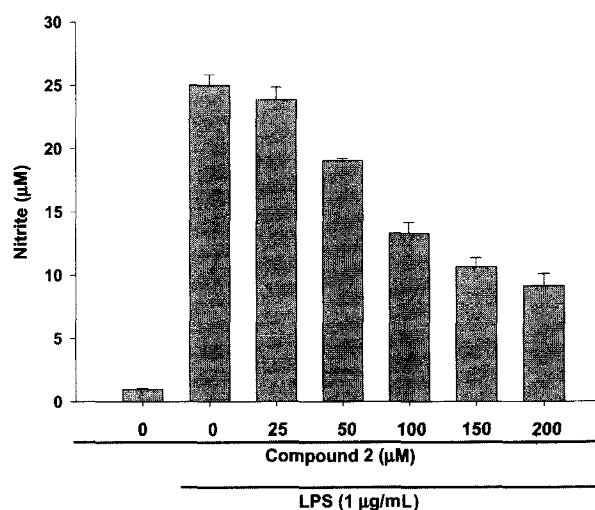

Fig. 3. Compound **2** inhibits the production of NO released into the media of LPS-activated RAW 264.7 cells. Media treated with compound **2** (25~200 μM) were collected after 18 h activation and the NO_2^- levels were assayed as described *in the materials and methods section.* Results are expressed as mean±S.D. of three experiments.

aminoguanidine, an iNOS specific inhibitor, inhibited production of NO by iNOS. However, compound **1** could not inhibit the NO production when treated after the completion of iNOS induction. Thus, compound **1** may be an inhibitor of iNOS induction by LPS in RAW 264.7 cells rather than an inhibitor of NOS. This postulation was reconfirmed in Western blot analysis. The cytosol of RAW 264.7 cells activated by LPS in the presence of compound **1** contained dose dependently reduced amounts of iNOS protein compared with LPS control group (Fig. 6). The

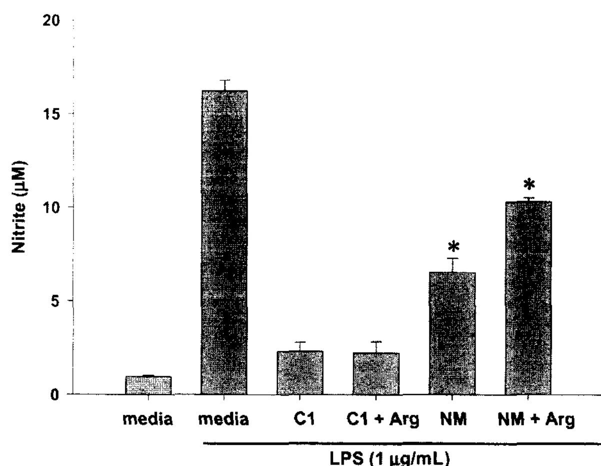

Fig. 4. The inhibition of NO production by compound **1** (C1; 15 μM) and L-NMMA (NM; 0.1 mM), and the effect of addition of L-arginine (Arg; 1 mM). The media were changed with fresh one 18 h after LPS-activation with compound 1 or L-NMMA. After further 18 h incubation with or without addition of L-arginine, the amounts of NO were determined. Results are expressed as mean±S.D. of three experiments. Significant difference between two groups, *p<0.05.

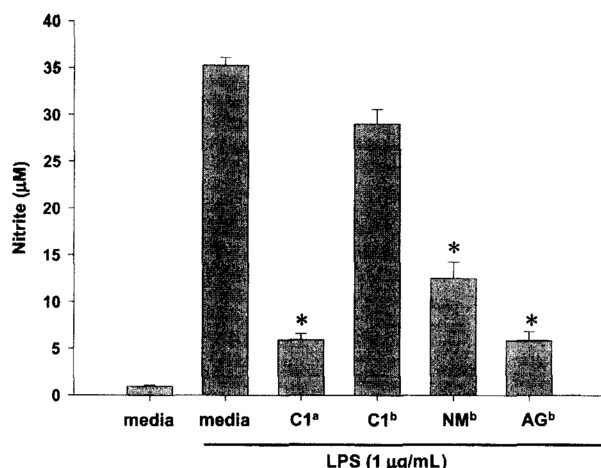

Fig. 5. The effects of compound **1** (C1; 15 μM), L-NMMA (NM; 0.1 mM) and aminoguanidine (AG, 1 mM) on the NO production in LPS-activated RAW 264.7 cells. All the media was changed with fresh one after 18 h LPS-activation. [a]Incubation with compound **1** during 18 h LPS-activation and wash out. [b]Incubation with effectors for another 18 h after LPS-activation. Nitrite assay was performed 36 h post LPS treatment. Results are expressed as mean±S.D. of three experiments. Significant difference from LPS control, *p<0.001.

exact mechanisms of biological activity of *C. zedoaria* were not fully disclosed in this report. We expect that compound **1** from this plant might have biological activities similar to curcumin such as inhibition of nuclear transcription factor kappa B (NF-κB) activation, because of their structural similarity. In combination with the inhibitory activity of iNOS (Matsuda *et al.*, 2001a, 2001b) and PGE_2 production (Hong *et al.*, 2002), *C. zedoaria* can

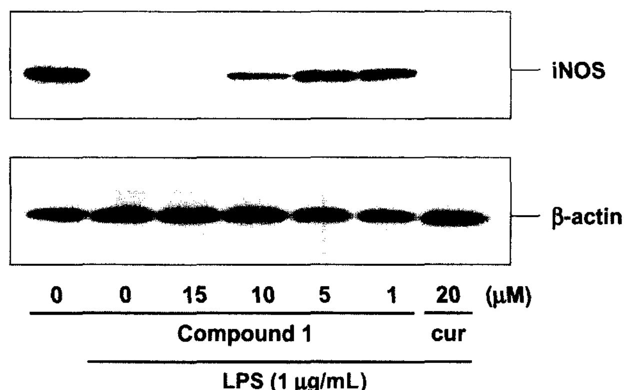

Fig. 6. Western blot analysis of iNOS in cytosolic fraction of RAW 264.7 cells (30 μg protein/lane). Cytosolic fractions were prepared as described in the materials and methods section. Lane 1: LPS-activated cells, lane 2: unactivated cells, lane 3-6 : LPS-activated cells in the presence of 15, 10, 5, 1 μM of compound **1**, lane 7 : 20 μM of curcumin. The expression of iNOS was compared with that of β-actin. Data are representative of three independent experiments with similar results.

be a good candidate for the development of anti-inflammatory drug.

In summary, diarylyheptanoid (**1**) and sesquiterpenoids (**2** and **3**) from *C. zedoaaria* were identified as inhibitors of iNOS in LPS-activated macrophages. These compounds can be the active principles that can explain the traditional use of *C. zedoaria* rhizome as anti-inflammatory drug. These may have potential use in the treatment of endotoxemia and inflammation that accompany the overproduction of NO.

ACKNOWLEDGEMENT

This research was supported by the Sookmyung Womens University Grants 2003.

REFERENCES

Achike, F. I. and Kwan, C. Y., Nitric oxide, human diseases and the herbal products that affect the nitric oxide signaling pathway. *Clin. Exp. Pharmacol. Physiol.*, 30, 605-615 (2003).

Bredt, D. S. and Snyder, S. H., Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, 87, 682-685 (1990).

Chi, Y. S., Cheon, B. S., and Kim, H. P., Effect of wogonin, a plant flavone from Scutellaria radix, on the suppression of cyclooxygenase-2 and the induction of inducible nitric oxide synthase in lipopolysaccharide-treated RAW 264.7 cells. *Biochem. Pharmacol.*, 61, 1195-1203 (2001).

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Anal. Biochem.*,

126, 131-138 (1982).

Hong, C. H., Noh, M. S., Lee, W. Y., and Lee, S. K., Inhibitory effects of natural sesquiterpenoids isolated from the rhizomes of *Curcuma zedoaria* on prostaglandin E_2 and nitric oxide production. *Planta Med.*, 68, 545-547 (2002).

Kim, E. J., Jin, H. K., Kim, Y. K., Lee, H. Y., Lee, S. Y., Lee, K. R., Zee, O. P., Han, J. W., and Lee, H. W., Suppression by a sesquiterpene lactone from *Carpesium divaricatum* of inducible nitric oxide synthase by inhibiting nuclear factor-kappaB activation. *Biochem. Pharmacol.*, 61, 903-910 (2001).

Kuroyanagi, M., Ueno, A., Koyama, K., and Natori, S., Structures of sesquiterpene of *Curcuma aromatica* SALISB. II. Studies on minor sesquiterpenes. *Chem. Pham. Bull.*, 38, 55-58 (1990).

Lowenstein, C. J., Glatt, C. S., Bredt, D. S., and Snyder S. H., Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, 89, 6711-6715 (1992).

Matsuda, H., Morikawa, T., Toguchida, I., Ninomiya, K., and Yoshikawa, M., Medicinal foodstuffs. XXVIII. Inhibitors of nitric oxide production and new sesquiterpenes, zedoarofuran, 4-epicurcumenol, neocurcumenol, gajutsulactones A and B, and zedoarolides A and B, from Zedoariae Rhizoma. *Chem. & Pharm. Bull.*, 49, 1558-1566 (2001a).

Matsuda, H., Morikawa, T., Toguchida, I., Ninomiya, K., and Yoshikawa, M., Inhibitors of nitric oxide production and new sesquiterpenes, 4-epi-curcumenol, neocurcumenol, gajutsulactones A and B, and zedoarolides A and B from Zedoariae Rhizoma. *Heterocycles*, 55, 841-846 (2001b).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55-63 (1983).

Nakayama, R., Tamura, Y., Yamanaka, H., Kikuzaki, H., and Nakatani, N., Two curcuminoid pigments from *Curcuma domestica. Phytochem.*, 33, 501-502 (1993).

Oshima, H. and Bartsch, H., Chronic infectious and inflammation process as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.*, 305, 253-264 (1994).

Ryu, J.-H., Son, H. J., Lee, S. H., and Sohn, D. H., Two neolignans from *Perilla frutescens* and their inhibition of nitric oxide synthase and tumor necrosis factor-α expression in murine macrophage cell line RAW 264.7. *Bioorg. Med. Chem. Lett.*, 12, 649-651 (2002).

Ryu, J.-H., Ahn, H., Kim, J. Y., and Kim, Y.-K., Inhibitory activity of plant extracts on nitric oxide synthesis in LPS-activated macrophages. *Phytother. Res.*, 17, 485-489 (2003).

Syu, W-J, Shen, C-C, Don, M-J, Ou, J-C, Lee, G-H, and Sun, C-M., Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zedoaria. J. Nat. Prod.*, 61, 1531-1534 (1988).

Thiemermann, C. and Vane, J., Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat *in vivo. Eur. J. Pharmacol.*, 182, 591-595 (1990).

Thomsen, L. L., Ching, L. M., and Baguley, B. C., Evidence for the production of nitric oxide by activated macrophages treated with the antitumor agents flavone-8-acetic acid and xanthenone-4-acetic acid. *Cancer Res.*, 50, 6966-6970 (1990).

Yoshihara, M., Yang, C., Zheng, C., Shibuya, H., Hamamoto, Y., Tanaka, N., and Kitagawa, I., Chemical conversion of (4S, 5S)-(+)-germacrone 4,5-epoxide, a plausible biogenetic intermediate found in the essential oil of zedoariae rhizoma from Yakushima, Japan. *Chem. Pharm. Bull.*, 34, 434-437 (1986).

Yoshioka, T., Fujii, E., Endo, M., Wada, K., Tokunaga, Y., Shiba, N., Hohsho, H., Shibuya, H., and Muraki, T., Antiinflammatory potency of dehydrocurdione, a zedoary-derived sesquiterpene. *Inflam. Res.*, 47, 476-481 (1998).