JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Suppression Effect of *Cinnamomum cassia* Bark-Derived Component on Nitric Oxide Synthase

HOI-SEON LEE,* BYUNG-SU KIM, AND MOO-KEY KIM

Research Center for Industrial Development of Biofood Materials and Institute of Agricultural Science & Technology, College of Agriculture, Chonbuk National University, Chonju 561-756, South Korea

The inhibitory effects of *Cinnamomum cassia* bark-derived material on nitric oxide (NO) production in RAW 264.7 cells was determined through the evaluation of NO production and expression of inducible nitric oxide and compared to the effects of three commercially available compounds, cinnamyl alcohol, cinnamic acid, and eugenol. The biologically active constituents of *C. cassia* extract were characterized as *trans*-cinnamaldehyde by spectral analysis. The inhibitory effects varied with both chemical and concentration used. Potent inhibitory effects of cinnamaldehyde against NO production were 81.5 and 71.7% at 1.0 and 0.5 μg/μL, respectively, and a 41.2% inhibitory effect was revealed at 0.1 μg/μL. However, little or no activity was observed for cinnamic acid and eugenol. Suppression effects of cinnamaldehyde on inducible nitric oxide synthase expression were revealed by Western blot analysis. As a naturally occurring therapeutic agent, *trans*-cinnamaldehyde could be useful for developing new types of NO inhibitors.

KEYWORDS: NO production; *Cinnamomum cassia*; cinnamaldehyde; inducible nitric oxide synthase; supression

INTRODUCTION

Nitric oxide (NO), an inorganic gas, plays a number of diverse physiological and pathological roles in mammalian systems, such as immune regulation (*1*), smooth muscle relaxation (*2*), neurotransmission (*3*), and inhibition of platelet aggregation (*4*). Generally, a regulated NO production has several essential roles, but unregulated NO production can cause acute lung disease, septic shock, and cell death through oxidative stress, disrupted energy metabolism, or DNA damage (*5*−*8*). NO is produced by three isoforms of nitric oxide synthase (NOS), two of which are constitutive forms and the other an inducible form (*6*). Constitutive forms are predominantly expressed in neurones (nNOS) and endothelial tissue (eNOS). The major function of constitutive NOS is to regulate vascular tone and to be retrograde, signaling across synapses. The inducible form (iNOS) can be upregulated considerably in immune cells and many other tissues, where it is required for the cellular immune response. iNOS produces much greater amounts of NO than constitutive NOS and appears to be the "pathophysiological" form of the enzyme because a high concentration of NO is associated with inflammatory diseases, mutagenesis, and formation of carcinogenic *N*-nitrosamines (*5*, *7*, *8*).

The study of NO has created considerable interest in the identification of inhibitors of NOS with potential clinical use in humans (*9*). However, early enthusiasm was tempered by reports in the literature testifying to the widespread vasoconstriction, elevation of serum cholesterol and triglyceride, lowering of serum level of nitrate, and secondary malignancies induced by NOS inhibitors when administered to both animals and humans (*10*, *11*). Decreasing efficacy and increasing concern over possible adverse effects of NOS inhibitors have brought about the need for the development of new types of selective alternatives with lower toxicity and more inhibitory effects. Plant extracts may be an alternative to currently used NOS inhibitors because they constitute a rich source of bioactive chemicals (*9*, *11*−*13*). Since many of them are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer agents of NOS inhibitors. Additionally, some of the plant-derived materials are found to be more effective against NOS than the current chemotherapeutic agents (*9*, *11*−*13*). Therefore, much effort has been focused on plants for potentially useful products as commercial NOS inhibitors or lead compounds. We already reported that, among 30 oriental medicinal plant species, methanol extract of *Cinnamomum cassia* bark revealed potent inhibitory response to NO production against iNOS (*12*). This plant species is not only important as a spice, but in East Asia is considered to have some medicinal properties, such as antibacterial, antipyretic, astringent, carminative, and stomachic agents (*14*−*16*). However, relatively little work has been carried out on the inhibitory responses of *C. cassia* bark-derived materials against iNOS, despite its excellent pharmacological action. To develop new and potentially safer types of iNOS inhibitors, this study has been performed to investigate the inhibitory effect of *C. cassia* bark-derived materials on iNOS

* Corresponding author. Phone: +82-63-270-2544. Fax: +82-63-270-2550. E-mail: hoiseon@moak.chonbuk.ac.kr.

10.1021/jf020751f CCC: $22.00
Published on Web 11/15/2002

in murine peritoneal macrophages after stimulation by lipopolysaccharide (LPS).

MATERIALS AND METHODS

Chemicals. Acrylamide, ϵ-amino-*n*-caproic acid, bovine serum albumin, brilliant blue G-250, cinnamyl alcohol, cinnamic acid, ethylenediamine acid, eugenol, leupeptin, lipopolysaccharide from *Escherichia coli* serotype O111:B4, *N*,*N*′-methylene-bis-acrylamide, sodium dodecyl sulfate, sodium nitrite, sulfanilamide, and trypsin inhibitor type II (soybean) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum, penicillin, RPMI 1640, skim milk dehydrate, and streptomycin were supplied by Gibco (Gaithersburg, MD). Goat anti-rabbit IgG(H+L)-AP conjugate was from Bio-Rad Laboratories (Hercules, CA), and rabbit anti-mouse macNOS was from Transduction Laboratories (Rockville, MD). All other chemicals were of reagent grade.

Isolation of Murine Peritoneal Macrophage Cells. Macrophages were isolated from peritoneal exudate cells following previous protocols (*17*). Thioglycolate-elicited peritoneal exudate cells were obtained from 8−10-week-old ICR male mice by intraperitoneal injection of 1 mL of Brewer thioglycolate broth (4.05% w/v) and lavage of the peritoneal cavity with 5−6 mL of RPMI 1640 medium 3 days later. The cells were washed twice and resuspended in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM glutamine, penicillin (200 IU/mL), and streptomycin (200 IU/mL). Peritoneal exudate cells were seeded at densities of 1×10^5 cells/well in 96-well tissue culture plates or 1×10^7 cells/dish on 6-cm tissue culture dishes, and the macrophages were allowed to adhere for 2 h at 5% humidified atmosphere. The nonadherent cells were removed by pouring off the medium and rinsing the wells twice with prewarmed medium. The adherent cells were incubated under the conditions appropriate for individual experiments.

Treatment of Macrophages with LPS. Peritoneal macrophages were incubated in 96-well tissue culture plates (1×10^6 cells/mL) or on 6-cm tissue culture dishes (1×10^7 cells/dish) with LPS (10 μg/mL) for 24 h at 37 °C in a 5% CO_2−air incubator. The supernatant was harvested and assayed for production of nitrite.

Isolation and Identification. The bark from *C. cassia* (3.6 kg), purchased as a commercially available product, was dried in an oven at 60 °C for 2 d, finely powdered, extracted twice with methanol (10 L) at room temperature, and filtered (Toyo filter paper No. 2, Japan). The combined filtrate was concentrated in vacuo at 35 °C to yield about 10.4% (based on the weight of the bark). The extract (20 g) was sequentially partitioned into hexane (3.9 g), chloroform (4.5 g), ethyl acetate (1.9 g), and water-soluble (9.7 g) portions for subsequent bioassay against iNOS. The organic solvent portions were concentrated to dryness by rotary evaporation at 35 °C, and the water portion was freeze-dried.

The hexane portion (10 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 500 g, 5.5 mm i.d. × 70 cm) and successively eluted with a stepwise gradient of hexanes−ethyl acetate (0, 10, 30, 50, 80, and 100%). The active 50% fraction (4.1 g) was chromatographed on a silica gel column and eluted with hexanes−ethyl acetate (2:1). Twenty-five column fractions were collected and analyzed by TLC (hexanes−ethyl acetate, 3:1). Fractions with similar TLC patterns were combined. The active fraction (2.4 g) was chromatographed on a silica gel column and eluted with hexanes−ethyl acetate (8:2). For further separation of the bioactive substance(s), preparative HPLC (Waters Delta Prep 4000) was used. The column was 29 mm i.d. × 300 mm Bondapak C_{18} (Waters), using methanol−water (3:7) at a flow rate of 10 mL/min and detection at 260 nm. Finally, a potent active principle (0.1 g) was isolated. Structural determination of the active isolate was based on spectral analysis. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer, and chemical shifts are given in ppm. UV spectra were obtained on a Waters 490 spectrometer, IR spectra on a Bio-Rad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Cell Stimulation and Samples Cotreatment. RAW 264.7 cells were stimulated with LPS (1 mg/mL) and IFN-γ (10 units/mL) with or without samples, as mentioned above. Supernatant was then harvested and assayed for production of nitrite. The cells incubated on 6-cm culture dishes were scrapped and collected. The cells were then resuspended in 500 μL of sonication buffer. Cells were disrupted by 10-s sonication, and the sonicate was centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was used as cytosol fraction in Western blot analysis.

Nitrite and Protein Determination. NO production was measured by estimating the stable NO metabolite, nitrite, in conditioned medium by the Griess reaction (*18*). Cell-free supernatant (100 μL) was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid:0.1% naphthylethylenediamine dihydrochloride = 1:1) and incubated at room temperature for 15 min. After incubation, the absorbance of the wells was determined by using a microplate reader (Multiscan MCC/340 P version 2.3) equipped with a 540-nm filter. Concentrations of nitrite were determined from a linear regression analysis of standards (sodium nitrite) generated for each plate. Protein contents in the cytosol were measured using the Bradford method (*19*), with bovine serum albumin as a standard.

SDS−Polyacrylamide Gel Electrophoresis and Western Blot Analysis. The sonicated cells (1 protein) were subjected to electrophoresis on 1.5-mm-thick 15% polyacrylamide gel. The separated proteins were transferred to PVDF membrane using Trans-Blot. The membrane was blocked for 30 min at room temperature with 5% skim milk in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20 and was then incubated with anti-iNOS antibody (1:2000 dilution) in blot buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% skim milk, and 0.05% Tween 20) overnight at 4 °C. After the membrane was washed twice in blot buffer, it was incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000 dilution) in blot buffer for 2 h at room temperature and then washed three times in blot buffer for 3 min and once in TBS for 20 min. The membrane was then incubated with alkaline phosphatase substrate for 1−10 min, and the immunoreactive bands were regarded as iNOS protein, molecular mass 130 kDa.

Cell Viability. After incubation, the medium was poured off, and macrophages were incubated with samples to concentrations of 0.1, 0.5, 1, and 5 mg/μL for 4 h at 37 °C. Medium was then removed, and 100 μL of DMSO was added to all wells to solubilize formazan crystals. Optical densities were measured at 540 nm with a microplate reader.

RESULTS AND DISCUSSION

The controlled production of NO has important roles in the human nervous, immune, and vascular systems. However, unregulated excessive production of NO may cause abnormal physical conditions or diseases. In this regard, *C. cassia* bark, used not only for flavoring foods but also as antiallergic and medicines (*16*, *20*), was investigated to evaluate and isolate the inhibitor of NO production. In primary screening, the inhibitory response of methanol extracts of *C. cassia* bark on NO production in RAW 264.7 cells after stimulation by LPS (1 μg/mL) and IFN-γ (10 units/mL) was tested at 10, 5, and 1 μg/μL using Griess assay (**Figure 1**). In a previous experiment, it was reported that a concentration of 0.1−10 μg/μL of *C. cassia* bark extract did not cause any cytotoxic effect through MTT assay (*21*). In a test with 10 μg/μL, a very strong inhibitory effect (91.3%) on NO production by the methanol extract of *C. cassia* bark was shown. Furthermore, a moderate inhibitory effect (51.8%) was detected at 1 μg/μL.

Based on the strong inhibitory effect of *C. cassia* bark extract, the inhibitory responses of each fraction from *C. cassia* bark extracts were evaluated at low concentrations of 0.5 and 1 μg/μL for the application (**Table 1**). In fractionation, guided by the inhibitory effect of NO production, the hexane fraction of methanol extracts showed more than 50% inhibitory effect at 0.5 μg/μL. Little or no activity was present in the chloroform, ethyl acetate, butanol, and water fractions. This result indicated that the active component for the inhibition of NO production may exist in the hexane fraction from the methanol extract of

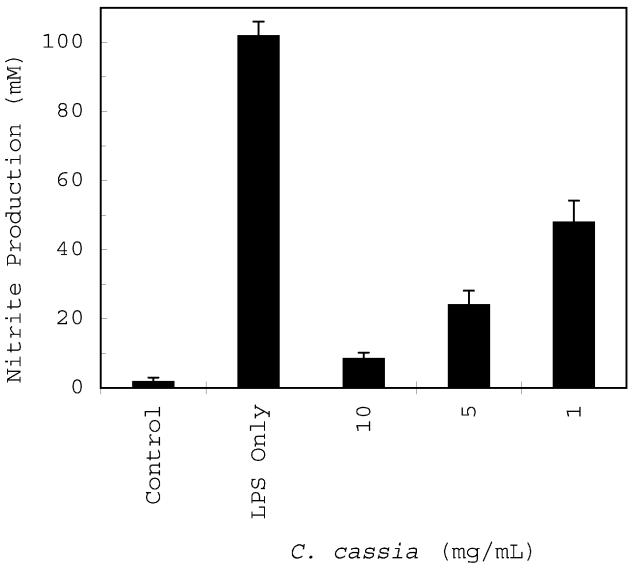

Figure 1. Inhibitory effect of *C. cassia* bark extracts on nitrite release from LPS-treated macrophages. Macrophages (1×10^5 cells/dish) were incubated with LPS (1 μg/mL) and IFN-γ (10 units/mL) on a 96-well tissue culture plate in the absence or presence of *C. cassia* bark extracts for 24 h. After incubation, NO content in the supernatant (100 μL) was measured using the Griess reagent. Results are the means ± S.E. of four separate experiments.

Table 1. Inhibitory Effects of Fractions of Methanol Extract from *C. cassia* Bark on Nitrite Release from LPS-Treated Macrophages[a]

	inhibitory effect (% control)	
fractions	0.5 μg/μL	1.0 μg/μL
hexane	49.9 ± 6.2	30.6 ± 1.2
chloroform	100.5 ± 4.1	102.4 ± 2.6
ethyl acetate	100.3 ± 2.8	101.8 ± 5.4
butanol	103.5 ± 4.8	103.2 ± 6.9
water	101.7 ± 4.1	100.5 ± 4.6

[a] Macrophages (1×10^5 cells/dish) were incubated with LPS (1 mg/mL) and IFN-γ (10 units/mL) on a 96-well tissue culture plate in the absence or presence of fractions of *C. cassia* bark extracts for 24 h. After incubation, NO content in the supernatant (100 mL) was measured using the Griess reagent. Results are the means ± S.E. of four separate experiments.

C. cassia bark. Therefore, the hexane fraction was purified by using silica gel column chromatography and HPLC, and the isolates were bioassayed. One active component was isolated. Structural determination of the isolate was made by spectroscopic analysis, including EI-MS and NMR, and by direct comparison with an authentic reference compound, and it was characterized as *trans*-cinnamaldehyde. The compound was identified on the basis of the following evidence: C_9H_8O (MW, 132); EI-MS (70 eV), *m*/*z* (relative intensity), M^+ 132 (3), 103 (2), 74 (83), 59 (100), 58 (75); IR (neat) max (cm^{-1}) 2920, 1680, 1630, 1130; ^1H NMR (CD_3OD, 400 MHz) δ 6.60 (dd, J = 8 and 18 Hz), 7.35 (d, J = 18 Hz), 7.1−7.7 (m), 9.52 (d, J = 8 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 195.6, 154.4, 135.0, 132.1, 129.9, 129.7, 129.5, 129.0, 128.9.

The inhibitory effects of cinnamaldehyde and other components of this plant species against NO production were determined (**Table 2**). Responses varied with the chemical and concentration tested. Cinnamaldehyde exhibited 71.7 and 41.2% inhibitory effect at 0.5 and 0.1 μg/μL, respectively, and cinnamyl alcohol showed 61.1 and 39.6% inhibitory effect at 1 and 0.5 μg/μL. However, little or no activity was observed for cinnamic acid and eugenol. The inhibitory effect of cinnamaldehyde was more potent than that of cinnamic acid, cinnamyl alcohol, and eugenol. These results indicate that the inhibitory effect of *C. cassia* extract against NO production is caused by cinnamaldehyde.

Table 2. Inhibitory Effects of *C. cassia* Bark-Derived Materials on Nitrite Release from LPS-Treated Macrophages[a]

	inhibitory effect (% control)		
compounds	0.1 μg/μL	0.5 μg/μL	1.0 μg/μL
cinnamaldehyde	59.8 ± 3.3	29.3 ± 4.1	19.5 ± 1.5
cinnamic acid	101.4 ± 3.2	100.6 ± 2.8	96.8 ± 5.4
cinnamyl alcohol	81.2 ± 4.7	61.4 ± 2.9	39.9 ± 3.2
eugenol	103.8 ± 5.5	100.6 ± 3.9	101.8 ± 4.2

[a] Macrophages (1×10^5 cells/dish) were incubated with LPS (1 mg/mL) and IFN-γ (10 units/mL) on a 96-well tissue culture plate in the absence or presence of fractions of *C. cassia* bark extracts for 24 h. After incubation, NO content in the supernatant (100 mL) was measured using the Griess reagent. Results are the means ± S.E. of four separate experiments.

Figure 2. Western blot analysis of iNOS in RAW 264.7 cells treated with *trans*-cinnamaldehyde isolated from *C. cassia*. Immunoblot. The cells were stimulated with LPS (1 μg/mL) plus IFN-γ (10 units/mL) and incubated for 16 h in the presence of *trans*-cinnamaldehyde. Cont$^-$, unstimulated; Cont$^+$, stimulated.

To clarify the mechanism of NO inhibition by *trans*-cinnamaldehyde, iNOS protein expression was analyzed in cellular extracts from RAW 264.7 cells stimulated with LPS and IFN-γ, with or without this inhibitor. In general, iNOS was sufficiently expressed by the stimulation of LPS and IFN-γ. **Figure 2** shows that the combination of LPS and IFN-γ was a strong inducer of iNOS protein expression in RAW 264.7 cells. However, *trans*-cinnamaldehyde isolated from *C. cassia* bark significantly decreased the iNOS expression dose-dependently in LPS/IFN-γ-stimulated RAW 264.7 cells. This component almost completely blocked iNOS protein expression at 20 μM. Furthermore, in the test with 10 μM *trans*-cinnamaldehyde, the suppression effect on iNOS expression appeared to compare well with that of positive control (**Figure 2**).

It is now apparent that NO generated by iNOS is cytotoxic or cytostatic at high concentrations. Excess NO causes many problems such as oxidative stress, disruption of energy metabolism, and calcium homeostasis. NO interacts with mitochondria in a number of ways that are central to its role in cell death (*22*, *23*). Externally produced NO diffuses easily into mitochondria and leads to inactivation of enzymes in the mitochondrial electron transport chain. NO binds directly to and inhibits several other heme enzymes, such as cytochrome oxidase, catalase, and cytochrom P-450, and reacts with iron to form iron−nitrosyl complexes in vivo (*24*, *25*). Excess NO also damages DNA, with $ONOO^-$ causing single strand breaks (*26*, *27*). DNA damage caused by NO activates p53, which leads to apoptosis independently of its transcriptional action (*28*). Recently, NO has been implicated in a number of pathological conditions, including graft-versus-host disease and experimentally induced neurological diseases (*29*). In most cases, the pathologies were significantly reduced through inhibiting NO synthesis in vivo by treating the animals with NO inhibitor derived from plants. In this study, the active component against iNOS isolated from *C. cassia* bark was identified as *trans*-cinnamaldehyde, although the inhibitory responses varied with

chemical and concentration tested. It has been reported previously that the *C. cassia* bark-derived materials including cinnamaldehyde, cinnamic acid, cinnamyl alcohol, and eugenol have antibacterial, antitumor, astringent, carminative, and stomachic effects (*14*, *15*, *30*−*32*). In this regard, it might be expected that *C. cassia* bark-derived cinnamaldehyde would be show at least one type of pharmacological action for the suppression of iNOS.

In conclusion, *trans*-cinnamaldehyde has a potent activity of suppressing excess NO production through the action of inhibitory responses against iNOS expression. Based upon our limited data and some earlier findings, the inhibitory action of cinnamaldehyde confirms its superiority and usefulness as an iNOS inhibitor, although its in vivo efficacy and clinical usefulness remain to be evaluated. Further research to identify the relationship between NO signal transduction and *trans*-cinnamaldehyde is in progress.

LITERATURE CITED

(1) Schneemann, M.; Schoedon, G.; Frei, K.; Schaffner, A. Immunovascular communication: activation and deactivation of murine endothelial cell nitric oxide synthase by cytokines. *Immunol. Lett.* **1993**, *35*, 159−162.

(2) Takehara, Y.; Nakahara, H.; Okada, S.; Yamaoka, K.; Hamazaki, K.; Yamazato, A.; Inoue, M.; Utsumi, K. Oxygen concentration regulates NO-dependent relaxation of aortic smooth muscles. *Free Radical Res.* **1999**, *30*, 287−294.

(3) Vizi, E. S. Nitric oxide in neurotransmission. *Trends Pharmacol. Sci.* **2001**, *22*, 595.

(4) Radomski, M. W.; Palmer, R. M.; Moncada, S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **1987**, *7*, 1057−1058.

(5) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−141.

(6) Forstermann, U.; Schmidt, H. H. H. W.; Pollack, J. S.; Sheng, H.; Mitchell, J. A.; Warner, T. D.; Nakane, M.; Murad, F. Isoforms of nitric oxide synthase. *Biochem. Pharmacol*. **1991**, *42*, 1849−1857.

(7) Marletta, M. A. Nitric oxide synthase structure and mechanism. *J. Biol. Chem*. **1993**, *268*, 12231−12234.

(8) Schmidt, H. H. W.; Lohmann, S.M.; Walter, U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim. Biophys. Acta*. **1993**, *1178*, 153−175.

(9) Philip, K. M.; Philip, A. B.-W. 7-Nitroindazole: an inhibitor of nitric oxide synthase. *Methods Enzymol*. **1996**, *268*, 393−398.

(10) Khedara, A.; Goto, T.; Kayashita, J.; Kato, N. Hypercholesterolemic effect in rats of a dietary addition of the nitric oxide synthase inhibitor, L-N-nitroarginine, by less synthesis of bile acids. *Biosci. Biotechnol. Biochem*. **1998**, *62*, 773−777.

(11) Lohinai, Z.; Benedek, P.; Feher, E.; Gyorfi, A.; Rosivall, L.; Fazekas, A.; Salzman, A. L.; Szabo, C. Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. *Br. J. Pharm*. **1998**, *123*, 353−360.

(12) Lee, H. S.; Lee, S. H.; Kim, Y. S.; Song, C. G.; Kim, M. K. Effect of oriental medicinal plants on nitric oxide synthase activity. *Food Sci. Biotechnol*. **2000**, *9*, 77−82.

(13) Colegte, S. M.; Molyneux, J. J. *Bioactive Natural Products: Detection, Isolation and Structure Determination*; CRC Press: Boca Raton, FL, 1993.

(14) Namba, T. *Coloured illustrations of wakan-yaku: the crude drugs in Japan, China and the neighbouring countries*; Hoikusha Publishing: Osaka, Japan, 1986.

(15) Lee, H. S.; Ahn, Y. J. Growth-inhibiting effects of *Cinnamomum cassia* bark-derived materials on human intestinal bacteria. *J. Agric. Food Chem*. **1998**, *46*, 8−12.

(16) Kurokawa, M.; Kumeda, C. A.; Yamamura, J.; Kamiyama, T.; Shiraki, K. Antipyretic activity of cinnamy derivatives and related compounds in influenza virus-infected mice. *Eur. J. Pharmacol.* **1998**, *348*, 45−51.

(17) Kirikae, T.; Kirikae, F.; Oghiso, Y.; Nakano, M. Micortubule-disrupting agents inhibit nitric oxide production in murine peritoneal macrophages stimulated with lipopolysaccharide or paclitaxel (Taxol). *Infect. Immun*. **1996**, *64*, 3379−3384.

(18) Biller, T. B.; Curran, R. D.; Stuehr, D. J.; West, M. A.; Bentz, B. G.; Simmons, R. L. An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis in vivo. *J. Exp. Med*. **1989**, *169*, 1467−1472.

(19) Bradford, M. E. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem*. **1976**, *42*, 248−254.

(20) Chopra, R. N.; Nayer, S. L.; Chopra, I. C. *Glossary of Indian Medicinal Plants*; Council of Scientific and Industrial Research: New Delhi, 1980; pp 51−55.

(21) Koh, W. S.; Yoon, S. Y.; Kwon, B. M.; Jeong, T. C.; Nam, K. S.; Han, M. Y. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. *Int. J. Immunopharmacol.* **1998**, *20*, 643−660.

(22) Bolanos, J. P.; Almeida, A.; Stewart, V.; Peuchan, S.; Land, J. M.; Clark, J. B.; Heales, S. J. R. Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. *J. Neurochem*. **1997**, *68*, 2227−2240.

(23) Packer, M. A.; Scarlett, J. L.; Martin, S. W.; Murphy, M. P. Induction of the mitochondrial permeability transition by peroxynitrite. *Biochem. Soc. Trans*. **1997**, *25*, 831−836.

(24) Kerwin, J. F.; Lancaster, J. R.; Feldman, P. L. Nitric oxide: a new paradigm for second messengers. *J. Med. Chem*. **1995**, *38*, 4343−4362.

(25) Butler, A. R.; Flitney, F. W.; Williams, D. L. H. NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron nitrosyls in biology: a chemist's perspective. *Trends Pharmacol. Sci.* **1995**, *16*, 18−22.

(26) Salgo, M. G.; Stone, K.; Squadrito, G. L.; Battista, J. R.; Pryor, W. A. Peroxynitrite causes apoptosis in rat thymocytes. *Biochem. Biophys. Res. Commun.* **1995**, *210*, 1025−1030.

(27) Wink, D. A.; Kasprzak, K. S.; Maragos, C. M.; Elespuru, R. K.; Misra, M.; Dunams, T. M.; Cebula, T. A.; Koch, W. H.; Andrews, A. W.; Allen, J.; Keefer, L. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **1991**, *254*, 1001−1003.

(28) Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **1997**, *88*, 323−331.

(29) Doyle, M. P.; Hockstra, J. W. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Chem*. **1981**, *14*, 351−358.

(30) Kim, M. K.; Lee, S. E.; Lee, H. S. Growth-inhibiting effects of Brazilian and oriental medicinal plants on human intestinal bacteria. *Agric. Chem. Biotechnol*. **2000**, *43*, 54−58.

(31) Kim, M. K.; Park, B. S.; Kim, B. S.; Lee, H. S. Growth-inhibiting effects of herb plants on human intestinal bacteria. *Agric. Chem. Biotechnol*. **2001**, *44*, 185−189.

(32) Kim, M. K.; Kim, S. Y.; Lee, H. S. Rat lens aldose reductase inhibitory activities of oriental medicinal plants. *Agric. Chem. Biotechnol*. **2002**, *45*, 84−88.

Received for review July 9, 2002. Revised manuscript received September 20, 2002. Accepted September 20, 2002. This research was supported by Research Center for Industrial Development of Biofood Materials, Chonbuk National University, Chonju, Korea. Research Center for Industrial Development of Biofood Materials is designated as a Regional Research Center appointed by the Korea Science and Engineering Foundation (KOSEF), Chollabukdo Provincial Government, and Chonbuk National University.

JF020751F