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Article

Chemical Composition of *Cinnamomum cassia* Leaf Oils and Suppression Effect of Cinnamyl Alcohol on Nitric Oxide Synthase

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The inhibitory effects of components derived from *Cinnamomum cassia* leaf on nitric oxide (NO) production in RAW 264.7 cells and on expression of inducible NO synthase (iNOS) were determined and compared to those of three commercially available components. Spectral analysis characterized the active constituent of this *C. cassia* extract as cinnamyl alcohol. The inhibitory effects varied with both chemical and concentration used. Cinnamyl alcohol showed inhibitory effects on NO production of 62.5%, 41.8%, and 22.1% at 1.0, 0.5, and 0.1 μg/μL, respectively. However, little or no inhibitory effect was observed for cedrene, cinnamic acid, copaene, eugenol, and limonene. Suppression effects of cinnamyl alcohol on iNOS expression were revealed by Western blot analysis. As a naturally occurring therapeutic agent, cinnamyl alcohol could be useful for developing new types of NO inhibitors.

Key words: Cinnamomum cassia, *cinnamyl alcohol, inducible nitric oxide synthase, NO production, NO suppression*

Nitric oxide (NO) plays diverse pathological and physiological roles in immune regulation [Schneemann *et al*., 1993], neurotransmission [Vizi, 2001], and smooth muscle relaxation [Takehara *et al*., 1999]. Regulated NO production has several roles in the normal human system, but unregulated NO production can cause DNA damage, cell death through oxidative stress, disrupted energy metabolism, and lung disease [Moncada *et al*., 1991; Marletta, 1993; Schmidt *et al*., 1993]. Regulated and unregulated NO is produced by the two constitutive NO synthases (cNOS) and by inducible NO synthase [Forstermann *et al*., 1991]. Constitutive NOS is expressed in endothelial tissue (eNOS) and in neurons (nNOS); it regulates vascular tone and is involved in retrograde signaling across synapses. Inducible NOS (iNOS) can be up-regulated in immune cells and many other tissues. iNOS produces much greater amounts of NO than cNOS does , and, due to the fact that excessive NO is associated with formation of carcinogenic *N*-nitrosamines and inflammatory diseases [Moncada *et al*., 1991; Schmidt *et al*., 1993], iNOS appears to be the "pathophysiological" form of the enzyme. Research into NO has led to considerable interest in the identification of NOS inhibitors with clinical applicability in humans [Philip and Philip, 1996]. Furthermore, the decreasing efficacy of current NOS inhibitors and increasing concern over their adverse effects indicate a need for the development of selective alternatives with lower toxicity and greater effectiveness.

Medicinal plants may be an alternative to currently used NOS inhibitors because they are a rich source of bioactive components [Colegte and Molyneux, 1993; Philip and Philip, 1996; Lohinai *et al*., 1998; Lee *et al*., 2000; 2002]. Some medicinal plants are pharmacologically active and free from adverse effects. Because of this, new classes of NOS inhibitors could be developed from them. Some of these plant-derived components have been found to be more effective against NOS than are current chemotherapeutic agents [Colegte and Molyneux, 1993; Philip and Philip, 1996; Lohinai *et al*., 1998; Lee *et al*., 2000; 2002]. Therefore, much attention has been focused on medicinal plants products that may be useful as NOS inhibitors. I and my colleagues already reported that cinnamaldehyde isolated from extract of *Cinnamomum*

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Abbreviations: IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopoly saccharide; NO, nitric oxide

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cassia bark revealed a potent inhibitory response to NO production against iNOS [Lee *et al*., 2002]. *C. cassia* bark is not only important as a spice, but is suggested to have some pharmacological properties, including antibacterial, anticoagulant, antidiabetic, astringent, and carminative [Namba, 1986; Lee and Ahn, 1998; Lee, 2007]. However, relatively little work has been carried out on other *C. cassia*-derived components' abilities to inhibit iNOS. To develop new types of iNOS inhibitors, this study investigated iNOS inhibition by some components of *C. cassia* in murine peritoneal macrophages after stimulation by LPS and IFN-γ.

Materials and Methods

Chemicals. Acrylamide, ε-amino-*n*-caproic acid, bovine serum albumin, brilliant blue G-250, cinnamaldehyde, cinnamyl alcohol, cinnamic acid, α-copaene, ethylene diamine acid, eugenol, leupeptin, limonene, lipopolysaccharide from *E. 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 (St. Louis, MO). Cadinene was obtained from Aldrich (Milwaukee, WI, USA). Fetal bovine serum, penicillin, RPMI 1640, skim milk dehydrate, and streptomycin were supplied by Gibco (Gaithersburg, NM). Goat anti-rabbit IgG(H+L)-AP conjugate was purchased from Bio-rad laboratories (Hercules, CA), and rabbit anti-mouse macNOS, from Transduction laboratories (Rockville, MD). All other chemicals were of reagent grade.

Murine peritoneal macrophage cells. Macrophages were purified from peritoneal exudate cells in accordance with established protocols [Kirikae *et al*., 1996; Lee *et al*., 2000]. Thioglycollate-elicited peritoneal exudate cells were acquired from 8- to 10-week old ICR male mice via intraperitoneal injection with 1 mL Brewer Thioglycollate broth (4.05% w/v) and lavage of the peritoneal cavity with 5 mL of RPMI 1640 medium 3 days later. The cells were washed twice and re-suspended 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 at 1×10^7 cells/dish on 6 cm tissue culture dishes and incubated 2 h in a 5% humidified atmosphere, to allow macrophages to adhere to the surfaces. The nonadherent cells were removed by pouring off the medium and rinsing the wells twice with pre-warmed medium. The adherent cells were incubated under the appropriate conditions for each of the individual experiments.

Extraction. Extracts were prepared from leaves of *C. cassia* (1 kg) purchased as a commercially available product. These were dried in an oven at 60°C for 2 days, finely powdered, then extracted twice with methanol (3 L), twice with ethanol (3 L), or twice with water (2 L) at room temperature and then filtered. The methanol and ethanol extracts were concentrated to dryness by rotary evaporation at 35°C, and the water extract was freeze-dried, for subsequent bioassay against NO production. Essential oil of *Cinnamomum cassia* leaves was purchased from Tairawhiti Pharmaceuticals Co. Ltd, New Zealand.

Gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the components of essential oil from *C. cassia* leaves was performed using a gas chromatograph (Shimadzu GC-2010)-mass spectrometer (Shimadzu GC-MS QP-2010, Shimadzu, Milan, Italy). The capillary column and temperature conditions for the GC-MS analyses were the same as described above for GC analysis. Helium carrier gas was used at a column head pressure of 26.9 kPa. The ion source temperature was 200°C, and the injector temperature was 170°C. The interface was kept at 280°C, and mass spectra were obtained at 70 eV. The effluent of the capillary column was introduced directly into the ion source of the mass spectrometer. The sector mass analyzer was set to scan from 50 to 500 amu every 0.5 sec. The components of the essential oil of *C. cassia* leaves were identified by comparison of mass spectra of each peak with those of authentic samples from a mass spectra library (The Wiley Registry of Mass Spectral Data, 6th ed.).

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 (1 μg/mL) for 24 h at 37°C in a 5% CO_2-air incubator [Lee *et al*., 2000]. The supernatant was harvested and assayed for nitrite production.

Cell stimulation and samples co-treatment. RAW 264.7 cells were stimulated with LPS (1 μg/mL) and IFN-γ (10 U/mL) with or without sample concentrations of cinnamyl alcohol [Lee *et al*., 2000]. The supernatants were subsequently harvested and assayed for nitrite production. The cells were incubated on 6 cm culture dishes and then scraped and collected. Next, the cells were resuspended with 500 μL of sonication buffer. The cells were disrupted via 10 sec of sonication, and the sonicates were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were used as cytosol fractions in Western blot analysis.

Nitrite and protein determination. NO production was assessed by estimating the stable NO metabolite, nitrite, in conditioned medium via the Griess reaction [Billiar *et al*., 1989; Lee *et al*., 2000]. Cell-free supernatants (100 μL) were mixed with 100 μL Griess

reagent (1% sulfanilamide in 5% phosphoric acid:0.1% naphthylethylenediamine dihydrochloride=1:1) and incubated for 15 min at room temperature. Following incubation, the absorbance of the wells was determined by means of a microplate reader (Multiscan MCC/ 340 P version 2.3, Labsystems, Finland) equipped with a 540 nm filter. Nitrite concentrations were determined via a linear regression analysis of standards (sodium nitrite) generated for each plate. Protein contents in the cytosol were measured via the Bradford method [Bradford, 1976], using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and western blot analysis. The sonicated cells were run on 15% polyacrylamide electrophoretic gels, 1.5 mm thick. The separated proteins were transferred to PVDF membranes using Trans-Blot [Lee *et al*., 2000; 2002]. The membranes were blocked for 30 min at room temperature with 5% skim milk containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20, and were subsequently incubated overnight, at 4°C, 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). The membranes were then washed twice in blot buffer and incubated for 2 h, at room temperature, with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000 dilution) followed by three washings in blot buffer for 3 min, and one in TBS for 20 min. The membranes were incubated for an additional 1-10 min with alkaline phosphatase substrate, and the immunoreactive bands were identified as iNOS protein, with a molecular weight of 130 kDa.

Cell viability. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan [Lee *et al*., 2000; 2002]. Thus, the cells were seeded at 1×10^5 cells/well in a 96-well plate and treated with LPS/IFN-γ and cinnamyl alcohol. After incubation for 20 hrs, the medium was removed by pouring off and macrophages were incubated with MTT (0.1 mg/mL) for 4 hrs at 37°C. Medium was then removed, and 100 μL of DMSO was added to solubilize formazan crystals and 25 μL of 0.1 M glycine buffer containing 0.1 M NaCl (pH 10.5) was then added. Optical densities were measured at 540 nm with a microplate reader.

Results and Discussion

The controlled production of NO plays important roles in mammalian nervous, immune, and vascular systems. However, excessive production of NO in mammalian systems may cause abnormal physical conditions or diseases, such as cell death, DNA damage, lung disease, or septic shock [Moncada *et al*., 1991]. In this study, materials derived from *C. cassia* leaves, used not only for flavoring foods but also as medicines [Chopra *et al*., 1980], were investigated to evaluate their inhibition of NO production. In primary screening, the inhibitory effects of essential oil, methanol extract, ethanol extract, and water extract of *C. cassia* leaves on NO production in RAW 264.7 cells after stimulation by LPS (1 μg/mL) and IFN-γ (10 U/mL) were tested at 2 and 1 μg/μL using Griess assay (Table 1). Essential oil, methanol extract, ethanol extract, and water extract of *C. cassia* leaves exhibited 66.2%, 50.6%, 28.8%, and 17.7% inhibition of NO production, respectively, at 1 μg/μL. In a previous experiment, it was reported that methanol extract of *C. cassia* bark at 1 μg/μL exhibited a pronounced inhibition of NO production, (51.3%) [Lee *et al*., 2002]. Thus, a more appreciable amount of the active component in the inhibition of NO production may be found in essential oil than in methanol extract, ethanol extract, and water extract of *C. cassia* leaves.

Table 1. Inhibitory effects of four extracts derived from *C. cassia* leaves on nitrite release by macrophages treated with LPS and IFN-γ

Extracts	Nitrite production (% Control)	
	1.0 μg/μL	2.0 μg/μL
Oil	33.8±4.1	19.7±3.1
Methanol	49.4±3.9	37.8±3.4
Ethanol	71.2±5.2	58.4±4.7
Water	82.3±4.4	78.1±5.6

Macrophages (1×10^5 cells/dish) were incubated for 24 h with LPS (1 μg/mL) and IFN-γ (10 U/mL) in a 96-well tissue culture plate in the absence or presence of the indicated *C. cassia* leaf extracts for 24 h. Results are the means±S.E. of three separate experiments.

The reported naturally occurring compounds from *Cinnamomum cassia* include cinnamaldehyde, benzaldehyde, 3-phenylpropionaldehyde, cinnamyl alcohol, salicylaldehyde, and 2-hydroxycinnamic acid [Lee and Ahn, 1998], and terpinene and pulegone from natural monoterpenes [Sánchez-Ramos and Castañera, 2001]. In previous reports, it has been noted that the bark-derived constituents of *C. cassia* (including cinnamaldehyde, cinnamic acid, cinnamyl alcohol, and eugenol) have antibacterial, anticoagulant, antitumor, astringent, carminative, and stomachic effects [Namba, 1986; Lee and Ahn, 1998; Lee *et al*., 2002; Lee, 2007]. In 2004, Kim *et al*. reported the main constituents of *C. cassia* bark oil were benzaldehyde, cinnamaldehyde, cinnamic acid methyl ester, cinnamyl acetate, cinnamyl alcohol, 4-hydroxy-

Table 2. Volatile compounds of *C. cassia* leaf-derived oil identified by GC-MS

Peak number	Compound	Mass spectral data[a]	Retention time (min)	Relative (%)
1	Limonene	39, 53, 68, 93, 107, 121, 136	29.52	9.3
2	Cinnamaldehyde	51, 77, 103, 131	45.12	70.3
3	Cinnamyl alcohol	39, 51, 78, 92, 105, 115, 134	49.19	5.7
4	Eugenol	55, 77, 103, 149, 164	51.86	5.8
5	Copaene	41, 69, 93, 119, 161, 204	54.14	3.0
6	(E)-Cinnamic acid	51, 77, 103, 148	56.53	0.9
7	α-Amorphene	41, 81, 93, 105, 119, 161, 204	57.01	1.1
8	Cadinene	41, 77, 91, 105, 119, 134, 161, 204	58.63	2.6
9	Cedrene	41, 69, 93, 105, 119, 161, 204	59.14	0.3
10	α-Calacorene	28, 115, 128, 142, 157, 200	61.03	0.7

[a]Major fragmentation ions; base peak (listed first) and other ions in decreasing order of relative abundance.

Table 3. Inhibitory effects of *C. cassia* leaf-derived components on nitrite release by macrophages treated with LPS and IFN-γ

Compounds	Nitrite production (% Control)		
	0.1 μg/μL	0.5 μg/μL	1.0 μg/μL
Cadinene	100.2±4.9	100.4±4.1	100.2±3.2
α-Cedrene	101.6±5.5	101.1±5.2	100.7±6.1
Cinnamaldehyde	60.2±4.1	20.1±3.7	19.9±1.9
Cinnamic acid	101.6±5.9	101.1±4.8	100.7±6.5
Cinnamyl alcohol	77.9±5.6	58.2±6.1	37.5±5.9
α-Copaene	100.8±3.6	100.5±3.3	100.1±2.9
Eugenol	102.2±3.7	101.3±5.2	100.7±5.7
Limonene	100.3±2.8	101.1±3.6	100.9±4.1

Macrophages (1×10^5 cells/dish) were incubated for 24 h with LPS (1 μg/mL) and IFN-γ (10 U/mL) in a 96-well tissue culture plate in the absence or presence of the indicated components derived from *C. cassia* bark. Results are the means±S.E. of three separate experiments.

Table 4. Cell viability of RAW 264.7 cells on treatment of LPS and cinnamyl alcohol during NO inhibitory experiment

Compound	Cell viability (% of Control)			
	8 mM	4 mM	2 mM	1 mM
Cinnamyl alcohol	109±8	106±7	105±7	103±9

Each value represents the mean±S.D. of four determinations.

benzaldehyde, 2-hydroxycinnamic acid, 3-phenyl-propionaldehyde, and salicylaldehyde [Kim *et al*., 2004]. Furthermore, Ahn *et al*. [2007] determined that the composition of the oil derived from the *C. cassia* leaves was limonene (17.0%), cinnamaldehyde (52.9%), cinnamyl alcohol (16.4%), eugenol (13.3%), and β-caryophyllene (0.4%). Because of the potent inhibitory effect of the essential oil of *C. cassia* leaves on NO production, the essential oil volatiles identified by GC-MS are presented in Table 2. Analysis led to the identification of 12 volatiles from essential oil from *C. cassia* leaves. The main constituents were α-amorphene (1.1%), cadinene (2.6%), α-calacorene (0.7%), cedrene (0.3%), cinnamaldehyde (70.3%), cinnamic acid (0.9%), cinnamyl alcohol (5.7%), copaene (3.0%), eugenol (5.8%), and limonene (9.3%). Together, cinnamaldehyde, cinnamyl alcohol, eugenol (5.8%), and limonene (9.3%) accounted for 91.1% of the essential oil derived from the *C. cassia* leaves. When the three reported compositions of *C. cassia* bark-derived oil were compared, the amounts of cinnamaldehyde and cinnamyl alcohol in the essential oil of our study were smaller (around 2.1-8.9% less).

The inhibition of NO production by some components derived from the essential oil of *C. cassia* leaves were determined (Table 3). Some components (α-cedrene, cinnamic acid, cinnamyl alcohol, α-copaene, eugenol, and limonene) were purchased as a commercially available product, because it is difficult to isolate 8 components from the essential oil of *C. cassia* leaves. Cinnamyl alcohol exhibited inhibitory effects of 62.5% and 41.8% at 1 and 0.5 μg/μL, respectively. However, little or no inhibitory effect was observed for cedrene, cinnamic acid, copaene, eugenol, or limonene. The inhibitory effect of cinnamaldehyde was more potent than that of cinnamic acid, cinnamyl alcohol, or eugenol. In a previous study, it was reported that the potent inhibitory effect of methanol extract of *C. cassia* bark on NO production was due to cinnamaldehyde [Lee *et al*., 2002]. Thus, current and previous results indicate the observed inhibitory effects of the essential oil of *C. cassia* leaves against NO production are likely attributable to cinnamaldehyde and cinnamyl alcohol. Furthermore, we determined cell viability by MTT assay to determine

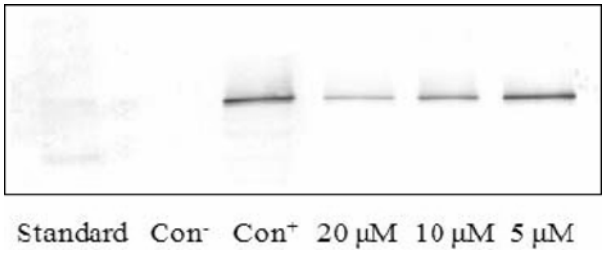

Fig. 1. Western blot analysis of iNOS in RAW 264.7 cells treated with cinnamyl alcohol. Immunoblot. The cells were stimulated with LPS (1 μg/mL)/IFN-γ (10 U/mL) and incubated for 16 h in the presence of cinnamyl alcohol. Protein was used at 50 μg/lane. Con^-= unstimulated control; Con^+=stimulated control; Standard= Standard protein.

whether inhibitory effects of cinnamyl alcohol on NO production were attributable to a nonspecific cytotoxicity (Table 4). In the presence of cinnamyl alcohol at concentration to 8 mM, cell viabilities were not different from those of LPS/IFN-γ-stimulated cells. The cytotoxicity data of cinnamyl alcohol in this study was determined to be similar with the data describing cinnamyl alcohol that was derived from *Cinnamomum cassia* barks [Lee *et al*., 2004].

To clarify cinnamyl alcohol's mechanism of NO inhibition, iNOS protein expression was analyzed in cellular extracts from RAW 264.7 cells stimulated with LPS/IFN-γ in the presence or absence of this inhibitor. In generally, iNOS was sufficiently expressed by the stimulation of LPS and IFN-γ. Fig. 1 showed that the combination of LPS/IFN-γ was a strong inducer of iNOS protein expression in RAW 264.7 cells. It also shows that cinnamyl alcohol derived from *C. cassia* leaves significantly decreased iNOS expression, in a dose-dependent manner, in LPS/IFN-γ-stimulated RAW 264.7 cells, blocking iNOS protein expression almost completely at 20 μM. However, for the test at only 5 μM, the result appears comparable to the positive control (Fig. 1). To clarify the inhibition of cinnamyl alcohol at iNOS protein expression, cinnamyl alcohol was used for the strong intensity at the high concentrations (Fig. 1).

Excess NO causes calcium homeostasis, disruption of energy metabolism, and oxidative stress. It also damages DNA, with ONOO- causing single strand breaks [Salgo *et al*., 1996]. To date, NO has been implicated in many pathological conditions, such as graft-versus-host disease and experimentally-induced neurological diseases [Doyle and Hockstra, 1981]. Such pathologies have been reduced via the inhibition of NO synthesis by treating the animals with NO inhibitor. In previous studies and in the current study, the active iNOS-inhibiting components from *C. cassia* bark and leaves were identified as cinnamaldehyde and cinnamyl alcohol, with the inhibitory responses varying with the chemical and concentration tested. In this regard, it might be expected that *C. cassia* bark-derived cinnamyl alcohol would be suggested an indication of at least one of pharmacological actions for the suppression effects against iNOS.

Cinnamyl alcohol has a potent ability to interfere with excess NO production through the action of inhibitory responses to iNOS expression. Based upon our limited data and some earlier findings, the inhibitory action of cinnamyl alcohol confirms its superiority and usefulness as an iNOS inhibitor, although *in vivo* efficacy and clinical usefulness remain to be evaluated. Further research to identify the relevant connections between NO signal transduction and cinnamyl alcohol is in progress.

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