COVER SHEET

TITLE: **Bioassay-Guided Purification and Characterization of Anti-Inflammatory Components in *Cinnamomum burmannii***

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ABSTRACT FORM

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Abstract

Excessive inflammatory response is implicated in many diseases such as arthritis, atherosclerosis, and cardiomyopathy. Previous studies have found that several cinnamon species could exhibit anti-inflammatory activities. Adopting the bioassay-guided purification technique, *trans*-cinnamaldehyde was identified to be the principle active component in *Cinnamomum burmannii*. At a dosage of 50 μM, *trans*-cinnamaldehyde could inhibit 50% production of nitric oxide by down-regulating the expression of iNOS. It also inhibited 75% of prostaglandin E_2 (PGE_2) production without changing cyclooxygenase-2 (COX-2) protein expression. It was concluded that *trans*-cinnamaldehyde was the major component in *C. burmannii* responsible for its anti-inflammatory activity through inhibition of NO and PGE_2 production.

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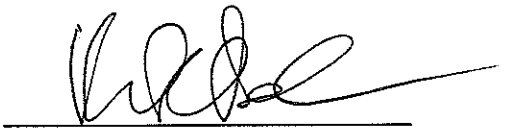

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12 January 2010

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Abstract

Excessive inflammatory response is implicated in many diseases such as arthritis, atherosclerosis, and cardiomyopathy. Previous studies have found that several cinnamon species could exhibit anti-inflammatory activities. Adopting the bioassay-guided purification technique, *trans*-cinnamaldehyde was identified to be the principle active component. At a dosage of 50 μM, *trans*-cinnamaldehyde could inhibit 50% production of nitric oxide by down-regulating the expression of iNOS. It also inhibited 75% of prostaglandin E_2 (PGE_2) production without changing cyclooxygenase-2 (COX-2) protein expression. It was concluded that *trans*-cinnamaldehyde was the major component in *C. burmannii* responsible for its anti-inflammatory activity through inhibition of NO and PGE_2 production.

Introduction

Inflammation is a normal physiological response to tissue injury or pathological infection. It is a complex process involving a multifactorial network of chemical signals to mediate the action. Macrophages can be activated by certain inflammatory agents, such as the lipopolysaccharide (LPS) on the cell wall of Gram-negative bacteria, to release proinflammatory cytokines to stimulate inflammatory response. An acute inflammatory response is essential for the repair of tissue injury and host survival from microbial infection. However, sustained or chronic (long-term) inflammation can result in development of many immune system diseases (*1-2*), including asthma, arthritis, atherosclerosis, cardiomyopathy, neurodegenerative disease, and even cancer (*3*).

Nitric oxide (NO) and prostaglandins (PGs) are proinflammatory cytokines and they play important roles to mediate both acute and chronic inflammation (*4-5*). NO is produced by nitric oxide synthase (NOS) as a by-product during the oxidative conversion of arginine to citrulline, while prostaglandins are generated from the metabolism of arachidonic acid by cyclooxygenase (COX). Constitutive synthesis of NO and prostaglandins from basal NOS and COX activities have little significance for inflammation. However, in the presence of inflammatory agents (e.g., LPS), inducible NOS (iNOS) and COX (COX-2) enzymes are expressed and they produce elevated amounts of cytokines to sustain the inflammatory response (*6*). Although physiological activities of iNOS and COX-2 provide short-term benefits to the cell during inflammation, an excessive and sustained expression of these enzymes and their cytokines are implicated in the pathogenic mechanisms of many inflammatory diseases as described above.

There is much interest in identifying chemical compounds from natural products that possess strong anti-inflammatory activity. One study found that the essential oil from cinnamon (*Cinnamomum osmophloeum*) exhibited anti-inflammatory activity in LPS-activated mouse macrophages (*7*). The production of NO was reduced but had no significant effect on PGE_2 production was observed. Another study reported that the extract from *Cinnamomi ramulus*, the young twig of *Cinnamomum cassia*, could suppress the expression of various genes related to the inflammatory response (e.g. *NOS2*, the gene encoding for iNOS) (*8*). These results provide compelling evidence to support the potential health-benefits of cinnamon, and provide incentive for more research to investigate the mechanistic basis of the anti-inflammatory action of cinnamon compounds.

In this study, a bioassay-guided approach was used to purify and identify the principle active compound from cinnamon (*Cinnamomum burmannii*) that could account for the abundance of anti-inflammatory activity in a cinnamon crude extract. Its anti-inflammation mechanism was characterized by examining the activities and expression levels of iNOS and COX-2, two major mediator proteins involved during an inflammation response.

Materials and Methods

1 Chemicals & Cell Culture

Cinnamon powder (*Cinnamomum burmannii*) was supplied by McCormick Inc. (USA) and kept in a sealed container at -20°C until use. All solvents were purchased from Fisher Scientific (Pittsburgh, PA). Lipopolysaccharide (LPS), mouse monoclonal anti β-actin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Goat polyclonal IgG anti COX-2, mouse monoclonal IgG_1 anti NOS2, donkey anti-goat IgG HRP, and goat anti-mouse IgG HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TRIzol reagent and PGE_2 enzyme immunoassay kit were purchased from EIA, Cayman Chemical (USA). Dulbecco's modified eagle medium (DMEM) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

To detect iNOS and COX-2 activities, mouse macrophage cells (RAW 264.7) were seeded at a density of 5×10^5 cells/well in a 96-well microtiter plate with 200 µL of Dulbecco's Modified Eagle Medium (DMEM) added. The medium was supplemented with 15% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The plate was incubated in a 5% CO_2 incubator at 37°C for 24 hours. The growth medium was decanted and replaced with fresh one containing LPS (1 µg/mL). All samples were dissolved in dimethylsulfoxide (DMSO) and added to the plate in a serially-diluted

fashion, where the final concentration of DMSO in each well was maintained at or below 0.25%. Controls with the sample alone (without LPS) were included in all cases. The plate was further incubated for 24 hours at 37°C before analyses. To isolate protein and RNA from the cultured cells, the macrophages were seeded at a density of 10^6 cells/well in a six-well plate, where each well contained 2 mL of DMEM. The plate was treated in the same manner as described above.

2 *Purification of Bioactive Compounds from Cinnamon*

2.1 Crude Acetone Extraction

Cinnamon powder (1 kg) was extracted with 10 L of aqueous acetone (80% in water) at 37°C for two hours in an orbital shaker. The slurry was centrifuged at 6000 rpm for ten minutes and the supernatant was decanted and filtered through Whatman #5 filter paper. The solvent was removed using a rotary evaporator (Buchi Rotavapor model R110, Switzerland) under reduced pressure at 40°C and the remaining water was removed by lyophilization for 72 hours. A reddish brown powder ("acetone crude extract") was obtained and stored at -20 °C.

2.2 Silica Gel-Column Chromatography

The compounds in the acetone crude extract were separated by silica gel-column chromatography. Acetone crude extract (10 g) and silica powder (50 g) were mixed in 200 mL of acetone. The solvent was removed and the residual powder was loaded onto a silica gel-packed column equilibrated with hexane. An elution was performed with a stepwise gradient from 90:10 to 50:50 hexane:ethyl acetate (500 mL of solvent used for each elution). The eluent was collected in separate test tubes with a fraction collector and the elution profile was monitored at 254 nm to allow for pooling of collected eluent into fractions. The solvent from each fraction was removed by evaporation and the obtained dry matter for the isolates were stored at -20 °C.

2.3 Preparative Thin-Layer Chromatography

One fraction obtained from the silica gel-column chromatography was further purified by preparative thin-layer chromatography (pTLC). About 50 mg of sample was dissolved in 500 µL of dichloromethane and loaded onto a preparative silica gel-TLC plate (1000 mm thickness). The plate was developed in an ascending mode in a saturated chamber for 45 minutes at room temperature, using 75:25 dichloromethane:hexane as the mobile phase solvent. The separated fractions were detected under UV light, scraped off the plate and extracted with acetone. The solvent was removed and the samples were stored at -20 °C until used.

2.4 Structural Determination by Spectroscopic Analyses

Nuclear Magnetic Resonance (NMR) and mass spectroscopy (MS) were performed

to identify the chemical structure of the purified compound isolated from cinnamon.

3 *Assessment of Anti-Inflammatory Activity of the Purified Isolate*

3.1 Inhibition of iNOS and COX-2 Activities

The ability of isolates to inhibit iNOS activity was assessed by measuring the amount of nitric oxide (NO) produced in LPS-activated mouse macrophage cells. Nitrite concentration was used as an indicator of NO level and was detected using the Griess reagent (1:1 mixture of 2 % sulfanilamide in 4 % phosphoric acid and 0.2 % naphthylethylenediamine in water). A 100 µL sample of culture supernatant was mixed with 100 µL of Griess reagent. The plate was incubated for ten minutes at ambient temperature with shaking and the absorbance at 542 nm was taken using an optical microtiter plate scanner (SPECTRA MAX plus, Molecular Devices, USA). The concentration of nitrite was calculated from a standard curve prepared with known concentrations of sodium nitrite solution. PGE_2 production was assayed using the PGE_2 enzyme immunoassay kit following the manufacturer's instructions.

The MTT assay was performed to determine cell viability in both bioassays. A 100 µL aliquot of MTT solution (0.05 mg/mL) in phosphate buffer saline (PBS) was added to each well after removing 100 µL of supernatant for determining NO/PGE_2 production. The plate was incubated at 37 °C for 1.5 hours, decanted, and refilled with 200 µL of DMSO to dissolve the blue formazan product. The plate was further incubated at 37°C for one hour with shaking and the absorbance at 550 nm was read.

3.2 Western Blot Analyses for iNOS and COX-2 Proteins

The cultured macrophages were washed twice with ice-cold PBS, harvested and lyzed in the RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS. Protease inhibitor (1X) (EMD Biosciences, Gibbstown, NJ) was added to the buffer to inhibit the activity of proteases in the cells. Cells were lysed on ice for 30 minutes, centrifuged (14000 rpm, 10 minutes), and the supernatants were collected. Protein concentration was determined by the BioRad protein assay reagent following the manufacturer's instructions. 50 µg of each protein sample was separated by running a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. After blocking the membrane with 5% milk for overnight, proteins of interest were probed with specific primary and secondary antibodies and detected by an enhanced chemiluminescence detection system (Thermo Scientific, MA) on X-ray films.

Results and Discussion

Compelling evidence in the literature has shown several cinnamon species, such as *Cinnamomum osmophloeum* and *Cinnamomum cassia*, possess strong anti-microbial and

anti-inflammatory activities (*9, 10, 11, 12*). In the US and many other countries, *Cinnamomum burmannii*, commonly referred as cinnamon stick, is also a popular and commonly used spice in foods. Compared to *C. osmophloeum* and *C. cassia*, *C. burmannii* has been subject to fewer studies on its anti-inflammatory activity. Hence, attempts were made to characterize the anti-inflammatory activity of *C. burmannii* by purifying and identifying its principle active component using a bioassay-guided fractionation technique.

In the purification procedures, acetone was used in the first step for crude extraction because of its wide solubility. Most phenolics (e.g. flavonoids) and essential oil in cinnamon could dissolve in acetone and be extracted. The acetone crude extract was then fractionated using silica gel-column chromatography with hexane and ethyl acetate. The effluent was monitored at 254 nm and an elution profile is shown in Figure 1. Four fractions were collected according to the peaks shown in the elution profile and the fifth fraction contained the residual solvent after F4. The anti-inflammatory effect for these fractions was examined using the NO inhibition bioassay. Yield of dry matter and estimated IC_{50} value (concentration of sample required to inhibit 50% of NO produced in LPS-stimulated macrophages) of each fraction are reported in Table 1. The results show that F3 and F5 were most active in inhibiting NO production and had the lowest IC_{50} values (<30 μg/mL) among other fractions. However, a greater yield of dry matter was obtained for the F3 fraction, implying the compound(s) contained in F3 existed in higher abundance in cinnamon than that in F5. This also suggests the compounds in F3 have greatest potential to confer health benefits when consuming cinnamon. Thus, efforts were placed on further purifying F3 and identifying the principle component(s) within this fraction that contributed most to its anti-inflammatory response. Using pTLC, a single band was isolated and its chemical identity was determined by nuclear magnetic resonance (NMR) and mass spectroscopy (MS). With the help of a graduate student in the lab, one-dimensional ^{1}H and ^{13}C-NMR and two-dimensional HMBC (Heteronuclear Multiple Band Coherence) and HSQC (Heteronuclear Single Quantum Coherence) were performed and the structure of *trans*-cinnamaldehyde was identified from the NMR spectra (data not shown). MS was also carried out and the spectrum consistently indicated that *trans*-cinnamaldehyde was the only compound present in the purified TLC band.

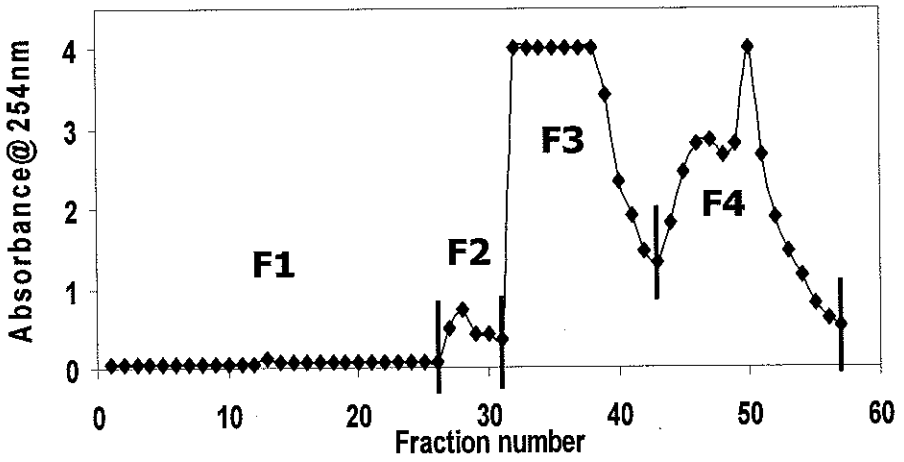

Figure 1. Elution profile of cinnamon extract on silica gel-column chromatography using hexane and ethyl acetate as solvents. Five fractions were obtained, four of which were pooled according to the above elution profile and the fifth fraction was the residual solvents collected after F4.

Table 1. Yield and the ability to inhibit NO production (expressed in terms of IC_{50}) of the five fractions collected from the silica gel-column chromatography.

	F1	F2	F3	F4	F5
Yield	<0.1%	0.47%	4.92%	2.08%	0.78%
IC_{50}(μg/mL)	>100	>100	<30	~60	<30

trans-Cinnamaldehyde is one of the major components in the essential oil of cinnamon and its anti-microbial activity has been well studied in the literature (*13-14*). Other potential bioactivities of *trans*-cinnamaldehyde include its cytotoxicity on human cancer cell lines (*15*) and hypoglycemic and hypolipidemic effects in diabetic rat (*16*). Not much information has been reported on the anti-inflammatory activity of *trans*-cinnamaldehyde except its ability to inhibit NO production and NOS activity (*7,17*). Nevertheless, the methods used to detect the amount of NO production in these two previous studies were not consistent and the IC_{50} values for NO inhibition and varied considerably. To better characterize the anti-inflammatory activity of *trans*-cinnamaldehyde, its ability to inhibit both NO and prostaglandin E_2 (PGE_2) production in LPS-activated macrophages was measured. These cytokines are produced by iNOS and COX-2, two major inflammatory mediators, respectively. Consequently, the expression levels of iNOS and COX-2 proteins in the stimulated cells were measured to look at the mechanism of the anti-inflammatory effect of *trans*-cinnamaldehyde.

Trans-cinnamaldehyde inhibited the production of NO effectively in a dose-dependent manner, with an IC_{50} value at around 50 μM (Figure 2). The protein expression level of iNOS also decreased with increasing concentration of *trans*-cinnamaldehyde. At 75 μM, *trans*-cinnamaldehyde completely inhibited the production of iNOS and thus no protein band was detected on the western blot. Yet, NO was still detected at this dosage, and it is likely that this level of NO was produced by iNOS during the early stage of LPS stimulation and accumulated in the culture supernatant before *trans*-cinnamaldehyde was maximally effective in suppressing the protein expression. At levels up to 120 μM, the cytotoxic effect of *trans*-cinnamaldehyde was not significant and the cell viability determined from the MTT assay maintained above 80% (data not shown). These results suggested that *trans*-cinnamaldehyde exhibited anti-inflammatory activity by suppressing the production of NO through down-regulation of iNOS protein expression.

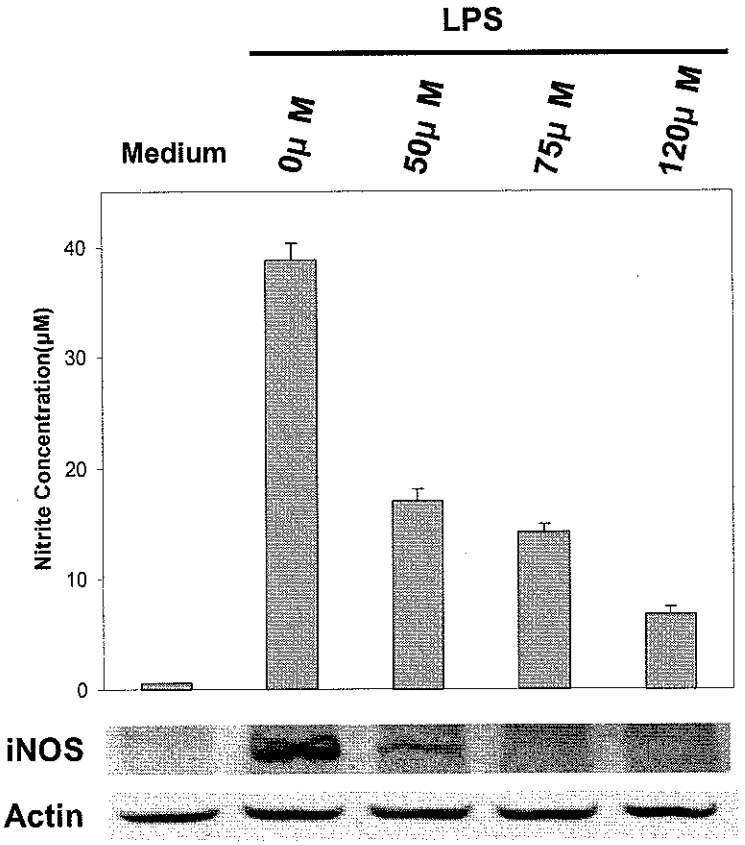

Figure 2. Anti-inflammatory response of *trans*-cinnamaldehyde isolated from *C. burmannii*. The cultured macrophages were stimulated by LPS, followed by the addition of an increasing concentration of *trans*-cinnamaldehyde (0-120 μM). Nitric oxide production was detected in terms of the amount of nitrite present in the supernatant. A control without LPS or *trans*-cinnamaldehyde (labeled as "Medium") was included. iNOS protein expression was determined by western blot. Actin was detected as a loading control.

Besides the production of NO, inflammation could also be mediated via the COX-2 pathway. COX-2 enzymes were expressed upon cellular stimulation by inflammatory agents and can metabolize arachidonic acid to act as mediators for inflammatory response. Prostaglandin E_2 (PGE_2) is a downstream product generated by COX-2 and thus the amount of PGE_2 production can be used to assess activity of COX-2 in LPS-stimulated cells. At 50 μM dosage of *trans*-cinnamaldehyde, expression level of PGE_2 was significantly inhibited by about 75% (data not shown), suggesting that *trans*-cinnamaldehyde was able to suppress COX-2 activity. However, at the same concentration, no reduction in COX-2 protein expression from the western blot was observed (Figure 3). Although *trans*-cinnamaldehyde failed to suppress COX-2 expression even up to 120 μM, it was able to inhibit PGE_2 production at 50 μM, implying that *trans*-cinnamaldehyde suppressed inflammatory response by directly inhibiting the activity of COX-2 instead of reducing the expression level of the COX-2 protein.

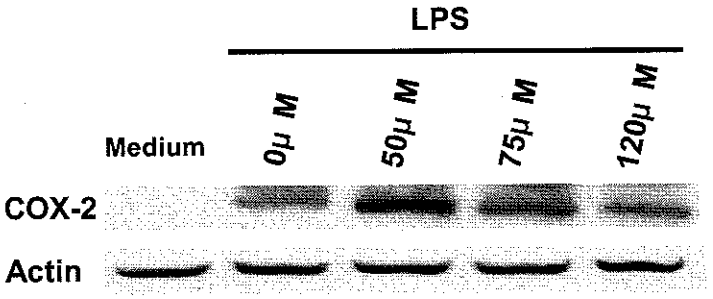

Figure 3. COX-2 protein expression of LPS-stimulated macrophages with increasing concentration of *trans*-cinnamaldehyde added (0-120 μM). Actin was detected as a loading control.

Collectively these results indicate that *trans*-cinnamaldehyde acts as an anti-inflammatory agent because of its inhibitory effects on both NO and PGE_2 production. It suppressed inflammation via two different mechanisms. It reduced the protein expression level of iNOS to inhibit the production of NO. In terms of the COX-2 pathway, *trans*-cinnamaldehyde significantly decreased the production of PGE_2, possibly by directly attenuating COX-2 enzyme activity. Beyond looking at the mechanistic basis of *trans*-cinnamaldehyde action, one could speculate on whether consuming *C. burmannii* itself could potentially confer health benefits through the action of *trans*-cinnamaldehyde. From the purification procedures shown in this paper, a 0.6% yield of *trans*-cinnamaldehyde from *C. burmannii* was obtained (yield of acetone crude extract: 22.2%; yield of F3 from silica gel-column: 4.9%; yield of purified fraction from pTLC: 57.2%). It appears to exist in relatively high abundance compared to many other phytochemicals present in cinnamon and possess the highest anti-inflammatory potential (Table 1). Hence, it is hypothesized that consumption of *C. burmannii* might be beneficial

to counter inflammation through the action *trans*-cinnamaldehyde. To prove this hypothesis, its anti-inflammatory activity *in vivo* and its potential to be used as an anti-inflammation drug need to be further researched.

In conclusion, *trans*-cinnamaldehyde was purified from *Cinnamomum burmannii* and was found to contribute significant anti-inflammatory activity. At 50 μM, *trans*-cinnamaldehyde could protect the cells from LPS-stimulated inflammation by reducing the expression level of iNOS protein and lowering the production of nitric oxide. It also decreased the generation of PGE_2, possibly by inhibiting the enzyme activity of COX-2, but had no change in COX-2 protein expression level. Via these two mechanisms, *trans*-cinnamaldehyde was identified to be the principle active component in *C. burmannii* responsible for its anti-inflammatory activity.

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