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Short Communication

Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs

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Abstract

In this study, chemical compositions of hydrodistilled essential oil and anti-inflammatory activities from the twigs of *Cinnamomum osmophloeum* Kaneh. were investigated for the first time. The chemical constituents of the twig essential oil were further analyzed by GC–MS and they were found to be L-bornyl acetate (15.89%), caryophyllene oxide (12.98%), γ-eudesmol (8.03%), β-caryophyllene (6.60%), T-cadinol (5.49%), δ-cadinene (4.79%), *trans*-β-elemenone (4.25%), cadalene (4.19%), and *trans*-cinnamaldehyde (4.07%). The effects of essential oil on nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages were also examined. Results of nitric oxide tests indicated that twig essential oil and its major constituents such as *trans*-cinnamaldehyde, caryophyllene oxide, L-borneol, L-bornyl acetate, eugenol, β-caryophyllene, *E*-nerolidol, and cinnamyl acetate have excellent activities. These findings demonstrated that essential oil of *C. osmophloeum* twigs have excellent anti-inflammatory activities and thus have great potential to be used as a source for natural health products.

Keywords: *Cinnamomum osmophloeum*; Twigs; Essential oil; *trans*-Cinnamaldehyde; Anti-inflammation

1. Introduction

Inflammatory response protects the host against tissue injury and microbial invasion. As such, this response should be short-lived, and failing can result in pathogenesis of many immunity-related diseases (Pulendran et al., 2001; Steinman, 2004). Today the treatment of inflammatory diseases involves mainly interrupting the synthesis or action of critical mediators that drive the host's response to injury. Although steroids and antihistamines have provided the main treatment for inflammatory diseases, they exist for the treatment of inflammation-driven diseases such as asthma, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, Crohn's disease, multiple sclerosis, and systemic vasculitis (Gayathri et al., 2007). An alternative approach to the development of novel therapeutics involving the endogenous mediators and mechanisms that switch off inflammation is being carried out in this study and it is thought that this strategy will bring in new possibilities for the future management of inflammation-based diseases.

Cinnamomum osmophloeum Kaneh. (Lauraceae) is an endemic tree that grows in natural hardwood forest of Taiwan at an elevation between 400 and 1500 m. This tree species has been of interested to researchers because the chemical constituents of its essential oil are similar to those of *Cinnamomum cassia* bark oil, known as cinnamon oil, which is commonly used in food and beverages (Ooi et al., 2006). Recent phytochemical analyses and biological screenings of *C. osmophloeum* have focused on the leaf essential oil components, which has shown excellent inhibitory effects on anti-bacteria, anti-termites, anti-mites, anti-mildew, anti-mosquito larvae, and anti-fungi (Chang et al., 2001; Chang and Cheng, 2002; Chen et al., 2002; Chen and Chang, 2002; Cheng et al., 2004, 2006). Furthermore, Chao

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et al. (2005) reported that essential oil of *C. osmophloeum* leaves at a dose of 60 μg/ml also exhibited effective inhibitory effects on IL-1β and IL-6 productions in LPS-stimulated macrophages. However, to the best of our knowledge, there is no prior study on the essential oil of *C. osmophloeum* twigs. In this study, chemical compositions of hydrodistilled essential oil obtained from *C. osmophloeum* twigs were analyzed by gas chromatography–mass spectrometry (GC–MS), and the effects of essential oil on nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages were investigated. In addition, the cytotoxicity of essential oil against human hepatoma cancer cell line, HepG2 cells, was also examined.

2. Methods

2.1. Plant material

The twigs of a 13-year-old *C. osmophloeum* Kaneh. were collected at the end of July 2004 from the Taiwan Sugar Company Research Center located in Nantou County in central Taiwan. Diameter of the twigs selected was below 1.5 cm. The species was confirmed by Dr.Yen-Ray Hsui of the Taiwan Forestry Research Institute and voucher specimens were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University).

2.2. Essential oil distillation

Twigs of *C. osmophloeum*, in triplicate, were subjected to hydrodistillation for 6 h in a Clevenger-type apparatus (Chang et al., 2001). The yellow-colored essential oil with characteristic odor was obtained and stored in airtight containers prior to further analysis.

2.3. Analysis of essential oil

Essential oil from twigs was analyzed by a PolarisQ Ion Trap GC/FID/MS^n system (Themo, USA), equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MS (Agilent J&W Scientific). The GC oven temperature was programmed from 80 °C, held 1 min, raised to 200 °C at 4 °C/min, and held for 5 min. The injector temperature was 250 °C; and the flow rate of carrier gas, helium, was at 1.0 ml/min; 1:10 split ratio. Diluted samples (1.0 μl, 1/100, v/v, in ethyl acetate) were injected manually in the split mode. The Kovats indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C_9–C_{25} on DB-5MS column. The major components of *C. osmophloeu*m twig oil were identified by co-injection with standards (wherever possible), confirmed with Kovats indices using the Wiley (V. 7.0) and National Institute of Standards and Technology (NIST) V.2.0 GC–MS library. The relative concentration of each compound in essential oil was quantified based on the peak area integrated by the analysis program.

2.4. Chemicals

Lipopolysaccharide (LPS), Greiss reagent, β-caryophyllene, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Quercetin, L-borneol, α-terpineol, *p*-allylanisole, *trans*-cinnamaldehyde, L-bornyl acetate, eugenol, cinnamyl acetate, *E*-nerolidol, caryophyllene oxide, and methyl sulfoxide (DMSO) were all purchased by Acros (Belgium). PGE_2 enzyme inmmunoassay kit was purchased from EIA, Cayman Chemical (USA). Dulbecco's modified eagle medium (DMEM) was purchased from Gibco BRL (USA).

2.5. Cell line and cell culture

RAW 264.7 cells, a murine macrophage cell line and HepG2 cells, a human hepatocellular liver carcinoma cell line were obtained from the Culture Collection and Research Center (CCRC), Hsinchu, Taiwan. Cells were cultured in 75- or 150-cm^2 flasks with Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a 5% CO_2 incubator at 37 °C.

2.6. Cytotoxicity assay on tumor cells (MTT assay)

This assay was performed according to the procedure reported by Mossmann (1983) with slight modifications. To measure the cytotoxicity of essential oil, caryophyllene oxide, and L-bornyl acetate in cell proliferation, the HepG2 cells (1×10^4 cells/well) were seeded into a 96-well plate in triplicate and pre-incubated for 3 h for cell adherence. First, 100 μl of fresh medium containing various concentrations of test samples were added into the cultures and incubated at 37 °C for 72 h under humidified air containing 5% CO_2. Following the removal of the medium from the wells, 100 μl of tetrazolium salt solutions (1 ml MTT in 10 ml DMEM) were added. After 4 h of incubation at 37 °C, the medium was removed and 100 μl of DMSO were added to dissolve the formazan crystals. Absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. The cell viability ratio (%) was calculated from the following equation: % viability = (absorbance of test sample/absorbance of control) × 100.

2.7. Anti-inflammatory activity assay

To investigate the anti-inflammatory activity of *C. osmophloeum* twig essential oils, NO and PGE_2 productions in LPS-stimulated RAW 264.7 cells were examined. For NO determination, RAW 246.7 cells were seeded in 96-well plates at a density of 2×10^5 cells/well and grown for 2 h for adherence. The cells were treated with test samples for 1 h and then incubated for 24 h in fresh DMEM with or without 1 μg/ml of LPS. The nitrite concentration

in the culture medium was measured as an indicator of NO production according to the Griess reaction (Kim et al., 1999). Briefly, 100 μl of cell culture supernatant were reacted with 100 μl of Griess reagent (1:1 mixture of 0.1% *N*-(1-naphthyl) ethylene-diamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, and absorbance at 540 nm was recorded using the ELISA reader.

For PGE_2 determination, RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 18 h. Cells were pretreated with 500 μM of aspirin for 3 h to inactivate endogenous cyclooxygenase-1 (COX-1) according to the method reported by Hwang et al. (2002). Then, cells were washed twice with phosphate buffered saline (PBS) and further incubated for 16 h in fresh DMEM with or without 1 μg/ml of LPS in the absence or presence of the test samples. After incubation, supernatants were collected to measure PGE_2 concentration with monoclonal antibody by ELISA as specified by the manufacturer.

2.8. Cell viability

The cell viability assay was determined on the basis of MTT assay as described above with a slight modification. After culture, supernatants were collected for NO or PGE_2 measurement, 100 μl of tetrazolium salt solutions (1 ml MTT in 10 ml DMEM) were added to each well, and then incubated for 1 h at 37 °C in a 5% CO_2 incubator. The medium was then aspirated, and the insoluble formazan product was dissolved in 100 μl of DMSO. The extent of MTT reduction was quantified by measuring the absorbance at 570 nm.

2.9. Statistical analysis

All results are expressed as mean ± SD ($n = 3$). The significance of difference was calculated by SAS Scheffe's test, and values < 0.05 were considered to be significant.

3. Results and discussion

3.1. Yield and constituents of essential oil

Hydrodistillation of *C. osmophloeum* twigs yields 0.08% (w/w) essential oil according to dry weight. Compared with the yields of essential oils from *C. osmophloeum* leaves, ranging from 0.1% to 2.9% (Cheng et al., 2006), the yield of essential oil from *C. osmophloeum* twigs was lower. A total of 24 compounds (Table 1) were identified in the twig essential oil, representing 97.62% of the total essential oil. The main constituents in twig essential oil were quantified to be L-bornyl acetate (15.89%), caryophyllene oxide (12.98%), γ-eudesmol (8.03%), β-caryophyllene (6.60%), T-cadinol (5.49%), δ-cadinene (4.79%), *trans*-β-elemenone (4.25%), cadalene (4.19%), and *trans*-cinnamaldehyde (4.07%) according to the results obtained from GC–MS and GC–FID analyses. Although the constituents of the leaf oil can be classified into different chemotypes according to their contents, most of the constituents are monoterpenes or belong to other groups, such as camphor, linalool, *trans*-cinnamaldehyde, and cinnamyl acetate (Cheng et al., 2006). On the other hand, in the twig essential oil, most of the constituents are sesquiterpenes. In this study, 35.63% of the constituents were identified as oxygenated sesquiterpene, followed by sesquiterpene hydrocarbons (29.31%), oxygenated monoterpene (23.15%), and others (9.53%).

3.2. Effects of essential oil on cytotoxicity of HepG2 cells

Hepatocellular carcinoma is one of the most common malignancies worldwide. The high incidence of liver cancer has been attributed to factors such as persistent infection with hepatitis virus and contact with hepatocarcinogens such as nitrosamines and aflatoxins (Henry et al., 2002). The cytotoxic effects of *C. osmophloeum* twig essential oil and its main components (L-bornyl acetate and caryophyllene oxide) were investigated using a MTT assay on HepG2 cells, a hepatoma liver cancer cell line. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells (Mossmann, 1983). At the dosage of 250 μg/ml, essential oil of *C. osmophloeum* twigs exhibited excellent inhibitory effects only with 36.6% of cells survived. However, no significant inhibition effects on cell survival were observed for both caryophyllene oxide and L-bornyl acetate, which are two major constituents in the twig essential oil (data were not shown).

3.3. Effects of essential oil and its constituents on NO production in LPS-stimulated RAW 264.7 cells

NO is an endogenous free radical species that is synthesized from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues. Small amounts of NO are important regulators of physical homeostasis, whereas large amounts of NO have been closely correlated with the pathophysiology of a variety of diseases and inflammation. After exposure to inducers, such as lipopolysaccharide (LPS) from Gram-negative bacteria, inducible NOS (iNOS) can be induced in various cells, such as macrophages, kupffer cells, smooth muscle cells, and hepatocytes, to trigger cytotoxicity, tissue damage, inflammation sepsis, and stroke (Marletta, 1993; Jiang et al., 2006). Thus, measuring of NO production may be a method for assessing the anti-inflammatory effects of plant extracts.

As for the inhibitory effects of *C. osmophloeum* twig essential oil on NO productions, data in Fig. 1 show that essential oil of *C. osmophloeum* twigs had the excellent inhibitory effect. In the presence of 25 μg/ml of essential oil, the inhibition of NO production was 68.8%. The IC_{50} value was 11.2 μg/ml. In addition, MTT assay revealed

Table 1
Yields and composition of essential oil from *C. osmophloeum* twigs

No.	Constituents	R.t.[a]	KI[b]	Area (%)	Identification[c]
1	L-Borneol	8.55	1172	1.34	MS, KI
2	α-Terpineol	9.19	1194	1.67	MS, KI
3	*p*-Allylanisole	9.36	1199	2.45	MS, KI
4	*trans*-Cinnamaldehyde	9.38	1272	4.07	MS, KI,
5	L-Bornyl acetate	11.40	1289	15.89	MS, KI, ST
6	Eugenol	14.01	1360	0.95	MS, KI
7	α-Copaene	14.70	1382	3.93	MS, KI
8	β-Caryophyllene	16.02	1426	6.60	MS, KI
9	Cinnamyl acetate	16.58	1445	2.74	MS, KI
10	α-Caryophyllene	17.02	1460	1.43	MS, KI
11	Curcumene	17.80	1486	1.67	MS, KI
12	δ-Cadinene	19.03	1528	4.79	MS, KI
13	α-Calacorene	19.59	1548	1.88	MS, KI
14	Elemicin	19.88	1558	0.85	MS, KI
15	*E*-Nerolidol	20.13	1566	1.05	MS, KI
16	Spathulenol	20.61	1582	2.75	MS, KI
17	Caryophyllene oxide	20.78	1588	12.98	MS, KI, ST
18	*trans*-β-Elemenone	21.18	1601	4.25	MS, KI
19	Unknown	21.50	1613	2.37	MS, KI
20	γ-Eudesmol	22.02	1633	8.03	MS, KI
21	Caryophylla-4(14), 8(15)-dien-5.alpha-ol	22.25	1641	3.74	MS, KI
22	δ-Cadinol	22.39	1646	3.51	MS, KI
23	T-Cadinol	22.75	1659	5.49	MS, KI
24	Cadalene	23.21	1676	4.19	MS, KI
25	Guaiol acetate	24.53	1720	1.37	MS, KI
Oxygenated monoterpene identified (%)				23.15	
Sesquiterpene hydrocarbons identified (%)				29.31	
Oxygenated sesquiterpene identified (%)				35.63	
Other (%)				9.53	
Identified components (%)				97.62	
Oil yield (%, w/wt)				0.08 ± 0.01	

[a] Retention time (min).
[b] Kovats index relative to *n*-alkanes (C_9–C_{25}) on a DB-5MS column.
[c] Identification based on comparison of the mass spectrum, Kovats index on a DB-5MS column in reference (Adams, 2001) and co-injection with authentic compounds.

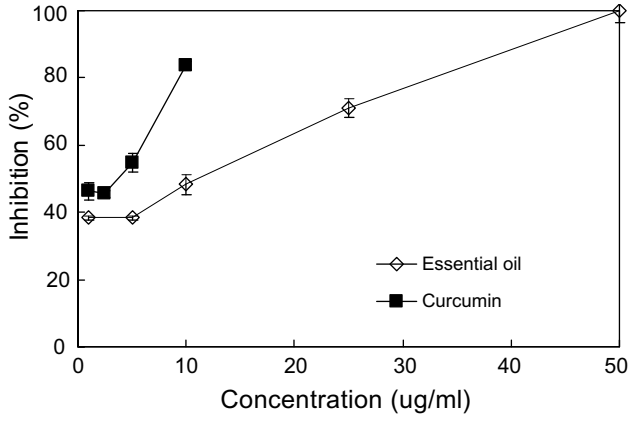

Fig. 1. Effects of essential oil from *C. osmophloeum* twigs and curcumin on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells. Results are mean ± SD ($n = 3$).

that concentrations up to 50 μg/ml produced no significant cytotoxic effects on cells treated with essential oil. According to the results reported by Lee et al. (2002), *Cinnamomum cassia* extract was found to possess significant inhibition of NO production, with an IC_{50} value is between 1 and 5 μg/ml. The result indicates that essential oil of *C. osmophloeum* twig has a good performance of inhibiting NO production. To understand the relationship between the constituents of *C. osmophloeum* twig essential oil and NO production inhibitory effects in RAW 264.7 macrophages, 10 constituents, namely L-borneol, α-terpineol, *p*-allylanisole, *trans*-cinnamaldehyde, L-bornyl acetate, eugenol, β-caryophyllene, cinnamyl acetate, *E*-nerolidol, and caryophyllene oxide were tested. Curcumin, a well known for its anti-inflammatory activity, was used in parallel as a positive control. *trans*-Cinnamaldehyde (59.9%) and caryophyllene oxide (54.0%) exhibited better NO inhibition effects than twig essential oil (48.3%) at the concentration of 10 μg/ml; while L-borneol (46.1%), L-bornyl acetate (45.7%), eugenol (46.2%), β-caryophyllene (50.9%), *E*-nerolidol (40.7%), cinnamyl acetate (48.1%) showed activity similar to that of twig essential oil. Nevertheless, α-terpineol (38.1%) and *p*-allylanisole (30.9%) were found to be the least active. In addition, MTT assay revealed no significant cytotoxic effects on cells treated with the 10 constituents at the dosage of 10 μg/ml.

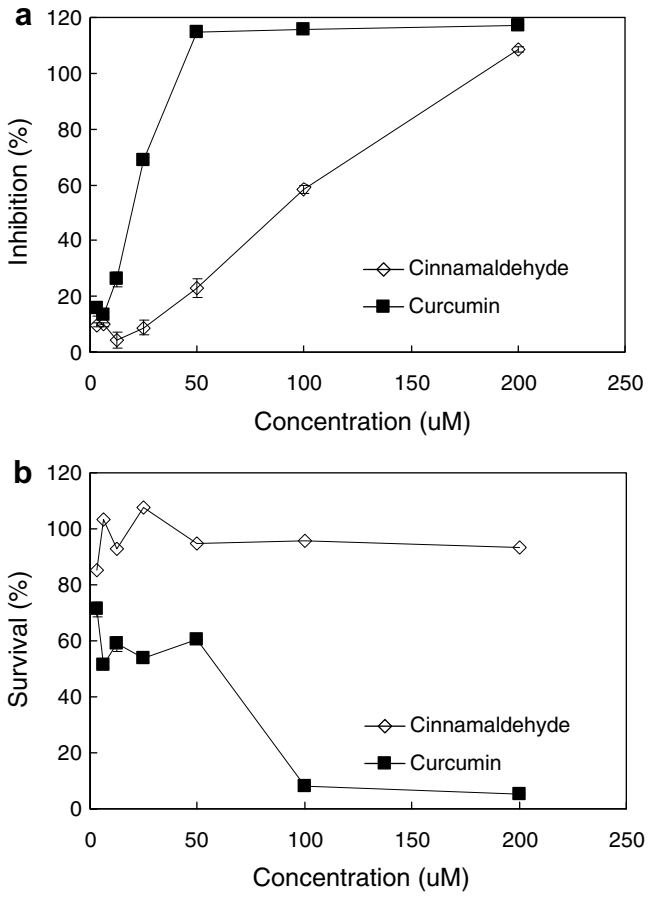

Fig. 2. Effects of *trans*-cinnamaldehyde and curcumin on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells. (a) represents the concentration-dependent inhibition of nitric oxide production and (b) indicates the cytotoxicty of pure compound on RAW 264.7 cells in the presence of LPS, measured by the MTT assay. Results are mean ± SD ($n = 3$).

Amongst the 10 compounds tested, *trans*-cinnamaldehyde exhibited the strongest activity. To examine further the inhibition of NO generation in LPS-stimulated RAW 264.7 cells, we selected different doses of *trans*-cinnamaldehyde. Fig. 2a shows that *trans*-cinnamaldehyde inhibited LPS-induced NO production in a concentration-dependent manner, with the IC_{50} value being 88.4 μM. Furthermore, at the dosage of 200 μM, the inhibition of NO production by *trans*-cinnamaldehyde was 100.8%. In addition, MTT assay revealed no significant cytotoxic effects on cells treated with *trans*-cinnamaldehyde at concentrations up to 200 μM (Fig. 2b). Lee et al. (2002) have also proved that *trans*-cinnamaldehyde has strong activity on suppressing NO synthase. Thus, *trans*-cinnamaldehyde might be a potential lead compound for the development of anti-inflammatory drugs.

3.4. Effects of essential oil and its constituents on PGE_2 production in LPS-stimulated RAW 264.7 cells

The activation of macrophages plays a critical role in the inflammatory process by releasing a variety of inflammatory mediators (Hwang et al., 2002). Besides NO production, PGE_2 was also an important inflammatory mediator involved in the pathogenesis (Surh et al., 2001). Thus, in addition to measuring NO productions, determining the inhibitory effects on the abnormal accumulation of PGE_2 was another method by which the anti-inflammatory effects of plant extracts can be assessed.

After induction of LPS, the accumulation of PGE_2 in RAW 264.7 cells increased from 72.7 to 655.6 pg/ml. However, indomethacin, the positive control and essential oil of *C. osmophloeum* twigs inhibited the LPS-induced accumulation of PGE_2 in a concentration-dependent manner. At a concentration of 10 μg/ml, essential oil of *C. osmophloeum* twigs significantly decreased almost 65% of the PGE_2 production in LPS-induced RAW 264.7 cells, while indomethacin exhibited 98% inhibition of PGE_2 production. At a concentration of 25 μg/ml, caryophyllene oxide and L-bornyl acetate decreased almost 18% and 28% of the PGE_2 production. The result reveals no significant inhibition effects of PGE_2 were observed for caryophyllene oxide and L-bornyl acetate, which are two major constituents in the twig essential oil.

In the anti-inflammatory assay, essential oil of *C. osmophloeum* twigs showed excellent inhibitory effects on PGE_2, but unfortunately, major constituents such as caryophyllene oxide and L-bornyl acetate, were not responsible for suppressing PGE_2 production. Our findings suggested that the excellent performance of twig essential oil might be attributable to the effects of minor constituents or synergetic effects among the constituents. To find out the key elements responsible for suppressing PGE_2 production and to clarify the mechanisms involved, further investigations are warranted.

4. Conclusion

This study demonstrated that essential oil of *C. osmophloeum* twigs has excellent anti-inflammatory activities and cytotoxicity against HepG2 cells. Furthermore, it also indicated that the constituents of *C. osmophloeum* twig essential oil such as *trans*-cinnamaldehyde, caryophyllene oxide, L-borneol, L-bornyl acetate, eugenol, β-caryophyllene, *E*-nerolidol, and cinnamyl acetate exhibited excellent anti-inflammatory activities in suppressing nitric oxide production by LPS-stimulated macrophages. The essential oil of *C. osmophloeum* twigs is worthy for further investigation, due to its excellent performance found in this study.

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