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RESEARCH ARTICLE

Anti-inflammatory activities of essential oils and their constituents from different provenances of indigenous cinnamon (*Cinnamomum osmophloeum*) leaves

Yu-Tang Tung, Pei-Ling Yen, Chun-Ya Lin, and Shang-Tzen Chang

School of Forestry and Resource Conservation, National Taiwan University, Taipei 106, Taiwan

Abstract

Context: Cinnamomum osmophloeum Kaneh. (Lauraceae) is one of the indigenous tree species in Taiwan. This tree species has been of interest to researchers because the chemical constituents of its essential oil are similar to those of *Cinnamomum cassia* Presl. bark oil, known as cinnamon oil, which is commonly used in foods and beverages.

Objective: The anti-inflammatory activities of the leaf essential oils and their major compounds from seven provenances of *C. osmophloeum* are investigated here for the first time.

Materials and methods: Chemical compositions of hydrodistilled essential oils obtained from *C. osmophloeum* leaves were analyzed by gas chromatography-mass spectrometry (GC-MS), and the effects of essential oils on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages were investigated.

Results: The leaf essential oils of cinnamaldehyde type and mixed type strongly inhibited NO production, with IC_{50} values ranging from 9.7-15.5 μg/mL. Furthermore, *trans*-cinnamaldehyde is responsible for the inhibitory activity of cinnamaldehyde type, and T-cadinol and α-cadinol are responsible for the inhibitory activity of mixed type.

Discussion and conclusion: These findings demonstrate that the leaf essential oils and their constituents of *C. osmophloeum* have excellent anti-inflammatory activities and thus have great potential as a source for natural health products.

Keywords: *Anti-inflammation; Cinnamomum osmophloeum; trans-cinnamaldehyde; essential oil; leaves*

Introduction

Nitric oxide (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 larger 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, thereby triggering 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.

Cinnamomum osmophloeum Kaneh. (Lauraceae) is a tree indigenous to natural hardwood forests of Taiwan at elevations between 400-1500 m. This tree species has been of interest to researchers because the chemical constituents of its essential oil are similar to those of *Cinnamomum cassia* Presl. bark oil, known as cinnamon oil, which is commonly used in foods and beverages (Ooi et al., 2006). Recent phytochemical analyses and biological screenings of *C. osmophloeum* have focused on the leaf essential oil

Address for Correspondence: Professor Dr Shang-Tzen Chang, School of Forestry and Resource Conservation, National Taiwan University, Taipei 106, Taiwan. Tel: +886 2 3366 4626; Fax: +886 2 2365 4520; E-mail: peter@ntu.edu.tw

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constituents, which have shown excellent antibacterial, antitermite, antimite, antimildew, antimosquito larvae, and antifungal effects (Chang et al., 2001; Chang & Cheng, 2002; Chen & Chang, 2002; Chen et al., 2002; Cheng et al., 2004, 2006). Furthermore, Chao 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 production in LPS-stimulated macrophages. Tung et al. (2008) also found that *C. osmophloeum* twigs strongly suppress NO synthase in LPS-stimulated macrophages. However, to the best of our knowledge, there is no prior study on the effects of provenances for leaf essential oils of *C. osmophloeum*. In this study, the leaf essential oils of *C. osmophloeum* from seven provenances were analyzed by gas chromatography-mass spectrometry (GC-MS), and the effects of essential oils and their constituents on nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages were investigated.

Materials and methods

Chemicals

Lipopolysaccharide (LPS), Greiss reagent and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Linalool, D-(+)-camphor, 3-phenylpropionaldehyde, *trans*-cinnamaldehyde, L-bornyl acetate, cinnamyl acetate, (-)-aromadendrene, caryophyllene oxide, curcumin, and methyl sulfoxide (DMSO) were all purchased from Acros (Geel, Belgium). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). α-Cadinol and T-cadinol were isolated from leaf essential oil of *C. osmophloeum*.

Plant material

The leaves of seven *Cinnamomum osmophloeum* provenances (COA–COG) were collected at the end of October 2005 from the Taiwan Sugar Company Research Center located in Nantou County in central Taiwan. The species were confirmed by Yen-Ray Hsui of the Taiwan Forestry Research Institute and voucher specimens (CO0109, CO4407, CO1709, CO0403, CO0902, CO0502, and CO0609) were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University).

Essential oil distillation

Leaves of *C. osmophloeum*, in triplicate, were subjected to hydrodistillation for 6 h in a Clevenger-type apparatus (Tung et al., 2008). The yellow essential oils with characteristic odor and hot water extracts were obtained and stored in airtight containers prior to analysis.

Analysis of essential oil

Essential oils from leaves were analyzed by a Trace GC Ultra (Thermo, Austin, TX) gas chromatograph coupled with a Polaris Q (Thermo, Austin, TX) ion-trap mass spectrometer, equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MS (Agilent J & W Scientific, USA). The mass spectrometer was operated in the electron-impact mode, with an ionization energy of 70 eV. The sector mass analyzer was set to scan from 50 to 400 amu. The GC oven temperature was programmed to start from 60°C, hold 1 min, rise to 220°C at 4°C/min, hold for 2 min, and rise to 250°C at 20°C/min, hold for 3 min. The injector temperature was 250°C; and the flow rate of carrier gas, helium, was at a 1 mL/min; 1:10 split ratio. Diluted samples (1 μL, 1/10000, 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_{19} on DB-5MS column. The major constituents of *C. osmophloeum* leaf oil were identified by co-injection with standards (wherever possible), confirmed with Kovats indices (Adams, 2007) 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.

Anti-inflammatory activity

Cell line and cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from the Culture Collection and Research Center (CCRC), Hsinchu, Taiwan. Cells were cultured at 37°C in a 5% CO_2 incubator and maintained in disks with DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Anti-inflammatory activity assay

To investigate the anti-inflammatory activity of the leaf essential oil from different *C. osmophloeum* provenances, NO production 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 4 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 LPS. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (Wu et al., 2008). Briefly, 100 μL cell culture supernatant was reacted with 100 μL 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 an ELISA reader.

Table 1. Composition of essential oils from *C. osmophloeum* leaves.

KI [a]	Compound	COA	COB	COC	COD	COE	COF	COG
937	α-Pinene	-	0.5	0.17	-	0.15	0.39	0.24
955	Camphene	-	0.4	0.13	-	0.13	0.31	-
966	Benzaldehyde	0.38	3.41	2.77	-	0.86	-	-
981	(1*S*)-(-)-β-Pinene	0.08	0.33	0.12	-	0.06	0.14	0.12
991	β-Myrcene	-	-	-	-	-	0.06	0.14
995	Mesitylene	0.08	0.14	-	-	-	-	-
1028	*p*-Cymene	-	-	-	-	-	0.52	-
1032	Limonene	-	0.25	0.08	-	0.11	0.61	0.33
1088	*cis*-Linalool oxide	-	-	-	-	-	-	0.18
1099	Linalool	0.22	-	0.07	-	0.13	0.09	96.28
1151	D-(+)-Camphor	-	-	-	-	0.06	56.63	-
1164	3-Phenylpropionaldehyde	5.35	6.72	3.35	-	0.65	-	-
1174	L-Borneol	0.1	0.11	-	-	-	0.35	-
1182	(+)-Terpinen-4-ol	-	-	-	-	-	1.04	-
1196	α-Terpineol	0.18	0.22	0.05	0.74	0.37	1.19	0.2
1198	4-Allylanisole	0.8	1.13	0.73	2.49	1.69	0.24	0.25
1219	*cis*-Cinnamaldehyde	0.37	0.35	0.53	-	0.08	0.08	-
1252	Chavicol	0.13	0.16	-	-	0.59	0.1	-
1273	*trans*-Cinnamaldehyde	72.93	62.64	77.21	-	9.09	0.35	0.38
1285	L-Bornyl acetate	3.84	4.83	1.11	21.98	2.88	31.33	-
1352	Eugenol	1.2	1.57	0.29	-	0.29	0.4	-
1370	Cyclosativene	-	-	-	0.67	-	-	-
1376	(-)-α-Copaene	0.25	0.44	0.13	4.34	2.3	0.49	-
1384	β-Bourbonene		0.06	0.08	-	0.05	-	-
1420	β-Caryophyllene	2.74	4.1	0.41	-	0.96	0.14	0.48
1433	Coumarin	-	-	-	3.64	1.36	0.72	0.25
1439	(+)-Aromadendrene	-	-	-	-	0.13	-	-
1445	Cinnamyl acetate	8.82	9.54	11.57	-	40.78	0.59	-
1456	α-Humulene	0.42	0.4	-	-	0.17	-	-
1460	(-)-Aromadendrene	-	-	0.09	5.52	0.78	-	-
1475	γ-Muurolene	-	0.05	-	2.66	0.26	-	-
1494	β-Selinene	-	0.03	-	0.59	-	-	-
1497	α-Muurolene	-	-	-	1.13	0.38	0.06	-
1512	γ-Cadinene	-	-	-	1.82	0.68	0.1	-
1517	δ-Cadinene	0.23	0.27	0.23	-	2.3	0.27	0.12
1520	*trans*-Calamenene	-	-	-	2.23	0.57	0.05	-
1541	α-Calacorene	-	-	-	0.58	0.28	-	-
1560	(-)-Nerolidol	0.09	0.09	-	-	0.27	0.12	-
1580	Caryophyllene oxide	1.06	1.25	0.18	5.62	6.2	0.8	-
1614	*epi*-cubenol	-	-	-	-	0.2	-	-
1640	T-Cadinol	-	-	-	10.9	5.49	0.64	-
1654	α-Cadinol	0.06	0.05	0.07	11.15	5.39	0.6	-

[a]Kovats index relative to *n*-alkanes (C_9-C_{19}) on a DB-5MS column.

Cell viability

The cell viability assay was determined on the basis of MTT assay, as described above with a slight modification. After culturing, supernatants were collected for NO measurement, 100 μL tetrazolium salt solution (1 mL MTT/10 mL DMEM) was 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.

Cluster analysis and principal components analysis

The cluster analysis and principal components analysis (PCA) were performed using multivariate statistical package (MVSP) software to identify relatively homogeneous groups of seven provenances (COA-COG) of *C. osmophloeum* based on the percentage composition of their essential

oil samples. Euclidean distance was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

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.

Results and discussion

Yields and constituents of essential oil

A total of 42 compounds of COA, COB, COC, COD, COE, COF, and COG were identified in the leaf essential oils, representing 99.3, 99, 99.4, 76.1, 85.7, 98.4, and 99%, respectively of the essential oils. Major compositions of seven essential oils are reported in Table 1. The leaf essential oils of COA, COB, and COC were found to contain mainly *trans*-cinnamaldehyde (72.9, 62.6, and 77.2%,

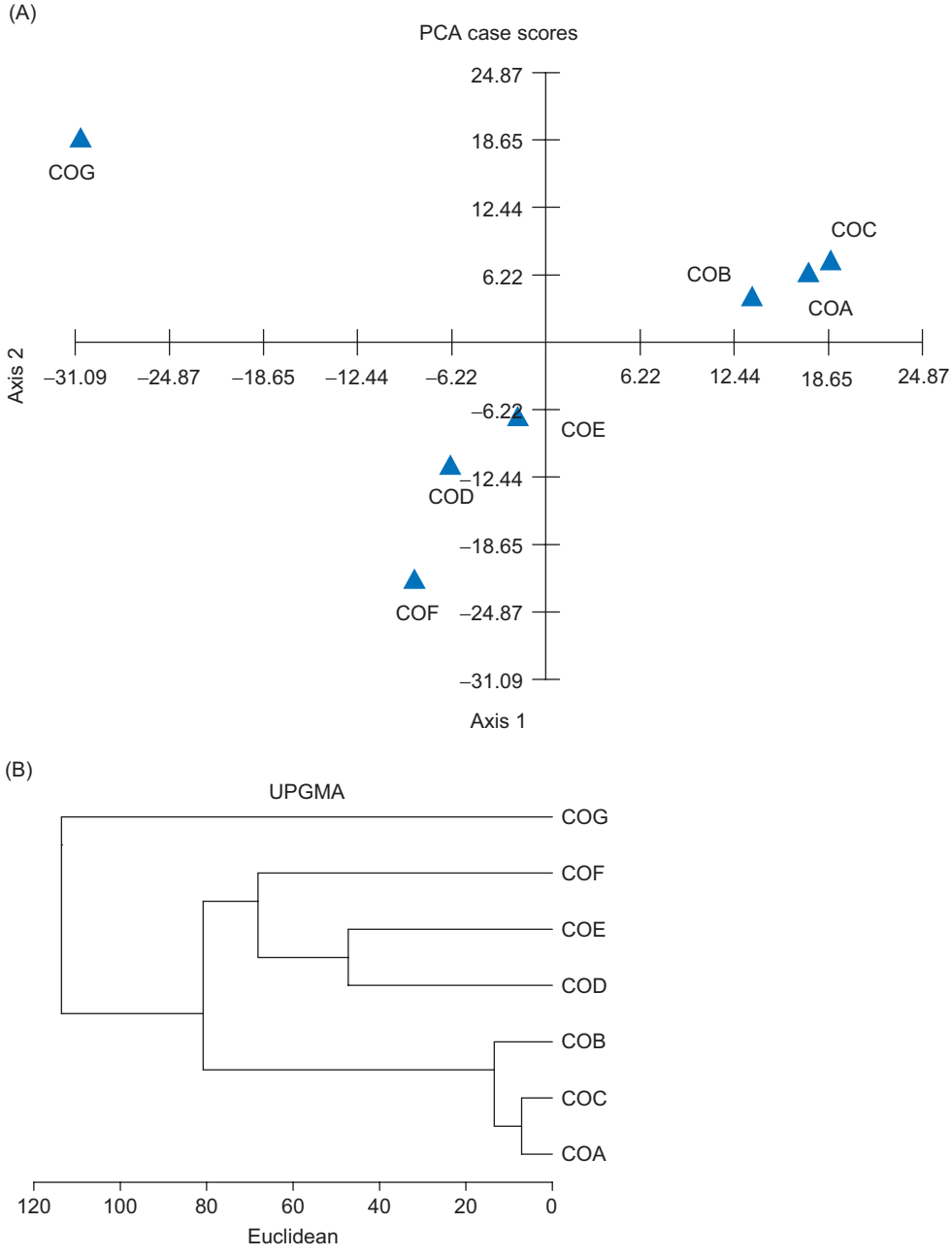

Figure 1. (A) Principal component analysis of essential oils from *C. osmophloeum* leaves, scatter plot of PC2 compared with PC1 dependence. (B) Dendrogram of cluster analysis in essential oils from *C. osmophloeum* leaves.

respectively). The major constituent of leaf essential oil from COF was D-(+)-camphor (56.6%). The major constituent of leaf essential oil from COG was linalool (96.3%). The leaf essential oil from COD was found to be rich in L-bornyl acetate (22%), α-cadinol (11.2%), T-cadinol (10.9%), caryophyllene oxide (5.6%), and (-)-aromadendrene (5.5%). The leaf essential oil of COE was found to be rich in cinnamyl acetate (40.8%), *trans*-cinnamaldehyde (9.1%), caryophyllene oxide (6.2%), T-cadinol (5.5%), and α-cadinol (5.4%). Principal component analysis (PCA) and cluster analysis were performed to detect the degrees of similarity of the compositions of the leaf essential oils analyzed (Figure 1). Different groups can be identified in the loading plots of PC1 and PC2. The leaf essential oils obtained from different geographical provenances were classified into four chemotypes: cinnamaldehyde type (COA, COB, and COC), camphor type (COF), linalool type (COG), and mixed type (COD and COE).

Effects of essential oils and hot water extracts from different provenances on NO production in LPS-stimulated RAW 264.7 cells

As for the inhibitory effects of the leaf essential oils and hot water extracts from four chemotypes of *C. osmophloeum* on NO production, data in Figure 2 and Table 2 show that the leaf essential oils had better inhibitory effect than the hot water extracts. The leaf essential oils of cinnamaldehyde type (COA, COB, and COC) and mixed type (COD and COE) showed excellent inhibitory effects. The IC_{50} values of cinnamaldehyde type (COA, COB, and COC) and mixed type (COD and COE) were 11.7, 9.7, 13.1, 11.6, and 15.5 μg/mL, respectively. However, the IC_{50} values of camphor type and linalool type were 65.8 and >100 μg/mL, respectively. In addition, MTT assay revealed that concentrations up to 100 μg/mL produced no significant cytotoxic effects on cells treated with the leaf essential oils. These results indicate that of the 4 chemotypes tested, the leaf essential oils of cinnamaldehyde type and mixed type inhibit NO production most strongly. Cinnamaldehyde type (COA, COB, and COC) contained more *trans*-cinnamaldehyde and also strongly inhibited NO production, consistent with our previous study showing that *trans*-cinnamaldehyde has excellent inhibitory activity on NO production (Tung et al., 2008). Furthermore, Lee et al. (2002) showed that *trans*-cinnamaldehyde strongly suppresses NO synthase.

Effects of the constituents on NO production in LPS-stimulated RAW 264.7 cells

To understand the relationship between the constituents of *C. osmophloeum* leaf essential oils and

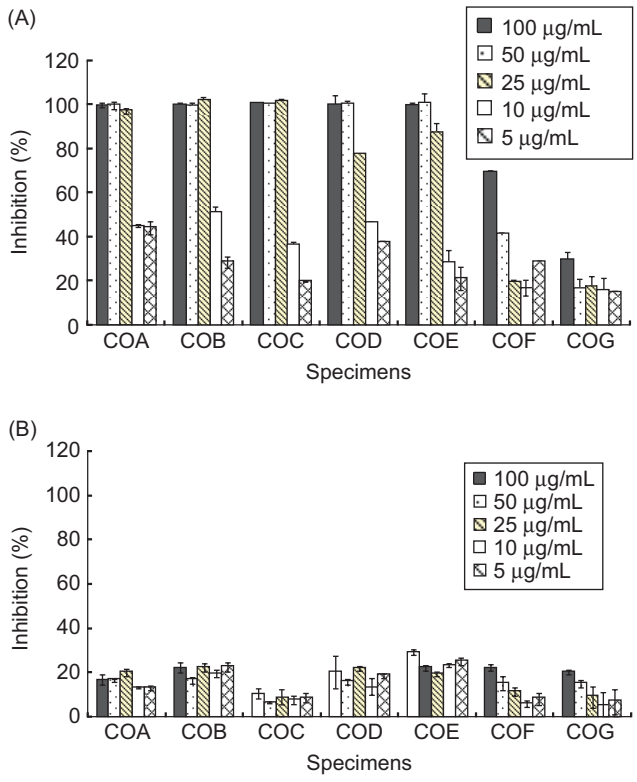

Figure 2. Effects of (A) essential oils and (B) hot water extracts from *C. osmophloeum* leaves on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells. Results are mean ± SD ($n = 3$).

Table 2. IC_{50} values (μg/mL) of essential oils and hot water extracts from *C. osmophloeum* leaves on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells.

Specimens	Essential oil	Hot water extract
COA	11.4	> 100
COB	9.7	>100
COC	13.1	>100
COD	11.6	>100
COE	15.5	>100
COF	65.8	>100
COG	>100	>100

inhibition of NO production in RAW 264.7 macrophages, ten constituents, namely linalool, D-(+)-camphor, 3-phenylpropionaldehyde, *trans*-cinnamaldehyde, L-bornyl acetate, cinnamyl acetate, (-)-aromadendrene, caryophyllene oxide, T-cadinol, and α-cadinol were tested. Curcumin, well known for its anti-inflammatory activity, was used in parallel as a positive control. As shown in Figure 3 and Table 3, *trans*-cinnamaldehyde, (-)-aromadendrene, caryophyllene oxide, T-cadinol, and α-cadinol exhibited strong NO production inhibitory effects. The IC_{50} values of these five compounds were 15.7, 16.5, 43.2, 20.8, and 14.0 μg/mL (118.6, 80.5, 195.9, 93.6, and 63.2 μM), respectively. Nevertheless,

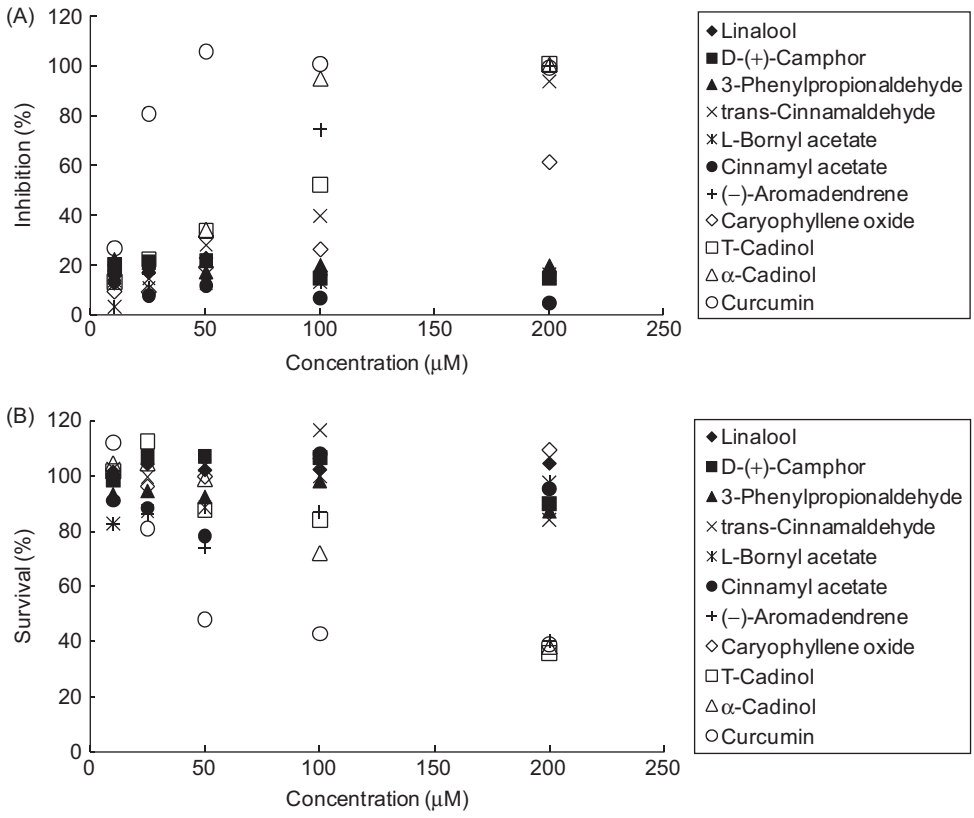

Figure 3. Effects of compounds from *C. osmophloeum* leaves and curcumin on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells. (A) represents the concentration-dependent inhibition of nitric oxide production; and (B) indicates the cytotoxicity of pure compound on RAW 264.7 cells in the presence of LPS, measured by the MTT assay. Results are mean ± SD ($n = 3$).

linalool, D-(+)-camphor, 3-phenylpropionaldehyde, L-bornyl acetate, and cinnamyl acetate were found to be the least active (IC_{50} value > 200 μM). In addition, MTT assay revealed no significant cytotoxic effects on cells treated with the ten constituents at the dosage of 100 μM (Figure 3B).

Although the three major compounds, 3-phenylpropionaldehyde, *trans*-cinnamaldehyde, and cinnamyl acetate, all exist in the leaf essential oils of cinnamaldehyde type (COA, COB, and COC), *trans*-cinnamaldehyde clearly is responsible for the excellent inhibition of NO production. Similar results in previous studies showed that in the major compounds of *C. osmophloeum* leaf essential oils *trans*-cinnamaldehyde performed best against bacteria, termites, mites, mildew, pathogens, mosquitoes, and fungi (Chang et al., 2001; Chang & Cheng, 2002; Chen et al., 2002; Chen & Chang, 2002; Cheng et al., 2004, 2006). Furthermore, of the major compounds of COD, (-)-aromadendrene, caryophyllene oxide, T-cadinol, and α-cadinol are responsible for the excellent inhibitory activity on NO production. In addition, *trans*-cinnamaldehyde, caryophyllene oxide, T-cadinol, and α-cadinol are responsible for the inhibitory activity on NO production of COE. Therefore, it is reasonable to conclude that T-cadinol and α-cadinol of mixed type (COD and COE) are mainly responsible for the inhibitory activity on NO production. However, the IC_{50} values of camphor type (COF) and linalool type (COG) were 65.8 and > 100 μg/mL, and the major constituents of leaf essential oils from COF and from COG were camphor (56.6%) and linalool (96.3%), respectively. Nevertheless,

Table 3. IC_{50} values (μM) of compounds from *C. osmophloeum* leaves on nitric oxide production of LPS-stimulated RAW 264.7 macrophage cells.

Compounds	IC_{50} values (μM)
Linalool	>200
D-(+)-camphor	>200
3-Phenylpropionaldehyde	>200
Trans-cinnamaldehyde	118.6 (15.7) *
L-Bornyl acetate	>200
Cinnamyl acetate	>200
(-)-Aromadendrene	80.5 (16.5)
Caryophyllene oxide	195.9 (43.2)
T-Cadinol	93.6 (20.8)
α-Cadinol	63.2 (14.0)

*Values in brackets are μg/mL.

D-(+)-camphor and linalool were found to be the least active (IC_{50} value > 200 μM).

Conclusions

According to GC-MS and cluster analyses the leaf essential oils from seven provenances and their relative contents were classified into four chemotypes: cinnamaldehyde type, linalool type, camphor type, and mixed type. It was also found that the major constituents of *C. osmophloeum* leaf essential oils such as *trans*-cinnamaldehyde, (-)-aromadendrene, caryophyllene oxide, T-cadinol, and α-cadinol had excellent anti-inflammatory activities in suppressing nitric oxide production by LPS-stimulated macrophages. However, the leaf essential oils of cinnamaldehyde type and mixed type had excellent inhibitory activity on NO production, with IC_{50} values ranging from 9.7 to 15.5 μg/mL. The essential oils of *C. osmophloeum* leaves and their active compounds are worthy of further exploration, due to their excellent performance found in this study.

Declaration of interest

The authors declare no conflict of interest.

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