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Study on the Antiinflammatory Activity of Essential Oil from Leaves of *Cinnamomum osmophloeum*

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The leaf essential oil from indigenous cinnamon (*Cinnamomum osmophloeum* Kaneh.) was investigated by gas chromatography−mass spectrometry, and 21 compounds were identified. The major constituents of leaf essential oil were the monoterpenes 1,8-cineole (17.0%) and santolina triene (14.2%) and the sesquiterpenes spathulenol (15.7%) and caryophyllene oxide (11.2%). In the antiinflammatory activity assay, we demonstrated that the essential oil has a higher capacity to inhibit proIL-1β protein expression induced by LPS-treated J774A.1 murine macrophage. At dosages of 60 μg/mL, essential oil clearly inhibited proIL-1β protein expression. Furthermore, a dose of 60 μg/mL of essential oil was effectively inhibitory for IL-1β and IL-6 production but not for TNF-α, suggesting that essential oil was bioactive in antiinflammation in vitro. This study is the first to report antiinflammatory activity of extracts obtained from the leaf essential oil of *C. osmophloeum*.

KEYWORDS: ***Cinnamomum osmophloeum*; leaf; essential oils; GC-MS; antiinflammatory activity; cytokines; macrophages**

INTRODUCTION

Indigenous cinnamon (*Cinnamomum osmophloeum* Kaneh.) (*Cinnamomum*), which belongs to the family Lauraceae, is an endemic tree that grows in Taiwan's natural hardwood forests at elevations between 400 and 1500 m. Many previous studies demonstrate the bioactivity of these forestry industry products. For example, Chang et al. found that the leaf essential oil of *C. osmophloeum* has an excellent inhibitory effect against bacteria, termites, mites, mildew, and fungi (*1*−*5*). Cheng et al. (*6*) reported that essential oil from the leaves of *C. osmophloeum* exhibited the strongest mosquito larvicidal activity. It is well-known that in the past, the essential oils from the plant were commonly used in folk medicine, food flavorings, and fragrance; their multiple bioactive functions have been examined and developed in recent years.

Most of the chemical constituents of plant essential oils belong to terpenoid compounds, including monoterpenes, sesquiterpenes, and their oxygenated derivatives. These low molecular weight (most below 300 g/mol) compounds easily diffuse across cell membranes to induce biological reactions. In recent years, there has been a tendency for applied studies of essential oils to focus on antimicrobial and the mosquito larvicidal activities as well as antiinflammatory bioactivity. Previous studies of antiinflammatory capacity found that the essential oils of some Lamiaceae can reduce carrageenan-induced hind paw inflammation in the rat (*7*−*9*). Hart et al. (*10*) demonstrated that tea tree oil would reduce the production in vitro of TNF-α, IL-1β, IL-8, IL-10, and PGE_2 by lipopolysaccharide (LPS)-activated human blood monocytes.

To further investigate the effects of the antiinflammatory activities of the essential oil from the leaves of *C. osmophloeum*, we examined the influence of this oil on the murine macrophage model (J774A.1 cell). The LPS or endotoxin is a well-known pathogen-associated molecular pattern, localized on Gram-negative bacteria cell walls (*11*). LPS activates macrophages by binding to toll-like receptor 4 and stimulates the production of inflammatory cytokines, including TNF-α, IL-1β, and IL-6 protein. While mediation of inflammation against pathogen infection by TNF-α, IL-1β, and IL-6 proteins could be beneficial to the host, overexpression of these cytokines may cause serious disease, including septic shock. Hence, suppression of TNF-α, IL-1β, and IL-6 protein production could aid in the treatment of septic shock. The IL-1β protein is secreted mainly from activated macrophages and is a central mediator of the cytokine network involved in countless biological functions and inflammation. Regulation of IL-1 expression in macrophages is a well-established model in previous studies (*12*−*14*).

In this study, leaf essential oil from *C. osmophloeum* was analyzed by gas chromatography−mass spectrometry (GC-MS)

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for its chemical composition, and the antiinflammatory activities of this leaf essential oil were investigated. In addition, as the above-mentioned cytokines play an important role in regulating cell growth and survival, including mitogenic and stress responses, we were prompted to investigate whether essential oil affects LPS-induced cytokine production. Herein, we present evidence that IL-1β and IL-6 protein production in macrophages are inhibited by leaf essential oil from *C. osmophloeum*. This is the first report to demonstrate that leaf essential oil from *C. osmophloeum* has antiinflammatory activities in macrophages. However, the efficacy and safety of this oil need to be further investigated if it is to be used as a source of natural agent in the future.

MATERIALS AND METHODS

Collection of Essential Oil from *C. osmophloeum* Leaf. The leaves of 13 year old *C. osmophloeum* were collected on August, 2003, from the Haw-Lin Experimental Forest located in Taipei county. The species was identified by Yen-Ray Hsui of the Taiwan Forestry Research Institute, and the voucher specimen was deposited at the laboratory of wood chemistry, School of Forestry and Resource Conservation, National Taiwan University. The fresh leaf oil of *C. osmophloeum* was obtained by using water distillation for 6 h, and their constituents were determined by GC-MS.

GC-MS Analysis. The mass spectrometer was equipped with a PoLaris Q mass selective detector in the electron impact ionization mode (70 eV). A Trace gas chromatograph was used and operated under the following conditions: RTx-5 capillary column (30 m × 0.25 mm; film thickness 0.25 μm); held at 80 °C for 1 min, raised to 200 °C at a rate of 4 °C/min, and held for 5 min; 250 °C injector temperature; carrier helium at a flow rate of 10 mL/min; 1:10 split ratio. Diluted samples (1.0 μL, 1/100, v/v, in ethyl acetate) were injected manually in the splitless mode. Identification of the major components of *C. osmophloeum* leaf oil was confirmed by comparison with standards, by spiking, and on the basis of their mass spectral fragmentation using the Wiley GC-MS library. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

Cell Culture. Murine macrophage J774A.1 cells were obtained from ATCC (Rockville, MD), propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured in a 37 °C, 5% CO_2 incubator, unless otherwise indicated. J774A.1 cells were pretreated with extracts [dissolved in dimethyl sulfoxide (DMSO)] for 30 min, followed by LPS (1 μg/mL) treatment for an additional 6 h. The sample was dissolved in DMSO, and the final DMSO concentration was 0.1% in all cultures containing this agent; the same amount of vehicle was added to the control cultures. The proIL-1β protein expression level was analyzed by Western blot using anti-IL-1β antibody as described by Hsu et al. (*13*).

Western Blot. To investigate the inhibitory effect of essential oil of the leaves from *C. osmophloeum* on proIL-1β production in LPS-stimulated J774A.1, the cells were pretreated with essential oil (0−60 μg/mL) for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for 6 h. The reaction was stopped by the addition of ice-cold phosphate-buffered saline (PBS) containing 5 mM Na_3VO_4. Cells were immediately pelleted at 4 °C and lysed with 100 μL of ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 205 mM pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 2 μg/mL leupeptin, and 1 mM PMSF) on ice for 10−15 min. Insoluble material was removed by centrifugation at 4 °C for 15 min at 12000*g*. The protein concentrations were determined using Bio-Rad protein assay. Samples were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride filter (Millipore Inc, Bedford, MA). Filters were incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20). These blocking steps were performed at room temperature for 1 h. Filters were incubated with primary anti-IL-1β antibody for 2 h. After they were washed three times in PBS with 0.1% Tween 20, filters were incubated with an HRP-conjugated secondary antibody directed against primary antibody. Blots were developed by an enhanced chemiluminescence Western blotting detection system (DuPont NEN Research Product Co., Boston, MA) according to the manufacturer's instructions. Histograms represent quantification by PhosphorImager of proIL-1β in J774A.1 cells with ImageQuaNT software from Molecular Dynamics.

Enzyme-Linked Immunosorbent Assay (ELISA). To investigate the inhibitory effect of the essential oil of the leaves from *C. osmophloeum* on TNF-α, IL-6, and IL-1β protein production from LPS-stimulated J774A.1 cells, the cells were pretreated with essential oil (0−60 μg/mL) for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for 6 or 24 h. The cytokines secretions in J774A.1 cells were measured following the protocol from R & D mouse TNF-α, IL-1β, and IL-6 ELISA System (R & D Systems, Inc). Briefly, the cells were incubated with essential oil at indicated times, and the cell culture medium was collected. For assaying, 50 μL of biotinylated antibodies reagent was added to anti-mouse TNF-α, IL-1β, and IL-6 precoated stripwell plate with 50 μL of supernatant from tested samples and incubated at room temperature for 2 h. After the plate was washed three times with washing buffer provided in kit components, 100 μL of diluted streptavidin-HRP concentrate was added to each well and the plate was incubated at room temperature for 30 min. The washing process was repeated, and 100 μL of premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition, 100 μL of provided stop solution was added to each well to stop the reaction, and the absorbance of the plate was measured by MRX microplate reader (Dynex Tech. Inc., VA) at 450−550 nm wavelength. The calculation of the relative absorbance units, and the IL-1β concentration for each sample, as well as the construction of standard curve of recombinant mouse TNF-α, IL-1β, and IL-6 calibration curves were carried out as described in the manual of R & D Systems.

Microculture Tetrazolium (MTT) Assay for Cell Viability. The cytotoxicity of the essential oil from the leaves of *C. osmophloeum* was assessed using the MTT assay (*15*, *16*). After they were cultured on 96 well plates for 24 h, the cells were washed twice and incubated with 100 μL of 1 mg/mL of MTT for 2 h at 37 °C. The medium was discarded, and 100 μL of lysis buffer was then added. After 30 min of incubation, the absorbance ($A_{550} - A_{690}$) was measured by spectrophotometry.

Statistical Analyses. Percent of mortality was determined and transformed to arcsine square-root values for analyses of variance. Significant differences ($P < 0.05$) were determined by the Scheffe test.

RESULTS AND DISCUSSION

Chemical Compositions of Essential Oil. Distillation of *C. osmophloeum* leaves yielded about 0.55% (v/w) essential oil based on dry weight. **Table 1** shows the compositions of the essential oil, which contains 21 compounds identified in the leaf essential oil from the *C. osmophloeum*, including 90.1% terpenoid compounds. The major constituents of leaf essential oil from *C. osmophloeum* were 1,8-cineole (17.0%), santolina triene (14.2%), spathulenol (15.7%), and caryophyllene oxide (11.2%), respectively, and a marked difference was observed between the same species or other species (*2*, *5*). In addition, according to our previous classification (*6*), the leaf essential oil from the *C. osmophloeum* was classified as a mixed type because of lack of a dominant compound.

Antiinflammatory Activity of Essential Oil. Several bioactivities of leaf essential oil from *C. osmophloeum*, except for the antiinflammatory activity, have been demonstrated in our previous studies (*1*−*6*). In addition, we have found that some essential oils containing the major constituents similar to those in the leaf oil of *C. osmophloeum* have a number of other bioactivities. For example, the essential oil of *Melaleuca leucadendron*, which contains 1,8-cineole (64.3%) as a major constituent (*16*), exhibited more effectiveness against Herpes simplex virus type 1 as studied in African green monkey kidney

Table 1. Chemical Compositions of Leaf Essential Oil from *C. osmophloeum*

chemical compounds	RT[a]	RI[b]	%	primary fragments in the mass spectrum
6-camphenol	4.29	1070	1.12	43, 55, 67, 77, 93*, 108, 126
1,8-cineole	5.17	1169	17.04	43, 55, 67, 81, 93*, 108, 125, 139, 154
cis-linalool oxide	5.98	1246	3.06	43, 67, 79*, 93, 109, 137, 155
trans-linalool oxide	6.33	1276	2.89	43, 67, 79*, 91, 109, 137, 155
santolina triene	6.54	1294	14.24	43, 55, 67, 79, 91*, 105, 121, 136
1-butenylidene-cyclohexane	8.35	1423	1.49	39, 67*, 79, 93, 107, 121, 136
sabinene hydrate	8.62	1440	1.00	43, 55, 71, 93*, 111, 136, 154
α-terpineol	8.96	1460	3.64	31, 43, 51, 55, 59, 67, 71, 93*, 105, 121, 136
α-campholenal	9.87	1511	1.24	43, 55, 67, 79, 93, 108*, 126
trans-verbenol	10.31	1535	4.20	31, 39, 51, 59, 67, 79, 91, 109*, 119, 137
cis-geraniol	10.66	1552	0.61	41, 53, 67, 91*, 105, 121, 139
citral	11.16	1577	6.14	41*, 53, 56, 67, 79, 91, 109, 137, 152
1,4,4-trimethylcyclohex-2-enecarboxylic acid	13.70	1685	1.09	39, 55, 81, 123*, 148, 168
germacrene-D	14.30	1708	1.62	41, 59, 105, 133, 161*, 204
geranyl acetate	14.43	1713	0.83	41, 67, 93*, 121
τ-cadinene	18.37	1840	0.61	39, 105, 133, 161*, 204
spathulenol	20.23	1891	15.70	39, 50, 67, 79, 91*, 105, 131, 145, 159, 173, 187, 205
caryophyllene oxide	20.40	1896	11.23	39, 51, 67, 79, 91*, 105, 119, 161, 187, 202
δ-cadinene	21.61	1926	0.69	39, 105, 13, 159*, 179, 202
T-cadinol	21.98	1935	0.79	39, 105, 133, 161*, 204
α-cadinol	22.35	1944	0.87	39, 57, 105, 133, 161*, 204
monoterpene hydrocarbons (%)			1.49	
oxygenated monoterpene (%)			57.1	
sesquiterpene hydrocarbons (%)			2.92	
oxygenated sesquiterpene (%)			28.6	
sum			90.1	
yield (%)			0.55	

[a] Retention time (min). [b] Retention index relative to *n*-alkanes (C_9–C_{22}) on RTx-5MS column.

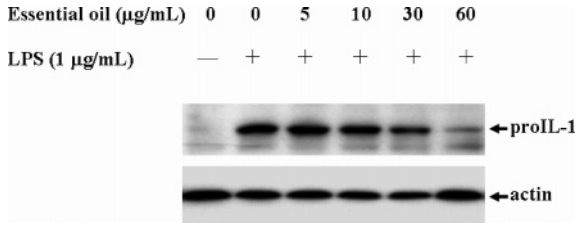

Figure 1. Essential oil of *C. osmophloeum* leaves inhibits proIL-1β protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of essential oil for 30 min prior to incubation with 1 μg/mL LPS for 6 h. The ProIL-1β protein expression level was analyzed by Western blot. One of four experiments is presented.

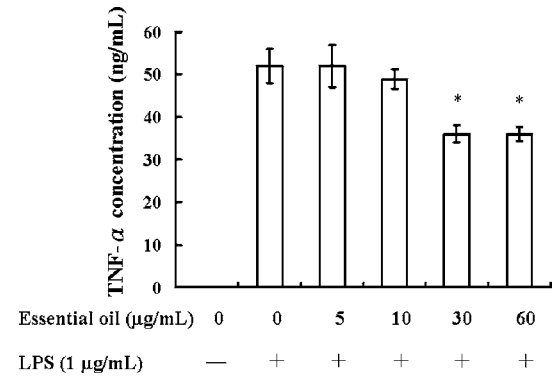

Figure 2. Essential oil of *C. osmophloeum* leaves inhibits TNF-α protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of essential oil for 30 min prior to incubation with 1 μg/mL LPS for 6 h. Culture media were assayed by using specific ELISA. The error bar for all experiments was mean ± SD ($n = 3$).

cells. Although the linalool oxide was a minor component (5.9%) in this essential oil, it has been proven to display antimicrobial and antiinflammatory bioactivies (*9*, *17*−*20*). Peana et al. also demonstrated that (−)-linalool not only could induce a significant decrease of acid-induced writhing but also possesses antiinflammatory activity in mice (*21*). Accordingly, the antiinflammatory activity of leaf essential oil from *C. osmophloeum* is worthy of investigation.

It is well-known that macrophages play an important role in regulating cell-mediated immune response. Besides the function of endocytosis, macrophages can be induced to secrete a series of cytokines such as IL-1β, IL-6, and TNF-α, which contribute to resistance against infective agents and tumor cells. In the present study, we utilized J774A.1 cells with a mouse model to observe the antiinflammatory capacity of the leaf essential oil from *C. osmophloeum* in LPS-stimulated cells. **Figure 1** shows that a dosage of 60 μg/mL of the essential oil could clearly inhibit proIL-1 protein expression when stimulated by 1 μg/mL LPS in J774A.1 cell.

Many essential oils have been used for treatment of inflammation, including some terpenoids that have been found to inhibit inflammatory cytokine (*22*, *23*). To investigate whether the leaf essential oil from *C. osmophloeum* exhibits immunomodulation activity on macrophages, J774A.1 cells were pretreated with the indicated concentrations of essential oil for 30 min and challenged with LPS for 6 h. As shown in **Figure 2**, bioactive TNF-α proteins were secreted from J774A.1 cells in response to the LPS challenge as measured by ELISA. Interestingly, LPS-induced TNF-α secretion was slightly reduced in macrophages pretreated with essential oil. Specifically, 52 ng/mL TNF-α was secreted from LPS-stimulated cells and LPS-induced TNF-α secretion was reduced to 36 and 35 ng/mL, by 30 and 60 μg/mL essential oil, respectively. In addition, pretreatment with essential oil also resulted in down-regulation of LPS-induced IL-1β protein secretion; cells were pretreated with essential oil for 30 min, followed by LPS challenge for 24 h. As shown in **Figure 3**, the leaf essential oil from *C. osmophloeum* showed a dose-dependent inhibitory effect on LPS-induced IL-1β protein secretion. A 94 pg/mL amount of IL-1β protein was secreted from LPS-stimulated cells, and this was reduced to 82, 78, 64, and 36 pg/mL by 5, 10, 30, and 60

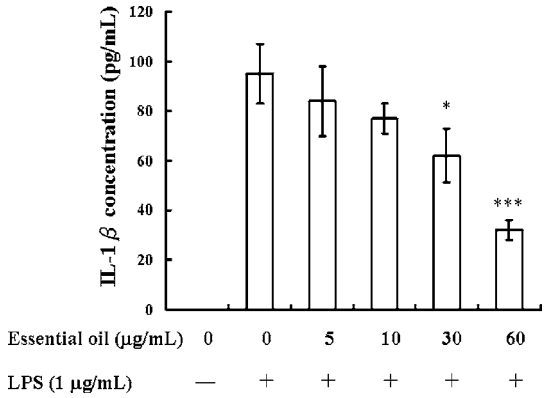

Figure 3. Essential oil of *C. osmophloeum* leaves inhibits IL-1β protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of essential oil for 30 min prior to incubation with 1 μg/mL LPS for 24 h. Culture media were assayed by using specific ELISA. The error bar for all experiments was mean ± SD ($n = 3$).

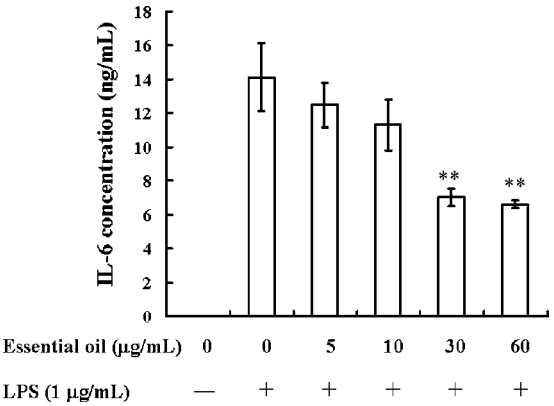

Figure 4. Essential oil of *C. osmophloeum* leaves inhibits IL-6 protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentrations of essential oil for 30 min prior to incubation with 1 μg/mL LPS for 6 h. Culture media were assayed by using specific ELISA. The error bar for all experiments was mean ± SD ($n = 3$).

μg/mL essential oil, respectively. Similar results have been observed in the Hart et al. studies (*10*); the essential oil of *Melaleuca alternifolia* at concentrations of 0.125% significantly suppressed LPS-induced production of TNF-α, IL-1β, IL-10 (approximately 50%), and PGE2 (approximately 30%) in human peripheral blood monocytes, and the terpinen-4-ol was found to be the main contributor to this inhibition. Several studies demonstrate that 1,8-cineole contributes more greatly to anti-inflammation in vitro (*24*, *25*).

Martin et al. (*7*), investigating the composition and anti-inflammatory activity of essential oil from *Bupleurum fruticescens*, found that the major components α-pinene (16.9%) and β-caryophyllene (30.6%) have obvious antiinflammatory activity in adrenolectomized rats. IL-6, which has been considered a proinflammatory cytokine, plays an important role in immune and inflammatory responses, such as TNF-α and IL-1β (*26*−*28*). Our study found that 60 μg/mL of essential oil could inhibit about 65% of IL-6 protein expression, as compared with macrophages mediated only by LPS (**Figure 4**). The results of our experiment are valuable as they imply that leaf essential oil from the *C. osmophloeum* has potential as an antiinflammatory agent.

Cytotoxicity in Macrophages. The effects of essential oil on murine macrophages were tested. Furthermore, after cells were treated with essential oil plus LPS for 24 h, no cytotoxic effect was observed, as measured by MTT assay (**Figure 5**). These results indicate that the inhibition of TNF-α, IL-1β, and IL-6 secretion by leaf essential oils from *C. osmophloeum* was not due to cell death. Although some studies have suggested an antiinflammatory activity of essential oil, some cytotoxicity to mammalian cells in vitro is associated (*29*−*31*). In the studies by Brand et al. (*32*), a dosage of 0.05% tea tree oil was very toxic to neutrophils and monocytes. To avoid toxicity in these cells, it was necessary to dilute the oil to 0.016%. In our present studies, the effective dosage of essential oil was below 0.016% (about 0.006%); at this concentration of oil, there was no toxicity in murine macrophages.

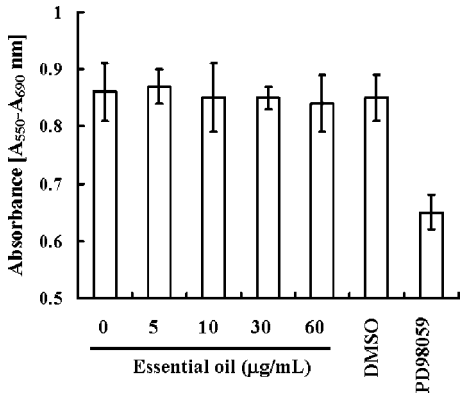

Figure 5. Effect of essential oil from *C. osmophloeum* leaves on J774A.1 cells viability. Cells were either treated with essential oil (0–60 μg/mL), DMSO vehicle, or PD98059 (100 μM) for 24 h, followed by incubation with the MTT reagent and precipitate solubilized, and the absorbance ($A_{550} - A_{690}$) was measured by spectrophotometry. The bar graph with the mean absorbance values of three separate experiments is shown. The error bar for all experiments was mean ± SD ($n = 3$).

In conclusion, the essential oil of *C. osmophloeum* exhibits clear antiinflammatory activities by LPS-stimulated macrophages, indicating that the major components 1,8-cineole, santolina triene, spathulenol, and caryophyllene oxide may play an important role in regulating cell-mediated immune response. The antiinflammatory activities and the signal transduction pathways of these individual constituents remain to be detailed in a further study.

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