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Anti-inflammation activity of fruit essential oil from *Cinnamomum insularimontanum* Hayata

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ABSTRACT

In this study, the fruit essential oil of *Cinnamomum insularimontanum* was prepared by using water distillation. Followed by GC–MS analysis, the composition of fruit essential oil was characterized. The main constituents of essential oil were α-pinene (9.45%), camphene (1.70%), β-pinene (4.30%), limonene (1.76%), citronellal (24.64%), citronellol (16.78%), and citral (35.89%). According to the results obtained from nitric oxide (NO) inhibitory activity assay, crude essential oil and its dominant compound (citral) presented the significant NO production inhibitory activity, IC_{50} of crude essential oil and citral were 18.68 and 13.18 μg/mL, respectively. Moreover, based on the results obtained from the protein expression assay, the expression of IKK, iNOS, and nuclear NF-κB was decreased and IκBα was increased in dose-dependent manners, it proved that the anti-inflammatory mechanism of citral was blocked *via* the NF-κB pathway, but it could not efficiently suppress the activity on COX-2. In addition, citral exhibited a potent anti-inflammatory activity in the assay of croton oil-induced mice ear edema, when the dosage was 0.1 and 0.3 mg per ear, the inflammation would reduce to 22% and 83%, respectively. The results presented that the fruit essential oil of *C. insularimontanum* and/or citral may have a great potential to develop the anti-inflammatory medicine in the future.

1. Introduction

Many plants of genus *Cinnamomum* have been applied in folk medicine for their interesting bioactivities. *Cinnamomum insularimontanum* Hayata (Lauraceae) is an endemic evergreen tree grown in Taiwan. It has a strong fragrance and has been used as a folk medicine in Taiwan for a long time (Kan, 1986). Recently, some scientific literatures have focused on the bioactivities of *C. insularimontanum*. Lin et al. had reported that the extract exhibited the antiviral activity (Lin et al., 2003). Actinodaphnine isolated from *C. insularimontanum*, which demonstrated a potent cytotoxicity against Mel-5, HL-60 and hepatoma Mahlavu cells (Stevigny et al., 2002; Hsieh et al., 2006).

As regards the fruits of *C. insularimontanum*, they emanate the strong characteristic fragrance naturally. To the best of our knowledge, there is hardly any study that has discussed the chemical composition of fruits essential oil as well as the pharmacological activity of *C. insularimontanum*. In our current study, the composition of fruit essential oil from *C. insularimontanum* was characterized by using the GC–MS analysis. Moreover, a nitric oxide (NO) inhibitory assay was performed to evaluate the anti-inflammatory activity of essential oil and its dominant compounds. The protein expression which is involved in the anti-inflammatory pathway was measured. Finally, the anti-inflammatory activity of fruit essential oil and active compound was further confirmed by the mice croton oil-induced ear edema by topical treatment in this study.

2. Methods

2.1. Plant materials and chemicals

The fruits of *C. insularimontanum* were collected in August 2006 at Aowanda national forest recreation located in the Nantou County, which is located in central Taiwan. The plants were authenticated by Dr. Yen-Hsueh Tseng (NCHU) and a voucher specimen (Y.S. Tseng 3125, TCF) was deposited in the herbarium of the same university. DMEM (Dulbecco's modified essential medium) and other cell-culture reagents including FBS (fetal bovine serum) were obtained from Gibco BRL (New York). LPS (lipopolysaccharide, *Escherichia coli* 0127:138), MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide], Griess reagent, Croton oil, Curcumin (≧80%) and Citral (95%) were purchased from Sigma–Aldrich (St. Louis). All other chemicals and solvents used in this study were of reagent grade or HPLC grade.

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2.2. Essential oil preparation

Fruit essential oil of *C. insularimontanum* (150 g) was extracted by hydro-distillation for 8 h by using a Clevenger-type apparatus. The yield (mL/kg) of essential oil was determined based on the dry weight of fruit. Essential oil was stored in the sample vials after deoxygenation with N_2 prior to analysis by gas chromatography (GC) and GC–mass spectrometry (GC–MS).

2.3. GC–MS analyses of essential oil

The compositions of fruit essential oil of *C. insularimontanum* were analyzed by GC–MS (HP G1800A; Hewlett–Packard, USA), equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J & W Scientific). The temperature program was as follows: 40 °C for 1 min, then increased by 4 °C/min to 260 °C and held for 4 min. The other parameters were as follows: injection temperature, 270 °C; ion source temperature, 280 °C; EI, 70 eV; carrier gas, He at 1 mL/min; injection volume, 1 μL; split ratio, 1:50; and mass range, *m*/*z* 45–425. Quantification was obtained from percentage peak areas from the gas chromatogram. Wiley (V. 7.0)/NBS (V. 2.0) Registry of Mass Spectral Database libraries search and/or authentic reference compounds and comparison of their Kovats index (Adams, 2001) were used for substance identification.

2.4. Nitric oxide inhibitory assay

The anti-inflammatory activity of essential oil and its dominant compounds was evaluated by using a nitric oxide (NO) inhibitory activity assay (Wang et al., 2003; Hsieh et al., 2007). Briefly, the marine macrophage cell line, RAW 264.7 cells, grown on a 75 cm^2 culture dish was seeded in 96-well plates at a density of 2×10^5 cells/well. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator as recommended by American Type Culture Collection (ATCC). Adhered cells were then incubated for 24 h with or without 1 μg/mL of LPS, in the absence or presence of essential oil or compounds. The nitrite concentration was measured using the supernatant from the RAW 264.7 cells by the Griess reaction (Schmidt and Kelm, 1996).

2.5. Determination of TNF-α production

The amounts of TNF-α were measured in cell-culture media using the ELISA (enzyme-linked immunosorbent assay) kits (Biosource, Camarillo, California). Briefly, RAW 264.7 cells macrophage cells were seeded in a 96-well cell-culture dish at a density of 2×10^5 cells/well and incubated with citral (1, 5, 10, and 20 μg/mL) in the presence or absence of LPS (1 μg/mL) for 18 h. The medium was collected and assayed for TNF-α using the ELISA kits. The medium was diluted with Reagent (1:2); 100 μL of each diluted sample was used for ELISA. Quantization of the ELISA results was detected by the absorbance at OD 450 nm using a Microplate Spectrophotometer (μQuant, Bio-Tek Instruments, Inc., Winooski, VT).

2.6. Preparation of whole cell, cytosolic and nuclear extracts

The preparation of whole cell extract was previously described (Wang et al., 2007). RAW 264.7 cells (2.5×10^5 cells/well) were seeded on 6 cm dishes and were treated with different concentrations of citral, stimulated with LPS (1 μg/mL) and incubated at 37 °C, 5% CO_2 for 16 h for COX-2 and iNOS detection. For the detection of p50 NF-κB, IκBα and IKK, the cells were only treated for 1 h. Curcumin 10 μg/mL (27.1 μM) was used as a reference compound. For whole cell extracts, cells were lysed in Mammalian Protein Extraction Reagent (Cayman chemicals, USA) and the lysates were centrifuged at 4 °C for 10 min to remove debris. The procedure for cytosolic and nuclear extraction was carried out by following protocol #78833 Nuclear and Cytoplasmic Extraction Reagents (NE-PER) kit (Pierce Biotechnology Inc., Rockford, USA). The protein content was measured at 595 nm by Bradford method (Bradford, 1976).

2.7. Western blot analysis

RAW 264.7 cells were incubated with or without various concentrations of citral and 1 μg/mL of LPS for detecting the expressions of iNOS, COX-2, IKK, IκB, and NF-κB. Whole cell, cellular and nuclear proteins were collected from treated and untreated cell extracts. 20 μg per lane of protein was loaded in 5–7% gradient SDS–PAGE to detect iNOS, IKK, IκB, NF-κB and COX-2 expression. After running at 300 mA for 90 min, the size-separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA) at 100 V for 1 h. The membranes were incubated in a blocking buffer (10% w/v skim milk in TBST buffer) for 1 h, and then incubated with anti-iNOS (1:1000) and anti-COX-2 (1:1000) (Cayman Chemical, Ann Arbor, MI), anti-NF-κB (1:500) (Abcam, Cambridge, UK), anti-IKK (1:1000) and anti-IκBα (1:1000) polyclonal antibody (Cell Signaling Technology, Danvers, USA), anti-actin (1:500) monoclonal antibody (Sigma, St. Louis). After washing two times with 0.1% TBST (TBS containing 0.1% Tween 20), the membranes were incubated with the anti-rabbit secondary antibodies conjugated with horseradish peroxidase and detected by the enhanced chemiluminescence reagents (ECL, Pierce Biotechnology, Rockford, IL).

2.8. In vivo anti-inflammatory activity assay

To estimate the inhibitory activity of fruit essential oil and citral against animal models of acute inflammation, croton oil-induced mice ear edema was performed according to the method described by us (Wang et al., 2007). Briefly, 10 μL acetone solution containing the 5% croton oil was applied topically to the right ear of a 4-week-old male ICR mice (25–28 g), obtained from BioLasco Co. (Taiwan). The left ear received an equal volume of acetone. Fruit essential oil and citral (100 and 300 μg/ear) were applied topically to the right ear about 60 min before the croton oil treatment. The left ear received the vehicle. As a reference, the non-steroidal anti-inflammatory drug (NSAID), indomethacin (300 μg/ear), was used. Six hours later, the mice were sacrificed and a plug (5 mm ϕ) was removed from both treated (right) and untreated (left) ears. The edematous response was measured as the weight difference between the two plugs.

2.9. Statistical analysis

Data are expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($^{*}p < 0.05$ and $^{**}p < 0.01$) between the positive control (LPS-activated cells) and treated cells were analyzed by Dunnett's test.

3. Results and discussion

3.1. Analysis of fruit essential oil

The yield of fruit essential oil of *C. insularimontanum* was 18 mL/kg. Essential oils are complex mixtures consisting of various compounds. Each of these compounds contributes to the beneficial or adverse activity of the essential oil. For this reason, it is necessary to elucidate the complete composition of an essential oil when investigating the viability of a specific application

(Buchbaure, 2000). GC–MS equipped with a capillary column is the most popular technique used to analyze the chemical ingredients of an essential oil. The fruit essential oil from the investigated *C. insularimontanum* is presented in Table 1. The most abundant constituent in essential oil was citral (35.89%), followed by citronellal (24.64%), citronellol (16.78%), α-pinene (9.45%), β-pinene (4.30%), limonene (1.76%), and camphene (1.70%). The main compounds, citral and citronellal, may present the characteristic odor of *C. insularimontanum*.

3.2. Anti-inflammatory activity of essential oil

Nitric oxide (NO), which is derived from the oxidation of L-arginine through three isoforms of nitric oxide synthase (NOS), namely neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses (Surh et al., 2001). High levels of NO cause a variety of pathophysiological processes including inflammation (MacMicking et al., 1997) and carcinogenesis (Ohshima and Bartsch, 1994). iNOS mainly exists in macrophages, it is expressed by stimulation with endotoxins, tumor necrosis factors or LPS. Pro-inflammatory agents, such as LPS, can significantly increase nitric oxide (NO) production in macrophages through activation of iNOS (Kojima et al., 2000; Park et al., 2000). In this study, the NO inhibitory activity of fruit essential oil of *C. insularimontanum* and citral was evaluated by using a LPS-stimulated RAW 264.7 cell assay. As shown in Fig. 1, the fruit essential oil and citral revealed the significant inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells treated with fruit essential oil and citral at dosages of 1–50 μg/mL caused a dose-dependent NO inhibitory activity. The 50% effective concentration (EC_{50}) for essential oil and citral was 18.68 and 13.18 μg/mL, respectively. Comparing with the EA soluble fraction of *Lindera erythrocarpa* (EC_{50} = 16.35 μg/mL) (Wang et al., 2007), fruit essential oil exhibited a stronger NO inhibitory activity, cytotoxicity. Citral was the major compound in the fruit essential oil which also presented a potent NO inhibitory activity. In the meantime, the test cells were healthy and viable determined by the MTT colorimetric assay (data not shown).

3.3. Mechanism elucidation for anti-inflammatory activity of essential oil

It is well known that NOS activity is induced by cytokines such as TNF-α (Drapier et al., 1998). Cumulative evidences indicate that an abnormality in the production or function of cytokines, such as TNF-α and IL-1β, plays an essential role in many inflammatory lesions (De Nardin, 2001). In congruence with these results, we found that the major compound, citral, in essential oil inhibits TNF-α in RAW 264.7 cells stimulated by LPS. The TNF-α levels were increased in the culture media of LPS-stimulated RAW 264.7 cells, and these increases were significantly decreased in a concentration-dependent manner by treatment with citral (Fig. 2A). Comparing with the curcumin, a well-known compound for its anti-inflammatory activity (Han et al., 2002), in the same assay, citral exhibits a strong TNF-α inhibition.

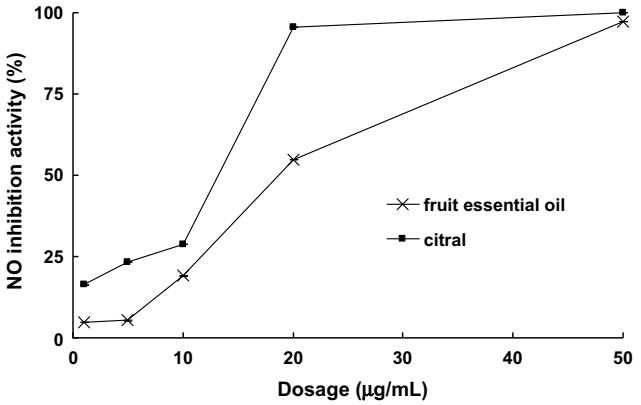

Fig. 1. Anti-inflammatory activities of fruit essential oil of *C. insularimontanum* and citral by using NO free radical inhibition assay. The data are representative of three experiments and as mean ± SE. Curcumin was used as a reference compound (E-C_{50} = 5.68 μg/mL).

Table 1
Composition of fruit essential oil from *C. insularimontanum*

No.	Compound	RT (min)	Concentration (%)	KI[a]	Identification[b]
1	α-Pinene	9.95	9.45	929	MS, KI, ST
2	Camphene	10.37	1.70	953	MS, KI, ST
3	β-Pinene	11.36	4.30	969	MS, KI, ST
4	β-Myrcene	11.82	0.86	983	MS, KI, ST
5	Limonene	13.16	1.76	1023	MS, KI, ST
6	Cineol	13.22	0.25	1031	MS, KI, ST
7	Citronellal	17.88	24.64	1153	MS, KI, ST
8	Borneol	18.26	1.29	1165	MS, KI, ST
9	4-Terpineol	18.56	0.11	1174	MS, KI, ST
10	Citronellol	20.84	16.78	1226	MS, KI, ST
11	Geraniol	21.38	1.49	1235	MS, KI, ST
12	Citral	22.24	35.89	1254	MS, KI, ST
13	Propanol	24.49	0.33	1385	MS, KI, ST
14	Caryophyllene	26.74	0.49	1419	MS, KI, ST
15	Elemol	30.62	0.66	1548	MS, KI, ST

[a] Kovats index on a DB-5MS column in reference to *n*-alkanes.
[b] MS, NIST and Wiley libraries and the literature; KI, kovats index; ST, authentic standard compounds.

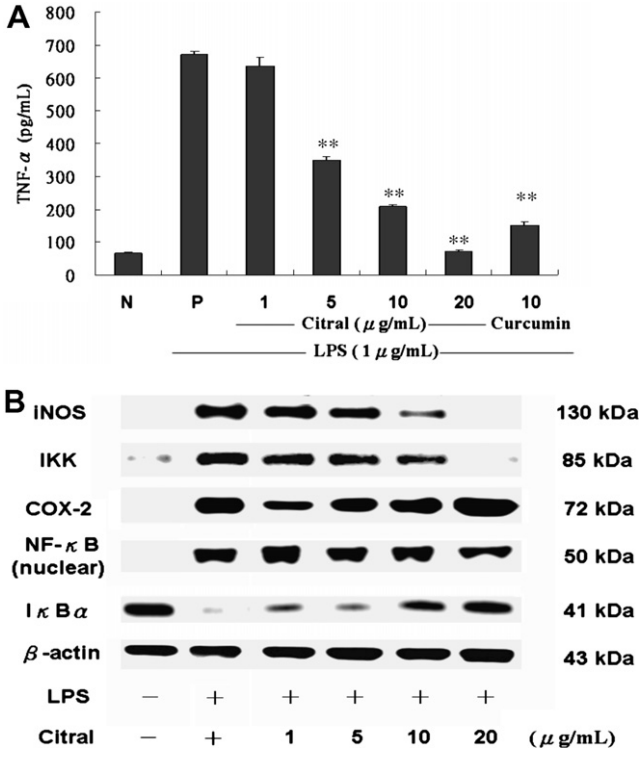

Fig. 2. Effects of citral on the expression of protein in LPS-stimulated RAW 264.7 cells. (A) TNF-α production. Values represent mean ± SE of three independent experiments. Statistical differences from positive control (LPS-activated cells) as analyzed by Dunnett's test (**$p < 0.01$). (B) iNOS, IKK, IκBα, NF-κB and COX-2 were determined by immunoblotting using specific antibodies.

Moreover, during immune and inflammatory responses, activated macrophages secrete increased amount of cytokines, eicosanoids such as prostaglandin E_2 (PGE_2), and reactive nitrogen species such as nitric oxide (NO) (Vane et al., 1994). Therefore, inhibition of NO and PGE_2 production from COX-2 and iNOS can effectively treat inflammatory diseases (Tamir and Tannenbaum, 1996; Surh et al., 2001). Whereas citral suppressed the LPS-induced NO production efficiently, its possible mechanisms of NO production inhibitory activity were further examined. First, the effects of citral on the expression of iNOS and COX-2 protein in LPS-stimulated RAW 264.7 cells were examined by using immunoblot analysis. After RAW 264.7 cells were stimulated for 16 h with 1 μg/mL LPS in the presence or absence of various concentrations (1, 5, 10, and 20 μg/mL) of citral, the total protein was prepared and quantized, then the immunoblot analysis was performed. Fig. 2B demonstrates that the citral exhibited an inhibitory activity in LPS-induced iNOS (130 kDa) protein expression in a dose-dependent manner; however, it could not find a significant suppression activity on COX-2 (Fig. 2B). It is known that iNOS and COX-2 are important enzyme mediators that regulate the inflammatory processes. Unsuitable expression of iNOS and COX-2 has been associated with the pathophysiology of certain types of human cancers as well as inflammatory disorders (Surh et al., 2001). According to the results, citral exhibited a significant NO inhibitory activity, which is mainly caused by the suppression of iNOS expression. However, it scarcely inhibits the expression of COX-2. The results indicate that citral is an anti-inflammatory principle of *C. insularimontanum* fruits, and it may specialty through suppress the activation of iNOS protein, and finally inhibit the inflammation. To further investigate the mechanism of the citral-mediated inhibition of iNOS and COX-2 transcription, the effect of citral on NF-κB pathway, which is known to transactivate iNOS and COX-2 (Surh et al., 2001), was examined. It is clear, in unstimulated cells, that NF-κB is localized in the cytosol by binding with IκB. When stimulated by LPS, IκB is phosphorylated by its inhibitor IκB kinase, ubiquitinated, and rapidly degraded *via* 26 S proteosome, thus releasing NF-κB (Sanchez-perez et al., 2002). According to our observation, citral could prevent the degradation of IκBα in LPS-induced inflammation response with a concentration-dependent manner, and Western blot analysis showed that LPS-induced p50 NF-κB levels in nuclear fractions were reduced by citral (Fig. 2B). It indicated that citral exhibited an inhibitory activity on the LPS-induced degradation of IκBα. Moreover, the translocations of the p50 NF-κB subunits to the nucleus were blocked in citral.

3.4. In vivo anti-inflammatory activity assay

Since the fruit essential oil and citral demonstrated the anti-inflammatory activity in cell assay, the anti-inflammatory activities of essential oil and citral were further evaluated by the inhibition of croton oil-induced ear edema in mice. Topical application of croton oil-induced edema on the ears of mice caused a significant increase in the ear plug weight of the right ear compared with the vehicle-treated left ear (data not shown). In comparison with the non-steroidal anti-inflammatory drug indomethacin, fruit essential oil and citral presented stronger and effective anti-inflammatory activity in the experimental animal model used, which induced a strong dose-dependent edema inhibition. Especially, citral showed a strong edema inhibitory activity at the same dosage (Fig. 3). The mouse ear plug weight was reduced by 33% after indomethacin treatment (dose 300 μg per ear). Moreover, fruit essential oil reduced the edematous response by 38% for 100 μg and by 77% for 500 μg per ear. Citral induced 22% and 83% edema reduction effects at 100 and 300 μg/ear, respectively. In summary, citral which is the essential oil and major compound of *C. insularimontanum* fruits presented a potent anti-inflammatory activity. To the best of our knowledge, it is the first report to demonstrate that a famous spice, citral, possesses a significant anti-inflammatory activity. The possible inhibitory mechanism for anti-inflammation might be caused by the blockage of NF-κB activation in RAW 264.7 macrophages. From the results of this investigation, valuable research references can be provided for the clinical medicine or pharmaceutical application in the future.

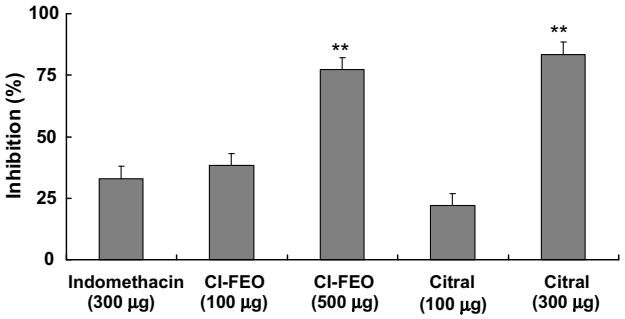

Fig. 3. Effects of fruit essential oil (FEO) and citral of *C. insularimontanum* prevent croton oil-induced ear edema in mice. The data are representative of six experiments and as mean ± SE. Indomethacin was used as a reference compound (300 μg per ear). Values represent mean ± SE of three independent experiments. Statistical differences from Indomethacin-treated control as analyzed by Dunnett's test ($^{**}p < 0.01$).

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