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Antiinflammatory Activity of *Lindera erythrocarpa* Fruits

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In this study, *in vitro* and *in vivo* antiinflammatory activities of fruits from *Lindera erythrocarpa* Makino were evaluated. The ethyl acetate soluble fraction derived from the ethanol extract of *L. erythrocarpa* fruits inhibited significantly nitric oxide (NO) production in lipopolysaccharide (LPS) induced NO in the murine macrophage cell line (RAW264.7) assay, the EC_{50} being 16.35 μg/mL. Four compounds, including lucidone (1), *cis*/*trans*-methylludicone (2), methyl linderone (3) and linderone (4) were identified from the active fraction based on the bioactivity-guided fractionation procedure. Of these lucidone possessed the strongest NO inhibitory activity with an EC_{50} value of 4.22 μg/mL. Furthermore, results from the protein expression assay demonstrated that lucidone suppressed iNOS and COX-2 protein expression in a dose-dependent manner. Lucidone also provided antiinflammatory activity in the croton oil-induced ear edema assay. When it was applied topically at a dosage of 0.5 and 1 mg per ear, the percent edema reduction in treated mice was 44% and 25%, respectively. The results obtained in this study indicated that lucidone has a good potential to be developed as an antiinflammation agent. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Lindera erythrocarpa; antiinflammation; fruit; lucidone.

INTRODUCTION

Plant secondary metabolites have provided an important source of drugs since ancient times and now around half of the practical drugs used are derived from natural sources. Many plants of Lauraceae have been applied in folk medicine for their interesting bioactivities. *Lindera erythrocarpa* Makino is one of the Lauraceae woody plants, which are distributed widely in Asia, including Taiwan, Japan, Korea and China. The fruit of *L. erythrocarpa* is used as a folk medicine as for its analgesic, digestive, diuretic, antidote and antibacterial properties (Liu *et al.*, 1973; Ichino *et al.*, 1988). However, little scientific study has been reported on the bioactivities of *L. erythrocarpa*. Oh and his coworkers (2005) demonstrated that four cyclopentenediones from the methanol extract of the fruits of *L. erythrocarpa* exhibited farnesyl protein inhibitory activity. In the current study of a bioactive investigation of Lauraceae woody plants indigenous to Taiwan, the extract from *L. erythrocarpa* fruits presented a significant antiinflammatory activity. Lucidone, an inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) inhibitor, was isolated and identified, following a bioactivity-guided fractionation procedure. The antiinflammatory activity of lucidone was further confirmed by the mouse croton oil-induced ear edema by topical treatment. The results of the current study are reported herein.

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MATERIALS AND METHODS

Plant materials and chemicals. The fruits of *L. erythrocarpa* were collected in June, 2006 at Chi-lan mountain located on the northeast of Taiwan island. The plants were authenticated by Mr Jeng-Chuang Yang (Specialist of Taiwan Forestry Research Institute) 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 and croton oil were purchased from Sigma-Aldrich (St Louis). All other chemicals and solvents used in this study were of reagent grade or HPLC grade.

Extract preparation, isolation and compound identification. 2.0 kg of fruit was extracted exhaustively with ethanol (EtOH). The total crude extract was concentrated under vacuum to yield a residue (124.3 g), and 100 g of crude extract was then suspended in water (1:1) and successively partitioned with *n*-hexane (*n*-hex) and ethyl acetate (EA), yielding a *n*-hex soluble fraction, EA soluble fraction and EA insoluble fraction. Each fraction was evaporated on a rotary evaporator, under reduced pressure, to remove organic solvent and then lyophilized until dry and weighed to determine the yield. The yields of *n*-hex soluble fraction, EA soluble fraction and EA insoluble fraction were 16.0%, 45.6% and 34.3%, respectively, of the total crude extract dry weight. As the EA soluble fraction presented potent nitric

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oxide (NO) inhibition activity, 15 g of EA soluble fraction was chromatographed on a silica gel (300 g) column, eluted with a gradient of *n*-hex/EA (95/5 to 100/0) to give a total of 12 subfractions (EA1–EA12). The active fraction (EA-5) was further separated by semi-preparative high performance liquid chromatography (HPLC) using a Cosmogel column (Comosil Co., 250 mm × 10 mm) eluted with *n*-hex/dichloromethane/EA solvent system to obtain compounds **1–4**. The structures of compounds **1–4** were then elucidated using spectroscopic analyses. The UV spectra of compounds tested were recorded on a Jasco V-550 spectrometer and IR spectra obtained from a Bio-Rad FTS-40 spectrophotometer. Electron-impact mass spectrometry (EIMS) and high resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-958 mass spectrometer and NMR spectra recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C). The amount of active compound (lucidone) in the EtOH extract was further analysed by HPLC. The peak area of lucidone in the chromatogram of the extract (with known loading concentration) was then defined and its content in the exudates was calculated based on the quantity calibrated from the standard calibration curve.

Nitric oxide inhibitory assay. Nitric oxide (NO) inhibitory activities of extract and compounds from the fruits of *L. erythrocarpa* were performed according to the method of Wang *et al.* (2003) and Hsieh *et al.* (2007). Briefly, RAW264.7 cells (a marine macrophage cell line) grown on a 75 cm^2 culture dish were 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 units/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 extracts or compounds. The nitrite concentration was measured using the supernatant from the RAW264.7 cells by the Griess reaction (Schmidt and Kalm, 1996).

Protein extraction and western analysis. Raw264.7 cells were incubated for 18 h with or without various concentrations of lucidone (**1**) and 1 μg/mL of LPS. The cells were washed with ice-cold PBS (calcium and magnesium-free phosphate buffered saline) and homogenized in 150 μL lysis buffer (Mammalian Protein Extraction Reagent, Pierce Biotechnology Inc., Rockford, IL). The protein concentration was determined by the Bradford method (Bradford, 1976). 20 μg per lane of protein was loaded in 5% to 7% gradient sodium dodecyl sulfate-polyacrylamide gels to detect iNOS 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 blocking buffer (10% w/v skim milk in TBST buffer) for 1 h, and then incubated with anti-iNOS and anti-COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI), anti-actin 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 (Pierce Biotechnology, Rockford, IL).

***In vivo* antiinflammatory activity evaluation.** To evaluate the inhibitory activity of lucidone against animal models of acute inflammation, mice croton oil-induced ear edema was performed according to the method performed by Innocenti and his coworkers (2005) with slight modifications. Briefly, 10 μL 5% croton oil in acetone was applied topically to the right ear of 4-week-old male ICR mice (25–28 g), which were purchased from BioLasco Co. (Taiwan). The left ear received an equal volume of acetone. The lucidone (0.5 and 1.0 mg/ear) was applied topically to the right ear about 1 h before the croton oil treatment. The left ear received vehicle only. Indomethacin (1.0 mg/ear) was used as a reference compound. 6 h after the application of the irritant agent, the mice were killed and a plug (5 mm Ø) was removed from both treated and untreated ears. The edematous response was measured as the weight difference between the two plugs.

RESULTS AND DISCUSSION

Nitric oxide (NO) is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses (Surh *et al.*, 2001). NO is derived from the oxidation of L-arginine through three isoforms of nitric oxide synthase (NOS), namely neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). 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, a LPS-stimulated RAW264.7 cell assay was employed to evaluate the NO inhibition activity of *L. erythrocarpa* extract. The ethanol extract of *L. erythrocarpa* was further divided into *n*-hex soluble, EA soluble and EA-insoluble fractions through liquid–liquid partition. Figure 1 shows

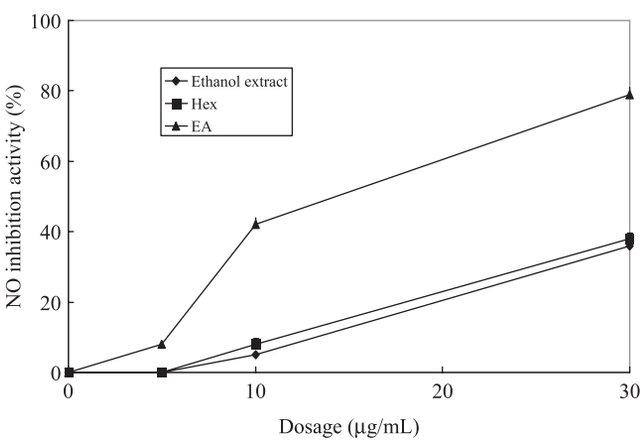

Figure 1. Effects of ethanol extract and its derived fractions from the fruits of *Lindera erythrocarpa* on LPS-induced nitric oxide (NO) production in activated RAW264.7 cells. ◆, ethanol extract; ■, *n*-hexane soluble fraction; ▲, ethyl acetate soluble fraction. The data are representative of three experiments and expressed as mean ± SE. Curcumin was used as a reference compound (EC_{50} = 5.68 μg/mL).

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the inhibitory effects of the ethanol extracts and its derived fractions on NO production in LPS-stimulated RAW264.7 cells. The NO levels in tested cells with and without LPS stimulation were 1.16 ± 0.08 μM and 32.98 ± 1.04 μM, respectively. After LPS-treated cells were co-incubated with *L. erythrocarpa* extracts, NO production was inhibited. The EA soluble fraction presented the strongest NO inhibitory activity, with a 50% effective concentration (EC_{50}) of 16.35 μg/mL; followed by the *n*-hex fraction and crude ethanol extract, the EC_{50} were 29.32 μg/mL and 35.40 μg/mL, respectively. Curcumin, a well-known antiinflammatory natural compound, was used as a reference compound, its EC_{50} value for LPS-induced NO production in activated RAW 264.7 cells was 5.68 μg/mL. The test cells were healthy and viable at a dosage of 50 μg/mL (for curcumin, at a dosage of 10 μg/mL), as determined by the MTT colorimetric assay (Mossmann, 1983) (data not shown).

Based on the results obtained from NO inhibitory assay, the EA soluble fraction was further separated into 12 subfractions (EA-1 to EA-12), then the same NO inhibitory assay was performed and EA-5 exhibited a potent antiinflammatory activity. Following HPLC purification, four compounds (**1**–4) were isolated and characterized from EA-5. According to the IR, mass and NMR analyses, compounds **1–4** were identified as lucidone (**1**) (Ng *et al.*, 1990), *cis*/*trans*-methylludicone (**2**) (Oh *et al.*, 2005), methyl linderone (**3**) (Syah *et al.*, 2005) and linderone (**4**) (Ng *et al.*, 1990) (Fig. 2). To quantify the amount of active component, lucidone, in the EtOH extract, the standard calibration curves (peak area vs concentrations) of lucidone, ranging from 1 to 50 μg/mL were established. According to HPLC analysis, the total content of lucidone was 6.50% in the EtOH extract of *L. erythrocarpa* fruit. The NO inhibitory activities of compounds **1–4** were further evaluated by assay of LPS-induced NO production in activated RAW264.7 cells. The dosage of each compound used was 10 μg/mL and curcumin was used as a reference compound. The results indicated that lucidone (**1**) possessed the strongest NO inhibitory activity of the compounds obtained from *L. erythrocarpa* fruits, it inhibited 89% NO production at a dosage of 10 μg/mL, followed by *cis*/*trans*-methylludicone (48%), methyl linderone (20%) and linderone (7%) (Fig. 3). Meanwhile, there was no cytotoxic effect shown by cells at a dosage of 10 μg/mL in this assay. According to our results,

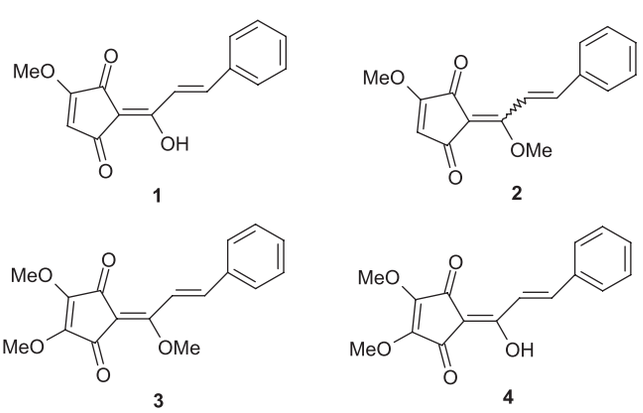

Figure 2. Structures of lucidone (**1**), *cis*/*trans*-methylludicone (**2**), methyl linderone (**3**) and linderone (**4**).

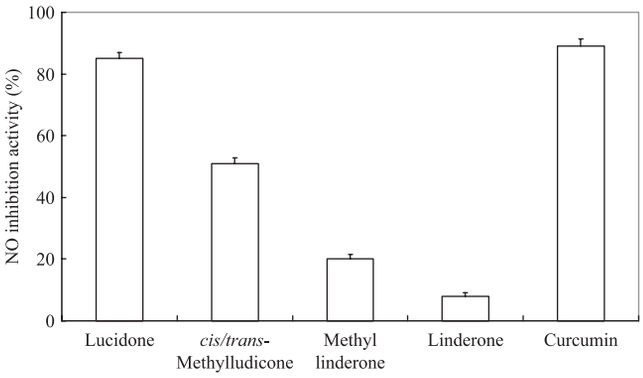

Figure 3. Inhibitory effects of compounds isolated from *Lindera erythrocarpa* at 10 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells. The data are representative of three experiments and expressed as mean ± S.E.

lucidone presented a significant NO inhibitory activity. The effect was comparable to that of curcumin (91%) used as a reference compound. After further investigation, the EC_{50} value determined for lucidone was 4.22 μg/mL, it was nearly equivalent to that of curcumin (5.68 μg/mL).

It has been well recognized that compounds which block the expression of both COX-2 and iNOS can effectively treat inflammatory diseases (Tamir and Tannenbaum, 1996; Surh *et al.*, 2001). Since lucidone (**1**) suppressed the LPS-induced NO production efficiently, its mechanisms of NO inhibition were explored and the effects of lucidone on LPS-induced iNOS and COX-2 protein expression were confirmed with immunoblot analysis. After RAW264.7 cells were stimulated for 18 h with 1 μg/mL of LPS in the presence of various concentrations (1, 5, 10, 20 μg/mL) of lucidone (**1**) or 10 μg/mL curcumin, total lysates and the immunoblot analysis was performed. As shown in Fig. 4, lucidone exhibited inhibitory ability in LPS-induced iNOS (130 kDa) and COX-2 protein (72 kDa) expression in a dose-dependent manner. As mentioned above, both iNOS and COX-2 are important enzyme mediators that mediate inflammatory processes. Improper expression of iNOS and COX-2 have been associated with the pathophysiology of certain types of human cancers as

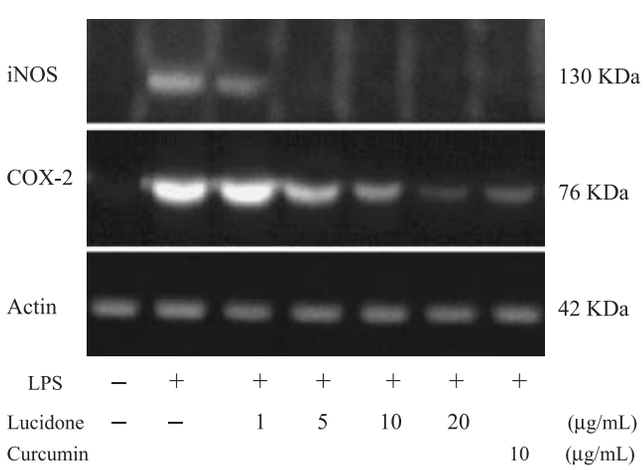

Figure 4. Effects of lucidone on the expression of protein in LPS-stimulated RAW264.7 cells. The cells were incubated with or without LPS (1 μg/mL) in the presence of the indicated concentrations of lucidone.

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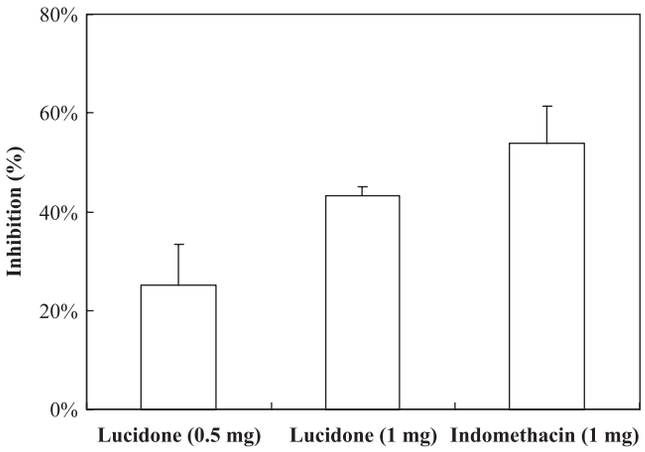

Figure 5. Antiinflammatory effect of lucidone on croton oil-induced ear edema. The data are representative of eight experiments and expressed as mean ± SE. Indomethacin was used as a reference compound (1 mg per ear).

well as inflammatory disorders (Surh *et al.*, 2001). According to the results obtained from the *in vitro* assay, lucidone exhibited a significant NO inhibitory activity, which is caused by suppression of iNOS expression. In the mean time, lucidone also inhibited the expression of COX-2. The activity presented in the cell level assay may indicate that lucidone is an antiinflammatory principle of *L. erythrocarpa* fruits.

Finally, the antiinflammatory activity of lucidone was further studied by the inhibition of croton oil-induced ear edema in mice. Topical application of croton oil-induced cutaneous inflammation 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). As a reference compound, indomethacin (dose 1 mg/ear) inhibited the changes in ear plug weight. The mouse ear plug weight was reduced by 51% after indomethacin treatment (Fig. 5). When lucidone was applied topically at 0.5 and 1 mg per ear, it provided an inhibitory effect on croton oil-induced ear edema formation in a dose-dependent manner (Fig. 5), the percent edema reduction was 44% and 25% for 1 mg and 0.5 mg lucidone treatment, respectively, indicating that lucidone possesses potent antiinflammatory activity. In summary, the EtOH extract of *L. erythrocarpa* fruits possesses potent antiinflammatory activity, and according to the results obtained from this investigation, the cyclopentenedione skeleton compound, lucidone, is a candidate for development as an antiinflammatory agent. Further epidemiological studies on this plant, for nutraceutical or pharmaceutical applications, are warranted.

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