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Cytotoxicity of Lignans from *Lindera erytherocarpa* Makino

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Abstract – Three lignans were isolated from a methanol extract of *Lindera erytherocarpa* Makino (Lauraceae) are evaluated in vitro cytotoxicity using three cancer cell line assay. The compounds were identified as methyllinderone (**1**), linderone (**2**), and kanakugiol (**3**) by spectroscopic methods. Amongst the compounds, methyllinderone (**1**) showed significant cytotoxicity against mouse melanoma (B16-F10), human acetabulum fibrosarcoma (HT1080), and choronic myelogenous leukemia (K562) cancer cell lines with ED_{50} values of 2.2, 2.5, 8.3 μg/ml, respectively.

Key words – *Lindera erytherocarpa* Mak., methyllinderone, linderone, kanakugiol, cytotoxicity

Introduction

Lindera erytherocarpa Makino belongs to the large family Lauraceae that consists of mostly trees or shrub from the warmer regions of the earth. *L. erythrocarpa* itself is commonly found in the base of mountain forests of the Far East. Previous studies of this species have shown that linderone, methyllinderone, lucidone, and methyllucidone as lignans ([a]Liu, 1973) and sitosterol-D-glucoside ([b]Liu, 1973), and geranyl acetate, caryophyllene as terpenes were isolated from the bark of *L. erythrocarpa* (Komae, 1972). In the course of our ongoing research in identifying cytotoxic compounds from plants sources, three lignans were isolated from the stem bark of *L. erythrocarpa* and its chemical structures were identified by previously spectroscopic data ([a]Liu, 1973).

Experimental

General procedures – Melting points were determined on Electrothermal Melting Point Apparatus 9100. IR spectra were measured on IR Report-100 infrared spectrophotometer (JASCO). HREIMS spectra were measured on JMS 700 Mass (JEOL). ^{1}H- and ^{13}C-NMR spectra were recorded on a DRX 300 MHz (Bruker) the chemical shifts being represented as part per million (ppm) referenced to solvent signal. Column chromatography was carried out using Kieselgel 60, 400-230 mesh, (Merck). TLC was performed on aluminium backed kieselgel 60 GF254 plates (Merck) developed with hexane/acetone/AcOH, 13:3:0.1, and spots were visualized under UV light and by 10 % sulfuric acid (in H_2O) followed by heating.

Plant material – The stem bark of *L. erythrocarpa* was collected in Mt. Jiri, Korea in Aug 2001 and identified by Prof. KiHwan Bae, Chungnam National University. A voucher specimen (CNU 606) deposited in the herbarium of the College of Pharmacy, Chungnam National University.

Extraction, fractionation and isolation – The fresh stem bark of *L. erythrocarpa* (500 g) was extracted with MeOH by reflux. The MeOH extract (10 g) was suspended in water and then partitioned successively with hexane, EtOAc, and BuOH. Among the solvent fractions, the EtOAc fraction exhibited the strongest cytotoxity with an ED_{50} value of 3.6 μg/ml against B16-F10 melanoma cell. Accordingly, the EtOAc fraction (1 g) was further subjected to column chromatography on a silica gel (4×30 cm, 70-230 mesh) eluting with hexane-acetone (gradient, 10:1 → 2:1). Five fractions were obtained based on the monitoring of their TLC (silica gel) pattern. Among the fractions, the fraction 3 (0.4 g) had the most significant cytotoxic activity with an ED_{50} value of 3.8 μg/ml against B16-F10 melanoma cell. The fraction 3 was purified by Recycling Preparative HPLC (LC-908, JAI) on an ODS-18 column (YMC-Pack ODS-AQ, 300×10 mm; mobile phase: 80% acetonitirle; flow rate: 5 ml/min; detection: 254 nm; recycling: twice) to give compound **1** (110 mg), **3** (91 mg), and **2** (200 mg).

Compound **1** – Pale yellow solid, mp 75°C. UV (MeOH) λ_{max} nm: 248, 364, HREIMS: $[M]^+$ *m/z* 300.0993 ($C_{17}H_{16}O_5$ requires *m/z* 300.0998). ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.93 (1H, d, *J*=15.9 Hz, H-α), 7.61 (2H, dd, *J*=7.8, 2.1 Hz, H-2, H-6), 7.55 (1H, d. *J*=15.9 Hz, H-β), 7.39 (3H,

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m, H-3, H-4, and H-5), 4.17 (6H, s, 2OC$\underline{H}_3$), 4.08 (3H, s, OC$\underline{H}_3$), ^{13}C-NMR (75 MHz, CD_3OD) δ: 188.3 (C-β′), 185.8 (C-1′), 166.5 (C-4′), 150.0 (C-4), 148.8 (C-2′), 142.5 (C-3′), 137.0 (C-1), 131.2 (C-β), 130.0 (C-3, 5), 129.2 (C-2, 6), 122.0 (C-α), 110.5 (C-5′), 64.7 (O$\underline{C}H_3$-C-β′), 60.5 (O$\underline{C}H_3$-C-2′), 60.4 (O$\underline{C}H_3$-C-3′).

Compound **2** – Yellow solid, mp 92.1-93.5°C. UV (MeOH) λ_{max} nm: 243, 363, HREIMS: $[M]^+$ *m/z* 286.0840 ($C_{16}H_{14}O_5$ requires *m/z* 286.0841). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.00 (1H, d, *J*=15.6 Hz, H-α), 7.93 (1H, d, *J*=15.6 Hz, H-β), 7.61 (2H, m, H-2, H-6), 7.40 (3H, m, H-3, H-4, and H-5), 5.96 and 5.94 (each s, tautomeric OH), 4.21 and 4.20 (3H, each s, tautomeric OC$\underline{H}_3$), 3.94 and 3.93 (3H, each s, tautomeric OC$\underline{H}_3$), ^{13}C-NMR data can not assigned due to a complicated signals from its tautomers.

Compound **3** – Yellow oil, UV (MeOH) λ_{max} nm: 208, 314, HREIMS: $[M]^+$ *m/z* 344.1259 ($C_{19}H_{20}O_6$ requires *m/z* 344.1260). ^{1}H-NMR (300 MHz, $CDCl_3$) δ : 10.11 (1H, s, OH), 7.92 (1H, d, *J*=15.5 Hz, H-α), 7.84 (1H, d, *J*=15.5 Hz, H-β), 7.63 (2H, m, H-2, H-6), 7.41 (3H, m, H-3, H-4, and H-5), 4.10 (3H, s, OC$\underline{H}_3$), 3.89 (6H, s, 2OC$\underline{H}_3$), 3.86 (3H, s, OC$\underline{H}_3$) ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 192.8 (C-β′), 149.4, 147.3, 146.2, 143.9 (C-β), 138.5, 137.1, 134.3, 130.6, 129.0 (C-3, 5), 128.5 (C-2, 6), 127.9 (C-α), 116.1 (C-1′), 61.5 (O$\underline{C}H_3$), 61.0 (O$\underline{C}H_3$), 60.9 (O$\underline{C}H_3$), 60.8 (O$\underline{C}H_3$).

Cytotoxicity assay – Aliquots of 2×10^5 cells were seeded into each well of a 96-well flat microtiter plates in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). The compounds dissolved in MeOH at various concentrations were added to culture and adjusted to a final concentration of 0.1% (v/v). Above cells were grown in a humidified atmosphere of 95% air and 5% CO_2, and the cells were seeded in plates after three passages. The cell viability was determined in the presence or absence of a compound or adriamycin, as a positive control, using the standard microculture tetrazolium (MTT) assay. Briefly, twenty-four hours after seeding, 100 μl new media or a test compound was added and the plate was incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After incubation for 4 h at 37°C, the medium was discarded and formazan blue formed in the cells was dissolved with 50 μl DMSO. Optical density was measured at 570 nm. The concentration required to reduce absorbance by 50% (ED_{50}) in comparison to control cells was determined.

Result and Discussion

Compound **1** had a molecular weight of 300, as identified by HREIMS ([M]+ at m/z 300.0993). The characteristic ^{1}H- and ^{13}C-NMR signals δ_H 7.93 (1H, d, *J*=15.9 Hz), δ_C 188.3, and δ_H 7.55 (1H, d, *J*=15.9 Hz), δ_C 185.8 were indicative of H-α and H-β, respectively. ^{1}H-NMR spectrum indicated five aromatic protons including mono substituted coupling pattern at δ 7.61 (2H, dd, *J*=7.8, 2.1 Hz) and 7.39 (3H, m), three methoxyl groups 4.17 (6H, s) and 4.08 (3H, s). In addition, δ_C 166.5, 150.0, 148.8 and 142.5 were appearance as four aromatic carbon signals on ^{13}C-NMR spectrum. Thus, the structure of **1** was determined to be 4,5-dimethoxy-2-(1-methoxy-3-phenyl-allylidene)-cyclopent-4-ene-1,3-dione (methyllinderone) (Fig. 1). This was confirmed by a physiochemical and spectral data comparison with published data (Leong, 1998). Compound **2** had a molecular weight of 286, as identified by HREIMS ([M]+ at m/z 286.0840). The characteristic ^{1}H- NMR signals δ_H 8.00 (1H, d, *J*=15.6 Hz) and δ_H 7.93 (1H, d, *J*=15.6 Hz) were indicative of H-α and H-β, respectively. ^{1}H-NMR spectrum indicated five aromatic protons including mono substituted coupling pattern at δ_H 7.61 (2H, m, H-2, H-6) and 7.40 (3H, m, H-3, H-4, and H-5), two methoxyl group signals appear at δ_H 4.21, 4.20 (3H, each s,) and 3.94, 3.93 (3H, each s) which were appear two peaks for one methoxyl group from tautomers (Fig. 2). As well as, one hydroxyl group appears at δ_H 5.96, 5.94 (each s, tautomeric OH) in ^{1}H-NMR spectrum. Thus, the structure of **2** was determined to be 4,5-Dimethoxy-2-(3-phenyl-acryloyl)-cyclopent-4-ene-1,3-dione (linderone) (Fig. 1). Compound **3** had a molecular weight of 344, as identified by HREIMS ([M]+ at m/z 344.1259). The ^{1}H- and ^{13}C-NMR spectra of **3** showed ketone peak at δ_C 192.8, hydroxyl peak at δ_H 10.11 (1H, s, OH), four methoxyl carbons at δ_C 61.5, 61.0, 60.9 and

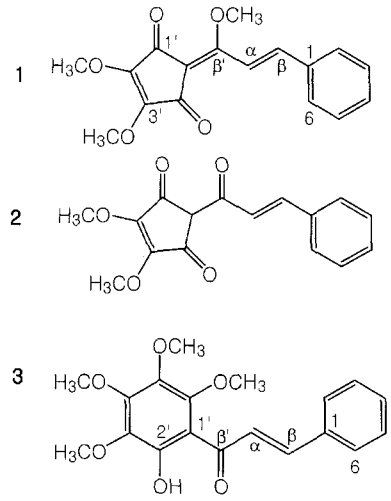

Fig. 1. Structures of methyllinderone (**1**), linderone (**2**) and kanakugiol (**3**).

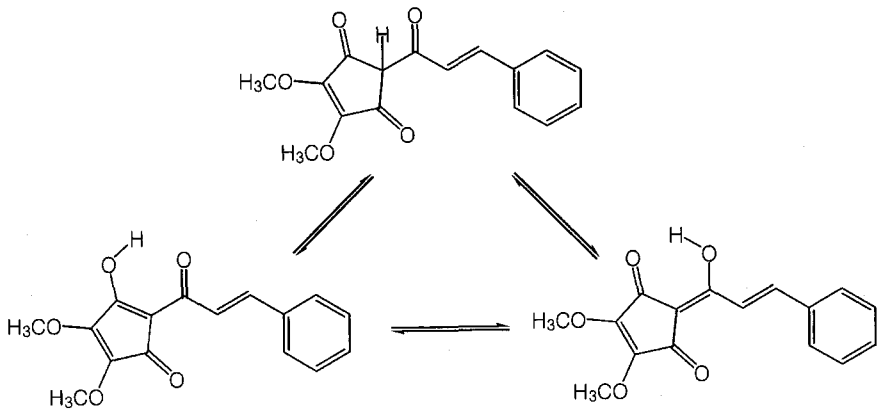

Fig. 2. Expectative tautomers of linderone (**2**).

Table 1. Cytotoxicity of compound **1-3**

Compound	ED_{50} (μg/ml)*		
	B16-F10	HT1080	K562
1	2.2 ± 0.9	2.5 ± 1.0	8.3 ± 1.8
2	10.3 ± 1.8	3.3 ± 0.8	12.0 ± 2.7
3	18.7 ± 2.1	7.2 ± 2.1	10.2 ± 1.0
Adriamycin	0.08 ± 0.01	0.05 ± 0.02	0.12 ± 0.04

*Results are mean of triplicate. B16-F10: mouse melanoma, HT1080: human acetalbulum fibrosarcoma, K562: human choronic myelogenous leukemia.

60.8, and δ_H 4.10 (3H, s), 3.89 (6H, s), and 3.86 (3H, s). The mono substituted aromatic hydrogen peaks appear at δ_H 7.63 (2H, m, H-2, H-6) and 7.41 (3H, m, H-3, H-4, and H-5). The twelve aromatic carbon peaks appear 149.4, 147.3, 146.2, 138.5, 137.1, 134.3, 130.6, 129.0 (2C), 128.5 (2C), and 116.1. Especially, 127.9 (C-α) and 143.9 (C-β) correlated to 7.92 (1H, d, *J*=15.5 Hz, H-α), 7.84 (1H, d, *J*=15.5 Hz, H-β) in HMQC spectrum. Thus, the structure of **3** was determined to be 1-(2-hydroxy-3,4,5, 6-tetramethoxy-phenyl)-3-phenyl-propenone (kanakugiol, **3**) (Fig. 1). All compounds were tested for their cytotoxicity against B16-F10, K562 and HT1080 tumor cell lines by MTT method. The results (ED_{50} values) are summarized in Table 1. Compound **2** and **3** showed weak inhibitory activity with ED_{50} values of 10~20 μg/ml against B16-F10 and K562 tumor cells, but methyllinderone (**1**) exhibited significant cytotoxic activity against B16-F10, HT1080 and K562 with ED_{50} values of 2.2, 2.5 and 8.3 μg/ml, respectively. These results suggested that the important cytotoxic compound was to be methyllinderone (**1**) in *Lindera erythrocarpa*.

Acknowledgments

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