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Short Communication

Inhibition of Low Density Lipoprotein-oxidation, ACAT-1, and ACAT-2 by Lignans from the Bark of *Machilus thunbergii*

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The bark of *Machilus thunbergii* was extracted with 80% aqueous methanol (MeOH), and the concentrated extract was partitioned using ethyl acetate (EtOAc), butanol (*n*-BuOH), and H_2O, successively. From the EtOAc fraction, five lignans were isolated through the repeated silica gel, octadecyl silica gel (ODS) and, Sephadex LH-20 column chromatography. Based on nuclear magnetic resonance (NMR), mass spectroscopy (MS), and infrared spectroscopy (IR) spectroscopic data, the chemical structures of the compounds were determined to be machilin A (1), machilin F (2), licarin A (3), nectandrin A (4), and nectandrin B, (5). This study presents comparative account of five lignans from *M. thunbergii* bark contributing inhibition of low density lipoprotein (LDL), ACAT-1, and ACAT-2. Compounds 2-5 showed varied degree of antioxidant activity on LDL with IC_{50} values of 2.1, 11.8, 15.3, and 4.1 μM. Compounds 1, 2, and 3 showed inhibition activity on ACAT-1 with values 63.4±6.9% (IC_{50}=66.8 μM), 53.7±0.9% (IC_{50}=109.2 μM), and 78.7±0.2% (IC_{50}= 40.6 μM), respectively, at a concentration of 50 mg/mL, and on ACAT-2 with values 47.3±1.5% (IC_{50}=149.7 μM), 39.2±0.2% (IC_{50}=165.2 μM), and 52.1±1.0% (IC_{50}=131.0 μM), respectively, at a concentration of 50 mg/mL.

Key words: ACAT-1, ACAT-2, LDL-oxidation, licarin A, machilin A, machilin F, *Machilus thunbergii*, nectandrin A, nectandrin B

Machilus thunbergii Siebold & Zuccarinii (Lauraceae) is a widely distributed tree used in Korean traditional medicine [Kim, 1984]. The cortex of the plant is used for treatment of leg edema, abdominal distension, and pain [Chung and Shin, 2000]. Several lignans and neolignans have been reported from the bark of this plant [Shimomura *et al*., 1987; 1988]. Previous studies on the bark of *M. thunbergii* reported nitric oxide synthesis inhibitory butanolides [Kim and Ryu, 2003]. Some lignans from the bark have been shown to be antioxidant [Yu *et al*., 2000], melanin biosysnthesis inhibitory [Li *et al*., 2003], caspase-3 activating [Park *et al*., 2004], and neuroprotective [Ma *et al*., 2004; 2009]. In this study we isolated 5 lignans and investigated on their inhibition effect on LDL-oxidation and ACAT-1 and ACAT-2 activities.

Oxidation of low-density lipoprotein (LDL) is considered as an early event in the development of atherosclerosis [Glass and Witztum, 2001]. Antioxidants such as probucol, N,N'-diphenyl phenylenediamine, and butylated hydroxy-toluene (BHT) have been shown to decrease the degree of oxidation and the extent of atheromatous lesions in animal models of atherosclerosis, but have side effects [Jialal and Devaraj, 1996]. Thus antioxidants from natural source are attractive alternatives. Cholesterol acyltransferase (ACAT) catalyses the acylation of cholesterol to cholesteryl ester and it exists in two isoforms, ACAT-1 and ACAT-2. ACAT-1 is in charge of foam cell formation in macrophages, whereas ACAT-2 controls the cholesterol absorption in intestinal mucosal cells [Rudel *et al*., 2001]. Therefore, ACAT inhibition is a useful strategy for treating hypercholesterolemia and atherosclerosis by the effect of lowering plasma cholesterol in humans [Lawrence and Gregory, 2000].

The dried bark of *M. thunbergii* was received from Green Flower Cosmetics Co., in 2009 and was identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU-090826) was deposited at the laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea. The dried and powdered bark of *M. thunbergii* (500 g) was extracted two times with 80% aqueous methanol (MeOH) [4 L] at room temperature. The MeOH extract was successively partitioned with water (2 L), ethyl

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acetate (EtOAc) [2 L×2], and *n*-butanol (1 L×2). The concentrated EtOAc extract (MTE, 16 g) was applied to a silica gel (Merck 60A, 70-230 mesh ASTM, Darmstadt, Germany) column chromatography (c.c.) (ϕ 6×14 cm) and was eluted with $CHCl_3$-MeOH-H_2O (20:3:1→17:3:1→9:3:1→6:4:1, 6 L of each) with monitoring by thin layer chromatography (TLC) to provide 20 fractions (MTE-1 to MTE-20).

Fraction MTE-2 [1.79 g, elution volume/total volume (V_e/V_t) 0.006-0.009] was subjected to the silica gel c.c. (ϕ 4×12 cm) eluted with *n*-hexane-EtOAc (10:1→5:1, 2.5 L of each), yielding 22 fractions (MTE-2-1 to MTE-2-22) and an isolated compound **1** [33 mg, V_e/V_t 0.019-0.020, TLC (SiO_2) R_f 0.20, *n*-hexane-EtOAc=10:1].

Subfraction MTE-2-17 (100 mg, V_e/V_t 0.218-0.356) was subjected to an octadecyl silica gel (ODS) c.c. (ϕ 2.5×2 cm) and eluted with *n*-hexane-EtOAc-MeOH-H_2O (4:2:4:2), yielding 5 fractions (MTE-2-17-1 to MTE-2-17-5). Then, MTE-2-17-3 (66 mg) subjected to a Sephadex LH-20 c.c. (ϕ 2×35 cm) eluted with *n*-hexane-EtOAc-MeOH-H_2O (4:2:4:2), yielding 5 fractions (MTE-2-17-3-1 to MTE-2-17-3-5). Further, MTE-2-17-3-3 (52 mg, V_e/V_t 0.40-0.56) was applied to the ODS c.c. (ϕ 2×2.5 cm) eluted with MeOH-H_2O (5:4), yielding 7 fractions (MTE-2-17-3-3-1 to MTE-2-17-3-3-7) and ultimately isolated compound **2** [18 mg, V_e/V_t 0.110-0.015, TLC (ODS F_{254S}) R_f 0.47, MeOH-H_2O=6:1] and compound **3** [19.5 mg, V_e/V_t 0.27-0.65, TLC (ODS F_{254S}) R_f 0.42, MeOH-H_2O=6:1].

Subfraction MTE-2-21 (107.5 mg, V_e/V_t 0.66-0.89) was applied to the ODS c.c. (ϕ 2.5×4 cm) eluted with MeOH-H_2O (4:1), yielding 18 fractions (MTE-2-22-1 to MTE-2-21-18) and ultimately isolated compound **4** [25.5 mg, V_e/V_t 0.14-0.18, TLC (ODS F_{254S}) R_f 0.55, MeOH-H_2O=6:1] and compound **5** [21.5 mg, V_e/V_t 0.08-0.10, TLC (ODS F_{254S}) R_f 0.65, MeOH-H_2O= 6:1].

Compound **1**: Colourless needle; m.p. 48-50°C; $[\alpha]_D^{20}$ =5.50° (*c*=0.10, $CHCl_3$); EI-MS *m/z* 326 $[M]^+$; IR (CaF_2 window, cm^{-1}) 2905, 1600, 1505, 1450; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.72 (2H, d, *J*=8.0 Hz, H-5, 5'), 6.67 (2H, br.s, H-2, 2'), 6.57 (2H, dd, *J*=8.0, 1.6 Hz, H-6, 6'), 5.90 (4H, s, OCH_2O×2), 2.70 (2H, dd, *J*=13.5, 4.8 Hz, H-7b, 7'b), 2.26 (2H, dd, *J*=13.5, 9.2 Hz, H-7a, 7'a), 1.75 (2H, m, H-8, 8'), 0.86 (6H, d, *J*=6.8 Hz, H-9, 9'); ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 147.34 (C-4, 4'), 145.35 (C-3, 3'), 135.50 (C-1, 1'), 121.72 (C-6, 6'), 109.23 (C-5, 5'), 107.87 (C-2, 2'), 100.64 (OCH_2O×2), 39.43 (C-7, 7'), 39.05 (C-8, 8'), 16.18 (C-9, 9').

Compound **2**: Colorless oil; $[\alpha]_D^{20}$ = –46.10° (*c*=0.10, $CHCl_3$); EI-MS *m/z* 342 $[M]^+$; IR (CaF_2 window, cm^{-1}) 3550, 2930, 1720, 1450, 1250; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.95 (1H, d, *J*=1.2 Hz, H-2), 6.88 (1H, d, *J*=1.2 Hz, H-2'), 6.87 (2H, dd, *J*=8.0, 1.2 Hz, H-6, 6'), 6.77 (2H, d, *J*=8.0 Hz, H-5, 5'), 5.93 (2H, s, OCH_2O), 5.61 (1H, s, OH, H-4'), 4.44 (2H, dd, *J*=6.4, 2.8 Hz, H-7, 7'), 3.87 (3H, s, OCH_3), 2.26 (2H, m, H-8, 8'), 1.00 (6H, d, *J*=6.4 Hz, H-9, 9'); ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 147.60 (C-3), 146.80 (C-4), 146.30 (C-3'), 144.84 (C-4'), 135.97 (C-1'), 133.81 (C-1), 119.80 (C-6'), 119.20 (C-6), 114.00 (C-5'), 108.80 (C-5), 107.88 (C-2'), 106.69 (C-2), 100.89 (OCH_2O), 87.35 (C-7'), 87.25 (C-7), 55.82 (OCH_3), 44.53 (C-8), 44.44 (C-8'), 12.94 (C-9), 12.93 (C-9').

Compound **3**: Colourless crystals; m.p. 102-104°C; $[\alpha]_D^{20}$ = –35.40° (*c*=0.10, $CHCl_3$); EI-MS *m/z* 326 $[M]^+$; IR (CaF_2 window, cm^{-1}) 3400, 2957, 1608, 1510, 1480, 1210; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.95 (1H, br.s, H-2), 6.87 (1H, d, *J*=7.2 Hz, H-5), 6.85 (1H, br.d, *J*=7.2 Hz, H-6), 6.76 (1H, br.s, H-6'), 6.74 (1H, br.s, H-2'), 6.33 (1H, dd, *J*=15.6, 1.6, H-7'), 6.08 (dq, *J*=15.6, 6.8 Hz, H-8'), 5.65 (1H, s, OH), 5.06 (1H, d, *J*=9.6 Hz, H-7), 3.85 (6H, s, OCH_3×2), 3.41 (1H, dq, *J*=9.6, 6.8 Hz, H-8), 1.84 (3H, d, *J*=6.8 Hz, C-9'), 1.34 (3H, d, *J*=6.8 Hz, C-9); ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 146.56 (C-3), 146.35 (C-4'), 145.61 (C-4), 143.95 (C-5'), 133.11 (C-3'), 132.04 (C-1'), 131.87 (C-1), 130.75 (C-7'), 123.37 (C-8'), 119.83 (C-6), 114.01 (C-5), 113.19 (C-2'), 109.06 (C-6'), 108.87 (C-2), 93.72 (C-7), 55.9 (OCH_3× 2), 45.60 (C-8), 18.42 (C-9'), 17.56 (C-9).

Compound **4**: Colourless oil; $[\alpha]_D^{23}$ = –8.9° (*c*=0.40, $CHCl_3$); EI-MS *m/z* 358 $[M]^+$; IR (CaF_2 window, cm^{-1}) 3540, 2950, 1610, 1250; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.95 (1H, d, *J*=1.6 Hz, H-2), 6.93 (1H, d, *J*=1.6 Hz, H-2'), 6.94 (overlapped, H-6), 6.91 (1H, dd, *J*=8.0, 1.6 Hz, H-6'), 6.87 (1H, d, *J*=8.0 Hz, H-5'), 6.83 (1H, d, *J*=8.0 Hz, H-5), 5.57 (1H, s, HO-4), 4.49 (1H, d, *J*=5.6 Hz, H-7'), 4.48 (1H, d, *J*=5.6 Hz, H-7), 3.85 (9H, s, OCH_3×3), 2.30 (2H, m, H-8, 8'), 1.01 (6H, d, *J*=6.7 Hz, CH_3-9, 9'); ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 148.79 (C-3), 148.30 (C-4), 146.37 (C-3'), 144.94 (C-4'), 134.71 (C-1'), 134.07 (C-1), 119.21 (C-6'), 118.50 (C-6), 114.03 (C-5'), 110.71 (C-5), 109.52 (C-2), 109.00 (C-2'), 87.26 (C-7'), 87.16 (C-7), 55.80 (OCH_3×2), 44.30 (C-8'), 44.26 (C-8), 12.96 (C-9'), 12.90 (C-9).

Compound **5**: Colourless oil; $[\alpha]_D^{23}$ =0° (*c*=0.4, $CHCl_3$); EI-MS *m/z* 344 $[M]^+$; IR (CaF_2 window, cm^{-1}) 3390, 3050, 1640, 1590, 1240; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 6.91 (2H, d, *J*=1.6 Hz, H-2, 2'), 6.87 (2H, dd, *J*=8.0, 1.6 Hz, H-6, 6'), 6.84 (2H, d, *J*=8.0 Hz, H-5, 5'), 4.47 (2H, d, *J*=6.4 Hz, H-7, 7'), 3.85 (6H, s, OCH_3×2), 2.30 (2H, m, H-8, 8'), 1.07 (6H, d, *J*=6.4 Hz, CH_3-9, 9'); ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 146.24 (C-3, 3'), 144.77 (C-4, 4'), 133.93 (C-1, 1'), 119.09 (C-6, 6'), 113.97 (C-5', 5'), 108.97 (C-2, 2'), 87.24 (C-7, 7'), 55.80 (OCH_3×2), 44.25 (C-8, 8'), 12.99 (C-9, 9').

LDL-oxidation assay was carried out using the method described in the literature [Lee *et al*., 2009], with BHT as a positive control. The activities of ACAT-1 and ACAT-2 were determined with the method developed by Brecher and Chan [1980] with slight modifications [Lee *et al.,* 2001], with oleic acid anilide (OAA) as a positive control. The assay data are

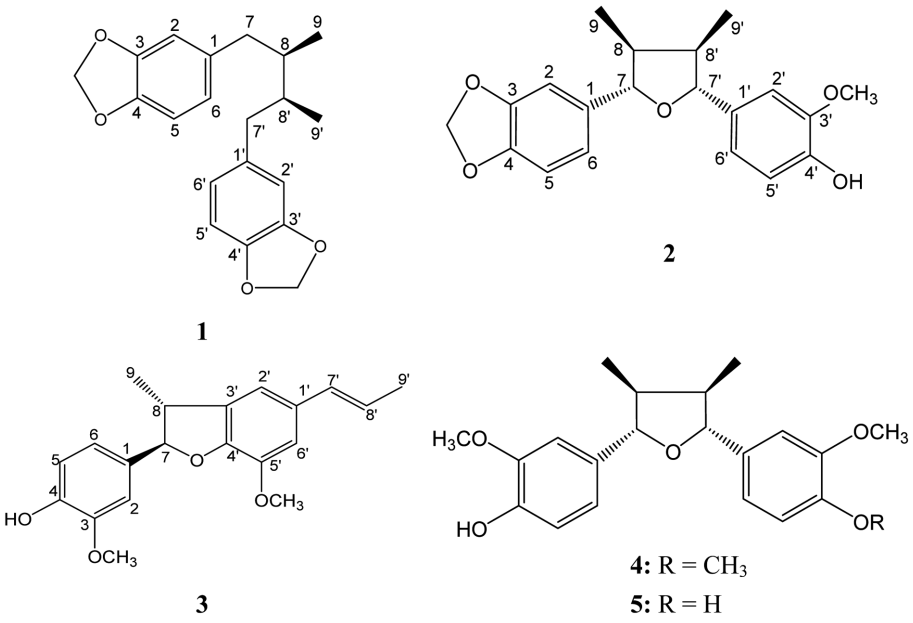

Fig. 1. Chemical structures of compounds 1-5 isolated from the bark of *M. thunbergii*.

Table 1. Inhibition activity of lignans from the bark of *M. thunbergii* on LDL-oxidation[1], ACAT-1[2], and ACAT-2[3]

compounds	LDL-oxidation Inhibition (%)			IC_{50} (μM)	ACAT-1 Inhibition (%)		ACAT-2 Inhibition (%)	
	10 μg/mL	5 μg/mL	2.5 μg/mL		50 μg/mL	IC_{50} (μM)	50 μg/mL	IC_{50} (μM)
1	-16.9±2.6	N.D	N.D	-	63.4±6.9	66.8	47.3±1.5	149.7
2	97.6±0.1	94.9	70.7±0.1	2.1	53.7±0.9	109.2	39.2±0.2	165.2
3	89.8±0.0	70.0	26.3±1.2	11.8	78.7±0.2	40.6	52.1±1.0	131.0
4	96.1±0.5	90.1	61.4±1.1	15.3	16.7±1.7	-	14.5±0.6	-
5	84.4±0.5	40.1	11.0±1.9	4.1	20.3±2.6	-	13.0±0.7	-

Data are means±SD (n=3).
[1]Positive control of LDL-Oxidation, BHT, showed 85.0±0.3% inhibition at 3.0 μM with IC_{50} value of 2.1 μM.
[2]Positive control of ACAT-1, OAA, showed 33.6±1.5% inhibition at 0.1 μM with IC_{50} value of 0.126 μM.
[3]Positive control of ACAT-2, OAA, showed 36.0±0.6% inhibition at 0.1 μM with IC_{50} value of 0.138 μM.

expressed as mean±SD of three replicated experiments.

In a search of biologically active materials in *M. thunbergii*, the barks were extracted with MeOH and partitioned into EtOAc, *n*-BuOH, and H_2O layers through solvent fractionation. Successive repeated silica gel, ODS, and Sephadex LH-20 c.c. of the obtained fractions led to isolation of five lignans. Structural identifications of these lignans were determined to be machilin A (**1**), machilin F (**2**), licarin A (**3**), nectandrin A (**4**), and nectandrin B (**5**) by interpretation of extensive spectroscopic data and comparison of data with those described in the literature [Shimomura *et al.,* 1987; 1988; Lee *et al*., 2009] (Fig. 1).

The oxidation of LDL cholesterol is an important step in the formation of atherosclerotic lesions [Steinberg *et al*., 1989; Diaz *et al*., 1997]. Evidence to support this hypothesis is based in part on observation that demonstrate associations between oxidized LDL cholesterol and both the presence of atherosclerotic lesions [Regnstrom *et al.,* 1992] and the progression of carotid artery atherosclerosis [Salonen *et al*., 1992]. In order to determine whether the compounds might be effective in the development of hypercholesterolemic or antiatherogenic agents, their potential for inhibiting LDL oxidation was evaluated. Compounds **2-5** showed varied degree of antioxidant activity with IC_{50} values of 2.1, 11.8, 15.3, and 4.1 μM, respectively. The key factor governing the antioxidant activity is attributed to phenolic hydroxyl. Among these values compounds **2** and **5** showed significant inhibition in comparison to the positive control, BHT, which had an IC_{50} value 2.1 μM. Compounds **2** and **5** showed more potent activity than saururin (IC_{50} 8.5 μM) and virolin (IC_{50} 4.3 μM) from *Saururus chinensis*, the compound **2** was also more effective than machilin D (IC_{50} 2.9 μM), [Ahn *et al.*, 2001]. This is first report of LDL oxidation activity for compounds **2-5**. Compounds **1**, **2**, and **3** showed significant inhibitory activity on ACAT-1 with values of 63.4±6.9, 53.7±0.9, and 78.7±0.2%, respectively, and on ACAT-2 with values 47.3±1.5, 39.2±0.2, and 52.1±1.0%, respectively, at a concentration 50 mg/mL. The positive control OAA inhibited ACAT-1 by 36.6±1.5% and ACAT-2 by 36.0±0.6% at 0.1 μM (Table1). Compounds **1** and **3**

showed more potent activity than meso-dihydroguaiaretic acid from *Myristica fragrans* with ACAT 60.0±1.2% at concentration 100 mg/mL and compound **2** showed more potent activity than syringing methyl ether from *M. fragrans* with ACAT 27.2±0.9% at concentration 100 mg/mL [Song *et al.,* 2004] and oleanolic acid from *Albizia julibrissin* with ACAT-1 52.5±0.7% and ACAT-2 22.0±2.6% at concentration 50 mg/mL [Baek *et al.*, 2010]. This is first report of ACAT-1 and ACAT-2 inhibitory activity for compounds **1-5**. Although compounds **1-5** had lower inhibition activity on ACAT-1 and ACAT-2 compared to positive control oleic acid anilide, the relative paucity of naturally occurring ACAT-1 and ACAT-2 inhibitors makes this report worth further investigation. There are few reports of LDL-oxidation inhibitors, ACAT-1, and ACAT-2 from the natural resources. The compounds **2**, **3**, **4,** and **5** exhibited similar activity for LDL-oxidation in comparison with the positive control. Compounds **1**, **2,** and **3** showed significant inhibitory activity on ACAT-1, and on ACAT-2. Therefore, the bark of *M. thunbergii* is used in oriental medicine in Korea can be useful source for treating hypercholesterolemia and atherosclerosis, and dementia. Among the active compounds, compound **2** appeared as the most potent inhibitor of LDL-oxidation with an IC_{50} value of 2.1 μM and compound **3** as the most potent inhibitor of ACAT-1 and ACAT-2 with IC_{50} value of 40.6 μM and 131.0 μM, respectively.

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