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Low-density lipoprotein (LDL)-antioxidant lignans from *Myristica fragrans* seeds

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Abstract—Six diarylbutane lignans **1**–**5** and one aryltetralin lignan **6** were isolated from the methanol (95%) extracts of *Myristica fragrans* seeds and then 7-methyl ether diarylbutane lignan **4** has proven to be new a compound. Their compounds **1**–**7** were evaluated for LDL-antioxidant activity to identify the most potent LDL-antioxidant **3** with an IC_{50} value of 2.6 μM in TBARS assay. Due to its potency, compound **3** was tested for complementary in vitro investigations, such as lag time (140 min at 1.0 μM), relative electrophoretic mobility (REM) of ox-LDL (inhibition of 80% at 20 μM and 72% at 10 μM), and fragmentation of apoB-100 (inhibition of 93% at 20 μM) on copper-mediated LDL oxidation. In macrophage-mediated LDL oxidation, the TBARS formation was also inhibited by compound **3**.

Since it has been observed that the diarylbutane lignans and neolignans including 8-*O*-4′-types have shown various biological activities such as cell adhesion inhibitory,[1] anti-inflammatory,[2] anti-plasmodial,[3] murine neuroleptic,[4] and anti-feedant activities,[5] we have concentrated our research goal to exhibit significant LDL-antioxidant activities. It is due to the fact that oxidized low-density lipoproteins (ox-LDLs) play important role in early stage of atherosclerosis.[6] Also, there has been increasing interest to intake antioxidant-rich foods, such as fruits, vegetables, and beverage plants.[7,8] Recently, we found that bioactivity-guided fractionation of the ethyl acetate extracts of *Saururus chinensis* led to new diarylbutane lignans, saururin and 2′-hydroxy dihydroguaiaretic acid, and a known 8-*O*-4′-type neolignan, machilin D, and virolin.[9,10] Sadhu et al. reported that neolignan, machilin C, showed an antioxidant activity as the DPPH radical scavenger.[11]

During search for new LDL-antioxidants from natural sources, we found that the MeOH (95%) extract of *Myristica fragrans* Houtt seeds at 20 μg/mL inhibited LDL-oxidation in 85%. Subsequent bioactivity-guided fractionation of the MeOH extracts led to the isolation of six compounds that were identified as six diarylbutane lignans **1**–**5** and one aryltetralin lignan **6** on the basis of their spectroscopic analyses, as shown in Figure 1.[12] Although the *M. fragrans* seeds usually used as spice and commonly known as nutmeg have anti-carcinogenic,[13] antioxidant, anti-inflammatory,[14] protein tyrosine phosphatase 1B (PTP1B),[15] and hepatoprotective activities,[16] their compounds **1**–**6** isolated from *M. fragrans* seeds have not been reported. In this study, we wish to describe the isolation, structure characterization, and biological activities of compounds **1**–**6** and **7** derived from **2** against LDL-oxidation. Antioxidant activities of LDL were detected by various tools, such as TBARS assay, conjugated diene formation, relative electrophoretic mobility (REM), fragmentation of apoB-100 on copper-mediated LDL oxidation, and macrophage-mediated LDL oxidation.

The structures of compounds **1**–**3**, **5**, and **6** were identified by comparing their spectroscopic data with the

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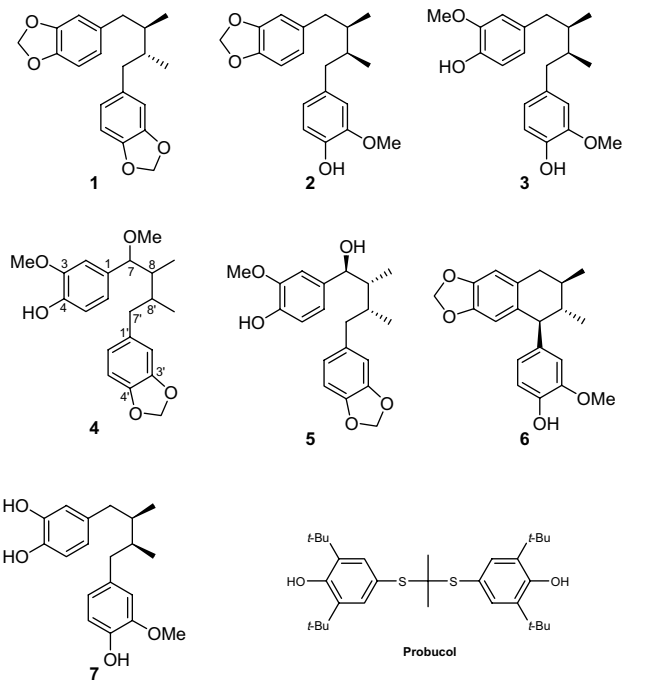

Figure 1. Compounds **1**–**6** isolated from the *M. fragrans* seeds, **7** derived from **2**, and probucol.

previously reported data.[17] Compound **4** was obtained as colorless oil having the molecular formula of $C_{21}H_{26}O_5$, and a molecular ion peak at *m*/*z* 358. It has the value of $[\alpha]_{20}^{D}$ −20° (*c* 0.25, $CHCl_3$) and the UV spectrum exhibited λ_{max} at 293 nm (CH_3OH). The IR spectrum showed the presence of hydroxyl (3448 cm^{-1}), aromatic C=C (1607 and 1509 cm^{-1}), and methylenedioxyl (930 cm^{-1}) groups. The exact structure of **4** was inferred from a detailed analysis of ^1H and ^{13}C NMR data, together with 2D NMR experiments (Table 1). The ^1H and ^{13}C NMR data with DEPT experiments showed the presence of 21 carbon atoms as two methoxy carbons [δ_C 56.2 (OCH_3-3) and 56.9 (OCH_3-7)], two methyl carbons [δ_C 11.2 (CH_3-9), 18.0 (CH_3-9′)], one methylene δ_C 37.8 (C-7′), one methylenedioxy group δ_C 100.9 (OCH_2O), eight methins [δ_C 36.7 (C-8′), 45.4 (C-8), 108.1 (C-5′), 109.1 (C-2), 109.5 (C-2′), 114.0 (C-5), 121.0 (C-6), and 122.0 (C-6′)], one oxygenated benzylic carbon δ_C 86.9 (C-7), and six quaternary carbons [δ_C 133.7 (C-1), 135.7 (C-1′), 145.3 (C-4), 145.6 (C-3′), 147.0 (C-3), and 147.6 (C-4′)]. The exact positions of the substituents were based on the results of HMBC experiments. In the HMBC spectral data, cross-peak at δ_H 3.19 (OCH_3-7) with C-7, δ_H 3.89 (OCH_3-3) with C-3, δ_H 5.59 (OH-4) with C-3, C-4, and C-5, and δ_H 5.89 (OCH_2O) with C-3′ and C-4′ showed existence of 4-hydroxy-3-methoxyphenyl and 3′,4′-methylenedioxyl groups. Furthermore, ^1H- and ^{13}C-long range correlation in HMBC spectrum showed cross-peaks between H-7 and C-1, C-2, and C-6, and between H-7′ a/b and C-1′, C-2′, and C-6′ (Table 1). Thus, based on all the above-obtained spectral data, the compound **4** was identified as 7-(4-hydroxy-3-methoxyphenyl)-7′-(3′,4′-methylenedioxyphenyl)-8,8′-lignan-7-methyl ether, which has proven to be a new compound.

Table 1. ^1H and ^{13}C NMR spectral data and HMBC correlations for compound **4** in $CDCl_3$[a]

Position	δ_H[b]	δ_C[c]	HMBC
1		133.7	H-2, H-5,
2	6.81 s	109.1	H-6, H-7
3		147.0	H-2, H-5, OH-4, OCH_3-3
4		145.3	H-2, H-5, H-6, OH-4
5	6.93 d (8.0)	114.0	OH-4
6	6.79 dd (1.2, 8.0)	121.0	H-2, H-7, OH-4
7	4.01 d, (7.2)	86.9	H-2, H-6, OCH_3-7, H-9
8	1.74 m	45.4	H-7, H-9, H-9′
9	1.04 d, (6.8)	11.2	H-7, H-8
1′		135.7	H-5′, H-7′ a/b
2′	6.46	109.5	H-6′, H-7′ a/b
3′		145.6	H-2′, H-5′, H-6′, CH_2O
4′		147.6	H-2′, H-5′, OCH_2O
5′	6.67 d (8.0)	108.1	
6′	6.44 dd (1.2, 8.0)	122.0	H-2′, H-7′ a/b
7′ a	2.73 dd (3.6, 13.6)	37.8	H-2′, H-6′, H-9′
7′ b	2.11 dd (11.2, 13.6)		
8′	1.56 m	36.7	H-7, H-9, H-7′ a/b, H-9′
9′	0.76 d (6.8)	18.0	H-7′ a/b
3-OCH_3	3.89 s	56.2	C-3
7-OCH_3	3.19 s	56.9	H-7
4-OH	5.59 s		
OCH_2O	5.89 dd (1.6, 4.4)	100.9	

[a] Chemical shifts are shown in the δ scale with *J* values (Hz) in parentheses. Assignments were made by ^1H–^1H COSY, HMQC, and HMBC data.
[b] Four hundred megahertz in $CDCl_3$ at 25 °C.
[c] One hundred megahertz in $CDCl_3$ at 25 °C. Multiplicity was established from DEPT data.

The six isolated compounds **1**–**6** and **7** derived from **2** were examined in vitro for their ability to protect human LDL against Cu^{2+}-induced peroxidation.[18] First of all, compounds **1**–**7** are measured in order to compare their LDL-antioxidant activities in the TBARS assay according to their structure–activity relationship (SAR). The results are summarized in Table 2. Among them, compound **3** bearing 3,3′-dimethoxy and 4,4′-dihydroxy groups showed the highest LDL-antioxidant activity with an IC_{50} value of 2.6 μM, whereas compound **1** protected with catecholic group at position 3,4 and 3′,4′ is not active. Compounds **2**, **4**, and **5** substituted with catecholic group

Table 2. LDL-antioxidant activities of compounds **1**–**6** isolated from *M. fragrans* seeds and **7** derived from 2

Compounds	Inhibition on LDL oxidation (TBARS)	
	20 μM	LDL (IC_{50}[a] μM)
1	0	NA[b]
2	64.5 ± 1.0	7.5
3	93.0 ± 5.9	2.6
4	28.9 ± 0.7	7.9
5	45.7 ± 1.7	6.3
6	40.9 ± 0.5	7.3
7	91.0 ± 0.5	3.6
Probucol[c]	92.0 ± 0.8	3.1

[a] In vitro antioxidant activity was measured using human plasma LDL (120 μg/mL). Data are shown as mean values of two independent experiments performed in duplicate.
[b] NA is not active.
[c] Probucol was used as a positive control.

at position 3,4 or 3′,4′ were less effective than **3**. Aryltetralin lignan **6** showed very similar LDL-antioxidant activity to **2**. Also, compound **7** derived from **2** was highly effective against LDL-oxidation with an IC_{50} value of 3.6 μM due to production of quinine intermediate from vicinal dihydroxy group that is responsible for releasing two electrons.[19] Then, probucol was used as a positive control in this assay (IC_{50} = 3.1 μM). These results may be rationalized that methoxy group on phenyl ring may stabilize the phenoxy radical formed to show more antioxidant activity against LDL, which agreed with earlier work.[20] On the basis of these results, **3** having the highest LDL-antioxidant activity was selected for further studies on detection of conjugated diene formation, REM of ox-LDL, and fragmentation of apoB-100 on copper-mediated LDL oxidation and macrophage-mediated LDL oxidation.

Oxidation of the LDL was determined by measuring the conjugated diene formation at 234 nm[21] and typical effect of the lignan **3** is shown in Figure 2. The formation of the conjugated diene during LDL oxidation represents the early peroxidation of the LDL. When LDL (120 μg protein/mL) was incubated with 5 μM $CuSO_4$, the lag time (the elapsed time before the onset of rapid formation of conjugated lipid hydroperoxides) was 59 min. However, when 1.0 μM of **3** was present during incubation, the lag time of LDL oxidation was extended to 140 min. The lag time in the presence of probucol (3.0 μM) was 67 min under the same condition, as shown in Figure 2. These data revealed that compound **3** extended more extensively the lag time than probucol.

The antioxidant protection of the lysine residues of LDL apoB-100 protein from Cu^{2+}-induced oxidation was evaluated with gel electrophoresis. Then, the effect of **3** on Cu^{2+}-mediated LDL oxidation was determined by REM assay.[22] The LDL was incubated with 5 μM $CuSO_4$ alone to induce the oxidation of LDL for 12 h to result from no protection of LDL oxidation. The mobility of LDL after treatment of compound **3** was reduced dose dependently. When treated with 20 and 10 μM of **3**, LDL oxidation was protected in 82% and 72%, respectively, compared to that of oxidized LDL, as shown in Table 3. As a result, compound **3** showed potent antioxidant activity against LDL. LDL oxidation is characterized by alterations in structure and biological properties not only of the lipids but also on apolipoprotein B (apoB), including early fragmentation of the protein which contains sensitive cysteine residues, followed in time by cross-linking by reactive aldehydes formed as end products of the oxidative chain.

The inhibition of the oxidative process was evaluated also by the fragmentation of apoB-100, a major component of LDL, through the electrophoretic analysis on 4% SDS–PAGE.[23] As shown in Table 4, the densitomet-

Table 3. Relative electrophoretic mobility (REM) on agarose gel of copper-oxidized LDL

Compounds (μM)	Distance (cm)	Protection (%)
Native LDL	3.9	100
Ox-LDL	0	0
3 (20)	3.2	82
3 (10)	2.8	72

LDL (120 μg/mL in PBS) was incubated for 12 h at 37 °C with 5 μM $CuSO_4$. After incubation, approximately 2.0 μg of LDL protein was loaded onto 0.7% agarose gel for electrophoresis. The gel was stained with Coomassie brilliant blue R-250 and subjected to densitometric scanning by Bio-Rad Model GS-800 with Bio-Rad Quantity One-4.4.0 software.

Table 4. Oxidative modification on SDS–PAGE of apoB-100[a]

Compounds (μM)	Area[b] (AU/mm)
Native LDL	14.0
Ox-LDL	0
3 (20)	13.0
3 (10)	7.6

[a] LDL (120 μg/μL in PBS) was incubated for 4 h at 37 °C with 5 μM $CuSO_4$ in the absence or presence of 20 and 10 μM of **3**. After incubation, approximately 2.0 μg of LDL protein was applied to SDS–PAGE (7.5%). After the electrophoresis, the gel was stained with Coomassie brilliant blue R250 and subjected to densitometric scanning by Bio-Rad Model GS-800 with Bio-Rad Quantity One-4.4.0 software.

[b] Areas of the peaks of the apoB-100 expressed as absorbance units per millimeter.

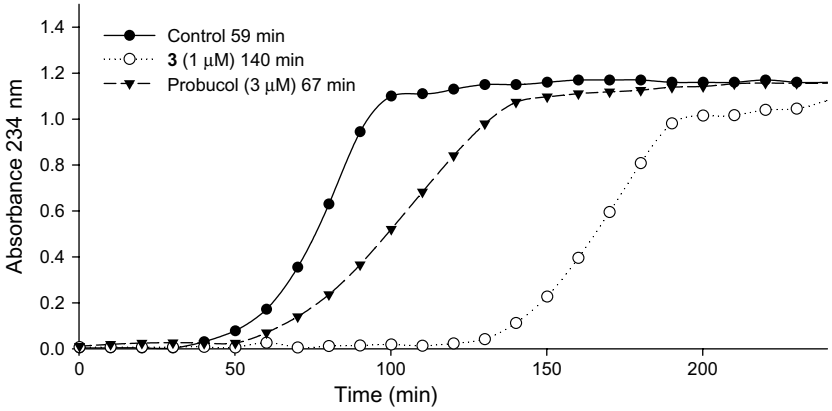

Figure 2. Effect of **3** on the conjugated diene formation. LDL (120 μg protein/mL) was incubated with 5 μM of $CuSO_4$ in the presence or absence of antioxidant, **3**. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Probucol was used as a reference antioxidant.

Table 5. Effects of **3** on macrophage-mediated LDL oxidation

Incubation conditions[a]	MDA nmol/mg LDL protein[b]
LDL + Cu^{2+}	110.0 ± 16.4
LDL + Cell + Cu^{2+} (control)	357.1 ± 2.4
LDL + Cell + Cu^{2+} + 1 μM **3**	306.3 ± 19.2
LDL + Cell + Cu^{2+} + 5 μM **3**	35.2 ± 7.9
LDL + Cell + Cu^{2+} + 1 μM BHA	357.0 ± 7.7
LDL + Cell + Cu^{2+} + 5 μM BHA	214.9 ± 18.6

[a] LDL (100 μg/mL) was incubated for 24 h at 37 °C in serum-free RPMI 1640 medium with 2 μM of Cu^{2+} in 12-well plate containing macrophages, in the absence (control) or presence of each 1 and 5 μM of compound **3**. BHA was used as a positive control.

[b] The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay. Data are shown as means ± SD ($n = 3$). $^{*}P < 0.01$ versus control.

ric values related to the areas of the bands of apoB-100 were expressed as absorbance units per millimeter (AU/mm). The band of apoB-100 on native LDL was evaluated at 14.0 AU/mm. In the presence of 20 and 10 μM of **3**, the percent of remaining apoB-100 against intact apoB-100 of native LDL was 93% and 54%, respectively. Thus, compound **3** has potent activity in the protection of apoB-100 fragmentation against copper-induced oxidation of LDL.

Next, we were interested in antioxidant activities of **3** on macrophage-mediated oxidation of LDL. After treatment of PMA to THP-1 cells, the morphology of the cells was differentiated into adherent cell as macrophage-like cells. In macrophage-mediated LDL oxidation, the compound **3** was also inhibited by the TBARS formation to a similar extent to that obtained in Cu^{2+}-induced LDL oxidation. LDL oxidation measured by MDA-like product formation was threefold higher in the presence of THP-1 macrophages (357.1 ± 2.4 MDA nmol/mg LDL protein) compared with incubation in the absence of cells (110.0 ± 16.4 MDA nmol/mg LDL protein). Therefore, antioxidant activity of **3** and BHT was tested by macrophage-mediated LDL oxidation with 2.0 μM $CuSO_4$. In presence of each 1.0 and 5.0 μM of **3**, the content of ox-LDL was 306.3 ± 19.2 and 35.2 ± 1.5 MDA nmol/mg LDL proteins, respectively. At the same concentration of BHT, it was 357 ± 7.7 and 214.9 ± 18.6 MDA nmol/mg LDL proteins, respectively. Antioxidant activities of **3** were about sixfold higher than that of BHT on macrophage-mediated LDL oxidation (Table 5).

In summary, we have discovered a new class of LDL-antioxidants. Six diarylbutane lignans **1**–**5** and one aryltetralin lignan **6** were isolated by bioassay-guided fractionation from the methanol extracts of *M. fragrans* seeds and then 7-methyl ether diarylbutane lignan **4** has a proven to be new compound. Among them, compound **3** exhibited the most potent LDL-antioxidant activity with IC_{50} value of 2.6 μM in TBARS assay. Therefore, compound **3** was tested with various tools to measure LDL-antioxidant activities such as lag time, REM of ox-LDL, and fragmentation of apoB-100 on copper-mediated LDL oxidation or in macrophage-mediated LDL oxidation. According to these results, compounds **2**, **4**, and **5** substituted with catecholic group at position 3,4 or 3′,4′ were less effective than **3**. Aryltetralin lignan **6** showed very similar LDL-antioxidant activity to **2**. As a result, methoxy group on the phenyl ring is necessary to stabilize phenoxy radical formed in order to show the potent LDL-antioxidant activity.

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12. *Procedure for extraction and isolation.* The dried of *M. fragrans* seeds (500 g) were chopped and extracted three times with 95% CH_3OH (3× 2 L) for 7 days at room temperature. The combined extract was evaporated to dryness under reduced pressure at a temperature below 45 °C. After filtration and concentration, the resultant extract (11.3 g) was separated by vacuum liquid chromatography (silica gel, 230–400 mesh, 60 g) using organic solvents as *n*-hexane, EtOAc, and MeOH. The different polarities afforded *n*-hexane (1.8 g), EtOAc (3.5 g), and MeOH (1.5 g) extracts, respectively. The EtOAc extract (3.5 g) was subjected to column chromatography (glass column 5 × 80 cm) over silica gel (230–400 mesh, 40 g), eluted with gradient mixtures of $CHCl_3$ (4 L) and $CHCl_3$/acetone, of increasing polarity (99:1 → 1:1), and finally with MeOH. Twenty-nine pooled fractions (F1–F29) were obtained after combining fractions with similar TLC profiles from this initial column chromatography. The fraction F4.4 (105 mg) was dissolved in *n*-hexane and loaded onto the top of a glass column packed with silica gel (230–400 mesh, 6 g) in hexane. Elution was carried out using mixtures of *n*-hexane:EtOAc and then increasing the polarity with EtOAc. Altogether, 200 fractions (10 mL) were collected and combined to give 10 major subfractions (F4.4.1–F4.4.10), based on the comparison of TLC profile

using $CHCl_3/CH_3CN$ (24:1) as a developing solvent. The colorless oil of compound **4** (3.1 mg) obtained from subfraction F4.4.7 was purified by Sephadex LH-20 column chromatography.

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