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Antioxidant lignans from *Larrea tridentata*

Hassan Abou-Gazar [a], Erdal Bedir [a,b], Satoshi Takamatsu [a], Daneel Ferreira [a,c], Ikhlas A. Khan [a,c,*]

[a] *National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, The University of Mississippi, University, MS 38677, USA*
[b] *Department of Bioengineering, Faculty of Engineering, Ege University, Izmir 35100, Turkey*
[c] *Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, MS 38677, USA*

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Abstract

Three lignans, (7*S*,8*S*,7′*S*,8′*S*)-3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan, *meso*-(*rel* 7*S*,8*S*,7′*R*,8′*R*)-3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan, and (*E*)-4,4′-dihydroxy-7,7′-dioxolign-8(8′)-ene, together with 10 known compounds, were isolated from the leaves of *Larrea tridentata*. The structures of the new compounds were determined primarily from 1D and 2D NMR spectroscopic analysis. Their antioxidant activities against intracellular reactive oxygen species were evaluated in HL-60 cells.

Keywords: *Larrea tridentata*; Zygophyllaceae; Lignans; Flavonoids; Antioxidant activity

1. Introduction

Larrea tridentata (Sesse' & Mocino ex DC.) Coville, also known as Larrea, chaparral, or creosote bush, is a shrubby plant belonging to the family Zygophyllaceae, which dominates some areas of the desert southwest in the United States and Northern Mexico, as well as some desert areas of Argentina (Sinnott et al., 1998). Tea brewed from the leaves of *L. tridentata* has been used in traditional medicine to treat digestive disorders, rheumatism, venereal disease, sores, bronchitis, chicken pox, and the common cold (Sinnott et al., 1998).

Phytochemical studies carried out on *L. tridentata* showed that it contains a series of lignans (Gisvold and Thaker, 1974; Konno et al., 1990), flavonoids (Chirikdjian, 1974; Sakakibara and Mabry, 1975; Sakakibara et al., 1976), condensed tannins (Hyder et al., 2002), triterpene saponins (Xue et al., 1988), and naphthoquinones (Luo et al., 1988). The extracts or constituents of *L. tridentata* have been reported to possess antioxidant (Gonzalez-Coloma et al., 1988), anti- HIV (Gnabre et al., 1995, 1996), antimicrobial (MacRae and Towers, 1984), enzyme inhibitory (MacRae and Towers, 1984; Sinnott et al., 1998), anti-tumor (MacRae and Towers, 1984), and anti-hyperglycemic (Luo et al., 1998) activities. The plant contains the powerful antioxidant, nordihydroguaiaretic acid (NDGA) which is suspected to contribute to the toxic effects associated with the consumption of chaparral products (Sinnott et al., 1998).

The overall objective of the project was to provide pure compounds for fingerprinting studies of creosote bush as part of our extensive program of standardizing traditional medicines and dietary supplements of plant origin. The isolation of three new lignans together with 10 known compounds, comprising six flavonoids and four lignans, is presented here.

* Corresponding author. Tel./fax: +1 662 915 7821.
E-mail address: rikhan@olemiss.edu (I.A. Khan).

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2. Results and discussion

The LC-ESI-MS spectrum of **1** exhibited molecular ions at *m/z* 331.1 $[M + H]^+$, 353.1 $[M + Na]^+$, 683.2 $[2M + Na]^+$; (HR-ESI-FTMS: *m/z* 329.1393 $[M-H]^-$), suggesting a molecular formula $C_{19}H_{22}O_5$, which was confirmed by the observation of three methyl, ten methine, and six quaternary carbon resonances in its ^{13}C NMR spectrum (Table 1). The results of comprehensive ^1H and ^{13}C NMR spectroscopic studies of **1**, and previous knowledge derived from metabolites isolated from *L. tridentata* (Konno et al., 1990), collectively suggested that compound **1** belonged to the 7,7′-epoxylignan class of natural products. Characteristic signals arising from the tetrasubstituted tetrahydrofuran ring system comprised two secondary methyl groups at δ 0.92 and 0.93, (each *d*, *J* = 5.0 Hz, 8- and 8′-Me, respectively), a two-proton multiplet at δ 1.63 due to the H-8 and H-8′ methine protons, and one proton doublets at δ 4.42 and 4.44 (each *J* = 9.0 Hz) due to the two oxymethine protons, H-7 and -7′. The ^1H NMR spectrum of **1** additionally showed two ABX systems for aromatic protons at δ 6.79 (*d*, *J* = 1.5 Hz), 6.71 (*d*, *J* = 8.0 Hz), and 6.62 (*dd*, *J* = 1.5, 8.0 Hz) and at δ 6.82 (*d*, *J* = 1.5 Hz), 6.86 (*d*, *J* = 8.0 Hz), and 6.73 (*dd*, *J* = 1.5, 8.0 Hz) implying that the aromatic rings each had a 1,2,4-trisubstitution pattern. The ^{13}C NMR spectrum of **1** supported the presence of four oxygenated aromatic carbons at δ 145.5, 145.0, 146.8, and 147.4. Complete assignment of the proton spin systems in **1** was achieved by DQF-COSY, while carbons were assigned from HMQC and HMBC spectra. The evaluation of the HMBC spectrum permitted the identification of the aromatic hydroxyl and *O*-methyl positions. The HMBC spectrum of **1** showed correlations between the methoxy protons at δ 3.74 and a quaternary carbon at δ 147.4 (C-4), and displayed crosspeaks from the latter to the H-2 and H-6 resonances (δ 6.82 and 6.73, respectively), indicating that the methoxy group was located at C-4. When taken in conjunction with the prominent HMBC crosspeaks from δ 6.73(H-6) and 6.82(H-2) to δ 87.6(C-7), this also differentiated the aromatic 3,4-dioxygenation pattern from an alternative 2,4-dioxygenated moiety (see Fig. 1).

The absolute configuration at C-7, C-7′, C-8, and C-8′ was assigned by a combination of 2D- NOESY data, analysis of coupling constants, and the comparison of optical rotation data with those of 7,7′-epoxylignans. The coupling constants between the H-7 and H-8, and H-7′ and H-8′ protons were *J* = 9.0 Hz, indicating a 7,8-*trans*-7′,8′-*trans* relative configuration. The strong nOe correlations between methyl protons (δ 0.92 and 0.93), and H-7 and H-7′ indicated that both H-7 and Me-8, and H-7′ and Me-8′ were cofacial. Comparison of the optical rotation of **1**, $[\alpha]_D^{25}$ -60° (*c* 0.001 in MeOH), with those of (7*S*,8*S*,7′*S*,8′*S*)-3,4,5-trimethoxy-3′,4′-methylenedioxy-7,7′-epoxylignan (Lopes et al., 1996), and talaumidin [(7*S*,8*S*,7′*S*,8′*S*)-3,4-methylenedioxy-4′-hydroxy-3′-methoxy-7,7′-epoxylignan] (Vieira et al., 1998) that also had negative optical rotations, taxumairin (Shen et al., 1997), 7,8-*trans*-7′,8′-*trans*-4,4′-dihydroxy-7,7′-epoxylignan (Arnone et al., 1988), and (+)-galbelgin [(7*R*,8*R*,7′*R*,-8′*R*)-3,4,3′,4′-tetramethoxy-7,7′-epoxylignan] (Takaoka et al., 1976) that showed positive optical rotations, strongly suggested that **1** possessed the *S* absolute configuration at all four stereocenters. The CD spectrum of compound **1** showed negative Cotton effects in both the 220–240 and 280–290 nm regions for the ^1L_a and ^1L_b aromatic transitions (UV absorptions at 230 and 282 nm), respectively. Similar Cotton effects were recorded for (7*S*,8*S*,7′*S*,8′*R*)-3,4,3′,4′-tetramethoxy-7,7′-epoxylignan (Prasad et al., 1995). Since the chirality at C-7 and C-7′ is expected to have a major influence on the electronic transition of the aromatic

Table 1
^1H and ^{13}C NMR data of **1** and **2**

C/H	**1**		**2**	
	δ (ppm), *J* (Hz)	δ (ppm)	δ (ppm), *J* (Hz)	δ (ppm)
1		135.7		133.9
2	6.82 *d*, (1.5)	113.9	6.79 *d*, (1.5)	114.0
3		146.8		145.3
4		147.4		144.8
5	6.86 *d*, (8.0)	112.3	6.73 *d*, (8.0)	115.6
6	6.73 *dd*, (8.0,1.5)	117.6	6.27 *dd*, (8.0,1.5)	117.7
7	4.42 *d*, (9.0)	87.6	4.42 *d*, (9.0)	87.8
8	1.63 *m*	50.8	1.63 *m*	51.0
1′		133.9		
2′	6.79 *d*, (1.5)	114.1		
3′		145.5		
4′		145.0		
5′	6.71 *d*, (8.0)	115.7		
6′	6.62 *dd*, (8.0,1.5)	117.9		
7′	4.44 *d*, (9.0)	87.9		
8′	1.63 *m*	50.9		
Me-8	0.92 *d*, (5.0)	14.0		14.4
Me-8′	0.93 *d*, (5.0)	14.0		
Ome	3.74 *s*	56.1		

Assignments confirmed by DQF-COSY, HMQC, and HMBC experiments.

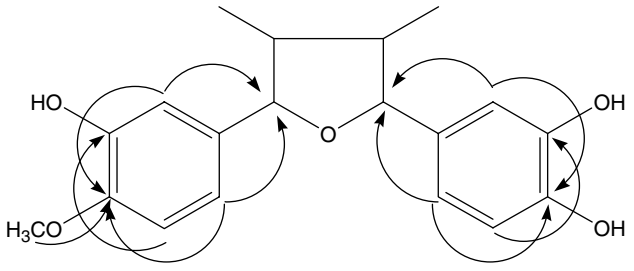


Fig. 1. Selected HMBC correlations of **1**.

chromophore, the structure of **1** may be defined as (7*S*,8*S*,7′*S*,8′*S*)-3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan (see Fig. 2).

In the LC-ESI-MS of **2**, the molecular ion was observed at *m*/*z* 316 (HR-ESI-FTMS: *m*/*z* 315.0562 [M–H]$^-$), 14 mass units lower than that of **1**, suggesting a molecular formula of $C_{18}H_{20}O_5$, and hence replacement of the *O*-methyl moiety with a hydroxyl group. Inspection of the ^{1}H and ^{13}C NMR spectra of **2** indicated typical spin patterns of a C_9-dimer which contained only eight protons (aromatic protons: 2 × ABX systems; δ 6.27, *dd*, J = 1.5, 8.0 Hz; 6.73, *d*, J = 8.0 Hz; and 6.79, *d*, J = 1.5 Hz; tetrahydrofuranoid protons: δ 0.94, *d*, J = 5.5 Hz, 1.63, *m*; 4.42, *d*, J = 9.0 Hz), and nine carbons. The symmetry shown by the ^{1}H and ^{13}C NMR spectral data was supported by the optical rotation value {$[\alpha]_D^{25}$ 0° (*c* 0.0012 MeOH)}, indicating that **2** was a *meso* diastereomer. There are two *meso* forms of 3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan. These two isomers can be distinguished based on the difference in the orientation of the methyl and aryl substituents of the tetrahydrofuran ring. The chemical shifts of the oxymethine (H-7/H-7′) and methyl protons, and of the carbons bearing the oxymethine protons (C-7/C-7′) are diagnostic in determining the *cis*/*trans* relationship between the methyl and the aryl substituents (Rimando et al., 1994). The methyl and the oxymethine protons of **2** displayed signals at δ 0.94 and 4.42, respectively, whereas the resonances of C-7/C-7′ and C-8/C-8′ were observed at δ 87.8 and 51.0, respectively, which verified that **2** is the 7,8-*trans*-7′,8′-*trans meso*-form. Compound **2** is thus *meso*-(*rel* 7*S*,8*S*,7′*R*,8′*R*)-3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan.

Compound **3** displayed a molecular ion at *m*/*z* 296 (HR-ESI-FTMS: *m*/*z* 295.0996 [M − H]$^-$), indicating a molecular formula of $C_{18}H_{16}O_4$. In the ^{1}H NMR spectrum of **3**, a vinylic methyl group (δ 2.00, *s*) and an AA′BB′ type aromatic system (δ 6.80, 7.58, both *d*, J = 8.5 Hz) were observed. The NMR profile (^{1}H:3 distinct signals, ^{13}C:7 distinct signals) and the molecular formula implied that compound **3** was also symmetrical.

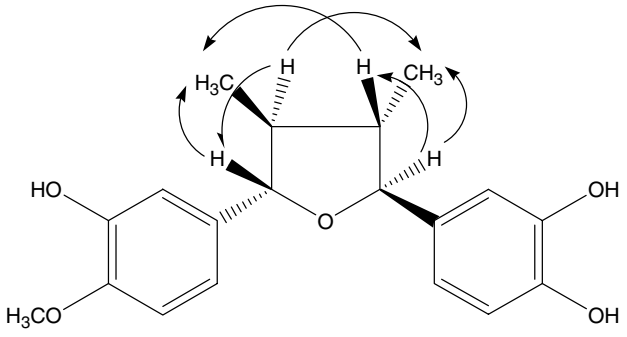
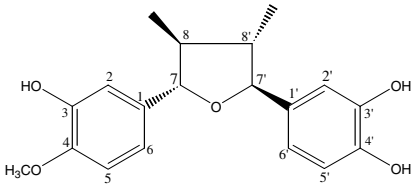
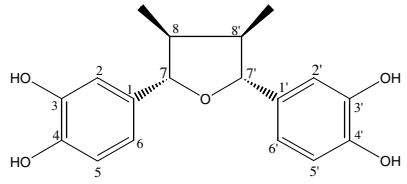


Fig. 2. Key nOe correlations of **1**.

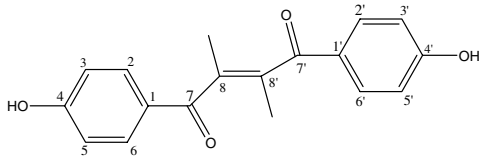
Inspection of the ^{13}C NMR spectrum of **3** showed a carbonyl carbon at δ 196.9 (2×) and a tetra-substituted double bond carbon at δ 138.5 (2×). When taken in conjunction with the HMBC correlations shown in Fig. 3, this data collectively indicated that **3** had a symmetrical 4,4′-dihydroxydioxolignene framework. The structure of this compound may hence be presented as either the (*E*)-4,4′-dihydroxy-7,7′-dioxolign-8(8′)-ene, **3a** (C-2 axis of symmetry) or the (*Z*)-isomer **3b** (mirror plane). Based on thermodynamic considerations, we favor the (*E*)-configured olefinic functionality as is expressed in structure **3a**. This is the second demonstration of the natural occurrence of the rare class of dioxolignene-type lignans, the only one previously reported being the 3,3′-dimethoxy analog of **3b** (Majumder and Bhattacharyya, 1975).



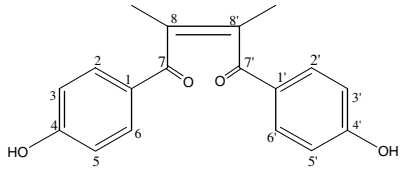
1



2



3a



3b

The flavonoids, herbacetin 3,8,4′-trimethyl ether (**4**) (Horie et al., 1998; Proksch et al., 1988), 5,7,4′-trihydroxy-3,8,3′-trimethoxyflavone (**7**) (Roitman and James, 1985), apigenin (**8**) (Gisvold and Thaker, 1974; Mabry

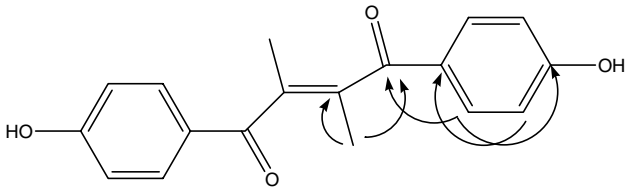


Fig. 3. Key HMBC correlations of **3**.

et al., 1970), (+)-dihydroisorhamnetin (**9**) (Pavanasasivam and Sultanbawa, 1975), 5,7,4′-trihydroxy-3,8-dimethoxyflavone (**10**) (Roitman and James, 1985), kaempferol-3-methyl ether (isokempferide) (**11**) (Star and Mabry, 1971; Wollenweber et al., 1972), and the lignoids 4-*epi*-larreatricin (**5**) (Konno et al., 1990), 3′-demethoxy-6-*O*-demethylisoguaicin (**6**) (Konno et al., 1990), 3″-hydroxy-4-*epi*-larreatricin (**12**) (Konno et al., 1990), and 3,4-dehydrolarreatricin (**13**) (Konno et al., 1990) were also isolated from the aerial parts of *L. tridentata*. These known compounds were identified on the basis of their LC-ESI-MS and NMR (^{1}H and ^{13}C) spectroscopic data in comparison with literature values. NDGA was identified via TLC comparison with a commercially available reference sample (Aldrich, 15, 761–9).

Earlier studies of plant-derived antioxidants have examined the reduction potential or radical-scavenging effects of natural products in solution-based chemical assays (Cavin et al., 1998; Westenburg et al., 2000).Fluorescent technology made it possible to evaluate antioxidants in live cells using specific probes such as 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The DCFH assay is based on the detection of intracellular respiratory burst activity in such phagocytic cells as neutrophils and macrophages (Rosenkranz et al., 1992; Rajbhandari et al., 2001).

The antioxidant effect of the new lignans **1**–**3**, together with the 10 known compounds **4**–**13** from *L. tridentata*, was evaluated using the DCFH method. Among the six lignans (Table 3), the epoxylignans possessing a tetrahydrofuran moiety, **1**, **2**, **5**, and **12** showed strong antioxidant activity in the range of IC_{50} 1.3–18.0 μg/mL, without cytotoxicity up to 10 μg/mL. The number and position of the hydroxy and methoxy substituents on the phenyl moieties may contribute to the antioxidant effect in an epoxylignan, especially **1** (IC_{50} 1.3 μg/mL) with its catechol aromatic functionalities and **12** (IC_{50} 1.9 μg/mL) compared to Trolox (IC_{50} 1.4 μg/mL). On the contrary, epoxylignans with a dihydrofuran ring, e.g., **13**, and oxidized compounds like **3a/3b**, did not show antioxidant activity, suggesting that both the tetrahydrofuran and phenyl moieties are important for activity. In addition, the destabilization effect of the benzylic carbonyl group on oxygen and carbon radicals at the aromatic ring would be detrimental to the antioxidant activity of compound **3**. 3′-Demethoxy-6-*O*-demethylisoguaicin (**6**) and its precursor, nordihydroguaiaretic acid (NDGA), also showed potent antioxidant activity with IC_{50} values of 1.6 and 0.7 μg/mL, respectively. Most recently, licarin A, (+)-guaiacin, (−)-isoguaiacin, and *meso*-dihydroguaiaretic acid from *Machilus thunbergii* were reported as neuroprotective lignans (Ma et al., 2004). Thus, the antioxidant activity of lignans may be partially responsible for neuroprotective effects. Flavonoids showed weak antioxidant activity at an $IC_{50} > 50.0$ μg/mL for **4**, 27.8 μg/mL for **8**, 21.1 μg/mL for **10** and 50.7 μg/mL for **11**, respectively. Compounds **7** and **9** were inactive as antioxidants.

We have thus isolated and identified 13 compounds, including the three new lignans **1**–**3**, which may be used individually or in combination to standardize *L. triden-*

Table 2
^{1}H and ^{13}C NMR data of **3**

C/H	Compound **3**	
	δ (ppm), *J* (Hz)	δ (ppm)
1		128.0
2	7.58 *d*, 8.5	132.0
3	6.80 *d*, 8.5	115.5
4		162.4
5	6.80 *d*, 8.5	115.5
6	7.58 *d*, 8.5	132.0
7		196.9
8		138.5
Me-8	2.00 *s*	17.3

Assignments confirmed by DQF-COSY, HMQC, and HMBC experiments.

Table 3
Antioxidant and cytotoxic effects of compounds **1**–**13** in HL-60 cells

Compound	Antioxidant assay IC_{50} (μg/mL)	XTT assay IC_{50} (μg/mL)
1	1.3 ± 0.4	17.3 ± 1.5
2	7.5 ± 2.0	>50.0
3	IA	>50.0
4	>62.5	>50.0
5	18.0 ± 2.5	>50.0
6	1.6 ± 0.4	13.6 ± 2.6
7	IA	>50.0
8[b]	27.8 ± 1.6	>25.0
9	IA	>50.0
10	21.1 ± 4.5	36.7 ± 4.4
11	50.7 ± 3.0	28.3 ± 3.5
12	1.3 ± 0.3	13.6 ± 0.8
13	>62.5	27.6 ± 0.4
NDGA[A,a,c]	0.7 ± 0.3	2.6 ± 0.2
Vitamin C[A,c]	1.9 ± 0.7	>10.0
Trolox[A,c]	1.4 ± 0.5	>10.0

Values are means of three independent determinations, ±SE.
IA, inactive.
[A] Used as standard.
[a] Nordihydroguaiaretic acid.
[b] Concentration tested, 5 mg/mL vs 10 mg/mL for other samples.
[c] Concentration tested, 2 mg/mL.

tata products that are being used in traditional medicine and/or as dietary supplements.

3. Experimental

3.1. General

Optical rotations were measured on an Autopol® IV Automatic polarimeter. CD spectra were recorded in MeOH on a JASCO J-17 spectrometer. IR spectra were recorded with an ATI Mattson Genesis Series FTIR spectrophotometer and UV spectra in MeOH on Hewlett–Packard 8453 spectrometer. The 1D and 2D NMR spectra were obtained on a Bruker Avance DRX 500 FT spectrometer operating at 500 and 125 MHz, respectively, for ^{1}H and ^{13}C measurements. The chemical shifts are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) and the coupling constants are in Hz (in parentheses). For the ^{13}C NMR spectra, multiplicities were determined by a DEPT experiment. LC–MS data were obtained using a Finnigan AQA ThermoQuest instrument in the ESI mode. HR-ESI-FTMS mass spectra were obtained using a Bruker BioApex FT-MS in ESI mode.

3.2. Chromatographic conditions

The following conditions were used: TLC, precoated Si 250F plates (Merck); developing system, $CHCl_3$/EtOAc/MeOH (60:30:10) and $CHCl_3$/MeOH/H_2O (90:10:0.5); visualization, vanillin/H_2SO_4; column chromatography, silica gel 230–400 mesh (Baker), Sephadex LH-20 (Amersham Biosciences) and silica gel RP-18 (Baker).

3.3. Plant material

Cut and sifted chaparral leaf (*L. tridentata*) was purchased from Frontier Natural Products Co-op. Norway, IA 52318 USA. The material was authenticated by the Quality Assurance Department of this company and a voucher specimen (1026) is deposited in the collection of NCNPR, University, MS.

3.4. Extraction and isolation

The plant material of *L. tridentata* (4.5 kg) was extracted by shaking with MeOH (5 × 6 L) at 45 °C for 4 h each. The dried MeOH extract (700 g) was suspended in H_2O (3 L) and partitioned (3×) with petroleum ether (4 L) (yield; 50 g) and then (3×) with $CHCl_3$ (4 L) (yield; 250 g). An aliquot of the $CHCl_3$ extract (80 g) was subjected to vacuum liquid chromatography (VLC) using silica gel (2 kg, 13 × 95 cm), eluting with hexanes, hexanes:EtOAc (90:10 →10:90), EtOAc, and MeOH to give 14 main fractions [Frs. A–N]. Fr. H gave a precipitate which afforded compound **4** (1.0 g). Fr. G was subjected to VLC [RP (C-18), 500 g, 10 × 30.5 cm] to give six main fractions (G_1–G_6). Fr. G_4 (620 mg) was subjected to column chromatography (Sephadex LH-20, 50 g, 2.5 × 45.7 cm) using MeOH to yield compounds **5** (20 mg) and **6** (200 mg). Fr. I (12 g) was applied to VLC [RP (C-18), 1 kg, 15 × 40.7 cm] and eluted with MeOH in H_2O (10–100%) to give 15 fractions. Fr. I_4 + Fr. I_5 (745 mg) was subjected to column chromatography (Sephadex LH-20, 130 g, 3.8 × 45.7 cm) using MeOH to yield compound **10** (150 mg), Fr. I_8 (382 mg) was chromatographed on Sephadex LH-20, (130 g, 3.8 × 45.7 cm) using MeOH to yield compound **11** (50 mg). Fr. I_{8b} (58 mg) was applied to VLC [RP (C-18), 50 g, 2.5 × 45.7 cm] and eluted with MeOH in H_2O (30–100%) to yield compound **12** (27 mg). Fr. I_{10} (2.24 g) was subjected to column chromatography (silica gel, 300 g, 5 × 61 cm) using $CHCl_3$:MeOH:H_2O (60:38:2) to yield 4 fractions. Fr. I_{10b} (160 mg) was chromatographed on Sephadex LH-20, (100 g, 3.8 × 45.7 cm) using MeOH to yield compounds **13** (47 mg) and **3** (6 mg). Fr. J (4.5 g) was applied to VLC [RP (C-18), 1 kg, 15 × 40.7 cm] and eluted with acetonitrile in H_2O (10–100%) to give 13 fractions. Fr. J_6 gave compound **7** (200 mg). Fr. J_9 (445 mg) was subjected to column chromatography (Sephadex LH-20, 100 g, 3.8 × 45.7 cm) using MeOH to yield compounds **7** (125 mg) and **8** (20 mg). Fr. J_{12} afforded compound **1** (276 mg). Fr. J_2 (217 mg) was subjected to column chromatography (Sephadex LH-20, 100 g, 3.8 × 45.7 cm) using MeOH to yield compound **2** (23 mg). Fr. K (4 g) was applied to VLC [RP (C-18), 1 kg, 15 × 40.7 cm] and eluted with acetonitrile in H_2O (10–100%) to give nine fractions. Fr. K_5 (130 mg) was subjected to column chromatography (silica gel, 60 g, 2.5 × 45.7 cm) using $CHCl_3$:MeOH:H_2O (60:38:2) to yield compound **9** (25 mg).

3.5. (7S,8S,7′S,8′S)-3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan (*1*)

Reddish-brown powder; $[\alpha]_D^{25}$ −60° (*c* 0.001, MeOH); CD$\{\theta\}_{230}$ −55.5, $\{\theta\}_{237}$ −57.9, $\{\theta\}_{287.7}$ −8.9; UV λ_{max} MeOH nm: 282, 230, 208; IR (KBr) ν_{max} 3350, 2957, 1614, 1513, 1443, 1374, 1274, 1024, and 757 cm^{-1}; ^{1}H and ^{13}C NMR: see Table 1; LC-ESI-MS: *m/z* 331.1 $[M + H]^+$; HR-ESI-FTMS: *m/z* 329.1393 $[M-H]^-$ ($C_{19}H_{21}O_5$, requires 329.1388).

3.6. meso-(rel 7S,8S,7′S,8′S)-3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan (*2*)

Off-white powder; $[\alpha]_D^{25}$ 0° (*c* 0.0012, MeOH); IR (KBr) ν_{max} 3360, 1637, 1516, 1458, 1364, 1281, 1026, and 792 cm^{-1}; ^{1}H and ^{13}C NMR: Table 1; LC-ESI-

MS: *m/z* 317 $[M + H]^+$; HR-ESI-FTMS: *m/z* 315.0562 $[M-H]^-$ ($C_{18}H_{19}O_5$, requires 315.1232).

3.7. 4,4′-Dihydroxy-7,7′-dioxolign-8(8′)-ene (3)

Off-white powder; IR (KBr) ν_{max} 3330, 1642, 1579, and 765 cm^{-1}; ^1H and ^{13}C NMR: see Table 2; LC-ESI-MS: *m/z* 297 $[M + H]^+$, HR-ESI-FTMS: *m/z* 295.0996 $[M-H]^-$ ($C_{18}H_{15}O_4$, requires 295.0970).

3.8. A microplate assay for the detection of oxidative products with DCFH-DA

Antioxidant activity was determined by the DCFH method reported previously (Takamatsu et al., 2003). Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C in 5% CO_2:95% air. The cell suspension (125 μL) was added to each well of a 96-well plate. After treatment with different concentrations of the test materials for 30 min, cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Furthermore, cells were incubated for 15 min after the addition of 5 μg/mL DCFH-DA (Molecular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyze DCFH-DA to 2′,7′-dichlorofluorescin (DCFH), and the ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA treated control incubations with and without the test materials. Levels of DCF were measured using a CytoFluor 2350, Fluorescence Measurement System (Millipore) with excitation wavelength at 485 nm (bandwidth 20 nm) and emission at 530 nm (band width 25 nm).

3.9. XTT assay for cytotoxicity in HL-60 cells

After sample (25 μL) exposure on cells for 48 h, the XTT assay was performed using the methods described by Scudiero et al. (1988). Briefly, 25 μl of XTT-PMS solution (1 mg/mL XTT solution supplemented by 25 μM of PMS) was added to HL-60 cells (2×10^4 cell in 225 μL in medium) in each well on the microplates. After incubating for 4 h at 37 °C absorbancy at 450 nm was measured by a microplate reader (reference absorbancy at 630 nm).

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