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Cytotoxic clerodane diterpenoids from the leaves of *Premna tomentosa*

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Abstract

Three clerodane diterpenoids, premnones A–C (**1**–**3**), were isolated from a chloroform-soluble fraction of *Premna tomentosa* along with four known flavonoids and three known triterpenoids. Among these isolates, premnones A–C exhibited cytotoxic activity when evaluated against a small panel of tumor cell lines. However, premnone A was found to be inactive when evaluated in a follow-up in vivo hollow fiber assay at the highest dose tested (50 mg/kg), using LNCaP, Lu1, and MCF-7 cells.

Keywords: *Premna tomentosa*; Verbenaceae; Clerodane diterpenoids; Premnones A–C; Flavonoids; Triterpenoids; Cytotoxicity; Hollow fiber assay

1. Introduction

The genus *Premna* L., comprised of 50–200 species, is distributed in tropical and subtropical Asia, Africa, Australia, and the Pacific islands (Kadereit, 2004). *Premna tomentosa* Willd. (Verbenaceae) is a deciduous tree with its shoots, leaves and inflorescence densely covered with a tawny yellow stellate tomentum (Devi et al., 2004b). It is used as a medicinal plant in India, for the treatment of diarrhea and stomach and hepatic disorders, where it is known as "Pudangainari" and "Krishnapalai" (Devi et al., 2004b). Previous studies have demonstrated that the leaves of *P. tomentosa* possess diuretic, anti-inflammatory, antinociceptive, and hypnotic effects, and a protective effect against acetaminophen-induced hepatotoxicity in the rat (Devi et al., 2004a,b,c). Phytochemical investigations on this plant have resulted in the identification of *dl*-limonene, β-caryophyllene, 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone, myricetin-7,3′,4′-trimethyl ether, and a di-*C*-glycosyl flavone, vicenin 3 (Balakrishna et al., 2003; Etti et al., 2005; Jyotsna et al., 1984; Lakshminarayen and Muthana, 1953). While there do not appear to have been any previous studies on the bioactive constituents of *P. tomentosa*, clerodane diterpenoids have been shown to be antibacterial and cytotoxic constituents of the genus *Premna* (Habtemariam et al., 1990, 1991, 1992; Habtemariam, 1995).

As a part of our ongoing program for the discovery of new anticancer agents of plant origin (Kinghorn et al., 2003), a chloroform-soluble extract of the leaves of

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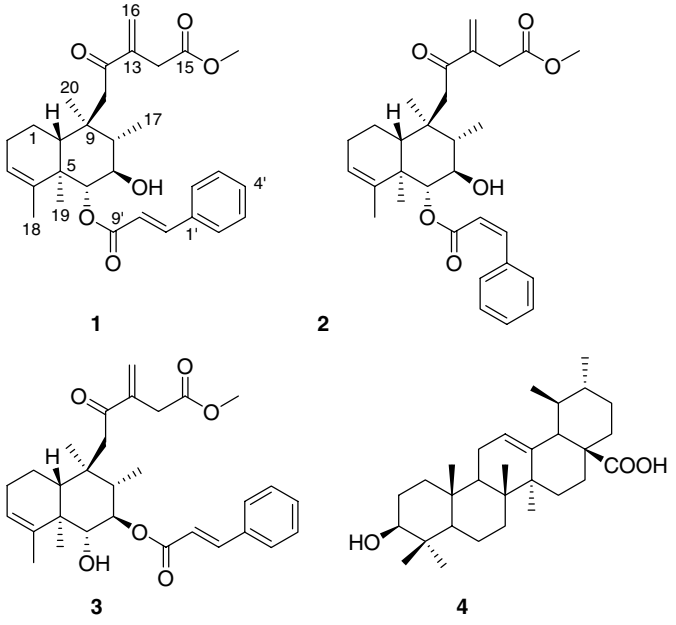
P. tomentosa was chosen because of its initial cytotoxic activity against a small panel of tumor cell lines. Bioactivity-guided fractionation of this extract led to the isolation of three new clerodane diterpenoids (**1**–**3**) and seven known compounds. Herein, the structure elucidation of these new substances and the biological testing of the isolates obtained are described.

2. Results and discussion

The fractionation and purification of a $CHCl_3$-soluble extract, with cytotoxicity assessment using the MCF-7 cell line, led to the isolation of three new clerodane diterpenoids, premnones A–C (**1**–**3**), four known flavonoids, and three known triterpenoids. The structures of the known compounds were identified by physical and spectroscopic data measurement ($[\alpha]_D^{22}$, ^{1}H NMR, ^{13}C NMR, DEPT, 2D-NMR, and MS) and by comparing the data obtained with published values, as chrysosplenetin (Sy and Brown, 1998), 3-*epi*-corosolic acid lactone (Ikuta et al., 2003), 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone (Bohlmann and Mahanta, 1979), 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone (Datta et al., 2000), 2α-hydroxyursolic acid (Taniguchi et al., 2002), 3,7,3′-trimethylquercetin (Valesi et al., 1972), and ursolic acid (**4**) (Jin et al., 2004).



Compound **1** was obtained as a colorless oil and its molecular formula, $C_{30}H_{38}O_6$, was deduced from the quasi-molecular ion peak at *m/z* 517.2566 (calcd. for $C_{30}H_{38}O_6Na$, 517.2560) in the HRESIMS. The ^{1}H NMR spectrum of **1** displayed signals for four methyl groups [δ_H 0.90 (*s*, Me-20), 1.06 (*d*, $J = 6.6$ Hz, Me-17), 1.25 (*s*, Me-19), and 1.61 (*s*, Me-18)], two isolated methylenes [δ_H 2.76 and 2.95 (*d*, $J = 16.3$ Hz, H_2-11), and 3.28 (*s*, H_2-14)], and an olefinic proton (δ_H 5.19, *brs*, H-3), which suggested that **1** is an analog of the clerodane diterpenoid, (5*R*,8*R*,9*S*,10*R*)-12-oxo-*ent*-3,13(16)-clerodien-15-oic acid, isolated previously from the genus *Premna* (Habtemariam et al., 1990). The observed differences were that two oxymethines at δ_H 3.46 (*t*, $J = 10.0$ Hz, H-7) and 4.88 (*d*, $J = 10.0$ Hz, H-6) were present in the ^{1}H NMR spectrum of **1**, instead of two methylenes in the known diterpenoid. Besides the ^{1}H and ^{13}C NMR signals of the diterpenoid unit, the signals of a *trans*-cinnamoyl moiety and a methoxy group were found in the ^{1}H and ^{13}C NMR spectra of **1** (Table 1). The connectivities of the diterpenoid unit and the other functional groups were established from its ^{1}H- ^{1}H COSY and HMBC spectra (Fig. 1). The location of a *trans*-cinnamoyl group was deduced to be at C-6 through an ester linkage by the correlation peak of H-6 (δ_H 4.88) and C-9′ (δ_C 168.8) in the HMBC spectrum. A methoxy group at δ_H 3.64 was affixed to C-15 based on the observed ^3J correlation of the resonances at δ_H 3.64 and δ_C 173.2 (C-15). The relative configuration of **1** was determined using ^{1}H NMR coupling constants and NOESY correlations. Thus, the coupling constant of 10.0 Hz between H-6 and H-7 enabled the protons at C-6 and C-7 to be positioned in a diaxial fashion. In the NOESY spectrum (Fig. 2), it was found that H-10 was not correlated to Me-19, but did exhibit NOESY correlations between H-6 and H-8, which indicated that a *trans*-fused bicyclic ring was present in **1**. The additional NOESY correlations between Me-17 to Me-20, and H-7 to Me-17, Me-19 and Me-20 made it possible to finalize the relative stereochemistry of **1**. Thus, the structure of **1** could be proposed as (5*R**,6*R**,7*R**,8*S**,9*R**,10*R**)-6-*O*-(*trans*-cinnamoyl)-7-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester, and this compound has been accorded the trivial name, premnone A.

The HRESIMS of **2** gave the same molecular formula as $C_{30}H_{38}O_6$, by observing the Na^+-adduct ion at *m/z* 517.2539 (calcd. for $C_{30}H_{38}O_6Na$, *m/z* 517.2560). The ^{1}H and ^{13}C NMR data of compound **2** were nearly identical to those of compound **1** except that a *cis*-cinnamoyl group was apparent in **2**, based on the coupling constants ($J = 12.5$ Hz) of H-7′ and H-8′, instead of the *trans*-cinnamoyl group present in **1**. The 2D-NMR data (HMQC, HMBC, and NOESY) obtained confirmed that the structure of **2** is otherwise the same as **1**. This compound, (5*R**,6*R**,7*R**,8*S**,9*R**,10*R**)-6-*O*-(*cis*-cinnamoyl)-7-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester, has been named premnone B.

A sodiated ion peak of **3** was observed in the HRESIMS at *m/z* 517.2576 $[M+Na]^+$, and corresponded to the molecular formula, $C_{30}H_{38}O_6$. Inspection of the ^{1}H NMR data of **3** revealed that a doublet proton (δ_H 3.48, H-6) appeared in the upfield region and a triplet proton (δ_H 5.09, H-7) was shifted to a more downfield position, when compared to the chemical shifts of H-6 (δ_H 4.88) and H-7 (δ_H 3.46) in **1**. This was consistent with analogous changes in the ^{13}C NMR signals of C-6 (δ_C 78.4) and C-7 (δ_C 78.9) in **3**. These

Table 1
^{1}H and ^{13}C NMR chemical shifts for compounds **1**–**3** in CD_3OD[a]

	1		**2**		**3**	
	δ_H, mult. (*J* in Hz)	δ_C[b]	δ_H, mult. (*J* in Hz)	δ_C[b]	δ_H, mult. (*J* in Hz)	δ_C[b]
1	1.70 *m*	20.0 *t*	1.65 *m*	20.0 *t*	1.69 *m*	20.0 *t*
2	1.97 *brs*	27.2 *t*	1.94 *brs*	27.2 *t*	1.95 *m*	27.2 *t*
3	5.19 *brs*	124.2 *d*	5.16 *brs*	124.1 *d*	5.17 *brs*	123.2 *d*
4		143.0 *s*		143.0 *s*		144.7 *s*
5		44.5 *s*		44.4 *s*		45.3 *s*
6	4.88 *d* (10.0)	82.5 *d*	4.79 *d* (9.5)	82.3 *d*	3.48 *d* (9.4)	78.4 *d*
7	3.46 *t* (10.0)	74.1 *d*	3.38 *t* (9.5)	73.9 *d*	5.09 *t* (9.4)	78.9 *d*
8	2.21 *dt* (10.0, 6.6)	43.0 *d*	2.18 *dt* (9.5, 6.7)	43.1 *d*	2.36 *dt* (9.4, 6.6)	41.1 *d*
9		42.9 *s*		42.9 *s*		43.1 *s*
10	2.05 *brd* (10.4)	46.1 *d*	1.98 *brd* (12.1)	46.1 *d*	1.96 *m*	45.9 *d*
11	2.95 *d* (16.3)	44.0 *t*	2.93 *d* (16.4)	44.0 *t*	3.02 *d* (16.5)	43.7 *t*
	2.76 *d* (16.3)		2.74 *d* (16.4)		2.70 *d* (16.5)	
12		201.9 *s*		202.0 *s*		202.0 *s*
13		145.1 *s*		145.2 *s*		145.1 *s*
14	3.28 *s*	38.1 *t*	3.27 *s*	38.1 *t*	3.29 *s*	38.1 *t*
15		173.2 *s*		173.3 *s*		173.3 *s*
16	6.29 *s*	128.2 *t*	6.28 *s*	128.2 *t*	6.30 *s*	128.3 *t*
	5.97 *s*		5.95 *s*		5.96 *s*	
17	1.06 *d* (6.6)	12.4 *q*	1.05 *d* (6.7)	12.4 *q*	0.89 *d* (6.6)	12.1 *q*
18	1.61 *s*	21.0 *q*	1.56 *s*	20.9 *q*	1.80 *s*	22.8 *q*
19	1.25 *s*	18.0 *q*	0.99 *s*	17.8 *q*	1.14 *s*	17.2 *q*
20	0.90 *s*	19.0 *q*	0.85 *s*	19.0 *q*	0.94 *s*	19.1 *q*
OCH_3	3.64 *s*	52.5 *q*	3.63 *s*	52.5 *q*	3.64 *s*	52.5 *q*
1′		135.9 *s*		136.6 *s*		136.0 *s*
2′	7.60 *m*	129.2 *d*	7.63 *m*	131.0 *d*	7.62 *m*	129.2 *d*
3′	7.39 *m*	130.0 *d*	7.31 *m*	129.0 *d*	7.40 *m*	130.0 *d*
4′	7.39 *m*	131.4 *d*	7.31 *m*	129.9 *d*	7.40 *m*	131.4 *d*
5′	7.39 *m*	130.0 *d*	7.31 *m*	129.0 *d*	7.40 *m*	130.0 *d*
6′	7.60 *m*	129.2 *d*	7.63 *m*	131.0 *d*	7.62 *m*	129.2 *d*
7′	7.68 *d* (15.6)	145.8 *d*	7.03 *d* (12.5)	144.6 *d*	7.73 *d* (16.0)	146.1 *d*
8′	6.58 *d* (15.6)	120.2 *d*	5.98 *d* (12.5)	121.6 *d*	6.60 *d* (16.0)	119.5 *d*
9′		168.8 *s*		168.0 *s*		169.1 *s*

[a] Chemical shifts refer to TMS ($\delta_H = 0$).
[b] Multiplicity was deduced from the DEPT and HMQC spectra.

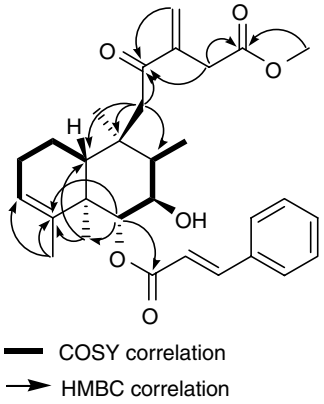


Fig. 1. Selected ^{1}H ^{1}H COSY and HMBC correlations of compound **1**.

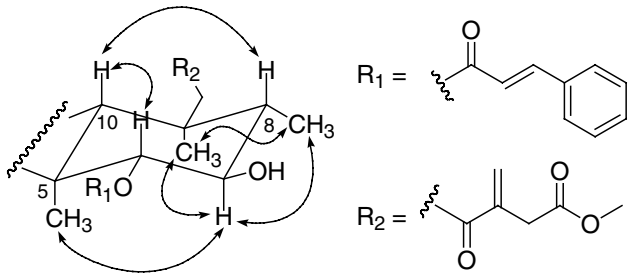


Fig. 2. Selected NOESY correlations of compound **1**.

observations implied that a cinnamoyl group was located at C-7 through an ester linkage rather than at C-6, as in **1**. This inference was supported from the HMBC correlation observed between H-7 (δ_H 5.09) and C-9′ (δ_C 169.1). Based on the interpretation of its 1D- and 2D-NMR data, compound **3** (premnone C) was identified as (5*R**,6*R**,7*R**,8*S**,9*R**,10*R**)-7-*O*-(*trans*-cinnamoyl)-6-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester.

Since initial extraction with ethanol as compared to methanol led to the isolation and structural determination of an analog of premnones A–C (**1**–**3**) with a free carboxylic acid unit from the leaves of *P. schimperi* (Habtemariam et al., 1990), it is possible that compounds **1**–**3** are extraction artifacts since they occur as methyl esters.

All the isolates obtained in this investigation from *P. tomentosa* leaves were evaluated for cytotoxicity against

Table 2
Cytotoxicity of compounds **1**–**4** against tumor cell lines[a,b]

	Cell line[c]			
	Lu1	LNCaP	MCF-7	HUVEC
1	1.2	1.8	0.7	1.0
2	4.1	5.0	3.3	7.0
3	1.2	1.7	0.9	0.9
4	8.9	9.3	5.5	4.9

[a] Results are expressed as ED_{50} values (μg/ml).
[b] Chrysosplenetin, 3-*epi*-corosolic acid lactone, 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone, 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone, 2α-hydroxyursolic acid, and 3,7,3′-trimethylquercetin were inactive against all cell lines ($ED_{50} > 5.0$ μg/ml).
[c] Key: Lu1 (human lung carcinoma); LNCaP (hormone-dependent human prostate carcinoma); MCF-7 (human breast carcinoma); HUVEC (human umbilical vein endothelial; non-tumorigenic).

three human cancer cell lines (Lu1, LNCaP, and MCF-7), and one normal cell line (HUVEC). Compounds **1**–**3** exhibited cytotoxic activity in the range of ED_{50} 0.7–7.0 μg/mL against these four cell lines (Table 2). Of the cytotoxic compounds (**1**–**3**), compound **1** was isolated in the largest amount from *P. tomentosa* leaves, and was chosen for evaluation in an in vivo hollow fiber assay, which is used as a secondary discriminator bioassay in our program on the discovery of plant antitumor agents (Kinghorn et al., 2003; Mi et al., 2002). However, using Lu1, LNCaP, and MCF-7 cells, compound **1** did not inhibit the growth of any cell line (<50%), using either the ip or sc sites, at doses of 6.25, 12.5, 25, and 50 mg/kg.

3. Experimental

3.1. General

Optical rotations were measured with a Perkin–Elmer 241 automatic polarimeter. UV spectra were obtained with a Beckman DU-7 spectrometer. IR spectra were run on an ATI Mattson Genesis Series FT-IR spectrophotometer. NMR spectroscopic data were recorded at room temperature on Bruker Avance DPX-300 and DRX-400 spectrometers with tetramethylsilane (TMS) as internal standard. Electrospray ionization (ESI) mass spectrometric analyses were performed with a 3-Tesla Finnigan FTMS-2000 Fourier Transform mass spectrometer, and electron impact (EI) ionization was performed with a Kratos MS-25 mass spectrometer, using 70 eV ionization conditions. A SunFire™ PrepC_{18}OBD™ column (5 μm, 150 × 19 mm i.d., Waters, Milford, MA) and a SunFire™ PrepC_{18} guard column (5 μm, 10 × 19 mm i.d., Waters) were used for preparative HPLC, along with two Waters 515 HPLC pumps and a Waters 2487 dual λ absorbance detector. Column chromatography was carried out with Purasil® (230–400 mesh, Whatman, Clilffton, NJ) and Diaion® HP-20 (Supelco, Bellefonte, PA). Analytical thin-layer chromatography (TLC) was performed on precoated 250 μm thickness Partisil® K6F (Whatman) glass plates, while preparative thin-layer chromatography was conducted on precoated 20 × 20 cm, 500 μm Partisil® K6F (Whatman) glass plates.

3.2. Plant material

The leaves of *P. tomentosa* were collected from Gunung Kancana, Cinta Asih village, Cianjur, West Java, Indonesia, in August 2003. The plant was identified by S. R., and a voucher specimen (collection number SR-CJR.30) has been deposited at the Herbarium Bogoriense, Bogor, Indonesia and at the Research Center for Chemistry, Indonesian Institute of Sciences, Serpong, Tangerang, Indonesia.

3.3. Extraction and isolation

The dried and milled leaves of *P. tomentosa* (614 g) were extracted by maceration with MeOH three times at room temperature, for up to 2 days each, and then evaporated in vacuo. The dried MeOH extract (71.8 g) was suspended with a mixture of MeOH–H_2O (9:1, 1 L) and partitioned sequentially with hexane (3 × 800 ml) and $CHCl_3$ (3 × 800 ml).

The $CHCl_3$-soluble partition was washed with 1% saline solution and concentrated under vacuum to yield a $CHCl_3$ extract (16.0 g), which exhibited cytotoxic activity (8.9 μg/ml) against the MCF-7 cell line. The $CHCl_3$-soluble fraction (16.0 g) was chromatographed over a silica gel column (70 × 600 mm), using a gradient of increasing polarity with $CHCl_3$ and MeOH as solvents and was fractionated into twelve sub-fractions (F01-F12). Cytotoxic activity of these 12 sub-fractions was monitored using the MCF-7 cell line, and two fractions (F02 and F03, 3.4 and 6.3 μg/ml, respectively) were deemed to be active. Fraction F02 (1.5 g) was subjected to column chromatography (55 × 150 mm) using Diaion HP-20 with MeOH and afforded seven sub-fractions (F0201-F0207). Ursolic acid (**4**) (8.0 mg; 0.0013% w/w) was precipitated from a MeOH solution of F0203 and the other MeOH-soluble constituents were purified by HPLC. This separation was conducted with MeOH–H_2O = 70:30, 7.0 ml/min, by isocratic elution for 30 min, and then increasing from 70:30 to 100:0 for 40 min, to afford compounds **1** (t_R 37.9 min, 40.0 mg; 0.0065% w/w), **2** (t_R 36.6 min, 12.0 mg; 0.0020% w/w), **3** (t_R 31.6 min, 12.0 mg; 0.0020% w/w), a mixture of chrysosplenetin and 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone (t_R 11.5 min), and a mixture of 3,7,3′-trimethylquercetin and 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone (t_R 18.6 min). The HPLC fractions were purified using preparative TLC (500 μm thickness layer, $CHCl_3$–MeOH = 20:1) and yielded chrysosplenetin (3.7 mg; 0.0006% w/w; R_f 0.58), 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone (3.9 mg; 0.0006% w/w; R_f 0.60), 3,7,3′-trimethylquercetin (6.0 mg; 0.0010% w/w; R_f 0.62) and 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone (4.0 mg; 0.0007% w/w; R_f 0.67). 3-*epi*-Corosolic acid lactone (3.0 mg; 0.0005% w/w) was obtained during

the purification of compound **1** using silica gel column chromatography ($CHCl_3$–MeOH = 30:1). Fraction F03 (675 mg) was subjected to silica gel column chromatography (26 × 350 mm, $CHCl_3$–MeOH = 30:1) and pooled into 10 sub-fractions (F0301-10). 2α-Hydroxyursolic acid (14 mg; 0.0023% w/w) was obtained as a white powder from F0308. Storage of compound **1** in acidic conditions (1 mg in 500 μl of 10% CH_3COOH in 2.5 ml MeOH for 24 h) did not lead to any conversion to **2** and **3**.

3.4. Premnone A (*1*)

Colorless oil, $[\alpha]_D^{22} + 24°$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.33) 277 (4.30) nm; IR ν_{max} (film) 3482, 2958, 1735, 1148 cm^{-1}; ^1H (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Table 1; HRESIMS *m/z*: 517.2566 $[M+Na]^+$ (calcd. for $C_{30}H_{38}O_6Na$, 517.2560).

3.5. Premone B (*2*)

Colorless oil, $[\alpha]_D^{22} + 7°$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.19) 273 (3.99) nm; IR ν_{max} (film) 3507, 2958, 1738, 1169 cm^{-1}; ^1H (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Table 1; HRESIMS *m/z*: 517.2539 $[M+Na]^+$ (calcd. for $C_{30}H_{38}O_6Na$ 517.2560).

3.6. Premnone C (*3*)

Colorless oil, $[\alpha]_D^{22} + 16°$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.33) 276 (4.27) nm; IR ν_{max} (film) 3508, 2956, 1738, 1171 cm^{-1}; ^1H (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Table 1; HRESIMS *m/z*: 517.2576 $[M+Na]^+$ (calcd. for $C_{30}H_{38}O_6Na$ 517.2560).

3.7. Cytotoxicity assay

Fractions were tested in the MCF-7 (human breast carcinoma) cell line and all isolates were evaluated in using the Lu1 (human lung carcinoma), LNCaP (hormone-dependent human prostate carcinoma), and MCF-7 cancer cell lines, and the HUVEC cell line (human umbilical vein endothelial) using established protocols (Likhitwitayawuid et al., 1993; Seo et al., 2001).

3.8. In vivo hollow fiber test

Compound **1** was evaluated in the in vivo hollow fiber model at doses of 6.25, 12.5, 25, and 50 mg/kg, using LNCaP, Lu1, and MCF-7 cells (Mi et al., 2002).

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