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Cytotoxic triterpenes from the twigs of *Celtis philippinensis*

Bang Yeon Hwang[a], Hee-Byung Chai[a], Leonardus B. S. Kardono[b], Soedarsono Riswan[c], Norman R. Farnsworth[a], Geoffrey A. Cordell[a], John M. Pezzuto[a], A. Douglas Kinghorn[a,*]

[a] *Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA*

[b] *Research Center for Chemistry, Indonesian Institute of Science, Serpong, 15310 Tangerang, Indonesia*

[c] *Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science, 16122 Bogor, Indonesia*

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Abstract

Two triterpene esters, 3β-*trans*-sinapoyloxylup-20(29)-en-28-ol (**1**) and 3β-*trans*-feruloyloxy-16β-hydroxylup-20(29)-ene (**2**), were isolated as cytotoxic constituents from the chloroform-soluble extract of the twigs of *Celtis philippinensis*, along with five known triterpenes, 3β-*O*-(*E*)-feruloylbetulin (**3**), 3β-*O*-(*E*)-coumaroylbetulin (**4**), betulin (**5**), 20-epibryonolic acid (**6**), and ursolic acid (**7**). The structures of **1** and **2** were assigned from their 1D and 2D NMR spectroscopic data. All isolates were evaluated for cytotoxicity against several human cancer cell lines.

Keywords: *Celtis philippinensis*; Ulmaceae; Twigs; Triterpenoids; 3β-*trans*-Sinapoyloxylup-20(29)-en-28-ol; 3β-*trans*-Feruloyloxy-16β-hydroxylup-20(29)-ene; Cytotoxicity evaluation

1. Introduction

As a part of an ongoing collaborative program to discover novel anticancer agents of plant origin (Kinghorn et al., 1999), the twigs of *Celtis philippinensis* Blanco (syn. *C. philippensis* Blanco; Ulmaceae) were collected in Indonesia and further investigated, since a $CHCl_3$-soluble extract exhibited cytotoxic activity against the KB (human oral epidermoid carcinoma) cell line. The genus *Celtis* (Ulmaceae) includes about 70 species of shrubs or trees, primarily distributed in the temperate and tropical regions of the Northern Hemisphere (Sargent, 1961; Li, 1963; Keay, 1989). *Celtis philippinensis* is a perennial woody tree or shrub widely distributed throughout tropical Africa, Asia, and Australia (Li, 1963; Keay, 1989), and no previous biological and phytochemical investigations on this plant have been reported. In the only two previous phytochemical reports on species of the genus *Celtis*, the presence of betulin, gallic acid, 3,3′-di-*O*-methylellagic acid, and moretenol was shown (Chari et al., 1968; Santa-Cruz et al., 1975).

Two triterpene esters, 3β-*trans*-sinapoyloxylup-20(29)-en-28-ol (**1**) and 3β-*trans*-feruloyloxy-16β-hydroxylup-20(29)-ene (**2**), and five known triterpenes, 3β-*O*-(*E*)-feruloylbetulin (**3**), 3β-*O*-(*E*)-coumaroylbetulin (**4**), betulin (**5**), 20-epibryonolic acid (**6**), and ursolic acid (**7**), were isolated from the twigs of *C. philippinensis* by bioassay-guided fractionation monitored by cytotoxicity toward the KB cell line. Among these, **1** and **2** were found to exhibit significant cytotoxicity in a small human tumor cell panel (Likhitwitayawuid et al., 1993; Seo et al., 2001). We report herein the isolation and structure elucidation of compounds **1** and **2**, and the cytotoxic evaluation of **1**–**7**.

2. Results and discussion

Compound **1** was obtained as a pale yellow amorphous powder. The molecular formula was established as $C_{41}H_{60}O_6$ from the HR-FAB–MS data at *m*/*z*

* Corresponding author. Tel.: +1-312-996-0914; fax: +1-312-996-7107.

E-mail address: kinghorn@uic.edu (A.D. Kinghorn).

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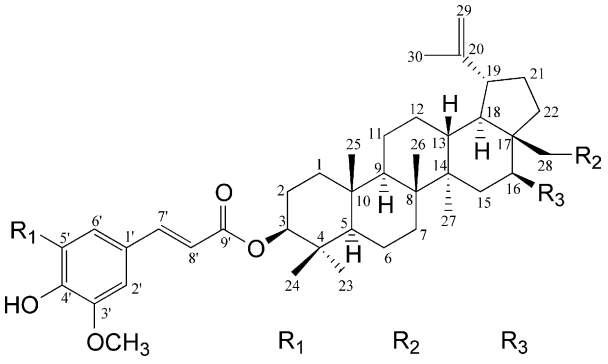
671.4228 $[M+Na]^+$ ($C_{41}H_{60}O_6Na$, calc. for 671.4288). This compound exhibited UV maxima at 225, 237, and 325 nm, suggesting the presence of conjugation in the molecule. The IR spectrum of **1** showed absorption bands for hydroxyl (3550–3100 cm^{-1}), α,β-unsaturated carbonyl ester (1694 cm^{-1}), and aromatic (1595 and 1515 cm^{-1}) functionalities.

The ^1H NMR spectrum of **1** revealed the presence of five tertiary methyl groups at δ_H 0.88, 0.90, 0.93, 0.99, and 1.04, one vinylic methyl at δ_H 1.69, two protons of an isopropenyl group at δ_H 4.59 and 4.69, two hydroxymethyl protons at δ_H 3.34 and 3.81, and a hydroxymethine proton at δ_H 4.62. Moreover, the presence of a *trans*-sinapoyl group in the molecule was suggested by a singlet aromatic resonance at δ_H 6.77 (2H, *s*), two olefinic protons as doublets with the same coupling constant of 15.8 Hz at δ_H 6.30 and 7.57, and two aromatic methoxyl groups at δ_H 3.93 (6H, *s*), in accordance with this ester unit being a symmetrically trisubstituted cinnamoyl moiety with *trans* stereochemistry (Haribal et al., 1999; Stochmal et al., 2001). The ^{13}C NMR spectrum of **1** showed signals for one ester carbonyl carbon at δ_C 167.0 (C-9′), four double bond carbons at δ_C 105.0 (C-2′ and C-6′), 144.6 (C-7′), and 116.6 (C-8′), and a terminal double bond at δ_C 109.8 (C-29). In addition, six methyl, two methoxy, 11 methylene, six methine, and 10 quaternary carbon signals were characterized by a DEPT experiment. These results suggested that **1** possesses an ester linkage between a lupene-type triterpene unit and *trans*-sinapic acid.

The ester substituent was placed at C-3 as a result of the downfield shifts observed for H-3 and C-3 in the ^1H and ^{13}C NMR spectra, respectively, compared with analogous data for betulin (Tinto et al., 1992), and the correlations observed between H-3 (δ_H 4.62) and C-2 (δ_C 23.9), C-4 (δ_C 38.1), C-23 (δ_C 28.0), C-24 (δ_C 16.7) and C-9′ (δ_C 167.0) of the *trans*-sinapic acid unit in the HMBC spectrum. The relative configuration of H-3 and the other spatial assignments for compound **1** were further supported by a NOESY experiment, wherein NOE enhancements were observed between H-3 (δ_H 4.62) and H-5 (δ_H 0.84) and H-23 (δ_H 0.90), and between H-28 (δ_H 3.34 and 3.81) and H-13 (δ_H 1.63) and H-19 (δ_H 2.39).

On alkaline hydrolysis, compound **1** afforded betulin (**5**) and *trans*-sinapic acid (Sakushima et al., 1994). Therefore, based on the above evidence, the structure of **1** was assigned as 3β-*trans*-sinapoyloxylup-20(29)-en-28-ol.

Compound **2** was obtained as a white amorphous powder. The molecular formula was established as $C_{40}H_{58}O_5$ from the HR-FAB–MS data at *m/z* 641.4146 $[M+Na]^+$ ($C_{40}H_{58}O_5Na$, calc. for 641.4182). This compound exhibited UV maxima at 218, 230, 293, and 324 nm, again suggesting the presence of conjugation in the molecule. The IR spectrum of **2** showed absorption bands for hydroxyl (3550–3100 cm^{-1}), α,β-unsaturated carbonyl ester (1692 cm^{-1}), and aromatic (1594 and 1514 cm^{-1}) functionalities.



	R_1	R_2	R_3
1	OCH_3	OH	H
2	H	H	OH

Its ^1H NMR spectrum showed signals for six tertiary methyl groups as sharp singlets at δ_H 0.80, 0.89, 0.90, 0.92, 1.00, and 1.05, one vinylic methyl at δ_H 1.69, and two protons of an isopropenyl moiety at δ_H 4.61 and 4.72. The presence of a *trans*-feruloyl substituent was supported by characteristic signals for three 1,2,4-trisubstituted aromatic protons at δ_H 6.91 (*d*, $J=8.2$ Hz), 7.04 (*d*, $J=1.5$ Hz), and 7.07 (*dd*, $J=8.2$, 1.5 Hz), two *trans*-oriented vinyl protons at δ_H 6.29 and 7.59 (each *d*, $J=15.9$ Hz), and an aromatic methoxy proton at δ_H 3.93 (3H, s). A cross peak between H-2′ and the aromatic OMe in the NOESY spectrum was used to locate the latter at position C-3′, indicating the nature of the ester group as a *trans*-feruloyloxy group (Siddiqui et al., 1997; Chang and Kuo, 1998). These results suggested that **2** is a lupene-type triterpene ester with a *trans*-ferulic acid unit. The ester substituent was placed at C-3 as a result of the downfield shifts observed for H-3 and C-3 in the ^1H and ^{13}C NMR spectra, respectively, compared with those of known lup-20(29)-ene-3β,16β-diol isolated from *Beyeria brevifolia* (Muell. Arg.) var. *brevifolia* Airy Shaw, *Nardophyllum lanatum* (Meyen) Cabr., and *Rhus taitensis* Guill. (Errington et al., 1976; Wenkert et al., 1978; Zdero et al., 1990; Yürüker et al., 1998), and from the correlations between H-3 (δ_H 4.61) and C-2 (δ_C 23.8), C-4 (δ_C 38.1), C-23 (δ_C 28.0), C-24 (δ_C 16.7) and C-9′ (δ_C 167.1) of the *trans*-ferulic acid unit observed in the HMBC spectrum.

The location of the remaining hydroxyl group was determined to be at C-16 on the basis of the HMBC correlations of the proton at δ_H 3.62 (H-16) and signals at δ_C 11.7 (C-28) and 44.1 (C-14). The relative configurations of H-3 and H-16 of compound **2** were further supported by a NOESY experiment, wherein NOE enhancements were observed between H-3 (δ_H 4.61) and H-5 (δ_H 0.81) and H-23 (δ_H 0.90), and between H-16 (δ_H 3.62) and H-18 (δ_H 1.41) and H-27 (δ_H 1.00) (Fig. 1). Therefore, based on the above evidence, the structure of

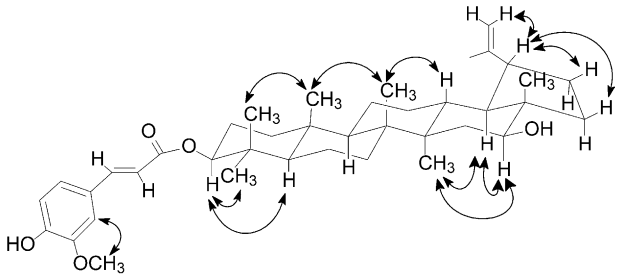


Fig. 1. Significant correlations observed in the NOESY spectrum of **2**.

2 was assigned as 3β-*trans*-feruloyloxy-16β-hydroxylup-20(29)-ene. At the conclusion of biological testing, an insufficient quantity of **2** remained to generate its triterpene alcohol and acid substituents by alkaline hydrolysis.

Five compounds of previously known structure were also isolated from the $CHCl_3$ extract of the twigs of *C. philippinensis*, as described in the Experimental, and were identified as 3β-*O*-(*E*)-feruloylbetulin (**3**) (Kuo et al., 1997), 3β-*O*-(*E*)-coumaroylbetulin (**4**) (Rashid et al., 1992), betulin (**5**) (Tinto et al., 1992), 20-epibryonolic acid (**6**) (Chang et al., 1996), and ursolic acid (**7**) (Lin et al., 1987). Their structures were identified by physical and spectroscopic methods (mp, $[\alpha]_D$, MS, ^1H and ^{13}C NMR) and by comparing the data obtained with those of published values.

As summarized in Table 1, compounds **1**–**7** were evaluated against a panel of human tumor cell lines (Likitwitayawuid et al., 1993; Seo et al., 2001). Compounds **1**, **2** and **7** exhibited significant cytotoxic effects with ED_{50} values in the general range of 5–15 μg/ml, whereas **3**–**6** were found to be weakly active or inactive (Table 1).

Table 1
Cytotoxic activity of compounds **1**–**7**

Compound	Cell line[a]					
	Lu1	Col2	KB	LNCaP	hTERT–RPE1	HUVEC
1	6.3	5.8	4.3	4.6	16.3	3.6
2	15.5	>20	5.2	7.7	5.1	9.0
3	>20	>20	7.7	>20	11.2	>20
4	18.1	>20	10.0	16.0	>20	13.8
5	>20	>20	>20	>20	>20	>20
6	>20	>20	9.9	10.9	>20	>20
7	11.8	9.0	6.6	6.7	13.2	4.1
Taxol (paclitaxel)	0.002	0.004	0.0004	0.004	0.02	0.09
Campto-thecin	0.01	0.02	0.008	0.01	0.08	0.09

[a] Results are expressed as ED_{50} values (μg/ml). Key to cell lines used: Lu1 = human lung cancer; Col2 = human colon cancer; KB = human oral epidermoid carcinoma; LNCaP = hormone-dependent human prostate cancer; hTERT–RPE1 = human telomerase reverse transcriptase–retinal pigment epithelial cells; HUVEC = human umbilical vein endothelial cells.

3. Experimental

3.1. General

Mps were determined using a Fisher-Johns melting point apparatus and are uncorr. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Beckman DU-7 spectrometer. IR spectra were recorded on a JASCO FT/IR-410 spectrometer. HR-FAB–MS were recorded on a VG 7070-HF mass spectrometer. ^1H and ^{13}C NMR data (including DEPT, HMQC, HMBC, NOESY and ^1H–^1H COSY spectra) were recorded at room temperature on a Bruker Avance DPX-300 and DRX-500 spectrometers with TMS as internal standard.

Column chromatography (CC) was conducted on silica gel (70–230 mesh, Merck, Darmstadt, Germany). TLC was performed on precoated silica gel 60 F_{254} (Merck, 0.25 mm layer thickness) plates. Visualization of the TLC plates was conducted at 254 and 365 nm and the vanillin-sulfuric acid spray reagent [1% vanillin containing 10% (v/v) sulfuric acid in EtOH] was used for detection. Prep. HPLC was performed using a Waters system with a 515 pump and 2487 UV detector.

3.2. Plant material

The twigs of *C. philippinensis* Blanco were collected at Kalteng, Indonesia, in October 1999 and identified by S. R. A voucher specimen (A4870) has been deposited at the Field Museum of Natural History, Chicago, IL.

3.3. Extraction and isolation

Dried twigs of *C. philippinensis* (977 g) were ground and extracted with MeOH (3 × 3 l) for 24 h by percolation. The extracts were combined and concentrated in vacuo at 40 °C. The concentrated extract was suspended in 90% MeOH and partitioned with petroleum ether (2 × 500 ml) to afford a petroleum ether soluble-extract (D001, 5 g). The aq. MeOH soln. (500 ml) was further partitioned with $CHCl_3$ (2 × 500 ml). The $CHCl_3$ extract was washed with 1% saline solution, and then evaporated, affording a $CHCl_3$ soluble-extract (D002, 9 g) and an aq. residue (D003, 21 g).

The $CHCl_3$-soluble extract, with 50% inhibitory activity at a concentration of 15.2 μg/ml against the KB cell line (human oral epidermoid carcinoma), was subjected to silica gel CC using $CHCl_3$–MeOH (100:0 → 2:1) mixtures, affording seven fractions (F004–F010). Of these, fractions F004–F007 showed cytotoxic activity (ED_{50} values of 5.6, 13.9, 11.5, and 16.1 μg/ml, respectively) and fractions F008–F010 were inactive (ED_{50} values of >20 μg/ml). Fraction F006 from this separation step was triturated with MeOH, yielding 20-epibryonolic

acid (**6**) [mp 278–280 °C, $[\alpha]_D^{20}$ −50.0° (MeOH, *c* 0.1)] (450 mg, 0.0461% w/w).

Fraction F004 [1.2 g, eluted with $CHCl_3$–MeOH (99:1)] was further chromatographed over silica gel with $CHCl_3$–Me_2CO (100:0 → 5:1) mixtures, and afforded the additional fractions F011–F016. Of these, fraction F013 [400 mg, eluted with $CHCl_3$–Me_2CO (30:1); ED_{50} value of 4.4 μg/ml] was further separated by silica gel CC using EtOAc–Me_2CO (50:1 → 2:1) yielding fractions F017–F026. Betulin (**5**) [mp 255–256 °C, $[\alpha]_D^{20}$ +28.0° (pyridine, *c* 0.1)] (18.2 mg, 0.0019% w/w) and 3β-*O*-(*E*)-feruloylbetulin (**3**) [mp 155–156 °C, $[\alpha]_D^{20}$ +21.0° ($CHCl_3$, *c* 0.2)] (12.5 mg, 0.0013% w/w) were isolated from F019 and F022, respectively, by recrystallization in MeOH. Prep. HPLC of fraction F023 (30 mg) (column: YMC J'sphere ODS–H80, 4 μm, 150 × 20 mm i.d., flow rate: 10 ml/min) using acetonitrile–H_2O (9:1) gave compound **1** (R_t 16 min, 6.5 mg, 0.0007% w/w).

Fractions F014 and F015 [300 mg, eluted with $CHCl_3$–Me_2CO (20:1, 10:1); ED_{50} values of 4.7 and 5.2 μg/ml, respectively] were combined and further subjected to silica gel CC eluted with mixtures of petroleum ether and Me_2CO to afford seven subfractions (F027–F032). Ursolic acid (**7**) [mp 242–244 °C, $[\alpha]_D^{20}$ +50.0° (pyridine, *c* 0.1)] (12.5 mg, 0.0013% w/w) was isolated from F029 by recrystallization in MeOH. Prep. HPLC of fraction F031 (25 mg) (column: YMC J'sphere ODS–H80, 4 μm, 150 × 20 mm i.d., flow rate: 10 ml/min) using acetonitrile–H_2O (9:1) afforded 3β-*O*-(*E*)-coumaroylbetulin (**4**) [mp 156–158 °C, $[\alpha]_D^{20}$ +25.0° ($CHCl_3$, *c* 0.2)] (R_t 18 min, 7.5 mg, 0.0008% w/w) and compound **2** (R_t 20 min, 2.5 mg, 0.0003% w/w).

3.4. 3β-trans-Sinapoyloxylup-20(29)-en-28-ol (1)

Pale yellow amorphous powder ($CHCl_3$–MeOH), mp 200 °C (dec), $[\alpha]_D^{20}$ +22.5° (MeOH, *c* 0.16). UV λ_{max} MeOH nm (log ε): 225 (4.10), 237 (4.12), 325 (4.15). IR ν_{max} (dried film) cm^{-1}: 3550-3100, 2943, 2872, 1694, 1633, 1595, 1515, 1455, 1336, 1284, 1114, 1016, 755. HR-FAB–MS *m/z*: 671.4228 ($C_{41}H_{60}O_6Na$, calc. 671.4288). ^1H NMR and ^{13}C NMR: Table 2.

3.5. Alkaline hydrolysis of 1

Compound **1** (4 mg) was refluxed with 5% KOH–MeOH solution (5 ml) under heating 70 °C for 7 h. The reaction mixture was diluted with H_2O (10 ml), and extracted with EtOAc to afford betulin (1.2 mg): mp 255–256 °C, $[\alpha]_D^{20}$ +28.5° (pyridine, *c* 0.1); exhibited comparable ^1H NMR spectral data to literature values (Tinto et al., 1992). The aq. layer was neutralized with 5% HCl and then extracted with CH_2Cl_2. The CH_2Cl_2 layer furnished *trans*-sinapic acid (0.5 mg): mp 198–200 °C; exhibited comparable ^1H NMR spectral data to literature values (Sakushima et al., 1994).

Table 2
^1H and ^{13}C NMR spectral data for compounds **1** and **2** (500/125 MHz, $CDCl_3$)

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	38.4 *t*[a]	1.06[b], 1.69[b]	38.5 *t*	1.04[b], 1.71[b]
2	23.9 *t*	1.66[b], 1.71[b]	23.8 *t*	1.67[b], 1.71[b]
3	80.9 *d*	4.62 *dd* (10.7, 5.2)	80.7 *d*	4.61 (overlapped)
4	38.1 *s*		38.1 *s*	
5	55.4 *d*	0.84[b]	55.5 *d*	0.81[b]
6	18.2 *t*	1.45[b], 1.54[b]	18.2 *t*	1.42[b], 1.56[b]
7	34.2 *t*	1.40[b], 1.42[b]	34.2 *t*	1.42[b]
8	41.0 *s*		41.0 *s*	
9	50.3 *d*	1.32[b]	49.9 *d*	1.29[b]
10	37.1 *s*		37.1 *s*	
11	20.9 *t*	1.21[b], 1.45[b]	20.9 *t*	1.23[b], 1.45[b]
12	25.2 *t*	1.11[b], 1.63[b]	24.7 *t*	1.08[b], 1.69[b]
13	37.3 *d*	1.63[b]	37.3 *d*	1.65[b]
14	42.7 *s*		44.1 *s*	
15	27.0 *t*	1.07[b], 1.70[b]	36.9 *t*	1.30[b], 1.57[b]
16	29.2 *t*	1.23[b], 1.92[b]	77.1 *d*	3.62 *dd* (10.9, 4.5)
17	47.8 *s*		48.6 *s*	
18	48.7 *d*	1.59[b]	47.7 *d*	1.41[b]
19	47.8 *d*	2.39 *ddd* (10.9, 10.9, 5.6)	47.6 *d*	2.50 *ddd* (10.8, 10.8, 5.6)
20	150.5 *s*		150.0 *s*	
21	29.7 *t*	1.41[b], 2.00[b]	29.9 *t*	1.35[b], 1.97[b]
22	34.0 *t*	1.06[b], 1.86[b]	37.7 *t*	1.30[b], 1.62[b]
23	28.0 *q*	0.90 *s*	28.0 *q*	0.90 *s*
24	16.7 *q*	0.93 *s*	16.7 *q*	0.92 *s*
25	16.2 *q*	0.88 *s*	16.22 *q*	0.89 *s*
26	16.0 *q*	1.04 *s*	16.0 *q*	1.05 *s*
27	14.7 *q*	0.99 *s*	16.17 *q*	1.00 *s*
28	60.6 *t*	3.34 *d* (10.8), 3.81 *d* (10.8)	11.7 *q*	0.80 *s*
29	109.8 *t*	4.59 *br s*, 4.69 *br s*	109.9 *t*	4.61 *br s*, 4.72 *br s*
30	19.1 *q*	1.69 *s*	19.3 *q*	1.69 *s*
1′	126.1 *s*		127.1 *s*	
2′	105.0 *d*	6.77 *s*	109.2 *d*	7.04 *d* (1.5)
3′	147.2 *s*		146.7 *s*	
4′	137.0 *s*		147.8 *s*	
5′	147.2 *s*		114.7 *d*	6.91 *d* (8.2)
6′	105.0 *d*	6.77 *s*	123.1 *d*	7.07 *dd* (8.2, 1.5)
7′	144.6 *d*	7.57 *d* (15.8)	144.4 *d*	7.59 *d* (15.9)
8′	116.6 *d*	6.30 *d* (15.8)	116.2 *d*	6.29 *d* (15.9)
9′	167.0 *s*		167.1 *s*	
3′-OCH_3	56.4 *q*	3.93 *s*	56.0 *q*	3.93 *s*
5′-OCH_3	56.4 *q*	3.93 *s*		

TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values (Hz) in parentheses. Assignments are based on ^1H–^1H COSY, HMQC and HMBC spectra.

[a] Carbon multiplicity.

[b] Multiplicity patterns were unclear due to signal overlapping.

3.6. 3β-trans-Feruloyloxy-16β-hydroxylup-20(29)-ene (2)

White amorphous powder ($CHCl_3$–MeOH), mp 168 °C (dec), $[\alpha]_D^{20}$ +18.7° (MeOH, *c* 0.15). UV λ_{max} MeOH nm (log ε): 218 (4.19), 230 (4.04), 293sh (4.02), 324 (4.17). IR ν_{max} (dried film) cm^{-1}: 3550-3100, 2945,

2870, 1692, 1594, 1514, 1453, 1381, 1268, 1174, 1014, 755. HR-FAB–MS *m/z*: 641.4146 ($C_{40}H_{58}O_5Na$, calc. 641.4182). ^1H NMR and ^{13}C NMR: Table 2.

3.7. Bioassay evaluation

The crude $CHCl_3$ extract of the twigs of *C. philippinensis*, sub-fractions of the extract, and compounds **1**–**7** were evaluated for cytotoxicity against a panel of human cancer cell lines, i.e., Lu1 (human lung cancer), Col2 (human colon cancer), KB (human oral epidermoid carcinoma), LNCaP (hormone-dependent human prostate cancer), hTERT–RPE1 (human telomerase reverse transcriptase–retinal pigment epithelial cells), and HUVEC (human umbilical vein endothelial cells), according to established protocols (Likhitwitayawuid et al., 1993; Seo et al., 2001). Taxol (paclitaxel) and camptothecin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The results are summarized in Table 1.

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