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Oleanane-type triterpenes from the flowers, pith, leaves, and fruit of *Tetrapanax papyriferus*

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Abstract

Four oleanane-type triterpenes, 3α,21β,22α-trihydroxy-11,13(18)-oleanadien-28-oic acid (**1**), 3-epi-papyriogenin C (**2**), 21-*O*-acetyl-21-hydroxy-3-oxo-11,13(18)-oleanadien-28-oic acid (**3**) and 3β-hydroxy-21-oxo-11,13(18)-oleanadien-28-oic acid methyl ester (**4**), together with four known triterpenes, were isolated from *Tetrapanax papyriferus* (Hook) K. Koch. Papyriogenin A (**8**) exhibited anti-HIV activity and low cytotoxicity in acutely infected H9 lymphocytes. Their structures were determined by analysis of spectroscopic data, including by 1D and 2D NMR.

Keywords: Tetrapanax papyriferus; Araliaceae; Oleanane-type triterpene

1. Introduction

Tetrapanax papyriferus (Hook) K. Koch (Araliaceae) is used as a traditional medicine in China to treat inflammation and dysentery (Giannattasio et al., 1996; Qian, 1996). Triterpenoids and saponins have been isolated from the leaves and roots (Amagaya et al., 1979; Mutsuga et al., 1997; Kojima et al., 1996). In our previous work, we isolated the flavonoids and benzoids from the flowers and fruit of this plant (Ho et al., 2005). Papyriogenin A and papyriogenin C from the leaves exhibit anti-inflammatory activities (Sugishita et al., 1982; Sugishita et al., 1983). Remarkable antihepatotoxic effects have been observed with papyriogenins and papyriosides from the leaves (Hikino et al., 1984). Studies have shown that extracts from *T. papyriferus* exhibit antithrombin activity (Chistokhodova et al., 2002). Herein, we describe the isolation and structural elucidation of four new and four known oleanane-type triterpenes.

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2. Results and discussion

The flowers, pith, leaves and fruit of *T. papyriferus* were sequentially extracted with methanol. Purification of the extract, produced four new oleanane-type triterpenes: 3α,21β,22α-trihydroxy-11,13(18)-oleanadien-28-oic acid (**1**), 3-epi-papyriogenin C (**2**), 21-*O*-acetyl-21-hydroxy-3-oxo-11,13(18)-oleanadien-28-oic acid (**3**) and 3β-hydroxy-21-oxo-11,13(18)-oleanadien-28-oic acid methyl ester (**4**), along with four known triterpenes: 3α-hydroxy-28-norolean-11,13(18),17(22)-trien-21-one (**5**) (Amagaya et al., 1979; Zorina et al., 1972), 28-norolean-11,13(18),17(22)-trien-3,21-dione (**6**) (Amagaya et al., 1979; Zorina et al., 1972), papyriogenin C (**7**) (Asada et al., 1980; Sugishita et al., 1983) and papyriogenin A (**8**) (Asada et al., 1980; Sugishita et al., 1983).

The molecular formula of compound **1** was deduced as $C_{30}H_{46}O_5$ by HR-EIMS. The IR spectrum revealed absorption bands of hydroxy (3450 cm^{-1}) and carboxylic (3500–2500, 1705 cm^{-1}) groups. The UV spectrum exhibited a conjugated diene moiety in methanol (λ_{max}) at 269, 250 and 239 nm. The ^1H NMR, ^1H–^1H COSY, ^{13}C

NMR, HMQC and HMBC spectra showed the presence of seven tertiary methyl and seven methylene signals, indicating that compound **1** was an oleanane-type triterpene (Table 1)(Asada et al., 1980; Sugishita et al., 1983). The presence of a disubstituted olefin at 11-C/12-C and a tetra-substituted double bond at C-13/C-18 was confirmed by analysis of the HMBC correlations. The (*Z*) geometry of the olefinic H-11/H-12 was evidenced by the coupling constant ($J = 10.4$ Hz). A carboxylic acid was observed at δ_C 176.5 and confirmed to be C-28 by analogy with similarly substituted triterpenes (Tan et al., 1999). In addition, the ^{1}H NMR resonances at δ 4.27 (1H, d, $J = 2.7$ Hz, H-22), 3.78 (1H, d, $J = 2.7$ Hz, H-21) and 3.42 (1H, d, $J = 2.4$ Hz, H-3) suggested three OH groups in compound **1**. The 3α,21β-dihydroxy group could be confirmed by comparing the ^{1}H NMR and ^{13}C NMR spectroscopic data with similarly substituted triterpenes (Asada et al., 1980; Dantanarayana et al., 1983; Zorina et al., 1972). The third hydroxyl group was positioned at C-22 (δ 85.5) on the basis of analysis of the HMBC experimental data, which showed a correlation between the C-22 carbon and the H-21 proton. The 22α-hydroxy configuration was determined from the coupling constant of $J_{21,22} = 2.7$ Hz and NOESY spectrum. The NOESY correlations of H-22 with H-21 and H-16e confirmed the structure of compound **1**. Thus compound **1** was determined as 3α,21β,22α-trihydroxy-11,13(18)-oleanadien-28-oic acid.

Table 1
^{13}C NMR data for compounds **1**–**4** (δ, ppm, in $CDCl_3$)

Position	1	2	3	4
1	31.7	31.3	33.4	31.4
2	25.6	26.5	33.9	26.1
3	77.8	74.3	217.1	74.4
4	38.8	38.9	37.7	39.8
5	48.5	53.4	54.5	54.4
6	19.4	19.2	19.3	19.5
7	30.1	30.0	31.9	31.5
8	39.3	38.8	39.3	40.1
9	54.3	54.3	53.4	53.6
10	38.9	38.6	38.3	40.3
11	129.3	126.8	128.3	126.7
12	124.3	125.5	125.4	125.6
13	139.7	132.5	137.8	136.4
14	42.3	42.0	41.2	42.5
15	25.3	24.9	24.9	25.7
16	28.5	28.5	29.7	30.1
17	48.5	48.2	47.4	48.5
18	122.5	130.3	126.0	131.5
19	37.9	36.7	40.2	39.3
20	36.6	47.2	38.1	46.4
21	80.3	218.0	109.9	217.4
22	85.5	36.7	38.5	40.4
23	28.4	27.1	29.7	27.1
24	18.1	22.1	21.9	22.4
25	16.4	16.0	16.1	16.4
26	17.7	17.4	17.4	18.3
27	25.3	25.0	25.8	25.9
28	176.5	179.0	175.2	176.6
29	28.4	28.8	27.8	28.2
30	22.9	23.4	24.3	24.1
CH_3CO			168.4, 22.7	
OMe				52.0

The molecular formula of compound **2** was determined as $C_{30}H_{44}O_4$ by HR-EIMS. The IR spectrum revealed hydroxy (3400 cm^{-1}), carboxylic (3500–2500, 1703 cm^{-1}) and carbonyl (1715 cm^{-1}) absorptions. The UV spectrum exhibited a conjugated diene moiety in methanol (λ_{max}) at 258, 246 and 234 nm. The ^{1}H NMR spectrum showed the presence of seven tertiary methyl groups, a hydroxymethine signal at δ 3.47 (1H, dd, $J = 10.2$, 4.2 Hz, H-3) and a disubstituted olefin at δ 6.48 (1H, dd, $J = 10.5$, 1.3 Hz, H-12), 5.60 (1 H, dd, $J = 10.5$, 2.3 Hz, H-11). The ^{13}C NMR showed a ketone group at δ 218.0 (C-21), a carboxylic group at δ 179.0 (C-28), four olefinic carbons at δ 132.5 (C-13), 130.3 (C-18), 126.8 (C-11), 125.5 (C-12) and a methine signal at δ 74.3 (C-3). The above data were similar to those of papyriogenin C (**7**) except for the C-3 configuration. The 3β-OH geometry was confirmed by comparing the chemical shift and coupling constant with similar substituted triterpenes (Asada et al., 1980; Hikino et al., 1984). Thus compound **2** was determined as 3β-hydroxy-21-oxo-11,13(18)-oleanadien-28-oic acid and name as 3-epi-papyriogenin C.

The HRFAB mass spectrum of compound **3** indicated a quasimolecular ion $[MH]^+$ at *m*/*z* 527.3395, corresponding to a molecular formula $C_{32}H_{46}O_6$ with ten units of unsaturation. The EIMS showed characteristic cleavage fragments at *m*/*z* 464 $[M–OH–COOH]^+$, 422 $[M\text{-}COOH\text{-}OAc]^+$, 421 $[M–OH–COOH\text{-}Ac]^+$, 406 $[M–OH–COOH\text{-}OAc\text{-}Me]^+$, indicating that **3** has carboxylic, hydroxyl and acetyl groups. The IR spectrum showed bands at 3400 (hydroxy), 1740, 1715 and 1700 cm^{-1} (carbonyl). The UV spectrum exhibited a conjugated diene moiety in methanol (λ_{max}) at 254, 245 and 232 nm. The ^{1}H NMR and ^{1}H–^{1}H COSY spectra showed the presence of seven tertiary methyl groups, some methylene signals [δ 3.16 (1H, dd, $J = 11.7$ Hz, H-19e), 2.70 (1H, d, $J = 16.0$ Hz, H-22e), 2.63 (1H, m, H-2e), 2.15 (1H, d, $J = 11.7$ Hz, H-19a), 2.12 (1H, m, H-2a), 2.10 (1H, d, $J = 17.2$ Hz, H-22a)] and a disubstituted olefin [δ 6.63 (1H, dd, $J = 10.4$, 2.7 Hz, H-12), 5.71 (1 H, dd, $J = 10.4$, 1.5 Hz, H-11)]. The ^{13}C NMR and DEPT spectra showed four olefinic carbons [δ 137.8 (C-13), 128.3 (C-11), 126.0 (C-18), 125.4 (C-12)], a carboxylic acid [δ 175.2 (C-28)] and a ketone signal [δ 217.1 (C-3)]. The NMR spectroscopic data of compound **3** were similar to those of 3,21-dioxo-11,13(18)-oleanadien-28-oic acid (papyriogenin A) except for the presence of an *O*,*O*-acetal moiety at δ 109.9 (C-21), an acetyl group at δ_C 168.4, 22.7, δ_H 2.11 and the absence of a ketone group. The HMBC correlations of C-21 to H-22e, H-19e and C-18 to H-22a, H-22e, H-19a, H-19e suggested that the hydroxy and acetyl groups were attached to C-21, whereas the correlations of C-3 to H-6a, H-6e and CH_3-24 established that the location of the ketone unit was at C-3. The structure of compound **3** was therefore confirmed to be 21-*O*-acetyl-21-hydroxy-3-oxo-11,13(18)-oleanadien-28-oic acid.

The molecular formula $C_{31}H_{46}O_4$ of compound **4** was determined on the basis of HR-EIMS. The IR spectrum revealed hydroxy (3400 cm^{-1}) and carbonyl (1725, 1710 cm^{-1}) groups. The UV spectrum exhibited a conjugated diene moiety in methanol (λ_{max}) at 258, 250 and 236 nm. The ^1H NMR spectrum showed the presence of seven tertiary methyl groups, a hydroxymethine [δ 3.44 (1H, dd, $J = 11.7$, 4.2 Hz, H-3)] and a disubstituted olefin [δ 6.47 (1H, dd, $J = 10.4$, 1.2 Hz, H-12), 5.66 (1H, dd, $J = 10.4$, 2.2 Hz, H-11)]. The ^{13}C NMR showed two carbonyl groups (δ 217.4, 176.6), four olefinic carbons (δ 136.4, 131.5, 126.7, 125.6) and a hydroxymethine signal (δ 74.4). The above data were very similar to those of 3-epi-papyriogenin C (**2**) except for the appearance of additional signals arising from a methoxyl group at δ_H 3.68 and δ_C 52.0. In the HMBC experiment, the methoxyl signal was correlated with C-28 (δ_C 176.6) indicating that the methoxyl group is attached to C-28. Therefore, the structure of compound **4** was characterized as 3β-hydroxy-21-oxo-11,13(18)-oleanadien-28-oic acid methyl ester.

Table 2 shows the anti-HIV activities of papyriogenin C (**7**) and papyriogenin A (**8**) with AZT included in the same experiment for comparison. The results of the HIV growth inhibition assay indicated that compound **8** displayed potent anti-HIV activity ($EC_{50} < 0.80$ μg/ml and TI value >11.2). The brine shrimp lethality assay is an effective and rapid assay method to screen compounds for potential cytotoxic activities. (Meyer et al., 1982). The observed lethalities, in compounds **1**–**2**, **6**–**8**, were comparable to that published for berberine chloride ($LD_{50} = 22.5$ μg/ml) and strychnine sulfate ($LD_{50} = 77.2$ μg/ml), and all compounds displayed low toxicity (LD_{50} values: 392–873 μg/ml) (Table 3) (Meyer et al., 1982; Tiew et al., 2002).

3. Conclusions

Papyriogenin A (**8**) showed anti-hepatotoxic and anti-inflammatory activities (Sugishita et al., 1982; Sugishita et al., 1983; Hikino et al., 1984) and also exhibited anti-HIV activity in this research. We find the replacing of the ketone group with hydroxyl group in position three showed no suppression.

Table 2
Anti-HIV activity of **7**–**8** in acutely infected H9 lymphocytes

Compounds	IC_{50} (μg/ml)[a]	EC_{50} (μg/ml)[b]	TI[c]
7	34.5	No suppression	–
8	8.91	<0.8	>11.1
AZT	500	0.0043	116,279

[a] Concentration that inhibits uninfected H9 cell growth by 50%.
[b] Concentration that inhibits viral replication by 50%.
[c] TI = therapeutic index IC_{50}/EC_{50}.

Table 3
Cytotoxicity of the compounds **1**–**2**, **6**–**8** by brine shrimp bioassay

Compounds	LD_{50} (μg/ml)
1	541
2	873
6	630
7	867
8	392

4. Experimental

4.1. General

Melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. EI-MS was recorded with a JMS-HX-100 instrument and FAB-MS with a JEOL LMS-SX 102 system. IR spectra were recorded on a JASCO FT-IR-110 infrared spectrophotometer. UV spectra were recorded on a Perkin Elmer Lambda 5 uv/vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-400 NMR and Bruker DMX-600 NMR spectrometer. Column chromatography was performed using silica gel (230–400 mesh, Merck), Sephadex LH-20 (Pharmacia Fine Chemicals) and Charcoal (Wako). Thin-layer chromatography (TLC) was conducted on precoated Kiesel gel 60 F_{254} plates (0.25 mm, Merck), spots were located by ultraviolet illumination and by spraying with 10% sulfuric acid followed by heating. MPLC was carried out on a Buchi MPLC system (pump, Buchi 688; detector, KAUER). HPLC was carried out on a Waters 1525 Binary HPLC system (RI detector, Waters 2410; UV detector, Waters 2487).

4.2. Plant material

The fresh flowers, pith, leaves and fruits of *Tetrapanax papyriferus* were collected in August 1995 in Miaoli county, Taiwan and then identified by Prof. C. M. Chen (Tp/1002a). Voucher specimens were deposited at the Department of Chemical Engineering, Ta-Hwa Institute of Technology, Hsinchu, Taiwan.

4.3. Extraction and isolation

The fresh flowers (ca. 16 Kg) were crushed and extracted with methanol (four times, each time 30 L) under reflux condition for 6–8 h. The crude extract was applied to a charcoal column, eluting with MeOH and CH_2Cl_2 to afford two fractions. The MeOH layer was concentrated to give a brown residue (150 g) and then subjected to chromatography on silica gel (CH_2Cl_2–MeOH gradient), following by MPLC (C-18, 40% H_2O/MeOH → MeOH) and HPLC (C-18, 40% H_2O/MeOH → MeOH) to give **1** (3.5 mg), **2** (2.1 mg), **5** (8.7 mg), **6** (4.5 mg), **7** (63.0 mg) and **8** (45.0 mg).

The fresh pith (ca. 2 kg) were extracted with methanol (three times, each time 5 L) under reflux condition for 6–8 h and concentrated to give a deep brown syrup (60 g). This syrup was partitioned between 1:1 EtOAc/H_2O. The

EtOAc layer was concentrated to give a brown residue (46 g) and then subjected to chromatography on silica gel eluting with *n*-hexane and increasing amounts of EtOAc (0–100%) to give three fractions. The first fraction was applied to Sephadex LH-20 column (H_2O–MeOH gradient) followed by HPLC (silica gel, CH_2Cl_2–MeOH gradient) to give 8 (11.2 mg). The second fraction was subjected to a Sephadex LH-20 chromatography (H_2O–MeOH gradient) followed by HPLC (silica gel, CH_2Cl_2–MeOH gradient; C18, H_2O–MeOH gradient) to give **1** (2.1 mg), **3** (9.5 mg), **4** (10.7 mg) and **7** (6.7 mg).

The fresh leaves (ca. 4 kg) were extracted with methanol (three times, each time 5 L) under reflux condition for 6–8 h and concentrated to give a deep brown syrup (120 g). This syrup was partitioned between 1:1 EtOAc/H_2O. The EtOAc layer was concentrated to give a brown residue (70 g) and then subjected to chromatography on silica gel eluting with *n*-hexane and increasing amounts of EtOAc (0–100%) to give three fractions. The first fraction was applied to a Sephadex LH-20 column (H_2O–MeOH gradient) followed by HPLC (silica gel, CH_2Cl_2–MeOH gradient) to give **6** (4.2 mg), **7** (3.1 mg). The second fraction was subjected to a Sephadex LH-20 chromatography (H_2O–MeOH gradient), followed by HPLC (silica gel, CH_2Cl_2–MeOH gradient; C18, H_2O–MeOH gradient) to give **1** (1.2 mg), **2** (5.4 mg), **3** (13 mg), **4** (5.6 mg), **7** (13.3 mg) and **8** (15.8 mg).

The fresh fruit (ca. 2 kg) were extracted with methanol (three times, each time 6 L) under reflux condition for 6–8 h and concentrated to give a deep brown syrup (65 g). This syrup was partitioned between 1:1 EtOAc/H_2O. The EtOAc layer was concentrated to give a brown residue (43 g) and then subjected to chromatography on silica gel (CH_2Cl_2–MeOH gradient), following by MPLC (C-18, 40% H_2O/MeOH → MeOH) and HPLC (C-18, 40% H_2O/MeOH → MeOH) to give **2** (6.8 mg), **7** (3.2 mg) and **8** (1.4 mg).

*4.4. 3α,21β,22α-Trihydroxy-11,13(18)-oleanadien-28-oic acid (**1**)*

White prisms, mp 231–232 °C (from CH_2Cl_2–MeOH), $[\alpha]_D^{20}$ −78.3 ($c = 0.35$, MeOH). HR-EIMS *m/z*: 486.3167 $[M]^+$, Calcd for $C_{30}H_{46}O_5$; 486.3345). EI–MS *m/z* (rel. int.): 486 $[M]^+$, (100), 406 (21), 394 (15), 255 (13), 189 (20), 95 (18), 43 (58), 41 (39). IR (KBr) ν_{max} cm^{-1}: 3500–2500 (br.), 3450, 2910, 2850, 1705, 1455, 1370, 1260, 1080, 900, 725. UV λ_{max}^{EtOH} nm (ε): 269 (24,600), 250 (26,700), 239 (25,400). ^1H NMR($CDCl_3$, 400.0 MHz) δ, ppm: 6.38 (1 H, dd, $J = 10.4$, 1.2 Hz, H-12), 5.75 (1H, dd, $J = 10.4$, 2.2 Hz, H-11), 4.27 (1H, d, $J = 2.7$ Hz, H-22), 3.78 (1H, d, $J = 2.7$ Hz, H-21), 3.42 (1H, d, $J = 2.4$ Hz, H-3), 2.57 (1H, d, $J = 16.0$ Hz, H-19e), 2.20 (1 H, m, H-16e), 2.08 (1H, m, H-9), 1.76 (1 H, d, $J = 16.0$ Hz, H-19a), 1.68 (1H, m, H-16a), 1.06 (3H, s, H-23), 0.95 (3H, s, H-29), 0.94 (3H, s, H-27), 0.88 (3H, s, H-30), 0.85 (3H, s, H-25), 0.83 (3H, s, H-26), 0.65 (3H, s, H-24).

*4.5. 3-Epi-papyriogenin C (**2**)*

White prisms, mp 215–216 °C (from CH_2Cl_2–MeOH). $[\alpha]_D^{20}$ +15.7 ($c = 0.85$, MeOH). HR EI–MS *m/z*: 486.3235 ($[M]^+$, Calcd for $C_{30}H_{44}O_4$; 486.3240). EI–MS *m/z* (rel. int.): 468 $[M]^+$ (100), 423 (23), 187 (37), 185 (16), 145 (17), 43 (23). IR (KBr) ν_{max} cm^{-1}: 3400–2500 (br.), 3400, 1715, 1703, 1460, 1380. UV λ_{max}^{EtOH} nm (ε): 258 (23,400), 246 (27,800), 234 (25,700). ^1H NMR($CDCl_3$, 400.0 MHz) δ, ppm: 6.48 (1H, dd, $J = 10.5$, 1.3 Hz, H-12), 5.60 (1H, dd, $J = 10.5$, 2.3 Hz, H-11), 3.47 (1H, dd, $J = 10.2$, 4.2 Hz, H-3), 2.60 (1H, d, $J = 13.7$ Hz, H-22e), 2.57 (1 H, m, H-19e), 2.43 (1H, d, $J = 13.7$ Hz, H-22a), 2.40 (1H, d, $J = 16.2$ Hz, H-19a), 2.12 (1H, m, H-9), 1.10 (3H, s, H-23), 1.04 (3H, s, H-29), 1.02 (3H, s, H-27), 1.01 (3H, s, H-30), 0.98 (3H, s, H-24), 0.82 (3H, s, H-26), 0.75 (3H, s, H-25).

*4.6. 21-O-acetyl-21-hydroxy-3-oxo-11,13(18)-oleanadien-28-oic acid (**3**)*

White prisms, mp 225–226 °C (from CH_2Cl_2–MeOH). $[\alpha]_D^{20}$ −69.5 ($c = 1.00$, $CHCl_3$). HR FAB–MS *m/z*: 527.3395 ($[M + H]^+$, Calcd for $C_{32}H_{47}O_6$; 527.3360). EI–MS *m/z* (rel. int.): 510 (19), 464 (24, $[M–OH–COOH]^+$), 422 (100, $[M–COOH–OAc]^+$), 421 (64, $[M–OH–COOH–Ac]^+$), 406 (62, $[M–OH–COOH–OAc–Me]^+$), 389 (36), 43 (62). IR (KBr) ν_{max} cm^{-1}: 3450–2500, 2910, 1790, 1760, 1715, 1455, 1360, 1205, 1040, 960. UV λ_{max}^{EtOH} nm (ε): 254 (23300), 245 (27300), 232 (24200). ^1H NMR($CDCl_3$, 400.0 MHz) δ, ppm: 6.63 (1H, dd, $J = 10.4$, 2.7 Hz, H-12), 5.71 (1H, dd, $J = 10.4$, 1.5 Hz, H-11), 3.16 (1H, dd, $J = 11.7$ Hz, H-19e), 2.70 (1H, d, $J = 16.0$ Hz, H-22e), 2.63 (1H, m, H-2e), 2.15 (1H, d, $J = 11.7$ Hz, H-19a), 2.12 (1H, m, H-2a), 2.11 (3H, s, OAc), 2.10 (1 H, d, $J = 17.2$ Hz, H-22a), 1.12 (6H, s, H-23, 29), 1.10 (3H, s, H-27), 1.03 (3H, s, H-30), 1.01 (3H, s, H-24), 0.97 (3H, s, H-26), 0.74 (3H, s, H-25).

*4.7. 3β-Hydroxy-21-oxo-11,13(18)-oleanadien-28-oic acid methyl ester (**4**)*

White prisms, mp 257–258 °C (from CH_2Cl_2–MeOH). $[\alpha]_D^{20}$ −92.5 ($c = 0.41$, $CHCl_3$). HR EI–MS *m/z*: 482.3376 ($[M]^+$, Calcd for $C_{31}H_{46}O_4$; 482.3396). EI–MS *m/z* (rel. int.): 486 $[M]^+$ (80), 423 (100), 405 (16), 213 (11), 185 (9), 43 (14). IR (KBr) ν_{max} cm^{-1}: 3400, 2945, 2850, 1725, 1710, 1455, 1380, 1360, 1200, 1140, 1040, 738. UV λ_{max}^{EtOH} nm (ε): 258 (24,300), 250 (28,900), 236 (26,700). ^1H NMR ($CDCl_3$, 400.0 MHz) δ, ppm: 6.47 (1H, dd, $J = 10.4$, 1.2 Hz, H-12), 5.66 (1H, d, $J = 10.4$. 2.2 Hz, H-11), 3.68 (3H, s, OCH_3), 3.44 (1 H, dd, $J = 11.7$, 4.2 Hz, H-3), 2.56 (1H, d, $J = 14.7$ Hz, H-22e), 2.49 (1H, d, $J = 14.7$ Hz, H-16e), 2.10 (1H, d, $J = 13.7$ Hz, H-19e), 1.90 (1H, m, H-16a), 1.60 (1H, m, H-22a), 1.10 (3H, s, H-23), 1.05 (3H, s, H-29), 1.04 (3H, s, H-27), 1.00 (3H, s, H-30), 0.98 (3H, s, H-24), 0.83 (3H, s, H-25), 0.76 (3H, s, H-26).

4.8. Anti-HIV assay

Anti-HIV activity was assayed by T cell line H9. The cell lines were cultured in RPMI medium 1640 containing 2 mM L-glutamine and heat-inactivated FCS, followed by culture in a T25 flask in a humidified incubator with 5% CO_2 and 37 °C. The cell line was only used in experiments when in log-phase of growth. Test samples were first dissolved in DMSO. The final drug concentrations used for test is 100, 20, 4 and 0.8 μg, but for active agents, additional dilutions were prepared for subsequent testing so that an accurate EC_{50} value could be achieved. As the test samples were being prepared, an aliquot of the T cell line, H9, were infected with HIV-1 strain IIIB while another aliquot was mock-infected with complete medium. The mock-infected was used for toxicity determinations (IC_{50}). The stock virus used for these studies had a $TCID_{50}$ value of 10^4 infectious units/ml. The appropriate amount of virus for a multiplicity of infection between 0.1 and 0.01 infectious units/cell was added to the first aliquot of the cells. The other aliquot of cells only received culture medium and then was incubated under identical conditions as the HIV-infected cells. After 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24-well-plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). Moreover, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 5% CO_2 and 37 °C for four days. Cell-free supernatants were collected on day four and tested by an in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter Counter on the mock-infected H9 cells which had either received culture medium (no toxicity) or test sample or AZT (Xia et al., 1999).

4.9. Brine shrimp lethality bioassay

The cytoxic effect of compounds **1**–**2**, **6**–**8** was evaluated by LD_{50} of brine shrimp lethality test. The compounds were dissolved in DMSO and five graded doses 62.5, 125, 250, 500, 1000 μg/ml, respectively were used for 5 ml sea water contained 10 brine shrimp nauplius in each group. The controls containing DMSO were included in each experiment. The number of survivors was counted after 24 h and LD_{50} was determined by probit analysis (Meyer et al., 1982). The experiment was carried out in quadruplicate and mean LD_{50} values were measured. Pure compounds with LD_{50} values >200 μg/ml were considered inactive (Othman et al., 2006).

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