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Cytotoxic Triterpenoids from the Fruits of *Ligustrum japonicum*

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Abstract – Medicinal plants are potential sources of anticancer agents screening. A large number of phytochemicals, including triterpenoids, have been reported to have significant cytotoxic effects on cancer cells. From the fruits of *Ligustrum japonicum* Thunb., thirteen triterpenoids (**1 - 13**) were isolated and evaluated for their cytotoxic activity against Hela and HL-60 cells. As results, **8** (oleanolic acid) showed significant effects on Hela with IC_{50} values of 5.5 μM, and moderate effects on HL-60 cells with IC_{50} values of 55.9 μM. Meanwhile, **10** (oleanderic acid) and **11** (3β-acetoxy-urs-12-en-28-oic acid) exhibited moderate inhibitory effects on Hela with IC_{50} value of 55.0 and 68.8 μM, respectively. Moreover, **10** showed cytotoxic effect on HL-60 cell line with IC_{50} value of 63.9 μM. To our knowledge, this is the first report that oleanderic acid was isolated from *L. japonicum* and investigated in cytotoxic effects on Hela and HL-60 cells.
Keywords – *Ligustrum japonicum*, Oleaceae, Triterpenoids, Cytotoxic activity

Introduction

Ligustrum japonicum Thunb. (Oleaceae) is a native evergreen shrub in Japan and Korea. The fruits of this plant have been used in Japanese traditional medicine as tonic.[1] Our previous study reported the isolation of triterpenoids, secoiridoids, and lignans from the fruits of *L. japonicum*.[2] A large number of phytochemicals, including triterpenoids, have been reported to have significant inhibitory activities against cancer cells. Triterpenoids, which consist 30 carbons and biosynthesized from isoprene units, have been considered as potentially cytotoxic compounds leading to complementary and alternative cancer treatments with low toxicities against normal cells.[3] In our continuing research, thirteen isolated triterpenoids were investigated for their cytotoxic activity.

Herein, we describe the isolation and structural elucidation of compounds **1** – **13** from *L. japonicum*, and evaluation of their cytotoxic effects on Hela (human cervix carcinoma) and HL-60 (human myeloid leukemia) cell lines.

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Experimental

General experiment procedures – Optical rotations were measured using a JASCO DIP 370 digital polarimeter. UV spectra were recorded using a Thermo spectrometer. IR spectra were recorded using a JASCO FT/IR-4100 spectrometer. Mass spectra were recorded using an AB SCIEX TripleTOF™ 5600 spectrometer. The 1D- and 2D-NMR spectra were determined using a Varian Unity Inova 400 MHz and a Bruker Ascend™ 500 MHz spectrometer with tetramethylsilane (TMS) as an internal standard; the chemical shifts were recorded in δ values (ppm). Silica gel (Merck, 63 - 200 μm particle size) and RP C-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was performed using Merck silica gel 60 F_{254} and RP C-18 F_{254} plates, and spots were visualized by spraying with 10% H_2SO_4 in ethanol, and then heating.

Plant material – The fruits of *L. japonicum* Thunb. used in this study were collected in Gyeongsan province, Republic of Korea in December 2015 and identified by Professor Byung Sun Min. A voucher specimen (CUD–14603) was deposited at the Herbarium of the College of Pharmacy, Daegu Catholic University, Korea.

Extraction and isolation – Air-dried fruits of *L. japonicum* (10 kg) were extracted with MeOH under reflux (10 L × 3 times). After concentrating *in vacuo*, the resulting extract (3.7 kg) was suspended in distilled water (4 L) and partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and BuOH, successively. The CH_2Cl_2-soluble fraction (337 g) was chromatographed over silica gel (CH_2Cl_2-EtOAc, 10 : 1) to obtain 12 fractions (LJC1 ~ LJC12). Then, fraction LJC3 (120.5 mg) was subjected on another silica gel column (*n*-hexane-acetone, 20:1) to yield compound **12** (4.5 mg). Fraction LJC4 (28.4 g) was fractionated by silica gel CC (CH_2Cl_2-*n*-hexane-EtOAc, 10 : 4 : 0.5) to produce 18 sub-fractions (LJC4.8 ~ LJC4.18). LJC4.8 (2.5 g) was subjected to RP C-18 silica gel CC (MeOH-H_2O, 3 : 1) to afford compound **10** (9.7 mg). Subsequently, LJC4.10 (510.4 mg) was purified by RP C-18 silica gel CC (MeOH-H_2O, 3 : 1 → 7 : 1) to obtain compounds **2** (2.4 mg), **6** (27.3 mg), **7** (8.4 mg), **9** (35.6 mg), **11** (17.3 mg), and **13** (29.3 mg). Fractionation of LJC4.18 (350.7 mg) by silica gel CC (*n*-hexane-EtOAc, 3 : 1) produced 23 sub-fractions (LJC4.18.1 ~ LJC4.18.23). LJC4.18.12 (89.7 mg) was chromatographed using an RP C-18 silica gel column (MeOH-H_2O, 2 : 1) to obtain compounds **1** (5.9 mg), **3** (63.2 mg), **4** (60.4 mg), **5** (6.1 mg), and **8** (9.7 mg).

3*β*,20,23-Trihydroxy-24,25,26,27-tetranordammarane (1) – White amorphous powder, $[\alpha]_D^{25}$ +25.7 (*c* 0.05, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε): 236 (3.14), 269 (1.93) nm; IR (KBr) ν_{max}: 3385, 2917, 1376 cm^{-1}; HR-ESI-MS *m/z*: 429.3327 $[M+Na]^+$ (calcd. for $C_{26}H_{46}O_3Na$: 429.3345); ^1H NMR (500 MHz, pyridine-d_5) δ (ppm): 1.64 (1H, dt, *J* = 10.4, 2.8 Hz, H-1_a), 0.94 (1H, m, H-1_b), 1.86 m (2H, m, H-2), 3.47 (1H, dt, *J* = 10.6, 5.4 Hz, H-3), 0.85 (1H, d, *J* = 11.5 Hz, H-5), 1.62(1H, m, H-6_a), 1.45 (1H, m, H-6_b), 1.57 (1H, m, H-7_a), 1.28 (1H, m, H-7_b), 1.38 (1H, td, *J* = 13.8, 3.4 Hz, H-9), 1.52 (1H, m, H-11_a), 1.25 (1H, m, H-11_a), 2.16 (1H, d, *J* = 12.2 Hz, H-12), 1.93 (1H, m, H-13), 1.60 (1H, m, H-15_a), 1.11 (1H, m, H-15_b), 1.87 (1H, m, H-16), 2.01 (1H, m, H-17), 0.99 (3H, s, H-18), 0.88 (3H, s, H-19), 1.48 (3H, s, H-21), 2.27 (1H, m, H-22_a), 2.01 (1H, m, H-22_b), 4.24 (1H, m, H-23_a), 4.31 (1H, m, H-23_b), 1.25 (3H, s, H-28), 1.07 (3H, s, H-29), 0.95 (3H, s, H-30); ^{13}C NMR (125 MHz, pyridine-d_5) δ (ppm): 40.0 (C-1), 25.9 (C-2), 78.6 (C-3), 40.1 (C-4), 56.9 (C-5), 19.3 (C-6), 36.2 (C-7), 41.2 (C-8), 51.6 (C-9), 37.9 (C-10), 22.4 (C-11), 28.6 (C-12), 43.1 (C-13), 51.0 (C-14), 32.1 (C-15), 28.8 (C-16), 51.8 (C-17), 16.2 (C-18), 17.0 (C-19), 75.4 (C-20), 26.8 (C-21), 42.7 (C-22), 59.8 (C-23), 29.1 (C-28), 16.8 (C-29), 17.2 (C-30).

3*β*-Hydroxy-22,23,24,25,26,27-hexanordammaran-20-one (2) – White powder; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.72 (1H, dd, *J* = 9.7, 1.9 Hz, H-5) 0.77 (3H, s) 0.84 (3H, s), 0.87 (3H. s). 0.97 (3H, s), 0.98 (3H, s), 2.13 (3H, s, H-21), 2.58 (1H, td, *J* = 10.9, 6.1 Hz, H-17), 3.19 (1H, dd, *J* = 11.2, 4.7 Hz, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 212.6 (C-20), 79.1 (C-3), 56.1 (C-5), 54.5 (C-17), 50.9 (C-9), 50.3 (C-14), 45.4 (C-13), 40.7 (C-8), 39.3 (C-1), 39.2 (C-4), 37.4 (C-10), 35.8 (C-7), 31.8 (C-15), 30.2 (C-21), 28.2 (C-28), 27.6 (C-2), 26.2 (C-16), 25.8 (C-12), 21.4 (C-11), 18.5 (C-6), 16.5 (C-18), 16.1 (C-29), 15.8 (C-18), 15.6 (C-19).

Isofouquierol (3) – White powder, ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.71 (1H, d, *J* = 10.4 Hz, H-5), 0.75 (3H, s, H_3-29) 0.82 (3H, s, H_3-19), 0.85 (3H, s, H_3-30). 0.94 (3H, s, H_3-18), 0.95 (3H, s, H_3-28), 1.10 (3H, s, H_3-21), 1.30 (6H, s, H_3-26, H_3-27), 2.16 (1H, m, H-17), 3.17 (1H, dd, *J* = 11.0, 5.1 Hz, H-3), 5.66 (2H, m, H-23, H-24); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 142.2 (C-24), 122.5 (C-23), 79.1 (C-3), 75.3 (C-20), 70.8 (C-25), 56.0 (C-3), 50.8 (C-9), 50.5 (C-14), 50.1 (C-17), 43.6 (C-22), 42.6 (C-13), 40.5 (C-8), 39.2 (C-1), 39.1 (C-4), 37.3 (C-10), 35.4 (C-7), 31.3 (C-15), 30.1 (C-26), 30.0 (C-27), 28.2 (C-16), 27.7 (C-28), 27.5 (C-2), 25.7 (C-21), 25.0 (C-12), 21.7 (C-11), 18.4 (C-6), 16.6 (C-19), 16.4 (C-30), 15.7 (C-18), 15.6 (C-29).

Fouquierol (4) – White powder, ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.72 (1H, d, *J* = 11.1 Hz, H-5), 0.76 (3H, s, H-29), 0.83 (3H, s, H-19), 0.87 (3H, s, H-30), 0.95 (3H, s, H-18), 0.96 (3H, s, H-28), 1.13 (3H, s, H-21), 1.72 (3H, s, H-27), 3.19 (1H, dd, *J* = 11.2, 5.0 Hz, H-3), 4.02 (1H, t, *J* = 6.2 Hz, H-24), 4.82 and 4.94 (2H, m, H-26); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 147.9 (C-25), 111.1 (C-26), 79.1 (C-3), 76.6 (C-24), 75.3 (C-20), 56.0 (C-5), 50.8 (C-9), 50.5 (C-14), 50.4 (C-17), 42.6 (C-13), 40.6 (C-8), 39.2 (C-1), 39.2 (C-4), 37.3 (C-10), 36.8 (C-22), 35.4 (C-7), 31.4 (C-15), 29.5 (C-23), 28.2 (C-16), 27.7 (C-28), 27.6 (C-2), 25.6 (C-21), 25.1 (C-12), 21.7 (C-11), 18.5 (C-6), 18.0 (C-27), 16.7 (C-19), 16.4 (C-30), 15.7 (C-18), 15.6 (C-19).

Hennadiol (5) – White powder, ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.68 (1H, d, *J* = 9.6 Hz, H-5) 0.75 (3H, s) 0.82 (3H, s), 0.96 (6H, s). 0.98 (3H, s), 2.88 (1H, td, *J* = 11.2, 4.7 Hz, H-19), 3.19 (1H, dd, *J* = 11.1, 4.9 Hz, H-3), 4.12 (2H, s, H-30), 4.92 (1H, s, H-29_a), 4.97 (1H, s, H-29_b); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 154.9 (C-20), 107.1 (C-29), 79.2 (C-3), 65.5 (C-30), 56.5 (C-19), 55.5 (C-5), 50.7 (C-9), 50.2 (C-18), 42.8 (C-17), 42.6 (C-14), 40.9 (C-8), 39.1 (C-22), 38.9 (C-4), 38.6 (C-1), 37.4 (C-13), 37.1 (C-10), 34.6 (C-16), 32.6 (C-7), 32.3 (C-28), 29.9 (C-21), 28.2 (C-23), 27.6 (C-2), 27.0 (C-15), 24.1 (C-12), 21.2 (C-11), 18.5 (C-6), 16.4 (C-25), 16.3 (C-26),

15.6 (C-24), 14.9 (C-27).

Betulin (6) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.68 (1H, d, J = 9.6 Hz, H-5), 0.76 (3H, s, H-24) 0.82 (3H, s, H-25), 0.96 (3H. s, H-23), 0.98 (3H, s, H-26), 1.02 (3H, s, H-27), 1.68 (3H, s, H-30), 2.38 (1H, td, J = 10.8, 5.8 Hz, H-19), 3.18 (1H, dd, J = 11.1, 4.9 Hz, H-3), 3.33 (1H, d, J = 10.8 Hz, H-28$_a$), 3.79 (1H, d, J = 10.8 Hz, H-28$_b$), 4.57 (1H, m, H-29$_a$), 4.68 (1H, d, J = 1.7 Hz, H-29$_b$); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 150.7 (C-20), 109.9 (C-29), 79.2 (C-3), 60.7 (C-28), 55.5 (C-5), 50.6 (C-9), 49.0 (C-19), 48.0 (C-17, C-18), 42.9 (C-14), 41.1 (C-8), 39.1 (C-1), 38.9 (C-4), 37.5 (C-10), 37.4 (C-13), 34.5 (C-7), 34.2 (C-22), 30.0 (C-21), 29.4 (C-16), 28.2 (C-23), 27.6 (C-2), 27.3 (C-15), 25.4 (C-12), 21.0 (C-11), 19.3 (C-30), 18.5 (C-6), 16.3 (C-25), 16.2(C-16), 15.6 (C-24), 15.0 (C-27).

Erythrodiol (7) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.73 (1H, d, J = 6.6 Hz, H-5). 0.79 (3H, s, H-24), 0.87 (3H, s, H-24), 0.89 (3H, s, H-29), 0.93 (3H, s, H-25), 0.94 (3H, s, H-30), 0.99 (3H, s, H-23), 1.16 (3H, s, H-27), 3.21 (1H, d, J = 11.0 Hz, H-28$_a$), 3.21 (1H, m, H-3), 3.55 (1H, d, J = 11.0 Hz, H-28$_b$), 5.19 (1H, t, J = 3.6 Hz, H-12); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 144.4 (C-13), 122.6 (C-12), 79.2 (C-3), 69.9 (C-28), 55.4 (C-5), 47.8 (C-9), 46.7 (C-19), 42.6 (C-18), 41.9 (C-14), 40.0 (C-1), 39.0 (C-4), 38.8 (C-17), 37.2 (C-8), 37.1 (C-10), 34.3 (C-21), 33.4 (C-29), 32.8 (C-7), 31.3 (C-22), 31.2 (C-20), 28.3 (C-15), 27.4 (C-2), 26.2 (C-23), 25.8 (C-27), 23.8 (C-30), 23.8 (C-11), 22.2 (C-16), 18.6 (C-6), 17.0 (C-26), 15.8 (C-24), 15.7 (C-25).

Oleanolic acid (8) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.75 (3H, s, H-26), 0.77 (3H, s, H-24), 0.90 (3H, s, H-29), 0.92 (3H, s, H-25), 0.93 (3H, s, H-30), 0.99 (3H, s, H-23), 1.13 (3H, s, H-27), 3.22 (1H, dd, J = 11.1, 4.7 Hz, H-3), 5.28 (1H, t, J = 3.5 Hz, H-12); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 183.5 (C-28), 143.8 (C-13), 122.9 (C-12), 79.3 (C-3), 55.5 (C-5), 47.9 (C-9), 46.8 (C-17), 46.1 (C-19), 41.8 (C-14), 41.2 (C-18), 39.5 (C-8), 39.0 (C-4), 38.6 (C-1), 37.3 (C-10), 34.0 (C-21), 33.3 (C-29), 32.9 (C-7), 32.7 (C-22), 30.9 (C-20), 28.3 (C-15), 27.9 (C-2), 27.4 (C-23), 26.2 (C-27), 23.8 (C-30), 23.6 (C-11), 23.2 (C-16), 18.5 (C-6), 17.4 (C-26), 15.8 (C-24), 15.5 (C-25).

Ursolic acid lactone (9) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.77 (3H, s, H-24), 0.90 (3H, s, H-25), 0.91 (3H, d, J = 7.7 Hz, H-29), 0.97 (3H, s, H-23), 0.98 (3H, d, J = 6.1 Hz, H-30), 1.04 (3H, s, H-26), 1.15 (3H, s, H-27), 1.94 (1H, brs), 2.12 (1H, td, J = 13.2, 5.8 Hz), 3.20 (1H, dd, J = 11.2, 5.1 Hz, H-3), 5.52 (1H, dd, J = 10.3, 3.1 Hz, H-11), 5.95 (1H, dd, J = 10.3, 1.4 Hz, H-11); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 180.1 (C-28), 133.6 (C-12), 129.0 (C-11), 89.9 (C-13), 79.0 (C-3), 60.8 (C-18), 54.9 (C-5), 53.2 (C-9), 45.3 (C-17), 42.1 (C-8), 41.9 (C-14), 40.4 (C-19), 39.1 (C-4), 38.5 (C-1), 38.3 (C-20), 36.5 (C-10), 31.5 (C-22), 31.4 (C-7), 31.0 (C-21), 28.0 (C-23), 27.2 (C-2), 25.7 (C-15), 23.0 (C-16), 19.4 (C-25), 19.1 (C-26), 18.1 (C-30), 18.0 (C-29), 17.9 (C-6), 16.3 (C-27), 15.1 (C-24).

Oleanderic acid (10) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.68 (1H, d, J = 9.6 Hz, H-5), 0.76 (3H, s, H-24), 0.83 (3H, s, H-25), 0.86 (3H, d, J = 6.9, H-29), 0.95 (6H, s, H-26, H-27), 0.97 (3H, s, H-23), 1.54 (3H, s, H-30), 2.12 (1H, td, J = 13.3, 4.4 Hz, H-15), 2.25 (1H, ddd, J = 13.6, 4.4, 2.6 Hz, H-16), 3.19 (1H, dd, J = 11.2, 5.0 Hz, H-3), 6.07 (1H, d, J = 7.6 Hz, H-21), 6.10 (1H, d, J = 7.6 Hz, H-22); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 175.7 (C-28), 138.6 (C-22), 134.0 (C-21), 83.9 (C-20), 79.2 (C-3), 55.6 (C-5), 50.7 (C-9), 48.4 (C-17), 47.5 (C-18), 44.8 (C-19), 42.4 (C-13), 41.5 (C-14), 40.8 (C-8), 39.1 (C-1), 39.0 (C-4), 37.4 (C-10), 34.2 (C-7), 28.2 (C-23), 27.6 (C-12), 27.5 (C-2), 27.1 (C-15), 25.7 (C-16), 21.3 (C-11), 21.2 (C-30), 19.9 (C-29), 18.5 (C-6), 16.5 (C-26), 15.9 (C-25), 15.6 (C-24), 14.3 (C-27).

3β-Acetoxy-urs-12-en-28-oic acid (11) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.76 (3H, s, H-26), 0.84 (3H, s, H-24), 0.85 (6H, d, J = 7.1 Hz, H-29), 0.86 (3H, d, J = 6.9 Hz, H-30), 0.95 (6H, s, H-25), 1.07 (3H, s, H-27), 2.04 (3H, s, H_3-acetyl), 4.50 (1H, dd, J = 9.3, 6.6 Hz, H-3), 5.22 (1H, brs, H-12); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 183.9 (C-28), 171.2 (C=O acetyl), 138.2 (C-13), 125.8 (C-12), 81.1 (C-3), 55.5 (C-5), 52.8 (C-18), 48.2 (C-17), 47.7 (C-9), 42.1 (C-14), 39.7 (C-19), 39.3 (C-20), 39.1 (C-8), 38.5 (C-1), 37.9 (C-4), 37.1 (C-10), 37.0 (C-22), 33.0 (C-7), 30.8 (C-21), 29.9 (C-23), 28.3 (C-15), 28.2 (CH_3-acetyl), 24.3 (C-16), 23.8 (C-2), 23.5 (C-27), 21.5 (C-11), 21.4 (C-30), 18.4 (C-6), 17.3 (C-26), 17.3 (C-29), 16.9 (C-24), 15.8 (C-25).

Ursaldehyde (12) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.71 (1H, dd, J = 11.5, 1.3 Hz, H-5), 0.77 (3H, s, H-24), 0.78 (3H, s, H-25), 0.87 (3H, d, J = 6.5 Hz, H-29), 0.92 (3H, s, H-26), 0.97 (3H, d, J = 6.9 Hz, H-30), 0.99 (3H, s, H-23), 1.09 (3H, s, H-27), 3.21 (1H, dd, J = 10.9, 4.9 Hz, H-3), 5.31 (1H, t, J = 3.7 Hz, H-12), 9.32 (1H, d, J = 1.2 Hz, H-28); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 207.7 (C-28), 138.0 (C-13), 126.4 (C-12), 79.2 (C-3), 55.4 (C-5), 52.8 (C-18), 50.4 (C-17), 47.8 (C-9), 42.4 (C-14), 40.0 (C-19), 39.2 (C-20), 39.0 (C-8), 39.0 (C-4), 38.9 (C-1), 37.2 (C-10), 33.3 (C-7), 32.1 (C-22), 30.4 (C-21), 29.9 (C-23), 28.4 (C-2), 27.4 (C-15), 27.1 (C-16), 23.5 (C-11), 23.5 (C-27), 21.3 (C-

30), 18.5 (C-6), 17.4 (C-26), 16.9 (C-24), 15.8 (C-29), 15.7 (C-25).

Uvaol (13) – White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.73 (1H, d, J = 10.5 Hz, H-5), 0.79 (3H, s, H-24), 0.80 (3H, d, J = 5.8 Hz, H-29), 0.94 (3H, d, J = 5.8 Hz, H-30), 0.95 (3H, s, H-25), 0.99 (3H, s, H-23), 0.99 (3H, s, H-26), 1.10 (3H, s, H-27), 3.19 (1H, d, J = 10.9 Hz, H-28$_a$), 3.22 (1H, dd, J = 11.0, 5.2 Hz, H-3), 3.52 (1H, d, J = 10.9 Hz, H-28$_b$), 5.13 (1H, t, J = 3.6 Hz, H-12); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 138.9 (C-13), 125.2 (C-12), 79.2 (C-3), 70.1 (C-28), 55.4 (C-5), 54.2 (C-18), 47.9 (C-9), 42.3 (C-14), 40.2 (C-8), 39.6 (C-19), 39.6 (C-20), 39.0 (C-1), 39.0 (C-17), 38.2 (C-4), 37.1 (C-10), 35.4 (C-22), 33.0 (C-7), 30.8 (C-21), 29.9 (C-16), 28.3 (C-23), 27.5 (C-2), 26.2 (C-5), 23.6 (C-11), 23.5 (C-27), 21.5 (C-30), 18.5 (C-6), 17.6 (C-29), 17.0 (C-26), 15.9 (C-24), 15.8 (C-25).

Cytotoxic activity – The cytotoxic activity assay was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hela and HL-60 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 15 mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 96-well plate at a density of 6×10^4 cells/mL. After reaching confluence (2×10^5 cells/mL), the cells were treated with the compounds. The compounds were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO was less than 0.1% (v/v). Various concentrations of tested compounds were prepared with serial dilutions. The experiment was allowed to proceed for 48 h at 37 °C in a humidified 5% CO_2 atmosphere. At the end of this period, supernatants were discarded. To minimize the interference of supernatant residue, the adherent cells were washed twice with Dulbecco's phosphate buffered saline (DPBS), and then 20 μL of MTT stock solution (5 mg/mL) was added to each well and the plates were further incubated for 3 h at 37 °C. DMSO (100 μL) was added to each well to solubilize the water-insoluble purple formazan crystals. After 1 h, the absorbance was measured at 570 nm with a microplate reader. Adriamycin, a commercial standard anticancer agent, was used as a positive control. The 50% reduction in cell number relative to the control (IC_{50}) was estimated visually. The results are presented as mean ± standard error of mean (SEM).[4]

Result and Discussion

From the CH_2Cl_2soluble fraction, thirteen triterpenoids (**1 - 13**) were isolated by repeated column chromatography (Fig. 1). Their chemical structures were elucidated as 3β,20,23-trihydroxy-24,25,26,27-tetranordammarane (**1**)[2], 3β-hydroxy-22,23,24,25,26,27-hexanordammaran-20-one (**2**)[5], isofouquierol (**3**)[6], fouquierol (**4**)[6], hennadiol (**5**)[7], betulin (**6**)[8], erythrodiol (**7**)[9], oleanolic acid (**8**)[10], ursolic acid lactone (**9**)[11], oleanderic acid (**10**)[12], 3β-acetoxy-urs-12-en-28-oic acid (**11**)[13], ursaldehyde (**12**)[14], and uvaol (**13**)[15] by comparison with reported spectroscopic data.

Compound **1** was obtained as a white amorphous powder. The ^{1}H NMR spectrum of **1** displayed signals for six tertiary methyl groups at δ_H 0.88 (3H, s, H_3-19), 0.95 (3H, s, H_3-30), 0.99 (3H, s, H_3-18), 1.07 (3H, s, H_3-29), 1.25 (3H, s, H_3-28), and 1.48 (3H, s, H_3-21); one oxygenated methine at δ_H 3.47 (1H, dt, J = 10.6, 5.4 Hz, H-3); and one oxygenated methylene at δ_H 4.24/4.31 (each 1H, m,

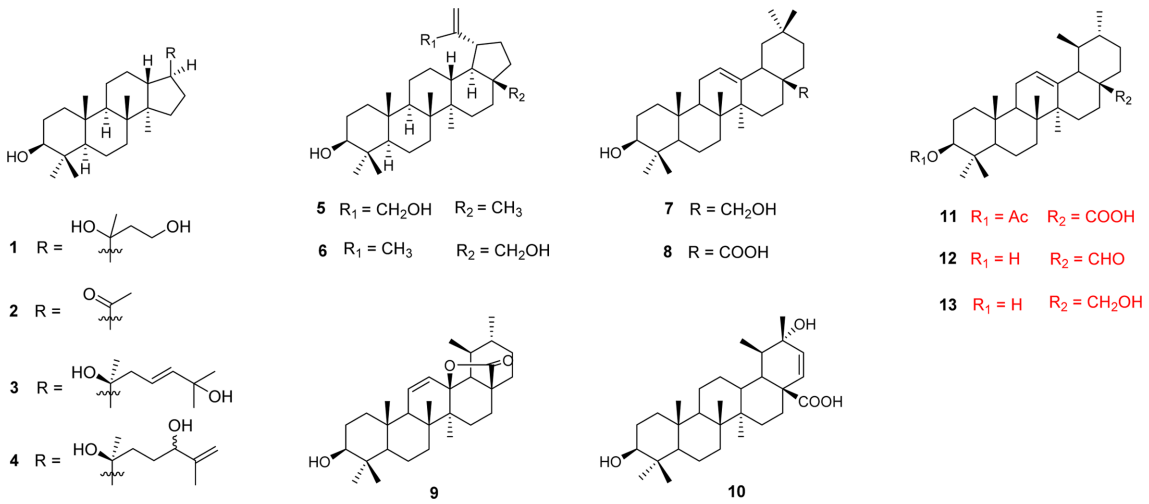

Fig. 1. Chemical structures of triterpenoids isolated from *L. japonicum*.

Table 1. Cytotoxic activities of compounds **1 - 13** against Hela and HL-60 cell lines.

Compds	IC_{50}, μM[a]		Compds	IC_{50}, μM[a]	
	Hela	HL-60		Hela	HL-60
1	> 100	> 100	**8**	5.5 ± 0.7	55.9 ± 1.2
2	> 100	> 100	**9**	> 100	> 100
3	> 100	> 100	**10**	55.0 ± 2.5	63.9 ± 2.8
4	> 100	> 100	**11**	68.8 ± 5.1	> 100
5	> 100	> 100	**12**	> 100	> 100
6	> 100	> 100	**13**	> 100	> 100
7	> 100	> 100	Adriamycin[b]	0.67 ± 0.5	0.24 ± 0.06

[a] The results are presented as mean ± SEM (n = 3).
[b] Positive control.

H-23). In particular, the protons belonging to the hydroxyl groups at C-3 and C-23 appeared as multiplets at δ_H 5.74 (1H, d, J = 5.4 Hz) and 6.12 (1H, t, J = 4.3 Hz) due to vicinal couplings to the hydrogens on the same carbons; whereas the resonance of the hydroxyl proton at quaternary carbon C-20 appeared as a singlet at δ_H 5.46. The ^{13}C NMR and DEPT spectra revealed twenty-six carbon resonances, including six methyls, ten methylenes, five methines, and five quaternary carbons, of which three oxygenated carbons were observed at δ_C 59.8, 75.4, and 78.6. By comparing the NMR data of **1** with those of fouquierol (20(*S*)-3β,20-dihydroxy-dammar-25-ene)[16], the structure of **1** was assigned as a dammarane-type triterpenoid derivative that lost the 2-methylpropyl moiety from the side chain. The location of the hydroxyl group at C-23 was confirmed by HMBC correlations from the signal at δ_H 4.24/4.31 (H_2-23) to the carbons at δ_C 75.4 (C-20) and 42.7 (C-22). The configuration of **1** was determined based on NOESY experiments and coupling patterns in the ^1H NMR spectrum. The NOE correlations between H-3 and H-5, H-3 and H_3-28 together with a large coupling constant of H-3 (J = 10.6 Hz) demonstrated that the oxymethine proton at this position is in the α (axial) orientation.[17] Compound **1** has been reported as a new compound in our previous study.[2]

Thirteen isolated triterpenoids (**1 - 13**) were evaluated for cytotoxic effects on Hela and HL-60 cell lines. As results, **8** (oleanolic acid) showed significant activities against Hela cells with IC_{50} values of 5.5 μM, and moderate activities against and HL-60 cells with IC_{50} values of 55.9 μM (Table 1). Oleanolic acid was reported as a common component from natural plants in the form of free acid or aglycones for saponin. It has been used in cosmetics and dietary supplements due to its pharmacological properties, such as hepatoprotective anti-oxidant, anti-inflammatory, and anti-tumor activities. Oleanolic acid was demonstrated to have anticancer activity on several cell lines, including Hep-G2 (human hepatocellular carcinoma), MCF-7 (breast cancer), HONE-1 (epithelial tumor), HL-60 (human myeloid leukemia), DU145 (human prostate cancer), and U87 (glioblastoma cancer).[18-20] In the present study, cytotoxic activities of this compound in Hela and HL-60 cells were confirmed with IC_{50} values of 5.5 and 55.9 μM, respectively (Table 1). Meanwhile, **10** (oleanderic acid) and **11** (3β-acetoxy-urs-12-en-28-oic acid) exhibited moderate growth inhibition on Hela cells with IC_{50} value of 55.0 and 68.8 μM, respectively. Moreover, oleanderic acid showed cytotoxic activity against HL-60 cell line with IC_{50} value of 63.9 μM. Oleanderic acid were first-isolated from *Nerium oleander* and showed moderate cytotoxic activity against Hep-G2 cells with IC_{50} value of 159 μM.[12] However, cytotoxic effects on Hela and HL-60 cells by oleanderic acid have been investigated in this study for the first-time. Our data indicated that oleanderic acid inhibits the growth of Hela and HL-60 cell lines with IC_{50} of 55.0 and 63.9 μM, respectively. Therefore, oleanolic acid, oleanderic acid, and 3β-acetoxy-urs-12-en-28-oic acid could be suggested as potential candidates for treatment of human cervix carcinoma and myeloid leukemia.

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